Genetic and Molecular Profiling of Neuroligin3, Neuroligin4X and Neuroligin4Y Genes in Autism Spectrum Disorder among the Population of North Karnataka

Thesis submitted to the BLDE (DU) University for the Partial Fulfilment for the award of the degree of

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IN
ALLIED HEALTH SCIENCES

(HUMAN GENETICS)

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Mr. Rajat Hegde

INDEX

LIST OF TABLES

LIST OF FIGURES

LIST OF ABBREVIATIONS

ASD- Autism Spectrum Disorder

DSM- Diagnostic and Statistical Manual of Mental Disorders

CDC- Center for Disease Control

CNV- Copy Number Variation

OMIM- Online Mendelian Inheritance in Man

NLGN3- Neuroligin 3

NLGN4X- Neuroligin 4-X

NLGN4Y- Neuroligin 4-Y

NRXN- Neurexin

SHANK- Multiple ankyrin repeat domains protein

DNA- Deoxyribo nucleic acid

RNA- Ribonucleic acid

SNP- Single nucleotide polymorphism

MiRNA- Micro Ribonucleic acid

mtDNA – Mitochondrial Deoxyribo nucleic acid

nDNA- nuclear DNA

EDTA- Ethylene Diamine Tetra Acetic acid

PCR- Polymerase Chain Reaction

TAE buffer- Tris Acetate EDTA buffer

µl – Micro litter

ml- Millilitre

^{o}C- degree Celsius

cDNA- Complimentary Deoxyribo nucleic acid

FA gel- Formaldehyde agarose gel

mM – milli molar

dNTP- Deoxy nucleotide triphosphate

GOI- Gene of interest

GAPDH- Glyceraldehyde-3-phospahte dehydrogenase

RT-PCR- Real time polymerase chain reaction

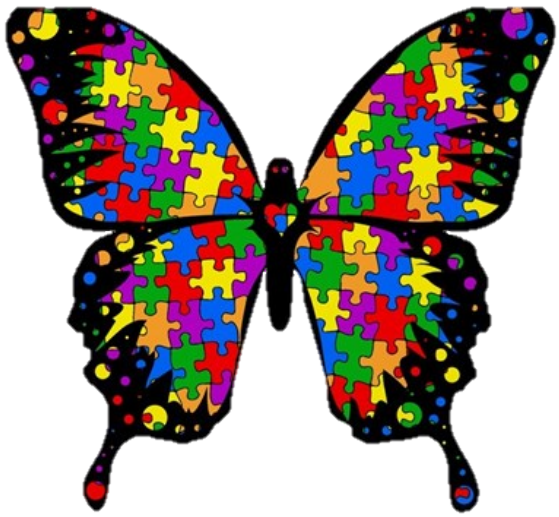
qPCR – Quantitative Polymerase chain reaction

Cq value- quantification cycle

NTC- no template control

Chapter 1

INTRODUCTION



1.1 General introduction

Autism Spectrum Disorder (ASD) or Autism (MIM 209850) is a complex heterogeneous neurodevelopmental disorder. It manifests before the age of three (1). Although autism can be diagnosed at any age, it is described as a "developmental disorder" because symptoms generally appear in the first two years of life. Diagnostic and Statistical Manual of Mental Disorders (DSM-5) is a diagnostic guide created by the American Psychiatric Association that healthcare providers use to diagnose different mental disorders/illnesses. People with ASD often have featured early onset dysfunctions in verbal and non-verbal communication, impairments in social interaction and repetitive and stereotyped behaviours and interests (2).

Autism is known as a "spectrum" disorder because there is wide variation in the type and severity of symptoms that individuals experience. Individuals of all genders, races, ethnicities, and economic backgrounds can be diagnosed with ASD. Although ASD can be a lifelong disorder, treatments and services can improve a person's symptoms and daily functioning. The previous version of the DSM, i.e. the DSM-4, included autism under the umbrella of pervasive developmental disorder and divided autism into five distinct categories ranging from Asperger's syndrome (often used to describe mild or high-functioning autism) to autistic disorder, which indicated severe autism. The 5 diagnostic ranges include autistic disorder, Asperger's disorder, Pervasive Developmental Disorder Not Otherwise Specified (PDD-NOS), Rett's disorder and Childhood Disintegrative Disorder (CDD). But the recent version of the DSM, i.e. DSM-V combines all of these into a spectrum of single diagnosis called autism spectrum disorder with different levels of severity (Fig.1) (3).

Level-1: Requires support : Level-1 ASD is the mildest form of autism. Children with level-1 ASD have difficulties communicating appropriately with others. For example, they may not say the right thing at the right time or be able to read social cues and body language. A person with ASD level 1 usually can speak in full sentences and communicate but has trouble engaging in back-and-forth conversations with others. They may try to make friends, but not be very successful.

Level-2: Requires substantial support: People with ASD level-2 have more obvious problems with verbal and social communication than those diagnosed with level -1. Likewise, they find it harder to change focus or move from one activity to the next.

Children with level-2 tend to have very narrow interests and engage in repetitive behaviours that can make it difficult for them to function in certain situations. For example, they may pace back and forth or say the same thing over and over again. A person diagnosed with ASD level-2 tends to speak in simple sentences and also struggles with nonverbal forms of communication.

Level-3: Requires very substantial support: Level-3 is the most severe form of autism. Children in this category have many of the same behaviours as those with levels 1 and 2 but to a more extreme degree. Problems expressing themselves both verbally and nonverbally can make it very hard to function, interact socially, and deal with a change in focus or location. Engaging in repetitive behaviours is another symptom of level 3 ASD. A person with ASD level 3 will have a very limited ability to speak clearly and will rarely start interactions with other people. When they do, they will do so awkwardly. Someone with level 3 will also respond only to very direct social approaches from other people.

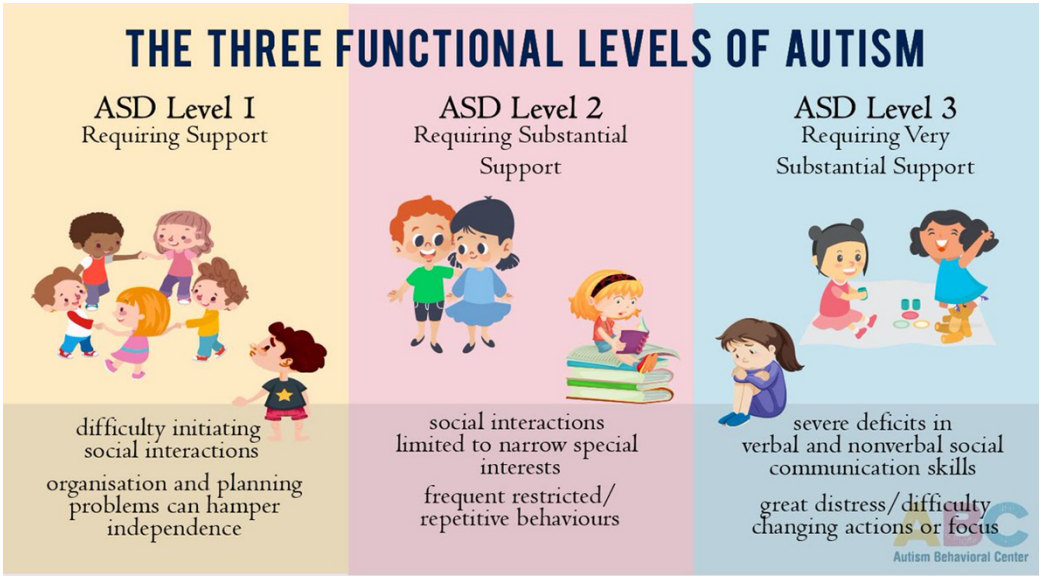


Fig.1.1 The three functional levels of Autism.

1.2 History

In 1908, psychiatrist Eugen Bleuler coined the term autism. He used it to characterise a schizophrenic patient who has closed himself off from the rest of the world. Bleuler coined the term "autism" from the Greek word "autós," which signified "morbid self-admiration and isolation inside oneself." Hans Asperger and Leo Kanner were pioneers in autism research. In the 1940s, they worked independently.

Kanner characterised seriously afflicted youngsters, whereas Asperger portrayed highly capable children. For the following three decades, their ideas were valuable to clinicians. Leo Kanner, an American child psychiatrist, investigated 11 children in 1943. The children displayed social difficulties, difficulty adapting to changes in habits, good memory, sensitivity to stimuli (notably sound), food resistance and allergies, great intellectual potential, echolalia (the tendency to repeat the speaker's words), and difficulties with spontaneous activity. Hans Asperger investigated a group of youngsters in 1944 while working on his own. Kanner's descriptions of his children were similarly accurate. The youngsters he investigated, on the other hand, did not have echolalia as a language issue and talked like adults. He also remarked that many of the kids were clumsy and didn't have the same fine motor abilities as other kids (4). It is important that Hans Asperger from Austria published a study on autism in 1944, while Kanner published his article in 1943 about ‘early infantile autism. Despite having no connection to one another, Kanner and Asperger worked on the same syndrome. Asperger's work was translated into English in 1981(5). In 1980 for the first time, "infantile autism" was recognised in the Diagnostic and Statistical Manual of Mental Disorders (DSM) and stated that the disorder is separated from childhood schizophrenia. Later autism was classified as a special education category by the federal government. Public schools began to recognise children on the autism spectrum and provide them with specialised treatment. In 2013 The DSM-5 combined all subtypes of autism into a single diagnosis of autism spectrum disorder (ASD). Asperger's Syndrome is no longer regarded as a distinct disorder. ASD is defined by two categories: 1) Impaired social communication and/or interaction. 2) Restricted and/or repetitive behaviours.

1.3 Historical overview from India

A. Ronald, a physician from Vienna who practised in Darjeeling, made the first mention of autism in Indian literature in 1944 and he termed as "abnormal children". Ronald provided an overview of the diagnosis, causes, characteristics, and treatment. In Indian literature, the term "autism" first emerged in 1959. Through during 1960s, dozens of publications used the term. Beyond that, the medical community knew very little about autism. In the late 1970s, a few organizations in India started autism diagnoses for children with abnormalities. By the early 1980s, there had been a slight rise in some professionals' "knowledge" of autism, to the point

where they were aware of its existence. However, it was not necessarily a real understanding because the expert's understanding was affected by the assumption that it was a kind of mental retardation or a mental disorder. Since the late 1980s knowledge about autism in India has witnessed a surge in comparison to earlier decades (6). In less than a decade, autism awareness in India has grown significantly in the field of diagnosis, treatment, educational opportunities, family participation, career options, human resource development, and legislation. But all these attempts to give an overview of the disorder were only able to highlight some of these aspects and cannot provide the depth or extent that the topic deserves which are still lacking and a lot of work needs to be carried out to understand the aetiology of autism (6).

1.4 Prevalence

Since the first epidemiological survey in 1960, a lot of data have been available for access which reveals that the condition is far more prevalent than previously assumed (7, 8). In recent years, cases of autism have increased drastically. Prevailing theories suggest that the rise is largely due to increased awareness and accurate diagnosis of autism rather than a massive increase in overall occurrences of autism. According to the Center for Disease Control (CDC), around 1% of the world's population has autism spectrum disorder and over 75,000,000 people are affected by autism spectrum disorder (9). The center for disease control announced in 2021 that the rate of autism in the U.S. during 2018 was 1 child in 44. This was a notable rise from rates given in scientific Americans, for 2016 (1 in 68, though other sources claim an even-higher 1 in 54 by age 8), 2008 (1 in 88) and 2000 (1 in 150) (10). Autism prevalence in various geographical populations around the world varies largely and the prevalence is measured as incidence per 1000 (Fig.3). The prevalence in the European population is 6.19, the Middle East population is 1.76, the prevalence in the overall Asian population is estimated to be 6.50, the prevalence in Australia and New Zealand is 3.15, North America is 7.17, Central and South America is 3.99 (7, 11). The following nations have the highest prevalence of ASD, in Europe and the Middle East 17.4, Poland 5.4 (12), West Pomerania, Poland 5.2 (13), Germany 6 (14), Denmark 12.6, Finland 7.7, 7.3 in the west, 4.8 in the east, and 31.3 in Iceland (15). Different regions of Italy, such as Tuscany has 11.5, Piemonte 4.2 (16), Emilia Romagna 4.3, and Abruzzo 8.0 (17), as well as other nations, such as Spain, showing

15.5 (16). Catalonia is 11.8 (18), Iran 7.1 (19), Oman 2.0 (20), Qatar 11.4 (21), and Lebanon 15.3 (22), Vietnam 10.8 (23), and Australia 14.1 and New Zealand 25.2 (24).

In Asian countries, the prevalence of autism also varies drastically. Previous epidemiological research on ASD prevalence in south Asian nations found substantial variations in prevalence, with Bangladeshi populations reporting 0.8 per cent, Indian populations 0.23 per cent, and Sri Lankan populations 1.07 per cent (25). In China, 2.8 (26), china Beijing 11.9 (27), Japan 19.0 (28), Nepal 3.4 (29), India has a population of 1.4 billion people, with 35% of them being children under the age of 14. In India, no large epidemiological research on the prevalence of autism has been conducted. However, extrapolating from the most current estimates we found that there were around 2.3 million children in India with autism spectrum disorder (30). The existing data on the prevalence of autism in India is insufficient. Autism prevalence also varies by location in India, with Kerala having a prevalence of 5.0 (31), Kolkata having a prevalence of 2.3 (32), and North West India having a prevalence of 1.5. In 2015, a population-based study in Himachal Pradesh, found a prevalence rate of 0.9/1000, with the rural population having a higher incidence rate (7). Until early 2015, the prevalence of ASD in India was almost unknown. Case reports, case series, retrospective chart reviews, qualitative investigations, and therapy studies are still the mainstays of ASD research in India. According to the authors of a case study Autism is not rare in India (33). Because of a severe lack of awareness and expertise regarding the illness among health professionals, it is commonly misdiagnosed (34, 35).

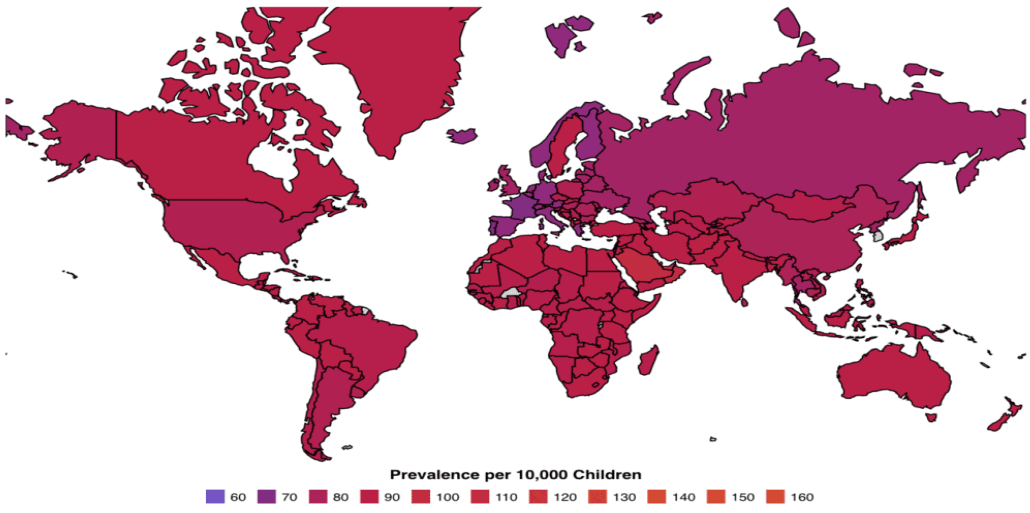


Fig.1.2 Global prevalence of autism

1.5 Aetiology of Autism

The aetiology of ASD is likely to be multifactorial, with both genetic and non-genetic factors (environmental factors) playing a role in the causation of autism. ASD can be syndromic or non-syndromic. Syndromic ASD is often associated with chromosomal abnormalities or monogenic alterations. ASD susceptibility is caused by a variety of genetic factors (polygenes), which when associated with an external trigger (environmental factor), would produce the behavioural framework associated with autism (Fig.3 &4) (36).

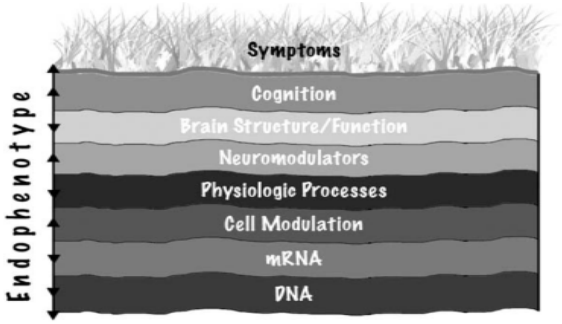


Fig.1.3 Representation of autism spectrum disorder endophenotype

1.5.1 Environment factors associated with ASD

Recent research suggested that, the environmental factors may account for up to 40–50% of the diversity in autism spectrum disorder risk. Environmental factors can influence individuals who are genetically susceptible to autism, which may explain why the prevalence of the condition is increasing so quickly (37). Environmental factors affect various stages of brain development, including the development and closure of the neural tube, cell migration and differentiation, the development of structures like cortical mini-columns, synaptogenesis, and myelination (38).

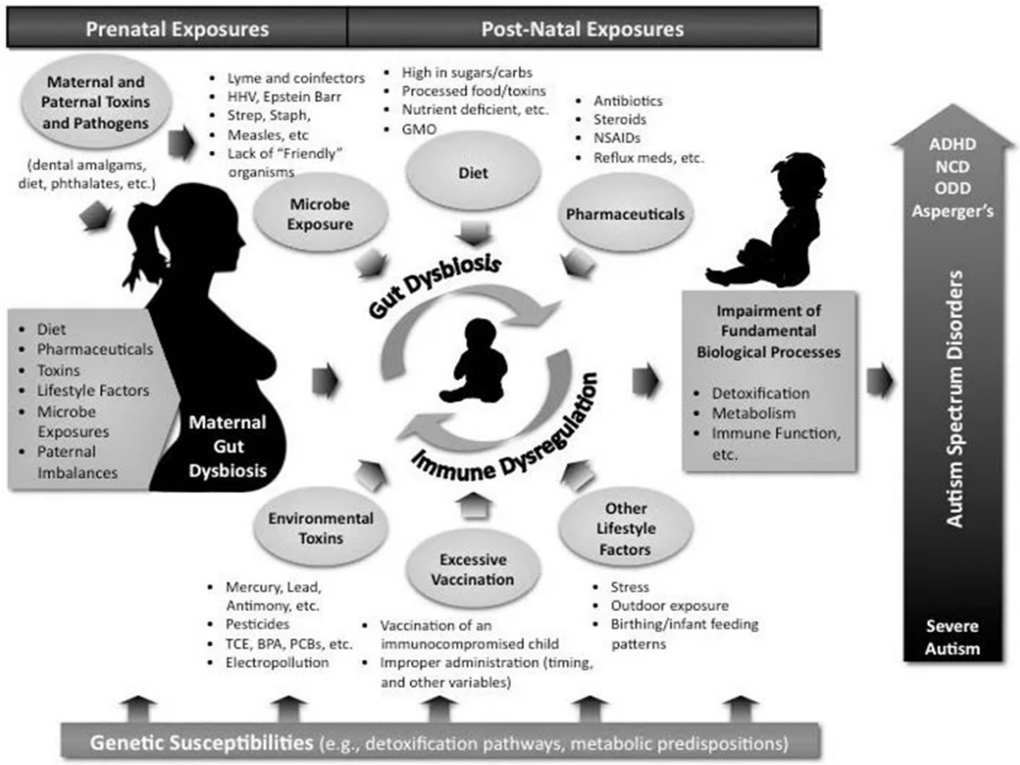


Fig.1.4 Aetiological factors associated with autism spectrum disorder

1.5.1.1 Prenatal environment

Several prenatal risk factors have been recorded for autism spectrum disorder. One of the main non genetic causes of autism has been identified as prenatal infection. Specifically, the flu, rubella, measles, herpes simplex virus, and bacterial infections may raise the likelihood of autism and other neurodevelopmental conditions in the offspring (39). Few animal studies have shown that, the mother's immune system activated by prenatal rubella or CMV exposure may significantly raise the risk for autism in mice (40). The most compelling environmental aetiology of autism is congenital rubella syndrome (41). Not only for autism but also for several psychiatric diseases with suspected neurodevelopmental roots, such as schizophrenia, infection-associated immunological responses in early pregnancy may have a greater impact on brain development than infections in late pregnancy (42). Teratogens are substances in the environment that cause birth defects. These teratogens include misoprostol, thalidomide, paracetamol, or valproic acid exposure to the embryo. Although there is only little scientific evidence to support such claims, some substances that are hypothesised to cause birth abnormalities have also been identified as potential risk factors for autism (43-45).Cognitive and developmental disabilities in humans have been strongly linked to several environmental agents such as Pesticides,

polybrominated diphenyl ethers (PBDEs), polychlorinated biphenyls (PCBs), polycyclic aromatic hydrocarbons (PAHs), manganese, mercury and lead have been linked to the development of autism (39). Elevated levels of inflammatory cytokines, abnormal maternal immune activation and autoimmune diseases can damage embryonic and fetal tissues, aggravating a genetic problem or damaging the nervous system that may lead to autism or any other neurodevelopmental disorders (46-47). Maternal obesity during pregnancy and prenatal maternal stress occurred when a pregnant woman is exposed to psychosocial or physical stress. This stress may be brought on by everyday occurrences or difficult environmental conditions that might also be the risk factor for the devolvement of autism (48). Among some of the mother's medical conditions that are linked to autism in children include metabolic syndrome, bleeding, gestational diabetes etc... Gestational diabetes is one of the major risk factors because, it affects fetal growth and it increases the chance of pregnancy complications (49-50). In addition, it affects motor development and leads to learning difficulties (51).

Maternal diet during gestation plays an important role in the development of the neural circuitry which controls behaviour, and determines the behavioural effects in the offspring (52). The incidence of ASD or autistic features in the offspring is influenced by various dietary factors consumed by the mother during pregnancy, such as FA, vitamin D, iron, and fatty acids (53). Recent studies have investigated the connection between prenatal substance abuse, such as chronic tobacco smoke, alcohol, or drug consumption, and ASD. High levels of alcohol intake during pregnancy have been linked to ASD in offspring, notably in those with fetal alcohol syndrome (54-55). The age of a parent at birth has also been linked to a child's likelihood of developing autism. Maternal and paternal ages greater than or equal to 34 have been linked in various studies to an elevated risk of autism in their offspring. Advanced maternal age may be linked with autism due to the increased risk of chromosomal abnormalities in ova or because of unstable trinucleotide repeats (56). Although it has been demonstrated that older mothers are more likely to experience obstetric difficulties, it is not known which of these complications may influence the risk of autism (57). The biological causes of this are unknown; potential explanations include an increased chance of chromosomal abnormalities, spontaneous mutations, and pregnancy problems.

1.5.1.2 Perinatal environment

Low birth weight, duration of the gestation period, and hypoxia during childbirth are among the perinatal and obstetric factors linked to autism (58). Autism spectrum disorder is more likely to develop in children who were exposed to complications before or during delivery, such as birth asphyxia and preeclampsia. A hypoxic-ischemic encephalopathy occurs when there is a lack of oxygen (hypoxia) or when there is a reduction in blood flow to the brain (ischemia) additionally, there is mounting evidence that air pollution exposure during pregnancy may increase the likelihood of developing autism (56). Other pregnancy related factors, including foetal distress, maternal hypertension, protracted labour, cord problems, low Apgar score, and caesarean delivery are thought to be related to hypoxia and have been linked to an increased risk of autism in some studies, but not all (58).

1.5.1.3 Postnatal environment

It has been suggested that a wide range of postnatal factors, such as gastrointestinal abnormalities, immune system abnormalities, allergies and exposure to medicines, infections, specific foods, or heavy metals can cause autism (59). It has been hypothesised that oxidative DNA damage and abnormalities of DNA repair are potential factors in the etiopathology of ASD (60). According to one hypothesis, toxins and oxidative stress may occasionally lead to autism. Evidence includes altered enzymes, increased oxidative stress indicators, and genetic influences on metabolic pathways (61). According to another hypothesis, stress causes damage to purkinje cells in the cerebellum after birth, and glutathione may be a key factor for this mechanism (62). Lower levels of total glutathione and higher amounts of oxidised glutathione are found in autistic children. These antioxidants might be helpful in the treatment of autism (63).

Mercury toxicity is also identified as one of the potential risk factors for autism. Mercury attaches to Cysteine Thiol which can harm brain cells. Mercury cannot bond to the Cysteine Thiol group due to glutathione. Children with autism have much lower amounts of glutathione than typically developing children, and they also have difficulty in excreting mercury (64). Children with autism may exhibit immunological deficiencies, sensory deficits, motor deficits, and behavioural abnormalities as a result of mercury exposure. Lead levels as less as 10μg/dl can cause aberrant learning and

defective neurobehaviors in autism. Lead exposure at high levels can result in long term brain damage, diminished cognition and learning, behavioural issues, attention deficit, decreased speech, and reduced social interactions (65). Aluminium is a neurotoxin and a potent immunological adjuvant and aluminium neurotoxicity may be explained by its role in causing oxidative stress and the release of DNase, a substantial DNA damage inducer (60). One of the most highly contested opinions about the causes of autism is the MMR vaccine theory. Concerns about the connection between vaccination and autism vary drastically (66). The Measles, Mumps, and Rubella (MMR) vaccine itself is the main cause of worry, followed by one of the preservatives used in several vaccines that cause autism and thirdly, receiving several vaccines at once can overwhelm the immune system and result in an immune compromise that causes autism (67). According to the Centre for Disease Control and Prevention, the Institute of Medicine of the National Academy of Sciences and the U.K. National Health Service there is no evidence of an association between the MMR vaccine and autism (68).

1.5.2 Genetic factors

Alterations in more than 1,000 genes have been linked to ASD, although many of these relationships have not been verified. Even though it is believed that the number of common gene changes influences the chance of having ASD, not everyone who carries one or more of these variations will be impacted. According to the estimation, genetic factors account for 40 to 80 per cent of the risk of causing ASD. Early twin studies estimated heritability to be around 90%, which means that more than 90% of a child's risk of developing autism is accounted for by genetics (69). As the heritability is estimated to be between 60 and 90% in later twin studies, this may be an overestimation. Evidence to date continues to point toward a significant genetic component and one of the recent studies estimated heritability at 83% (70). Many of the non autistic co-twins had social or learning challenges. Siblings who are adults have a 30% chance of sharing one or more traits from the more generalised autism phenotype (71).

Four distinct categories of candidate genes for ASD (table.1) are listed below;

1. Rare: genes linked to uncommon monogenic types of ASD. This class of allelic variations includes uncommon polymorphisms and single gene disruptions/mutations that are directly associated with ASD.

2. Syndromic: Genes linked to disorders in which a significant group exhibits symptoms of autism.

3. Association: genes with common mutations that confer a small risk for ASD and have been identified from genetic association studies of ASD derived from unknown cause (known as “idiopathic ASD”).

4. Functional: Genes that do not belong to any of the other genetic categories yet have biological roles important to ASDs.

Of these four gene categories, Rare and Syndromic genes contain the strongest evidence of links to aetiology of ASD. Association genes lack replication of their relationship to ASD, and Functional genes have no documented direct link to ASD

Table.1.1 Different categories of genes linked to autism spectrum disorder

Genetic Category	Number of Genes	Genes
Rare	81	*ANKRD11, A2BP1, APC, ASTN2, AUTS2, BZRAP1, C3orf58, CA6, CACNA1H, CADM1, CENTG2, CNTN4, CNTNAP2, CNTNAP5, CXCR3, DIAPH3, DLGAP2, DPP10, DPP6, DPYD, EIF4E, FABP5, FABP7, FBXO40, FHIT, FRMPD4, GALNT13, GLRA2, GRPR, HNRNPH2, IL1RAPL1, IMMP2L, JMJD1C, KCNMA1, KIAA1586, MBD1, MBD3, MBD4, MCPH1, MDGA2, MEF2C, NBEA, NLGN1, NLGN3, NLGN4X, NOS1AP, NRXN1, ODF3L2, OPHN1, OR1C1, PARK2, PCDH9, PCDH10, PCDH19, PDZD4, PLN, PPP1R3F, PSMD10, PTCHD1, RAB39B, RAPGEF4, RB1CC1, REEP3, RFWD2, RIMS3, RPL10, RPS6KA2, SCN1A, SCN2A, SEZ6L2, SH3KBP1, SHANK2, SHANK3, SLC4A10, SLC9A9, ST7, SUCLG2, TMEM195, TSPAN7, UBE3A, WNK3*
Syndromic	21	*ADSL, AGTR2, AHI1, ALDH5A1, ARX, CACNA1C, CACNA1F, CDKL5, DHCR7, DMD, DMPK, FMR1, MECP2, NF1, NTNG1, PTEN, SLC6A8, SLC9A6, TSC1, TSC2, XPC*
Association	84	*ABAT, ADA, ADORA2A, ADRB2, AR, ARNT2, ASMT, ATP10A, AVPR1A, C4B, CACNA1G, CCDC64, CDH10, CDH22, CDH9, CTNNA3, CYP11B1, DISC1, DLX1, DLX2, DRD3, EN2, ESR1, ESRRB, FBXO33, FEZF2, FOXP2, FRK, GABRA4, GABRB1, GABRB3, GLO1, GPX1, GRIK2, GRIN2A, GRM8, GSTM1,HLA-A, HLA-DRB1, HOXA1, HRAS, HS3ST5, HSD11B1, HTR1B, HTR3A, HTR3C, INPP1, ITGA4, ITGB3, LAMB1, LRFN5, LRRC1, LZTS2, MACROD2, MARK1, MET, MTF1, MYO16, NOS2A, NPAS2, NRCAM, NRP2, NTRK1, NTRK3, OXTR, PER1, PIK3CG, PITX1, PON1, PRKCB1, PTGS2, RELN, RHOXF1, SLC1A1, SLC25A12, SLC6A4, STK39, SYT17, TDO2, TPH2, UBE2H, VASH1, WNT2*

Functional	23	*ALOX5AP, ASS, CACNA1D, CADPS2, CBS, CD44, CNR1, DAB1, DAPK1, DCUN1D1, DDX11, EGR2, F13A1, FLT1, ITGB7, MAOA, MAP2, OPRM1, RAI1, ROBO1, SDC2, SEMA5A, TSN*

The genetic background of ASD is diverse in the mode of inheritance (inherited vs. de novo variation), frequency (common vs. rare) type of variation (single nucleotide, indel, or copy number variation (CNV) and mode of action (dominant, recessive, or additive) (72). Recorded genetic abnormalities of autism can be classified into the following categories; cytogenetically visible chromosomal abnormalities (5%), copy number variants (i.e., sub microscopic deletions and duplications) (10–20%), and known single-gene disorders (5%). Cytogenetic abnormalities have been recorded on almost every chromosome, although only a few occur with a high frequency suggesting the location of a specific autism gene (73). Duplications, deletions, translocations, and inversions are among the sub microscopic structural chromosomal abnormalities defined as copy number variations, which can occasionally extend over a few kilobases (74). CNV may be inherited or it can develop spontaneously (75). These abnormalities may have an impact on many genes, but not all of them are necessarily disease causing. Studies have revealed that autistic people have a higher burden of rare and genic CNVs, which link these variants to the pathogenesis of ASD (76). CNV is now recognised as a very significant risk factor for ASD susceptibility and current estimate suggest that these variations are directly responsible for 10% of ASD cases (table.2) (77).

Tuberous sclerosis and Fragile X syndrome are frequently associated with autism (78-79). Abnormal mRNA translation results in increased protein synthesis underlying both diseases' fundamental pathophysiological mechanisms and has been linked to autism (79-80). Indeed, autism is highly related to mutations in the genes encoding proteins involved in the cellular machinery regulating synaptic protein synthesis (*FMR1, TSC1/2, EIF4E*, and *PTEN*) (80). Based on autism diagnosis approximately 1–3% of children can have fragile X syndrome with the expansion of the CGG trinucleotide repeat in the *FMR1* gene. Additionally, it has been revealed that a significant number of children being investigated for autism have *FMR1* pre-mutations (55–200 CGG repeats) (81). According to molecular studies, RNA toxicity to the neurons and gene silence, this disrupts neural connection, with these two

potential mechanisms through which the *FMR1* gene may cause the autistic phenotype (81-82).

While 25–50% of intellectually disabled individuals with tuberous sclerosis complex (TSC), OMIM# 191100, meet the diagnostic criteria for autism, only 1.1–1.3% of individuals with an initial diagnosis of ASD have TSC (83). Early onset infantile spasms and temporal lobe tumours on magnetic resonance imaging examination increase the chance that children with TSC2 mutations will also develop autism. Recurrence chances may be much higher for families with children who have autism due to TSC than those with children who have autism for unknown reasons (84).

The most common chromosomal abnormalities in autism are maternally derived 15q duplications of the imprinted Prader Willi/Angelman syndrome which are found in 1-3% of cases (85). Cytogenetically apparent duplications occasionally result from the segregation of a maternal chromosomal translocation, but they most frequently take the form of a de novo isodicentric 15q chromosome. These duplications appear to be mediated by unequal homologous recombination involving low copy repeats (LCR) which are clustered in the region, similar to the deletions identified in the majority of cases of Angelman (OMIM# 105830) and Prader-Willi syndrome (OMIM# 176270) (86). Autism occurs more frequently than expected in children with Down syndrome (OMIM # 190685) and 45, X Turner syndrome (OMIM # 300082). In one Down syndrome study, 7% of individuals had autism (87). It is unclear why there is a connection between Down syndrome and autism. Moreover, there are more Turner syndrome individuals who meet the diagnostic criteria for autism (88). Although other sex chromosome abnormalities (47, XXX, 47, XXY, and 47, XYY) have also been linked to autism but they does not appear to be a strong correlation for autism (89).

The relationship between autism and the *PTEN (*phosphatase and tensin homolog) macrocephaly syndrome is especially remarkable. The PTEN gene was first identified as a tumour suppressor gene linked to the *PTEN* hamartoma tumour syndrome, a broad spectrum of diseases that includes the Cowden syndrome (OMIM# 158350), the Bannayan-Riley-Ruvlacaba syndrome (OMIM# 153480), the Proteus syndrome (OMIM# 176920), and the Lhermitte-Duclos disease (OMIM# 158350) (73). More

recently, a subset of people with autism and macrocephaly have been identified to have heterozygous *PTEN* gene mutations. PTEN haploin activity has been demonstrated to impact synaptic plasticity and neuronal survival during brain development. Although it is unknown how P*TEN* causes autism, studies suggested that this may be linked to the regulation of the phosphoinositide 3-kinase pathway (90-92).

A mutation in the *CACNA1C* gene at 12p13.3 develops Timothy syndrome, OMIM# 601005, an autosomal dominant calcium channel condition that is characterised by severe QT prolongation, syndactyly, cardiac abnormalities, dysmorphic features, developmental delays, and autistic symptoms (93). Timothy syndrome, autism and congenital sensory night blindness, and *CACNA1H* gene mutations all suggest that changes to ion channel function may contribute to autism (94). Joubert syndrome is an autosomal recessive disorder distinguished by cognitive impairment, behavioural issues, abnormal breathing, abnormal eye movement, and partial or total agenesis of the cerebellar vermis, which is seen as the "molar tooth sign" on MRI. The *AHI1* gene, which codes for the "jouberin" protein, appears to be connected to OMIM# 213300, a subtype of the Joubert syndrome (95). The monozygotic (MZ) twin with the more severe cerebellar impairment in a case of monozygotic (MZ) twins with Joubert syndrome had autism, indicating that some conditions may have the potential to result in the autism phenotype when other brain regions or circuits are damaged (96).

Genes with common mutations confer a small risk for ASD which is present in >1% of the total population. Common variants with small effects were suggested to act collectively in the development of autism with complex traits. The most recurrently recorded genes include the gamma aminobutyric acid A (*GABA*) receptor, beta 3 (*GABRB3*); oxytocin receptor (*OXTR*); reelin (*RELN*); serotonin transporter (SLC6A4); N-methyl-D-aspartate receptor (*NMDA; GRIN2B*); arginine vasopressin receptor 1A (*AVPR1A*); engrailed homeobox 2 (*EN2*); integrin, beta 3 (platelet glycoprotein IIIa, antigen CD61; *ITGB3*); met protooncogene (hepatocyte growth factor receptor; MET); and contacting-associated protein-like 2 (*CNTCAP2*) genes (72).

The homeobox gene Engrailed-2 (*EN-2*) plays a crucial role in the formation of the mid-brain and cerebellum. It is the human equivalent of the drosophila engrailed gene and is located on the long arm of chromosome 7 (7q36). Simultaneous with the development of cerebellar precursor cells is a temporal and geographical pattern of engrailed gene expression. As a result, it has been claimed that it is crucial to establish the proper cell number in the cerebellum (97). Genome wide linkage studies relate to the *MET* gene, which is found in the 7q31 candidate gene domain for autism. Because *MET* encodes a receptor tyrosine kinase important in neuronal growth and organisation, as well as immunological and gastrointestinal functioning to which autism has been associated with these abnormalities. *MET* is therefore a good functional candidate for autism. The *MET* promoter region variants exhibit a high association with autism (98).

In addition to clinically identifying known genetic disorders which may predispose to the development of autism, intense efforts have been directed to identifying genes that specifically cause or increase the risk of developing autism. The methods used include both large genome wide association studies and investigation of candidate genes. Many of these genes which are associated with ASD are present in circadian entrainment, which further indicates a heterogeneous complex genetic aetiology for autism. There have been various monogenic forms of autism identified and each one accounts for less than 1% of the total ASD population. It has been demonstrated that the frequency of *HOXA1* gene allelic variants was significantly higher in people with autism (7p15). This gene is only expressed during the third week after conception during the formation of neural tube. *HOXA1* and *HOXB1* play a crucial role in the development of the foetal caudal medullary structures. They also appear to be partially linked to the development of the superior olivary, facial, and abducens nuclei. According to some studies on the origin of autism, *HOXA1* may play a part in autism tendencies and is linked to the early stages of brain stem development. On the other hand, several studies have not found a strong connection between *HOXA1* gene variations and autism (72).

A crucial role in protein translation of mTOR is performed by the eukaryotic translation initiation factor 4E gene (EIF4E), which is found on chromosome 4q21–q25 (99). Interestingly, autistic-like behaviours, increased neuroligin translation, and higher neuronal excitation/suppression ratio was observed in eIF4E over-expressed

mice. However, these abnormalities recover with pharmacological inhibition of eIF4E activity or down-regulation of neuroligin 1 (100). The serotonin pathway was one of the early targets for candidate gene investigations of autism. It was implicated by pharmacological evidence and recurrent reports of higher levels of platelet serotonin (5HT) in around 25% to 30% of cases with autism (101). The majority of studies looking at the *SLC6A4* locus suggest its involvement in autism, although results have not focused on a particular allele or consistently observed a relationship with the same variant (102). A protein that is encoded by reelin (*RELN*) regulates intercellular connections involved in neuronal migration and placement throughout the development of the brain (103). *RELN* is present in the 7q22 chromosomal region, where multiple studies have identified a suggestive or substantial association with autism. Variations in *RELN* may increase the risk of autism, based on both population and family based association studies (104). The recent genetic data point to the possibility that abnormal Ca2+ homeostasis during neurodevelopment may be the cause of at least some ASD cases (105). Additionally, numerous genetic investigations have discovered genes linked to autism that encode proteins that are either directly or indirectly regulating intracellular Ca2+ levels or controlled by cytosolic Ca2+ transients. Ion channels, receptors, and Ca2+ regulated signalling proteins are some of these molecules, which are frequently important for CNS development (93). Gain-of-function mutations in Cav1.2 (*CACNA1C*), an L-type voltage-gated Ca2+ channel, cause Timothy syndrome, a multisystem illness that includes autism and mental retardation (85). The incomplete form of X-linked congenital stationary night blindness (*CSNB2*) is caused by mutations in the L-type voltage-gated Ca2+ channel Cav1.4 (*CACNA1F*), where gain of function mutations frequently result in *CSNB2* along with cognitive impairment and either autism or epilepsy, whereas loss of function mutations do not result in CSNB2 inactivation along with neurological symptoms (106-107). In a boy with autism, a balanced translocation disrupting one copy of the *KCNMA*1 gene has been discovered, leading to a more depolarized resting membrane potential and less effective modulation of neuronal excitability (108). One ASD patient had the R1902C mutation in the SCN2A gene, which lowers the gene's affinity for binding calmodulin and destabilises the inactivation gate, encouraging persistent channel activity during depolarization (109).

Synaptogenesis risk genes include those which encoding cell adhesion proteins viz, neuroligins, neurexins, and glutamate receptor signalling protein SH3 and multiple ankyrin repeat domains 3 (*SHANK3*). Several studies have discovered that neuroligins, *SHANK*, and neurexin genes, which encode proteins essential for synapse formation, maturation, and stabilisation, harbour the mutations that cause autism phenotype. The Neuroligin family contains 5 genes, among which *only NLGN3, NLGN4*, and *NLGN4Y* genes have been found to harbour mutations possibly responsible for causing autism. Autism related synaptic network gene is neurexin. The neurexin family contains three highly conserved *NRXN1, NRXN2* and *NRXN3*. Each gene has two separate promoters: alpha Neurexins are transcribed from a promoter region upstream of exon 1, while beta neurexin are transcribed from a downstream, intragenic promoter, resulting in a shorter form of neurexin (110). Neurexins encode a highly polymorphic family of neuronal proteins that interact with neuroligins to promote synaptic functioning (85). Evidence for neurexin involvement in autism comes from several recent investigations. Three members of the *SHANK* gene family include *SHANK1, SHANK2*, and *SHANK3* which encode scaffolding proteins necessary for the proper development and functioning of neural synapses. *SHANK3* (22q13.3) is expressed in the cerebral cortex and cerebellum (111). And it is present at excitatory synapses where it binds to postsynaptic neuroligins in synaptogenesis. Several studies recorded genomic deletions, CNVs and rare mutations encompassing the SHANK3 locus in 0.85% of all ASD individuals (85) Cadherins (CDHs) and protocadherins (PCDHs); A class of glycosylated transmembrane proteins which control neuronal movement, cell-cell adhesion, synaptic remodelling, synapse development, and spine morphology (112). Several GWA studies on different populations recorded common variants between CDH8 and CDH10 genes (5p14.1) and recent large genomic deletions in the CDH13 gene (5p14.1) (113). Few studies reported that ASDs have also been linked to homozygous deletions in PCDH10 and CNVs in PCDH9 (114). Synaptic vesicle cycling proteins synapsin-1(SYN1) and synapsin-2 (SYN2); synapsins are the families of presynaptic phosphoproteins which account for 9% of the total vesicle protein and they can regulate neurotransmitter release and neurite development (115). Synapsin 1, Synapsin 2, and Synapsin 3 are family members of the synapsis gene which are located on chromosomes Xp11.23, 3p25.2, and 22q12.3 respectively in mammals. Synapsin 1 mutation (Q555X, A51G, A550T, and T567A) are a large French Canadian family with epilepsy

and ASDs. Synapsis 2 also has been discovered as an autism risk factor gene (116). Contactins (CNTNs) are one of the members of the immunoglobulin superfamily. CNTNs are glycosylphosphatidylinositol-anchored neuronal membrane proteins and play crucial roles in synapse formation, plasticity, and axon growth and guidance (117). Studies suggested that deletions and duplications in *CNTN4* may be involved in ASDs and some familial studies showed mutations in *CNTN5* and *CNTN*6 were also been found in autism (118). Other major genes which are recorded to be associated with autism are ion transport proteins such as sodium voltage gated channel alpha subunit 2 (*SCN2*A), calcium voltage-gated channel subunit alpha1 E(*CACNA1E*), calcium voltage gated channel auxiliary subunit beta 2 (*CACNB*2), potassium voltage gated channel subfamily Q members 3 and 5 (KCNQ3 and *KCNQ5*), potassium voltage gated channel subfamily D member 2 (*KCND2*), synaptic Ras GTPase activating protein1 (*SYNGAP1*), and gamma aminobutyric acid type A receptor gamma3 subunit (*GABRG3*) (119, 111, 120-122).

1.5.3 Epigenetics of autism spectrum disorder

Epigenetics is the study of heritable variations in gene activity which are not caused by an alteration in the DNA sequences. According to growing evidence from recent studies, Epigenetic factors contribute significantly more to the pathogenesis of ASD than was previously believed. Several epigenetic mechanisms for the onset and development of ASD have been proposed. These mechanisms include miRNA, histone modifications, and DNA methylation (Fig.5) (123-124).

1.5.3.1 DNA methylation

DNA methylation is one of the important epigenetic mechanisms for controlling gene expression in response to environmental stimuli without alterations in DNA sequence (125). Methyl groups are produced by the methylation pathway for a variety of purposes, including the methylation of genes, which can lead to epigenetic modifications that switch genes on and off. When S-adenosylmethionine (SAM) donates a methyl group (SAH). SAH can be transferred to homocysteine, which can be further converted to glutathione through the sulfuration pathway or re-methylation to methionine. Reduced SAM/SAH ratio in patients with ASD is a sign of methylation dysregulation. Additionally, the methylation pathway requires folate and vitamin B12. The methylation pathway is believed to be severely affected by the SNP in the

MTHFR gene (123). There are 15 epigenetically altered genes which are identified to associated with ASD viz. *EN2, UBE3A, NLGN3, OXTR, MECP2, AFF2, BCL2, GABA, SLC6A4, RORA, NRXN1, AUTS2, SOX7, RELN,* and *SHANK3*) have been recorded (126).

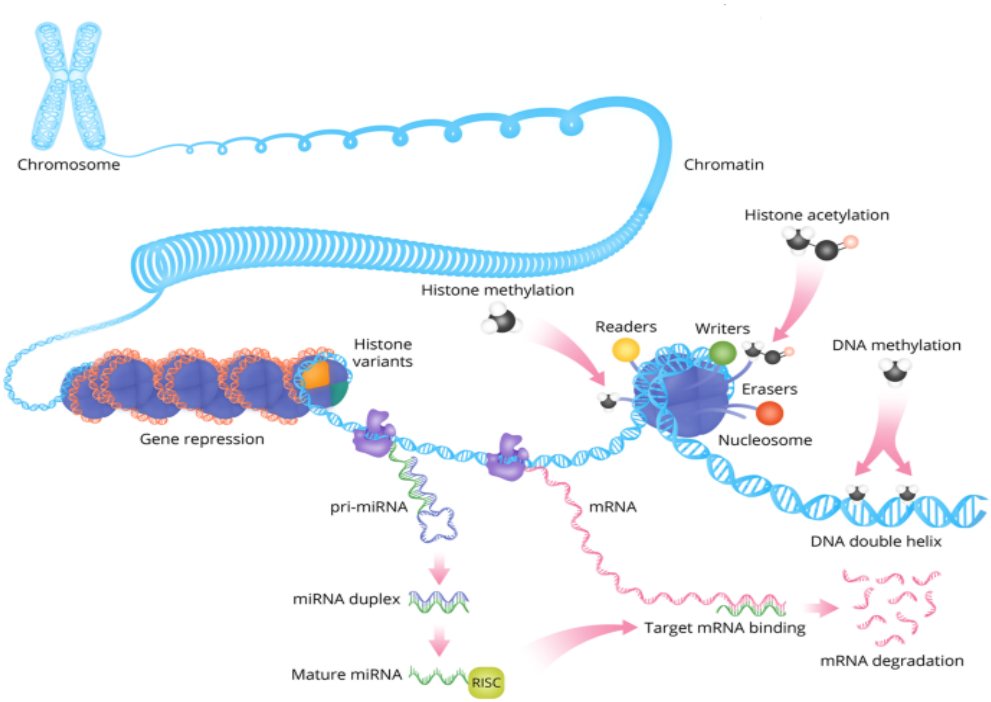


Fig.1.5 Mechanism of epigenetic regulation

1.5.3.2 Histone modifications

The association between ASD and histone modification and chromatin modification has not been extensively studied. Histone modification plays a role in the development of ASD (127). A study by Kim *et al.,* established that denovo mutations in the non-coding regions affect the chromatin interactions and are involved in the aetiology of Autism Spectrum Disorder (128).

1.5.3.2 miRNA

Small non-coding RNA molecules (18–24 nucleotide) known as miRNAs are transcribed in a tissue specific manner to control the expression of their target genes. It is essential for several biological processes, such as metastasis, carcinogenesis, differentiation, ageing, and cell survival (129). Genome desert regions contain miRNAs that are transcribed from multiple locations throughout the genome, either intragenic or intergenic locus. The primary purpose of miRNAs is to post

transcriptionally control the expression of genes (130). The function of miRNA in the aetiology of ASD has been determined by several of studies over the past ten years, and some of these studies found altered expression of several miRNAs in autistic people. Some of the miRNAs associated with ASD are retrieved from mirNet database viz, Has-miR-432, Has-miR-181d, Has-miR-128-3b, Has-miR-106a, Has-miR-155, Has-miR-381, Has-miR-598, Has-miR-431, Has-miR-146a and Has-miR-106-b.

1.6 Metabolic conditions

Metabolic disorders are caused by aberrant chemical processes (amino acid, carbohydrate, or lipid breakdown) that disrupt the body's metabolism. Because most metabolic disorders are autosomal recessive, they are more frequent in nations with high rates of consanguinity, where they cause major morbidity and mortality (131). The reported prevalence of inherited metabolic diseases among ASD patients is 2–5% (132). Several metabolic abnormalities have been linked to autism at a greater incidence than in the general population.

1.6.1 Mitochondrial dysfunction

Mitochondrial dysfunction is one of the most frequent metabolic disorders in autistic persons. Recent research has linked mitochondrial dysfunction to autism, which has a prevalence rate of 5% in people with autism. Because the mitochondria are the powerhouse of the cell and provide the majority of cellular energy, they play an important role in many cellular activities, particularly those requiring a lot of energy, such as the brain. As a result, mitochondria are vulnerable to a wide range of stressors, which explains how several variables may contribute to a consistent behavioural pattern in autism (133). Biochemical factors related to mitochondrial function are commonly abnormal in autism (133-134). Only a small percentage (1%) of autistic children associated with mitochondrial illness. Atypical clinical characteristics such as oculomotor abnormalities, dysarthria, ptosis, hearing impairments, hypertonia, and movement difficulties are present in these children (133, 135).

About 20% of autistic children with biochemical and clinical symptoms of the mitochondrial disease have genetic and genomic abnormalities affecting their mtDNA

or nuclear DNA (nDNA) (85); each mtDNA mutation or chromosomal rearrangement is recorded in 0.1% of all cases. nDNA chromosomal rearrangements may have an impact on mitochondrial function, including deletions in 15q11–q13 (cytochrome C oxidase subunit 5A, COX5A), 13q13–q14.1 (mitochondrial ribosomal protein 31, MRPS31), 4q32–q34.68 (electron-transferring-flavoprotein dehydrogenase, ETFDH), 2q37.3 (NADH dehydrogenase ubiquinone 1 alpha subcomplex 10, NDUFA10) (136). Mitochondrial dysfunction can occur as a consequence of a wide range of factors, such as dysreactive immunity and altered calcium (Ca2C) signalling (135), increased nitric oxide and peroxynitrite (137), propionyl CoA , malnutrition, vitamin B6 or iron deficiency, toxic metals (138), elevated nitric acid (139), oxidative stress , and exposure to environmental toxins, such (140). Medications like valproic acid (VPA), which impairs oxidative phosphorylation (141), and neuroleptics are additional causes of mitochondrial distress. Lactate, pyruvate, lactate-to-pyruvate ratio, carnitine (free and total), quantitative plasma amino acids, ubiquinone, ammonia, CD, AST, ALT, CO2 glucose, and creatine kinase (CK) are markers of mitochondrial dysfunction in children with ASD (Fig.6) (133).

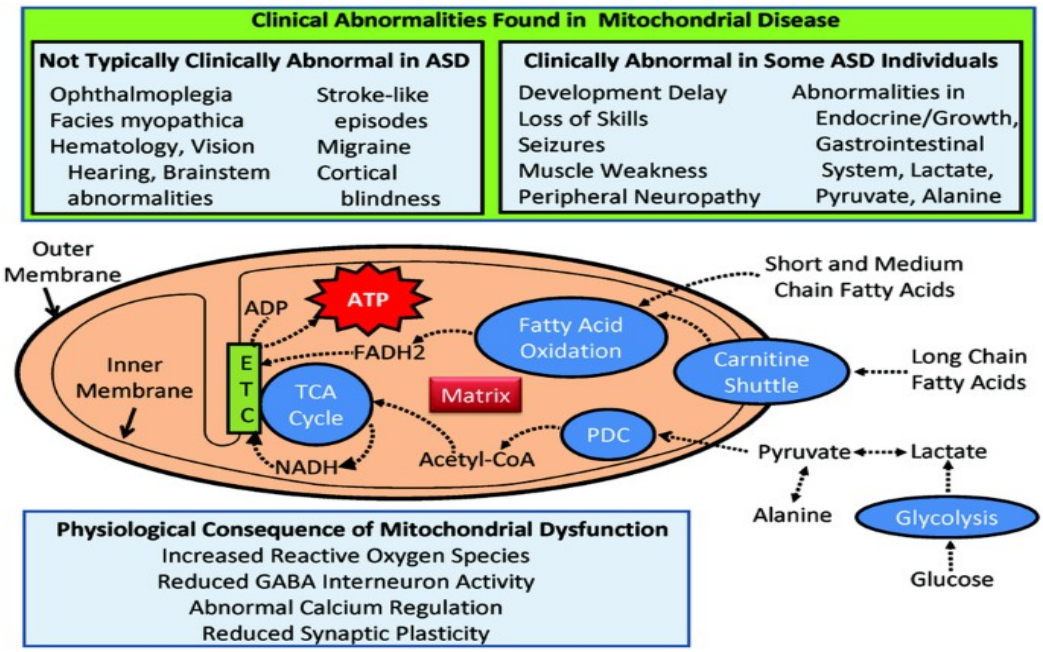


Fig.1.6 Mitochondrial dysfunction in autism spectrum disorder

Some other metabolic disorders which may be rarely associated with autism spectrum disorder are listed below;

Disorders of amino acid metabolism: Phenylketonuria (untreated), Homocystinuria and Branched-chain ketoacid dehydrogenase kinase deficiency.

Disorders of g-aminobutyric acid metabolism: Succinic semi-aldehyde dehydrogenase deficiency.

Disorders of cholesterol metabolism: Smith-Lemli-Opitz syndrome

Disorders associated with cerebral folate deficiency: Folate receptor 1 gene mutations and Dihydro-folate reductase deficiency

Disorders of creatine transport or metabolism: Arginine glycine amidino-transferase deficiency, Guanidino acetate methyl transferase deficiency and X-linked creatine transporter defect.

Disorders of carnitine biosynthesis: 6-N-trimethyl lysine dioxygenase deficiency

Disorders of purine and pyrimidine metabolism: Adenylosuccinate lyase deficiency, Adenosine deaminase deficiency, Cytosolic 5'-nucleotidase superactivity, Dihydropyrimidine dehydrogenase deficiency and Phosphoribosyl pyrophosphate synthetase superactivity

Lysosomal storage disorders: Sanfilippo syndrome (mucopolysaccharidosis type III) **Biotinidase deficiency** and **Urea cycle defects**

1.7 Gastrointestinal disorders

Gastrointestinal disorders (GI) are the one which affect the gastrointestinal tract. There are 2 types of GI disorders viz., Functional GI and Structural GI (142). Recent studies showed a potential association between autism and gastrointestinal disorders. According to case studies from paediatric gastroenterology clinics, children with autism may have an increased prevalence of gastrointestinal symptoms such as constipation, persistent loose stools, abdominal pain, and gaseousness/bloating (143). According to recent research on the gut brain axis, the gut contains millions of nerve cells that eventually connect to form the enteric nervous system. The gut brain axis is made up of the enteric nervous system (ENS) and the central nervous system (CNS), which are primarily connected by the vagus nerve (144). The autonomic nervous

system, enteric nervous system, neurotransmitters, hormones, and immunological responses all play a role in the communication between the gut and the brain (145). The gut microbiota of Healthy individuals shows a significant amount of microbial diversity. Some bacteria from the *Firmicutes* and *Bacteroidetes* phyla, predominate in the total microbial community (146). In autistic patients, Strati *et al*. reported an increase in the *Firmicutes/Bacteroidetes* ratio, which correlated to a decrease in the relative abundance of *Bacteroidetes*. Furthermore, they observed an increase in *Collinsella, Corynebacterium, Dorea,* and *Lactobacillus* and a decrease in the abundance of *Alistipes, Bilophila, Dialister, Parabacteroides,* and *Veillonella*. Due to the close connection between gut microbiota and the brain, it's crucial to understand the biochemical functions of GMB in the growth and function of the brain (147, 148).

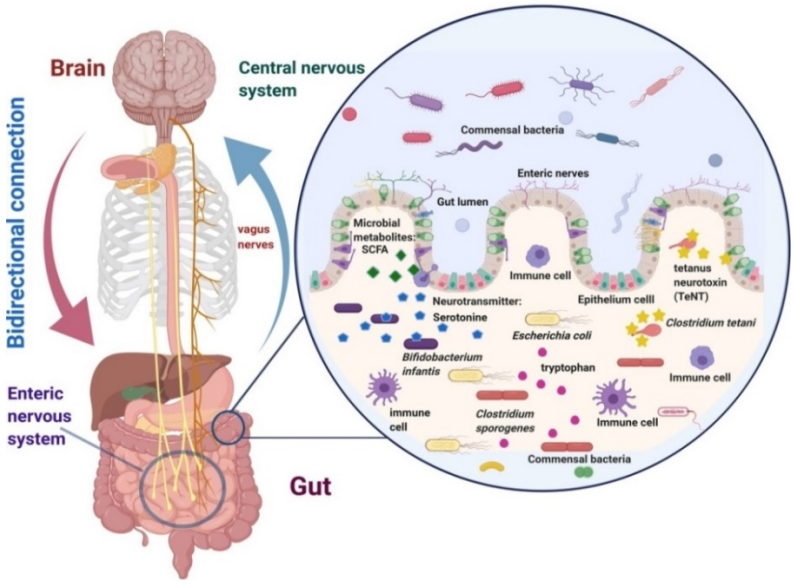


Fig.1.7 Schematic diagram of microbiota of gut brain axis

Megamonas, Megasphaera, and *Barnesiella* were the three most prevalent bacteria recorded in ASD. Lower levels of *Bacteroides vulgatus* and greater levels of *Eggerthella lenta* and *Clostridium botulinum* were associated with the disruption of glutamate metabolism. Several studies suggest that ASD with GI problems exhibit behavioural symptoms such as anxiety, self-injury, and violence (126). The typical symbiotic interaction between the gastrointestinal tract and gut bacteria has a significant impact on gut homeostasis (126). According to Xu *et al*. children with ASD had a high percentage of *Faecali bacterium* and a low percentage *of Bifido bacterium, Bacteroides, Enterococcus*, and *Escherichia coli* (149). Short-chain fatty acids (SCFAs), have gained more attention in recent years as researchers investigate

the role of gut microbiota metabolites in the pathophysiology of ASD. Propionic acid, butyrate, acetic acid, and valeric acid constitute the majority of SCFAs (126). PPA is one of the main SCFAs produced by *Clostridia, Bacteroides,* and *Desulfovibrio* in autistic children. Getachew *et al.,* reported that PPA disrupts GI function in a way that causes anomalies to manifest in individuals with ASD. PPA can lead to reversible behavioural, neuroinflammatory, metabolic, and epigenetic changes that resemble those shown in an animal model of autism spectrum disorder (150). One of the most significant SCFAs is butyrate (BT), which has been suggested as a neuroprotectant and positively regulates mitochondrial activity. Histone deacetylases (HDAC), blood-brain barrier (BBB) control, and inhibition of intestinal pro inflammatory macrophage function are the three main functions of BT. BT producing bacterial taxa are subsequently very less in autistic people (Fig.7) (151).

1.8 Immune dysregulation

Studying the complex pattern of interactions between various cell types from the innate and adaptive branches of the immune system is necessary to understand how immunological dysfunction in ASD may result in behavioural abnormalities. Effects of CNS function are mediated by a variety of immunological factors. Subgroups of T-cells and NK-cells may display altered activity and a reduced response to stimulation (152). Some cytokines can suppress neurogenesis and stimulate neuron death. Others can promote the development and multiplication of neurons and oligodendrocytes. Synaptic trimming and scaling can be facilitated by complement proteins and microglia, whereas brain reactive autoantibodies can change the development and functions of neurons. When numerous immune system components are dysregulated, these networks can result in modifications to neurodevelopment and behaviour (152). Reactive oxygen species, a defective blood brain barrier and traumatic brain injury are factors that increase the likelihood of activating brain microglia. In the majority of cases, ASD is associated with abnormal cytokine profiles, and increases in plasma cytokines are thought to be associated with the severity and regressive development of autistic and behavioural symptoms. Altered TGF-beta, CCL2, CCL5, IgM and IgG classes of immunoglobulin circulating levels are associated with a reduction of behavioural scores. Additionally, a Th1/Th2 imbalance has been identified, which may also contribute to the pathophysiology of autism (Fig.8) (152).

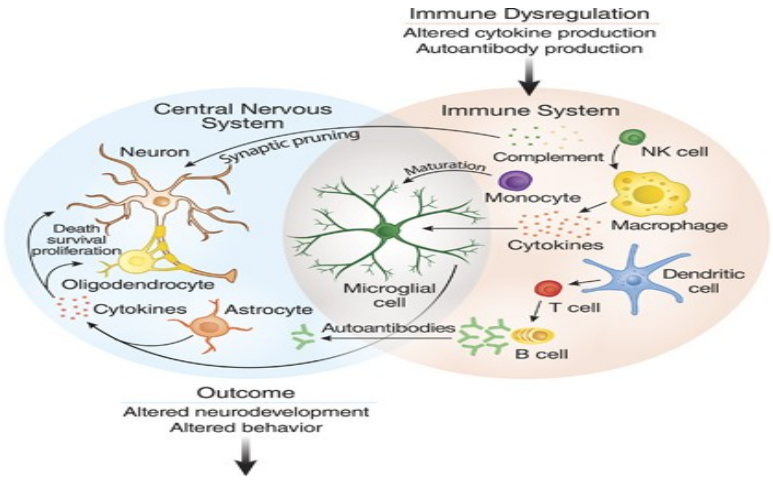


Fig.1.8 immune system deregulations related to autism

1.9 Synaptogenesis

Synapses are the asymmetric cell to cell connection that allow for the controlled passage of an electric or chemical signal from a presynaptic neuronal cell to a postsynaptic target cell (eg; a neuron or muscle) (153). Synapses are distinct subcellular junctional structures and typically consist of a presynaptic terminal, a postsynaptic target, and the synaptic cleft aligning pre and post synaptic specialisations (Jin Y *et al.,* 2005). The presynaptic terminal is characterized by a cluster of synaptic vesicles surrounding the electron dense membrane specializations. The postsynaptic site contains densely compacted ion channels and signals transduction molecules. Depending on the species and neuronal types, both the pre and post synaptic specialisations exhibit varying characteristics (154). En passant, where synaptic boutons form along the axon shaft, and terminaux**,** where synaptic boutons form at the ends of axon branches, are the two main ways that synapse formation occurs (Fig.9) (154).

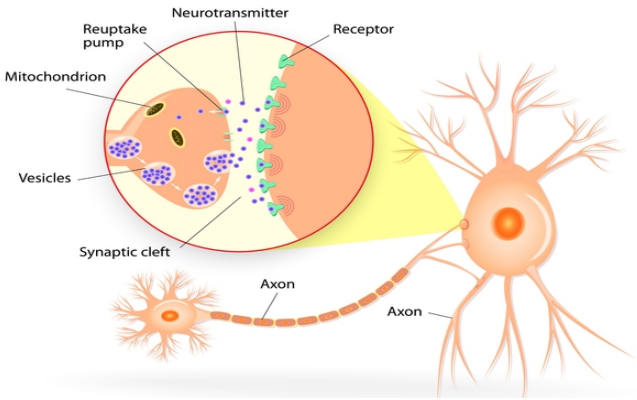


Fig.1.9 Structure of a typical chemical synapses

All neural processing including higher functions like learning and memory are dependent on adequate synapse function. In response to action potentials, synaptic vesicles release neurotransmitters (such as amino acids, amines, peptides, and acetylcholine) into the synaptic cleft (155). The majority of the genes associated with ASD are involved in the development and functions of the brain. These genes involved in a wide variety of fundamental cell functions, including chromatin remodelling, metabolism, and mRNA translation, may have an effect on neuronal processes, including neurogenesis, neuron migration, axon guidance, dendrite outgrowth, and synaptic formation and function. (156). Multiple studies have revealed that alterations in genes encoding cell adhesion molecules, scaffolding proteins, and proteins involved in synaptic transcription, protein synthesis, and degradation may be attributed to synaptic dysfunction, including synapse formation and elimination, synaptic transmission, and synaptic plasticity which further leads to functional and cognitive impairments such as autism spectrum disorder (Table.2) (157).

Table.1.2 Critical ASD candidate genes and major molecular functions in the synapse

	Gene s	**Location**	**Major molecular functions in synapses**
Cell adhesion molecules	*NRXN1*	2p16.3	Synaptogenesis, synaptic transmission, synaptic plasticity
	NRXN2	11q13.1	=//=
	NRXN3	14q24.3-q31.1	=//=
	NLGN1	3q26.31	Synaptogenesis, synaptic transmission, synaptic plasticity
	NLGN2	17p13.1	=//=
	NLGN3	Xq13.1	=//=
	NLGN4	Xp22.32-p22.31	=//=
	NLGN4Y	Yq11.221	=//=
	CNTNAP2	7q35-q36.1	Synaptogenesis, synaptic transmission
	CDH8	16q21	Synaptogenesis, synaptic transmission
	CDH11	16q21	=//=
	CNTN4	3p26.3-p26.2	Synaptogenesis
	CNTN6	3p26.3	=//=
	PCDH10	4q28.3	Synaptic elimination

Scaffolding proteins	*DLG4*	17p13.1	Synaptogenesis, synaptic elimination, synaptic plasticity
	GPHN	14q23.3-24.1	Synaptogenesis
	SHANK1	19q13.33	Synaptogenesis, synaptic transmission, synaptic plasticity
	SHANK2	11q13.3-q13.4	=//=
	SHANK3	22q13.33	=//=
	HOMER1	5q14.1	Synaptogenesis, synaptic plasticity
Regulators of protein synthesis	*FMR1*	Xq27.3	Synaptogenesis, synaptic transmission, synaptic elimination, synaptic plasticity
Regulators of protein degradation	*CYFIP1*	15q11.2	Synaptogenesis, synaptic transmission, synaptic plasticity
	TSC1	9q34.13	Synaptogenesis, synaptic plasticity
	TSC2	16p13.3	Synaptogenesis, synaptic plasticity
	EIF4E	4q23	Synaptogenesis, synaptic transmission, synaptic plasticity
	EIF4EBP2	10q22.1	Synaptogenesis, synaptic transmission, synaptic plasticity
	PTEN	10q23.32	Synaptogenesis, synaptic plasticity
	SYNGAP1	6p21.32	Synaptogenesis, synaptic plasticity
	UBE3A	15q11.2	Synaptogenesis, synaptic transmission, synaptic elimination
Chromatin remodelling	*MECP2*	Xq28	Synaptogenesis, synaptic plasticity
chromatin transcription	*MEF2C*	5q14.3	Synaptic elimination

1.9.1 Neurexin/Neuroligin complex and Autism

Neurexins are a class of presynaptic transmembrane proteins encoded by *NRXN1, NRXN2*, and *NRXN3*. Each of the three genes produces a long -Nrxn and a short Nrxn primary protein isoform using two separate promoters. (157.) Neurexin contains a single transmembrane domain and is usually located on the presynaptic membrane. The intracellular cytoplasmic portion interacts with exocytosis related

proteins, whereas the extracellular portion interacts with proteins in the synaptic cleft, most notably neuroligin (158). The α-Neurexins and β-neurexins have similar intracellular domains while their extracellular domains are different. In terms of structure, α-Nrxs made of six laminin/neurexin/sex-hormone (LNS) globular domains and three interspersed extracellular epidermal growth factor (EGF) like repeats, with these alternating structures serving to bind the proteins to the cell surface via a rigid and highly O-linked glycosylated stalk and a transmembrane domain. α-Nrxs also contains a short cytoplasmic tail domain made of cytoskeletal adapter protein interaction sites and a C-terminal PSD-95, DLG1, ZO-1 (PDZ) binding motif (159). The shorter β-Nrxs are missing any EGF-like domain and only contain the sixth extracellular LNS domain, which is followed by splicing in N-terminal α-Nrx sequences and the LNS6 domain. The transmembrane and intracellular tail domains are still present in the truncated proteins generated by γ-Nrx transcripts but lack the extracellular LNS or EGF-like domains (159). The PDZ (postsynaptic density (PSD)-95/discs large/zona-occludens-1) domains of CASK and Mint bind to synaptotagmin and the C terminus of the short intracellular region of both types of neurexins. These interactions result in the formation of connections between fusion proteins and intracellular synaptic vesicles. (160). Neurexins are therefore essential in the construction of the pre and postsynaptic machinery. The alternative splicing of six sites (SS1–SS6) can increase the structural and functional heterogeneity of Nrx, with SS1, SS2, SS3, and SS6 being present only within α-Nrxs, while SS4 and SS5 are found in both α- and β-Nrxs (161).

Neuroligins are a family of cell adhesion molecules on the postsynaptic membranes that bind to the laminin/neurexin/sex hormone (LNS) binding domain of Nrxn through the extracellular cholinesterase-like domain. They are encoded by five genes in the human genome: *NLGN1, NLGN2, NLGN3, NLGN4*, and *NLGN4Y*. Locations of NLGNs gene in the human genome are as follows: 3q26 (*NLGN1*), 17p13 (*NLGN2*), Xq13 (*NLGN3*), Xp22.3 (*NLGN4* or *NLGN4X*), and Yq11.2 (*NLGN4Y*). The molecular weights of NLGN1 is 93.835 kDa, NLGN2 is 90.820 kDa, NLGN3 is 93.895 kDa, NLGN4X is 91.915 kDa, and NLGN4Y is 92.021 kDa.(162) *NLGN1* and *NLGN4X* are expressed on excitatory synapses and where it binds to PSD-95 (158). While *NLGN2* is expressed in inhibitory synapses and binds to gephyrin and *NLGN3* is expressed on both excitatory and inhibitory synapses (158).

Complete NLGNs are made up of an N-terminal domain, an extracellular globular cholinesterase-like domain, a highly O-glycosylated stalk domain, a single pass transmembrane helical domain, and a short cytoplasmic tail (163). The C-terminal domain of NLGN can bind to the PDZ domains of postsynaptic scaffold proteins, including PSD-95 and gephyrin, which are engaged in the attachment of functional surface receptors and signalling proteins, and the extracellular acetyl cholinesterase like domain of *NLGN* allows them to interact with presynaptic NRXN in an activity dependent manner (161). Two distinct splice sites (splice site A and splice site B) also contribute to the formation of different *NLGN* transcript isoforms. Since these splice sites are situated in the cholinesterase-homologous domain which is responsible for interaction with *NRXN*s, which further suggests that the splicing of *NLGN*s has a dynamic effect on interaction with *NRXN*s (Fig.11) (163).

Neuroligins are extremely dynamic and are controlled by protein-protein interactions and post translational changes. Protein kinase A (PKA), tyrosine kinases, and calcium/calmodulin dependent protein kinase 2 (*CaMKII*) phosphorylate *NLGN1* to control its activity at excitatory synapses (164,165). Additionally, a recent study demonstrated that the interactions between *NLGN1* and Kalirin7, a Rho GEF, mediate the synaptogenic characteristics of *NLGN1* (166). The activity of *NLGN2* in inhibitory synapses is regulated by phosphorylation, which alters how it binds to inhibitory scaffolding proteins (167,168). Proteases can cleave NLGN3 to decrease its function at synapses (169). *NLGN*3's extracellular cleaved fragment has been discovered to be a powerful mitogen in brain tumours, which is an important discovery (170). Finally, protein kinase C (PKC) can phosphorylate NLGN4X to improve excitatory synapses (169). NLGNs make up a significant class of dynamic proteins with numerous functions. Several Deletions or point mutations in the *NLGN* genes have been associated with ASD, the majority of which map to the extracellular protein domain and just a small number to the stalk and intracellular domains. Three out of the five neuroligin genes (*NLGN3, 4X&4Y*) are found on the sex chromosomes may provide a rationale for the prevalence of ASD. Multiple investigations have connected mutations in the genes encoding neuroligin3 and 4 on the X chromosome and the gene encoding neuroligin 4Y on the Y chromosome to autism spectrum disorder (171).

Neurexin binds to Neuroligin which is its postsynaptic partner to form a Ca2+ dependent NRXN/NLGN complex, involved in synaptogenesis and necessary for

efficient neurotransmission. After the initial encounter of an axon with a target cell, Neurexin and Neuroligin jointly recruit essential synaptic components such as neurotransmitter receptors and scaffolding proteins to promote synaptic assembly, maturation and differentiation (172).

As synapses are formed during synaptogenesis, they differentiate into one of two categories: excitatory or inhibitory. Excitatory synapses increase the likelihood of triggering an action potential in the postsynaptic neuron and are often glutamatergic, or in which the neurotransmitter glutamate is released. Inhibitory synapses reduce the likelihood of triggering an action potential in the postsynaptic neuron and are often GABAergic or in which the neurotransmitter GABA is released (173). The E/I ratio (excitatory/inhibitory) which measures the ratio of excitatory to inhibitory synaptic input. which is crucial for neurons, especially during early development. It is believed that autistic spectrum disorders may be influenced by an imbalance in the E/I ratio (173). For proper circuit development and synaptic plasticity, E/I balance is very crucial and its disruption impairs information processing and social function, leading to the pathophysiology of ASD and many other neurodevelopmental illnesses (173,174).

For the first time in Indian autistic population, present study was undertaken to evaluate the role of neuroligin3, neuroligin4X and neuroligin 4Y genes in autistic population among North Karnataka region of India.

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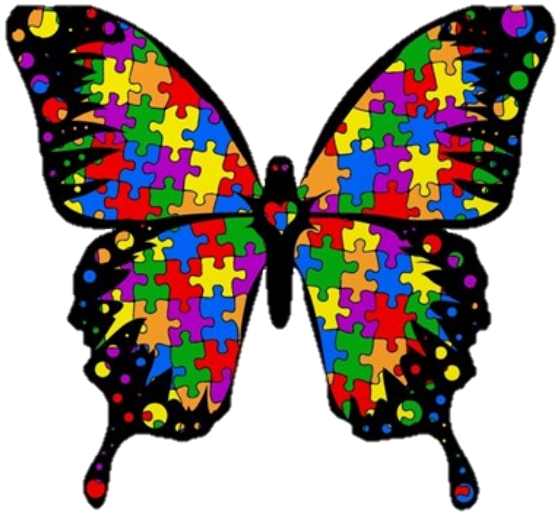
Chapter 2

AIMS AND OBJECTIVES



2.1 AIM: Screening of Genetic markers for Autism Spectrum Disorder.

2.2 OBJECTIVES

1. Identification of frequency of the Autism Spectrum Disorder in North Karnataka Population.
2. Identification of the role of Neuroligin family genes in the Autism Spectrum Disorder.
3. Identification of mutations in major genes involved in Autism Spectrum Disorder.
4. Screening for Biomarker for Autism Spectrum Disorder, in terms of Novel mutations/SNP's/ Insertions or Deletions.

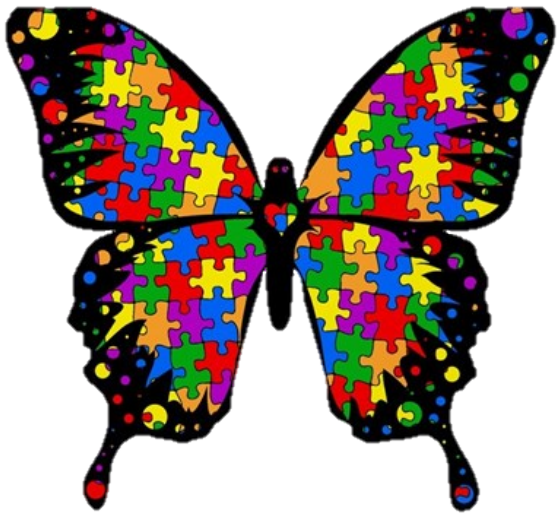
2.3 RESEARCH HYPOTHESIS

Autism is a heterogenous complex neurodevelopmental disorder and thought to arise from functional changes in neural circuitry and to be associated with an imbalance between excitatory and inhibitory synaptic transmission. NRXN and NLGN act as trans-synaptic cell adhesion molecules that mediate essential signalling between pre and postsynaptic specializations, signalling that performs a central role in the brain's ability to process information and that is a key target in the pathogenesis of cognitive diseases.

NLGNs are of particular interest due to their potential role in synapse function and neuron to neuron recognition. Since 2005, several attempts were made in different populations around the globe after initial finding in 2003 to establish the potential role of Neuroligin genes in autism spectrum disorder but the majority of the outcomes are inconclusive or lack of substantial support. Moreover, the potential involvement of NLGN genes in Indian autistic population is still unavailable. Hence, it is hypothesised that *NLGN3*, *NLGN4X* and *NLGN4Y* play a major role in the aetiology of Autism Spectrum Disorder.

Chapter 3

REVIEW OF LITERATURE



3.1 International

Bailey A. et al., has studied the clinical, genetic, neuropsychological and neurobiological perspectives of Autism and stated that Autism includes three main types of behavioural problems i.e. social abnormality, language abnormalities and stereotypes repetitive patterns of behaviour. The modal age to concern about autism is at age 2 at which there is a failure in language development. Numerous studies have shown that mental retardation is present in three-quarters of autistic patients. Several systematic studies have shown that epilepsy develops in about a fifth to a third of autistic individuals. Quite a wide range of pharmaceutical drugs have been tried on autism but there is no response is observed. Numerous studies have shown that autism is much more common in males and male to female ratio is 4:1. Autism is also associated with other genetic disorders like fragile x syndrome and tuberose sclerosis. Some autistic cases have been found in association with PKU (1).

The term "autism spectrum disorder," or "ASD," refers to a phenotype that includes the less severe conditions Asperger syndrome and pervasive developmental disorder, not otherwise defined (PDD-NOS), Rett syndrome, childhood disintegrative disorder and Kanner's syndrome.

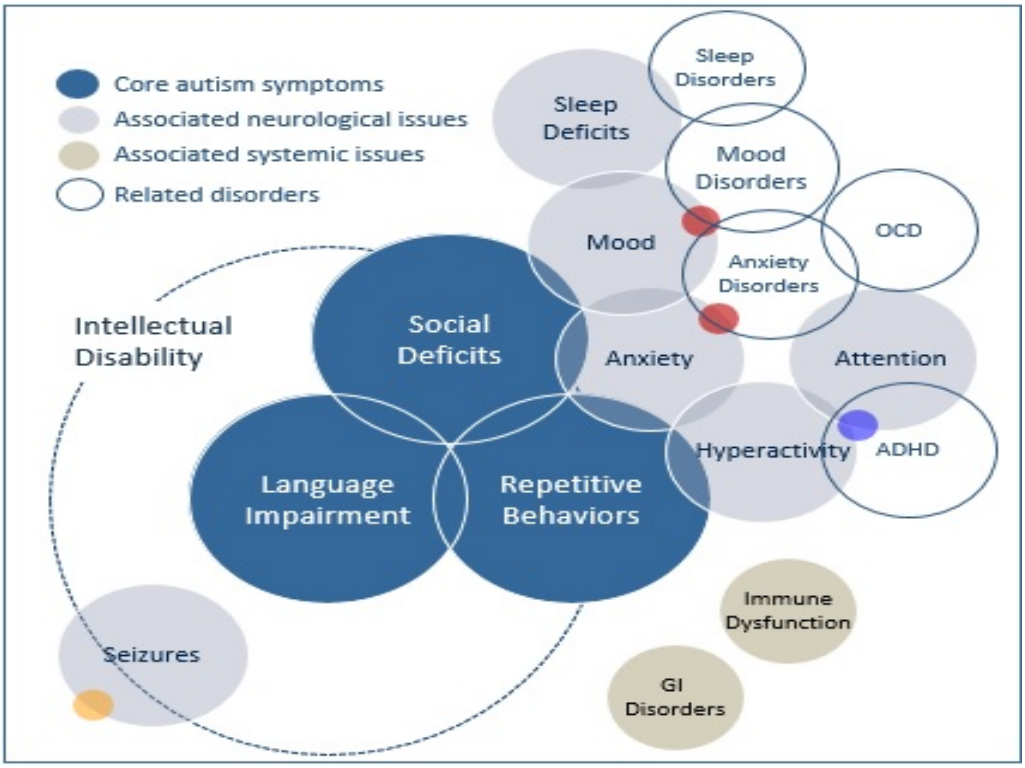


Fig.3.1 Clinical features of autism spectrum disorder

The Diagnostic and Statistical Manual of Mental Disorders, (DSM-V) (American Psychiatric Association, 1994) contains various diagnostic criteria for autism. Patients with autism, on the whole, have a qualitative impairment in social interaction, as evidenced by difficulties with nonverbal behaviours such as eye-to-eye gaze, facial expression, body postures, and gestures, failure to form appropriate peer relationships, and a lack of social sharing or reciprocity. Patients experience communication problems, such as a delay in the development of spoken language or a complete lack of it. Even in individuals who develop competent speech, there is still a significant handicap in initiating or maintaining a conversation, as well as stereotyped or idiosyncratic language use. Patients' behaviour, interests, and activities are also confined, repetitive, and stereotyped, with obsessive fixation with specific hobbies and rigid adherence to routines.

Kanner (1943) coined the phrase "an inherent incapacity to make the normal, naturally given affective contact with individuals" to describe infantile autism. Kanner (1943) observed that the child's conduct was aberrant from infancy onwards in the majority of instances, implying the presence of an inborn, probably genetic, abnormality (2).

According to Smalley (1997), mental retardation is present in around 75 percent of cases with autism, seizures are present in 15 to 30 percent of cases, and electroencephalographic abnormalities are present in 20 to 50 percent of cases. Furthermore, 15 to 37 percent of autism patients have a coexisting medical issue, with 5 to 14 percent having a recognised genetic illness or chromosomal abnormalities. Fragile X syndrome, tuberous sclerosis, 15q duplications and untreated phenylketonuria are the four most prevalent connections. Disruptions in a shared neurobiological circuit, common susceptibility genes, or genes in linkage disequilibrium might all explain significant phenotypic relationships (3).

With a male: female ratio of 3-4:1 in idiopathic autism and a rise in this ratio as the IQ of the affected persons increases, the autism spectrum disease has a notable sex bias (4) According to Folstein and Rutter, roughly 2% of siblings are affected, and speech delay is prevalent in sibships with autistic children. Folstein and Rutter (1977) observed 36 percent concordance among MZ twins and no concordance among DZ twins in a cohort of 21 same-sex twin pairs, 11 monozygotic (MZ) and 10 dizygotic

(DZ), in which at least one had infantile autism. For cognitive impairments, MZ couples had an 82 percent concordance rate while DZ pairs had a ten percent concordance rate. A biologic danger capable of causing brain injury was discovered in 12 of the 17 pairings discordant for autism. The researchers observed that infantile brain damage, alone or in conjunction with a genetic susceptibility, can cause autism. There was no suggestion of an inheritance pattern (4).

A complicated chromosomal rearrangement was documented by Lopreiato and Wulfsberg (1992) in a 6.5-year-old kid with autism and was otherwise normal except minor dysmorphism. Chromosomes 1, 7, and 21 were rearranged in every cell examined: 46, XY, -1, -7, -21, t (1;7;21) 7pter-q11.23::7q36.1-qter; 21pter-q22.3::7q11.23-q36.1-qter; 21pter-q22.3::7q11.23-q36.1::1pter-p22.1 (5). Vincent et al. (2006) described two autism-affected boys who shared a paracentric inversion of chromosome 4p, inv(4)(p12-p15.3), inherited from an unaffected mother and maternal grandfather. A closer look at the proximal breakpoint on 4p12 revealed that it contained a cluster of GABA receptor genes, including GABRA4 gene (137141), which has been linked to autism (6). In 94 families with 174 individuals with autism, Maestrini et al., (1999) found no correlation or linkage to the GABRB3 gene (7).

Jamain et al. (2003) discovered a C-to-T transition in the NLGN3 gene, which tends to result in an arg451-to-cys (R451C) substitution in a conserved region within the esterase domain of the protein, in a Swedish family in which one brother had X-linked autism and another had X-linked Asperger syndrome. The mutation was passed down from the mother and was not found in any of the 200 controls. According to Jamain et al. (2003), the mutation may alter the neuroligin protein's binding to its presynaptic partners, neurexins and disrupts crucial synaptic function(8). The R451C alteration in NLGN3 results in intracellular retention of mutant proteins, according to Chih et al. (2004) (9). Jamain et al. (2003) also discovered a frameshift mutation (1186T) in the NLGN4 gene in a Swedish family with X-linked autism and X-linked Asperger syndrome, resulting in a stop codon at position 396 and premature termination of the protein well before transmembrane region. The mutation was found in the mother but not in an unaffected sibling or 350 healthy people. Chih et al. (2004) found that a mutation in NLGN4 that caused premature termination at aspartate-396 resulted in mutant proteins being retained intracellularly. In hippocampal neurons, overexpression of wildtype NLGN3 and NLGN4X protein encouraged the

development of presynaptic terminals, while the disease associated mutation caused loss of synaptic function. The scientists speculated that neurodevelopmental problems in autism spectrum disorders and mental retardation could be caused by a synaptic cell adhesion molecule which is not working properly.

Laumonnier et al. (2004) discovered a 2-bp deletion, 1253delAG, in the fifth exon of the NLGN4 gene in all affected family members of a large French family with X-linked intellectual developmental impairment, with or without autism or pervasive developmental disorder. The loss resulted in a frameshift and a premature stop codon, and the protein was anticipated to be 429 amino acids long, a 50% truncation. The deletion was not found in healthy men in the family, and obligate carrier females were heterozygous for the mutation. Mutations in the NLGN4 gene have been linked to a wide range of symptoms (10).

Yan J. et.al (2005) has scanned the coding regions and associated splice junction of NLGN4 gene for better understanding of the relationship of NLGN4 gene and autism. They studied 148 unrelated patients with autism including 76 Midwest us Caucasian and 72 Portuguese Caucasian (122 males, 26 females), 48 patients without autism with 24 Midwest us Caucasian patients with ADHD and 24 Midwest US Caucasian with bipolar disorder and 48 Portuguese healthy controls mutation analysis showed putative missense mutation in NLGN4 gene in four separate autistic patients, G99S and K378R were found in unrelated Portuguese patient, V403M and R704C were found in unrelated Midwest patients. Mutation analysis of 48 healthy Portuguese controls and sequencing of 288 healthy including 96 Portuguese and 192 Midwest we Caucasians have not shown the mutation hence they concluded that missense changes in NLGN4 gene contribute to the Autism (11).

Lawson Yuen et al. (2008) discovered a hemizygous deletion in the NLGN4 gene spanning exons 4, 5, and 6 in a boy with autism and mental retardation as well as a motor tic. It was predicted that the 757-kb loss would result in a severely shortened protein. The loss was also found in the patient's 9-year-old brother, who had Tourette syndrome and attention deficit hyperactivity disorder with modest cognitive abnormalities. The carrier's mother suffered from a learning handicap, depression, and anxiety. According to Lawson-Yuen et al. (2008), NLGN4 mutations are linked to a wide range of neuropsychiatric diseases (12).

NLGN3 and NLGN4 have been linked to autism, according to Yan et al. (2008). Investigators analysed the NLGN4Y gene in 290 males having autism and 45 males with mental retardation based on this information, and found one missense mutation (ile679 to val; I679V) in an autism patient. The child's father, who had learning problems, also had the variation. The I679 residue is extremely conserved, and the variation was found in none of the 2,986 control Y chromosomes. There were no functional studies of the variation or examinations of patient cells. Ylisaukko-oja et al. (2005) found no NLGN4Y gene variations in 30 Finnish autism cases, including 26 men and four females (13).

In 2006 Talebizadeh and co to evaluated the NLGN3 and NLGN4X gene by screening cDNA for NLGN3 and NLGN4 from lymphoblastoid cells from autistic individuals. A novel splice variant lacking exon4 of NLGN4X and exon 7 of NLGN3 gene was detected which reduces the functional activity of the encoded NLGN4 protein and truncated NLGN3 product might have a regulatory role causes reduction of the mature protein contributes to autism. So not only missense or frameshift or deletion variants, but other forms of genetic modifications like splice variants are also take part in the abnormal function of neuroligin in the causation of autism (14).

Xu X and co in 2014 studied variation analysis of NLGN3 and NLGN4X gene and recorded four novel missense variants p.G426S (NLGN3), p.G84R, p.Q162 K and p.A283T (NLGN4X) in highly conserved extracellular non catalytic acetyl cholinesterase domain which is very crucial for binding to neurexin protein and triggering synapse activity. They concluded that NLGN3 and NLGN4X genes are involved in the pathogenesis of autism in the Chinese population and suggested functional analysis of these variants to confirm the results of Insilico predictions. This Chinese studies again supported the heterogenetic nature of neuroligin 3 and neuroligin4X in the autism population (15).

Males are more likely to have autism spectrum disorder (ASD), although it's unclear why this sex bias exists. The X-linked cell adhesion molecule NLGN4X is the site of many mutations that cause ASD or intellectual impairment. The X-Y pair that NLGN4X belongs to have an average sequence homology of 97% with NLGN4Y. Nguyen TA and co demonstrated that NLGN4Y has substantial abnormalities in maturation, surface expression, and synaptogenesis controlled by a single amino acid

variation from NLGN4X using biochemistry, electrophysiological, and imaging. In addition, they found a group of NLGN4X mutations that phenocopy NLGN4Y and were related with ASD around the crucial amino acid. They also suggested that the functional losses seen in NLGN4X mutations linked with ASD cannot be made up for by NLGN4Y. Finally, they concluded a possible pathogenic mechanism for the male bias in NLGN4X associated ASD (16).

Despite the fact that the underlying cause of ASD is largely unknown, recent developments in genome sequencing have made it possible to identify a number of related genes. Synaptic proteins like cell adhesion molecules have a particularly high association with ASD. It's interesting that many extensive genome sequencing studies do not include sex chromosomes, which causes the focus of ASD research to move to autosomal genes. However, numerous genes, including good candidates, on the X chromosome encode synaptic proteins. Here, Nguyen TA et al., 2020 discussed research on NLGN3 and NLGN4, two postsynaptic adhesion molecules from the neuroligin (NLGN) family. There are several isoforms of neuroligins (NLGN1-4), and they are sex and autosomal linked. The X chromosome contains the sex-linked genes NLGN3 and NLGN4, which were among the first few genes to be connected to ASD and intellectual disability (ID). On the Y chromosome, there is also the less well studied human neuroligin NLGN4Y, which forms an X-Y pair with NLGN4X. We'll go through new research on these neuroligin isoforms' synapse function in rodent models and differentiated neurons originating from humans, as well as the intriguing challenges that lie ahead for our knowledge of ASD/ID (17).

Several efforts have been made after 2013 to detect clinically important genetic variants in autism through whole genome/exome sequencing. Whole genome/exome sequencing has proven to be a powerful tool for understanding the genetic architecture of human diseases (18). According to exome and whole-genome sequencing (WGS) studies, ASD risk genes may be involved in dysregulation in different molecular processes, such as chromatin modifications, synaptogenesis, RNA splicing, signalling pathways, gene expression regulation, neuronal communication, cytoskeletal organisation, and cell cycling (19, 20). The study of ASD genetic risk factors is now being accelerated by big genomics data, bioinformatics, and experimental innovation, particularly the common variations and those variants in non-coding and regulatory regions.

3.2 National

The prevalence of ASD is increasing dramatically, even though there is currently no sufficient data available from India about the risk factors for ASD in India. The findings came from a study by Mamidala MP et al. for epidemiological assessment on prenatal, perinatal, and neonatal risk factors of ASD in India showed, Prenatal factors like foetal distress, gestational respiratory infections, and advanced maternal age are associated with ASD with an odds ratio of 1:8, while perinatal and neonatal risk factors like labour complications, preterm birth, delayed birth cry, birth asphyxia, and neonatal jaundice are associated with AS (21).

According to a study by Guhathakurta S and co, autistic symptoms in children with intellectual impairment were largely caused by genetic causes. The 5 hydroxy tryptamine (serotonin) receptor 2A (HTR2A) gene was a potential gene that has been linked to a number of neuropsychiatric illnesses. But this was only recorded American and Korean populations, not in Indian population (22).

According to a study by Naushad *et al.,* autistic children have a distinctive plasma amino acid profile with higher levels of glutamic acid and asparagine and lower levels of phenylalanine, tryptophan, methionine, and histidine, which can be used as a diagnostic fingerprint and disrupts the production of the neurotransmitter serotonin (23*)*

Serotonin influences the behaviour and serotonin deficiency one of the probable factors of autism susceptibility in ASD pathogenesis. In a population-based investigation by Verma D et al., the X chromosome enzyme monoamine oxidase A (MAOA), which is involved in the breakdown of serotonin, was found to be strongly related with autism spectrum disorder. Males are at higher risk because to the haplotyping relationship, which demonstrated distinct effects in males and females (24).

No published data on Chromosomal microarray analysis and exome sequencing on ASD from India. India with a multi ethnic population of 1.4 billion and predicted high burden of the disease, a systematic detailed study on ASD involving Chromosomal microarray analysis and WES are necessary.

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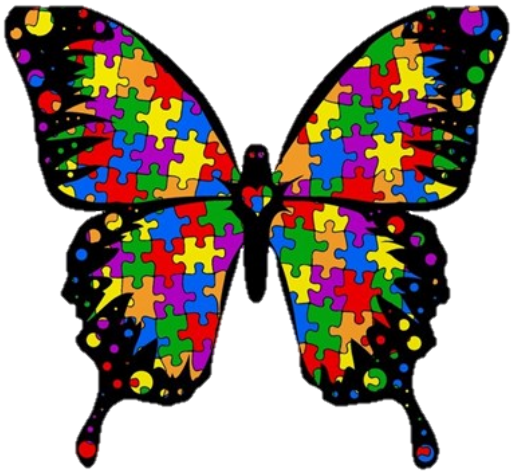
Chapter 4

MATERIALS AND METHODS



4.1 Study design: Complete enumeration

4.2 Sample size

All autistic children from the 42 special schools of all the districts of North Karnataka were screened and included for the study, due to following limitations;

- Prevalence of autism in India is not clear.
- Total diagnosed autistic population size in India is very less (<1%) and exact data is not available.
- No alternate major sources of data other than special schools are available
- Along with special schools, medical colleges, practicing clinicians and psychiatrists also helped in sample collection.

4.2.1 Inclusion criteria: Below 18 years of age and free of any neurological, psychiatric disordered autistic children were included in the study

Exclusion criteria: Associated medical conditions such as fragile-X syndrome, metabolic disorders and chromosomal aberrations were excluded from the study. Autistic children those who were not physically fit and/or not willing to give the samples were excluded from the study.

4.2.2 Institutional Ethical Clearance (IEC)

Ethical approval for the study was obtained from the Institutional ethical committee of Shri B.M Patil Medical College, Hospital and Research Centre, BLDE (Deemed to be University), Vijayapura (Ref No: BLDE (DU/IEC/337/2018-19).

4.3 Source of data

All the autistic children from the special schools of all the districts of North Karnataka were screened as per Diagnostic and statistical manual of mental disorders V (DSM-V, American Psychiatric Association, 2000)

(https://www.psychiatry.org/psychiatrists/practice/dsm) and International

Classification of Diseases-10 (ICD-10, WHO)

(https://www.who.int/classifications/icd/icdonlineversions/en/) and allowed for the study. Along with these, medical colleges, practicing clinicians and psychiatrists also helped during screening and collection of samples. The clinical data were collected as described in the enclosed data collection form.

4.4 Sample collection

Special schools and hospitals of all the 12 districts of North Karnataka viz, Belgaum, Bijapur, Bagalkot, Bidar, Bellary, Gulbarga, Yadagiri, Raichur, Gadag, Dharwad, Haveri, Koppal were included in the study. Total of 42 Schools and Hospitals were included for the collection of clinical samples. 1-2ml of peripheral blood sample was collected after taking the informed written consent from parents or guardian of the affected child. Sample was collected in EDTA (Ethylene Diamine Tetra Acetic acid) coated vacutainer with the help of clinicians and samples were stored at 4^0C during the fieldwork and later kept in -20 ^{0}C until further analysis.

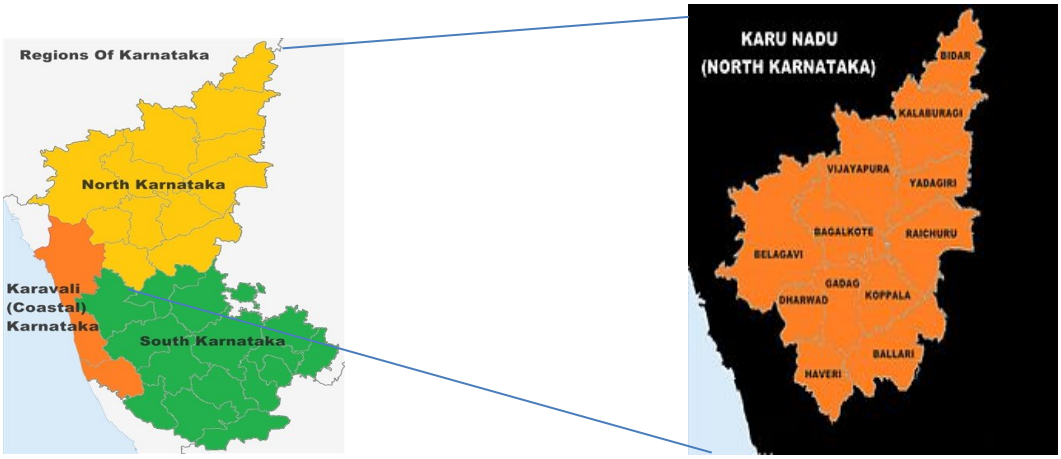


Fig.4.1 Karnataka map highlighting districts of North Karnataka region

4.5 Mutation Analysis

4.5.1 Genomic DNA isolation

Genomic DNA was isolated from all collected blood samples of autism spectrum disorder cases by using DNeasy Blood and Tissue (QIAgen, Germany) kit method. The isolated DNA samples were analysed for quality as well as quantity and later stored at -80^0C until further analysis.

DNeasy Blood & Tissue Kits are designed in such way that it can be used for variety of sample sources such as fresh or frozen animal tissues and cells, blood, or bacteria for rapid purification of genomic DNA. The DNeasy membrane integrates simple micro spin technology with the binding capabilities of a silica-based membrane. In the presence of high chaotropic salt concentrations, which hydrate molecules in solution with water, DNA binds to the DNeasy membrane. In DNeasy

Blood & Tissue process, buffer conditions are created to permit precise DNA adsorption to the silica membrane and the best possible removal of impurities and enzyme inhibitors. No phenol or chloroform extraction, no alcohol precipitation, as well as minimal handling are required for purification. This makes DNeasy Blood & Tissue Kits ideal for processing several samples simultaneously.

Brief protocol of Genomic DNA isolation as per manufacturer's guidelines

1. 20ul of proteinase k was pipetted into 1.5 ml eppendrof tube. 100ul of blood sample was added and adjusted the volume to 220ul with phosphate buffer saline.

   Optional: In cases of extraction of RNA free genomic DNA then add 4ul of RNase A (100 mg/ml) and incubate for 2 min at room temperature (15–25°C).

2. 200ul of buffer AL was added to the mixture. We ensured that ethanol had not been added to Buffer AL. Mix thoroughly by vortexing and incubated the blood sample at 56^0C for 10 min.
3. Further 200ul of absolute ethanol was added and mixed thoroughly by vortexing.
4. Transferred the mixture into DNeasy mini spin column placed in a 2ml collection tube. Centrifuged at 8000rpm for 1 min. Flow through and collection tube was discarded.
5. Spin column was placed in a new collection tube and 500ul of buffer AW1 was added. The reaction mixture was centrifuged for 1 min at 8000rpm. Flow through and collection tube was discarded.
6. Spin column was placed in a new collection tube and 500ul of buffer AW2 was added. The reaction mixture was centrifuged for 3 min at 14,000 rpm. Flow through and collection tube was discarded.
7. Spin column was transferred in to a new 1.5 ml eppendrof tube.
8. DNA was eluted by adding 200ul AE buffer to the spin column tube and reaction was incubated for 1 min at room temperature. After incubation it was centrifuge for 1 min at 8000 rpm.
9. Eluted DNA was stored in a new 1.5 ml eppendrof tube at -20^0C until further analysis.

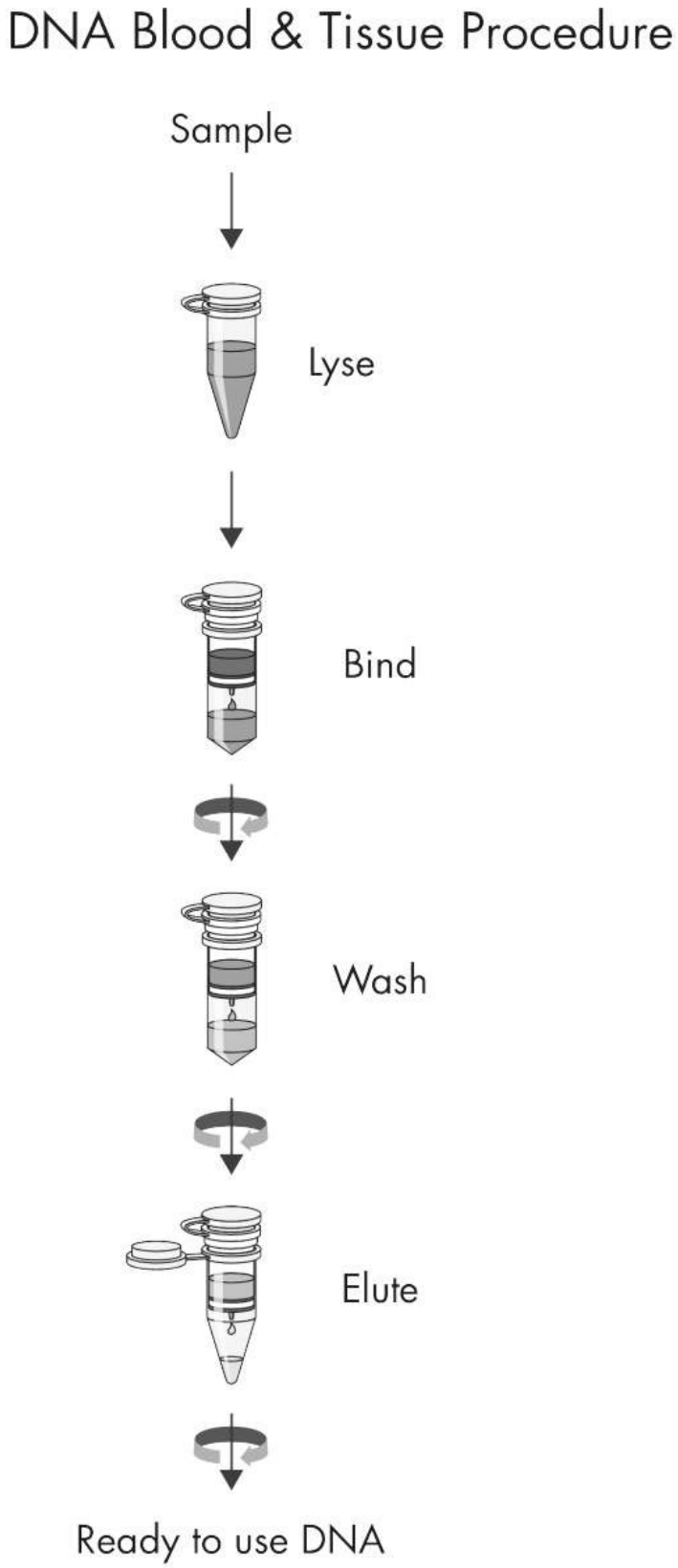


Fig.4.2 Schematic representation of DNA isolation of protocol from DNeasy blood and tissue kit

4.5.2 Quantity analysis of isolated genomic DNA

Concentrations of all the extracted DNA samples were measured using Nanodrop UV spectrophotometer. It measures DNA, RNA and Protein concentration and sample purity also. The ratio 260/280 measures the purity of the samples. Pure DNA sample gives 260/280 ratio ~1.8, for pure Protein 260/280 ratio is ~1.6 and for RNA 260/280 ratio is~2.

Procedure

1. 1-2ul of deionized water was pipetted out to cover the surface, lever arm and optical surface were cleaned with tissue paper in between each measurement (it is important to wipe to prevent the sample carry over residue).
2. Required nucleic acid molecule was selected from nanodrop software 3000.
3. Blank measurement was carried out by loading 1ul of de-ionized water.
4. Once the blank reading was noted down, optical surface was carefully cleaned with a tissue.
5. Since the buffer does not contribute to absorbance at 260, it is always made sure that the instrument is set to zero.
6. Similarly, 1-2ul of the DNA sample to be analysed was loaded. The quantity of DNA is displayed on the screen and also the quality is measured using absorbance at 260/280.

4.5.3 Quality analysis of isolated genomic DNA

The quality of isolated genomic DNA was determined by using agarose gel electrophoresis. 0.8% Agarose gel was prepared in 1X TAE buffer and Agarose was dissolved by heating, the mixture was allowed to cool at 55^0C and 1µl of Ethidium Bromide was added. The melted Agarose was poured into gel casting tray with comb making sure that no air bubbles were trapped in the gel. After solidifying, comb was removed without causing any damaged to the wells.

The tray was placed in electrophoretic tank containing 1X TAE buffer till the gel was completely submerged. 4-5µl of DNA was mixed with Bromophenol blue and loaded into the wells of Agarose gel and was run at 50-100v for 2 hours. The gel was observed under UV gel documentation system. The intact double stranded DNA forming thick single band of high molecular weight confirms the presence of good quality of DNA. Based on the band quality, the DNA samples were used for further process.

Composition of TAE buffer

50X TAE Stock Solution:

242g Tris Base (MW=121.1) was added to 600 mL of ddH20 and the Tris base was mixed with stir bar to dissolve completely. 57.1 mL Glacial Acetic Acid and 100 ml 0.5 M EDTA (MW=292.2438) was added and mixed well. Final volume was brought to 1L with ddH20. The buffer was stored at room temperature until further use.

Note: Final (1X) working concentration: 20ml of 50X TAE buffer was taken and made the volume up to 1 litre with ddH20.

4.5.4 Primer designing

In the polymerase chain reaction (PCR), a primer is a short, single-stranded DNA sequence. Pair of primers hybridize with the sample DNA and identify the area of the DNA that will be amplified in the PCR technique.

The sequence of the primer was generated using "Bioinformatics Primer Designing Tool" for the neuroligin family genes. These designed primers were confirmed through in Silico PCR method. Primer-3 is widely used, freely available web-based program (https://bioinfo.ut.ee/primer3/). This tool analyses the target regions and recommend forward and reverse oligonucleotides. Many of the factors which affect the primers specificity and sensitivity like product size, primer length, Tm, GC content, GC clamps and dimer formation can be analysed and adjusted as per the user requirements.

Procedure

1. Home page of NCBI was opened using http://www.ncbi.nlm.nih.gov.
2. Nucleotide reference sequence of human NLGN3, NLGN4X and NLGN4Y genes were searched.
3. FASTA format was accessed and converted the sequence in to FASTA format.
4. Region of interest in nucleotide sequence was marked using Genbank format.
5. Simultaneously primer 3 input web-based program was opened in a new tab (https://bioinfo.ut.ee/primer3/).
6. Selected regions and the product size depending upon the length of the sequence were entered and GC% content was set as 50percent.
7. Pick primer option was selected and the primer output was obtained.
8. Forward and reverse primer in the nucleotide sequences was marketed from primer 3 output.
9. Further in silico PCR was opened in a new tab to evaluate the amplification efficiency of designed primers.
10. Left and right primer sequences were entered.
11. BLAST option was selected and clicked on enter.
12. Further product size and amplification efficiency was compared with primer 3 output.

4.5.5 Polymerase Chain Reaction (PCR)

PCR is a simple, effective, enzymatic assay, which allows the amplification of a specific DNA portion/fragment from a complex DNA/pool of DNA. Dr. Kary Mullis, who discovered the PCR, stated “select the portion of DNA you're interested in and have as much of it as you want" (Mullis, 1990). PCR can be performed using DNA from different sources include variety of tissues and organisms, like peripheral blood, skin, hair, saliva, and microbes.

PCR assay requires essential components such as template DNA, primers, nucleotides, and DNA polymerase. The DNA polymerase is the key enzyme which adds individual nucleotides together to form the PCR product during elongation/extension. The nucleotides include four bases adenine, thymine, cytosine, and guanine (A, T, C, G). These nucleotides act as a building block. The primers are short DNA fragments with around 20 to 30 bases in length and sequence complementary to the target DNA that is to be amplified. The primers act as starting point for the synthesis of DNA.

The PCR involves three cyclic conditions

Denaturation: Denaturation is the first step in the PCR cycle and it occurs usually at 90℃-95℃ for about 0.5 to 2 minutes. Denaturation breaks the hydrogen bonds between the two strands of DNA and converts it into a single-stranded DNA. Thus, generated single strands act as a template for the production of new copy of DNA strands.

Annealing: In annealing, the primers bind to the complementary sequence of the template DNA and annealing occurs at 50-65℃ for around 30-60 seconds.

Elongation: During elongation/extension Taq Polymerase enzymes adds the nucleotide bases to the 3' end of the primer. Elongation occurs at 68-72℃ for 30-60 seconds.

These three steps are repeated for 30-40 times in order to obtain a large number of copies of DNA of interest in a very short time period.

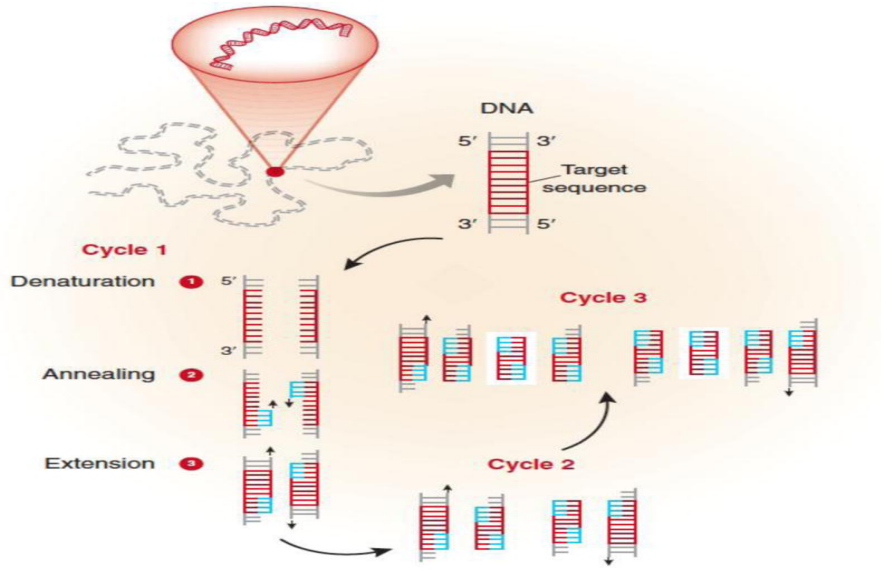


Fig.4.3 Graphical representation of PCR

NLGN3, NLGN4X and *NLGN4Y* genes were amplified using standardised PCR cycling conditions and master mix composition for all the isolated Genomic DNA from autistic children.

PCR master mixture composition (For 10µl)

- Molecular Biology grade water - 7.35µl
- Taq buffer - 1.0µl
- dNTP"s – 0.2µl
- Forward primer – 0.2µl
- Reverse primer - 0.2µl
- Template - 1.0µl
- Taq polymerase – 0.05µl

Thermo cycling condition for PCR

- Initial Denaturation of genomic DNA at 95^0C for 30 sec.
- Denaturation at 95^0C for 30sec.
- Primer annealing for ……...for 60sec.
- Primer extension at 72.0^0C for 60 sec.
- Repeated for to 35 cycles.
- Final extension 72.0^0C for 7min.
- Hold at 4^0C.

Note: annealing temperature is primer specific

4.5.5.1 Agarose gel electrophoresis of PCR products

The quality of amplified product was checked on 1.5-2.0% Agarose gel. The 1.5% of agarose gel was prepared in 100 ml of 1X TAE buffer and Agarose was dissolved by heating; the mixture was allowed to cool at 55^0C and 1.5-2.0 µl of Ethidium Bromide was added. The melted Agarose with the Ethidium Bromide was poured into the gel tray. After solidifying, comb was removed without causing any damage to the wells. The casting tray with a gel was placed in electrophoretic tank containing 1X TAE buffer till the gel was completely submerged.

The 5µl of PCR product was mixed with bromophenol blue and loaded into wells. 100bp/50bp ladder was used as molecular size marker. The initially gel was run

at 50volts till bands come out from wells and then it was changed to 80 volts and run for 1-1.5 hour. The bands were observed under UV documentation and compare with the ladder. Based on the band quality and the proper size the PCR Products were used for further downstream process.

4.5.6 DNA sequencing

A DNA primer complementary to the template DNA (the DNA to be sequenced) was used as the starting point for DNA synthesis during Sanger sequencing. The polymerase extends the primer by adding the complementary deoxynucleotide triphosphates (dNTPs) A, G, C, and T to the template DNA strand. Four dideoxynucleotide triphosphates (ddNTPs) ddATP, ddGTP, ddCTP, and ddTTP were labelled with a different fluorescent dye and were employed to stop the synthesis reaction in order to identify which nucleotide is incorporated into the chain of nucleotides.

An oxygen atom was removed in the ribonucleotide of ddNTPs in comparison to dNTPs, making it incapable of forming a bridge with the subsequent nucleotide. Depending on the various chain-terminating nucleotides, the reaction products were inserted into four lanes of a single gel and subjected through gel electrophoresis after being synthesised. The DNA sequence was thus defined based on their sizes.

Sanger sequencing analysis of all the targeted genes viz., *NLGN3, NLGN4X* and *NLGN4Y* for collected autistic samples were performed using ABI 3500 Sanger sequencing platform using Big Dye terminator version 3.1 cycle sequencing kit (Applied Biosystems, USA).

Brief procedure for DNA sequencing is as follows

4.5.6.1 Cycle sequencing

Cycles sequencing of the PCR products were carried out according to the annealing temperature of the primers (Any one of the primers i.e., forward primer was used during cycle sequencing).

The cycle sequencing master mixture consisted of following constituents: For 10 µl

- Molecular Biology grade water- 6.5 µl
- Big Dye buffer- 1.3 µl
- Big Dye- 1.0 µl
- Template (PCR product)- 1.0 µl

- Primer (Forward)- 0.2 µl

The cycle sequencing cycling conditions were as follows:

- Initial Denaturation at 96^0C for 60 sec
- Denaturation at 96^0C for 10 sec
- Primer annealing at ….. for 10 sec
- Primer extension at 72^0C for 10 sec
- Repeated for to 35 cycles
- Final extension 72^0C for 5min
- Hold at 4^0C

Note: annealing temperature is primer specific

4.5.6.2 Sequencing clean-up

To remove the unbounded florescent dNTPs of terminator sequencing reaction, to each sample 2 µl of 3M sodium acetate, 50 µl of 100% ethyl alcohol were added and incubated at room temperature for 15 min to precipitate the DNA. The samples were centrifuged at 4000 rpm for 30 min at 4^OC. The supernatant was discarded and the reaction plate was centrifuge in reverse manner at 300 rpm for 20 sec. 100 µl of 75% alcohol was added to each sample and centrifuged at 4000 rpm for 15 min at 25 ^{O}C. The supernatant was discarded and plate was centrifuged in reverse manner at 300 rpm for 20 sec to remove the alcohol completely. The plate was dried at room temperature until the last drop of alcohol dripped off.

10 µl of Hi-Di formamide was added to each well of the sample plate. The samples were heated to 96^OC for 15 min and immediately cooled to 4^OC to denature and linearise the cycle sequencing products.

4.5.6.3 Sequencing run

Sample information sheets which contain analysis protocol along with the sample details were prepared and imported into data collection software. The processed PCR products were loaded in the ABI 3500 Sanger sequinning platform (Applied Biosystem, USA) for sequencing.

4.5.6.4 Sequence quality check

After completion of sequencing reaction, the quality of the generated sequence was checked by using Sequence analysis software v5.2 (Applied Biosystem, USA).

The software programme has a base caller algorithm that perform base calling for pure and mixed base calls. It provides Quality Values (QV) for every single base and sample scores for the assessment of average quality values of the bases in the clear range sequence for the sample. The QV is per base estimate of the base caller accuracy.

4.5.6.5 Sequence alignment and analysis

The generated sequences were aligned to their respective reference sequences with the use of SeqScape v2.5 software (Applied Biosystem, USA). The reference sequence for the gene studied was obtained from NCBI Genbank data base.

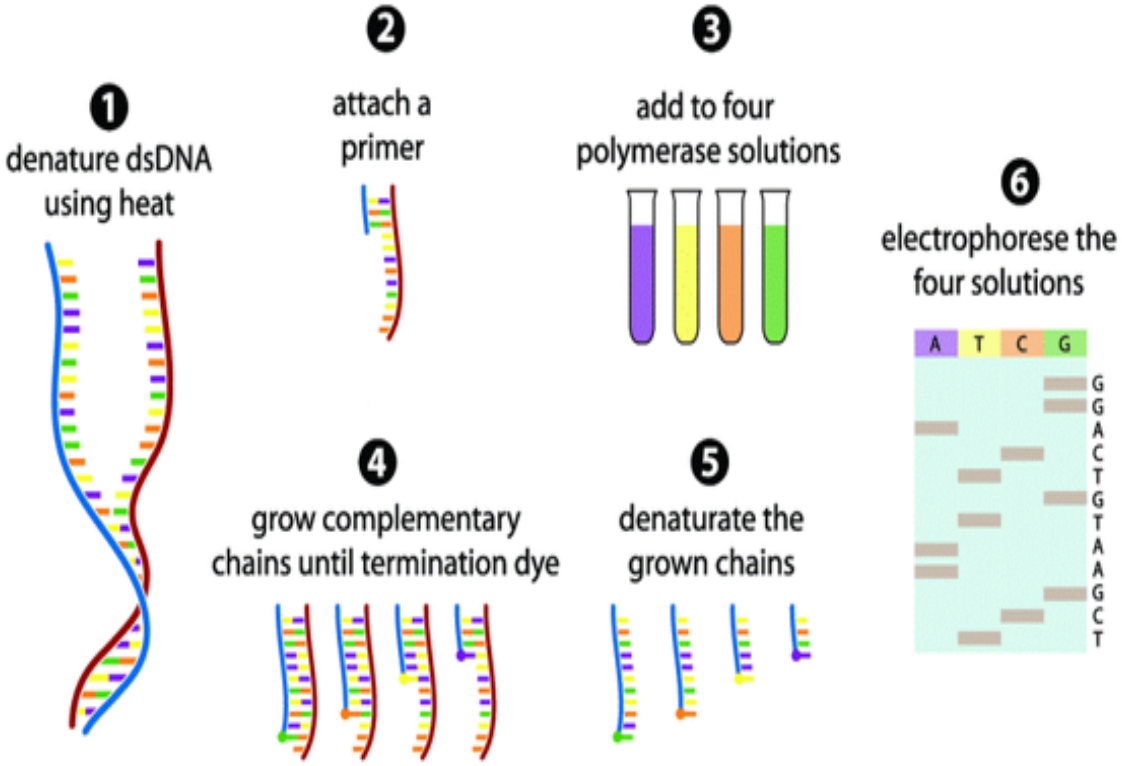


Fig.4.4 The Sanger sequencing method (adapted from Gauthier 2008).

4.6 Gene expression analysis

4.6.1 RNA isolation

Total RNA was isolated from peripheral blood of selected autistic children using QIAamp RNA blood mini kit (QIAGEN, Germany) as per the manufacturer's instructions. QIAamp RNA blood mini kit is a solid phase extraction technique to bind and extract the RNA within filter-based spin columns. Spin column uses membrane which contains glass fiber or silica to bind nucleic acids. A buffered

solution with RNase inhibitors and a significant amount of chaotropic salt is used to lyse the samples. During centrifugation lysate will pass through the membrane and RNA will bind to the silica membrane at appropriate pH. In the following step, membrane with residual proteins and salt will be removed with flow-through. RNA is subsequently eluted with RNase-free water, as RNA is stable at a slightly acidic environment.

Brief procedure

1. One volume of human whole blood was mixed with 5 volumes of buffer EL in an appropriately sized tube (not provided with kit). (For optimal results, the volume of the mixture (blood + Buffer EL) should not exceed 3/4 of the volume of the tube to allow efficient mixing. For example, 5 ml of Buffer EL was added to 1 ml of whole blood, and mixed in a tube which had a total volume of ≥8 ml).

2. Reaction was incubated for 10–15 min on ice. During incubation mixture was vortexed briefly for 2 times.

3. The cloudy suspension became translucent during incubation, indicating lysis of erythrocytes. (If necessary, incubation time can be extended to 20 min).

4. Reaction was centrifuged at 400xg for 10 min at 4°C, and supernatant was completely removed and discarded. Leukocytes form a pellet after centrifugation.

5. Buffer EL was added to the cell pellet (2 volumes of buffer EL was used per volume of whole blood used in step 1). Resuspended the cells by vortexing briefly. (For example, 2 ml of buffer EL was added to per 1 ml of whole blood used in step 1).

6. Reaction was centrifuged at 400xg for 10 min at 4°C and supernatant was completely removed and discarded. (Note: Incomplete removal of the supernatant will interfere with lysis and subsequent binding of RNA to the QIAamp spin column, resulting in lower yield).

7. Buffer RLT was added to the pelleted leukocytes according to Table 1. The mixture was vortexed briefly. When not using healthy blood, number of leukocytes should be taken into consideration to determine the volume of buffer RLT required. Buffer RLT disrupts the cells. No cell clumps should be visible before you proceed to the homogenization step. Vortexing or pipetting was done to remove any clumps.

Table 4.1 Volumes of Buffer RLT used for sample lysis

Buffer RLT* (µl)	Healthy whole blood (ml)	No. of leukocytes
350	Up to 0.5	Up to 2 x 106
600	0.5 to 1.5	2 x 106 to 1 x 107

Ensure β-ME (or DTT) is added to Buffer RLT

8. Lysate was directly pipetted into a QIAshredder spin column in a 2 ml collection tube (provided) and centrifuged for 2 min at maximum speed to homogenize. QIAshredder spin column was discarded and homogenized lysate was saved. To avoid aerosol formation, pipet was adjusted to ≥750 µl to ensure that the lysate can be added to the QIAshredder spin column in a single step.

9. One volume (350 µl or 600 µl) of 70% ethanol was added to the homogenized lysate and mixed briefly by pipetting. Do not centrifuge. A precipitate may be formed after the addition of ethanol. This will not affect the QIAamp procedure.

10. Sample was carefully pipetted, including any precipitate which may have been formed, into a new QIAamp spin column in a 2 ml collection tube (provided) without moistening the rim. Further reaction was centrifuged for 15 s at ≥8000 x g (≥10,000 rpm). Maximum loading volume was 700 µl. If the volume of the sample exceeds 700 µl, successively load aliquots onto the QIAamp spin column and centrifuge as above. Discard flow-through and collection tube.

11. QIAamp spin column was transferred into a new 2 ml collection tube (provided). 700 µl buffer RW1 was added to the QIAamp spin column and

centrifuged for 15s at ≥8000 x g (≥10,000 rpm). Flow-through and collection tube was discarded.

12. QIAamp spin column was placed in a new 2 ml collection tube (provided). 500 µl of Buffer RPE was added into the QIAamp spin column and reaction mixture was centrifuged for 15 s at ≥8000 x g (≥10,000 rpm). Flow-through and collection tube was discarded.

13. QIAamp spin column was carefully opened and 500 µl of Buffer RPE was added. Reaction mixture was centrifuge at full speed (20,000 x g, 14,000 rpm) for 3 min.

14. Recommended: Place the QIAamp spin column in a new 2 ml collection tube (not provided) and discard the old collection tube with the filtrate. Centrifuge at full speed for 1 min. This step helps to eliminate the chance of possible Buffer RPE carryover.

15. QIAamp spin column was transferred into a 1.5 ml micro centrifuge tube (provided) and 30–50 µl of RNase-free water (provided) was added directly onto the QIAamp membrane. Further it was centrifuge for 1min at ≥8000 x g (≥10,000 rpm) to elute. Repeat if >0.5 ml whole blood (or >2 x 106 leukocytes) has been processed.

16. Eluted total RNA was stored in -80^{O}C until further use.

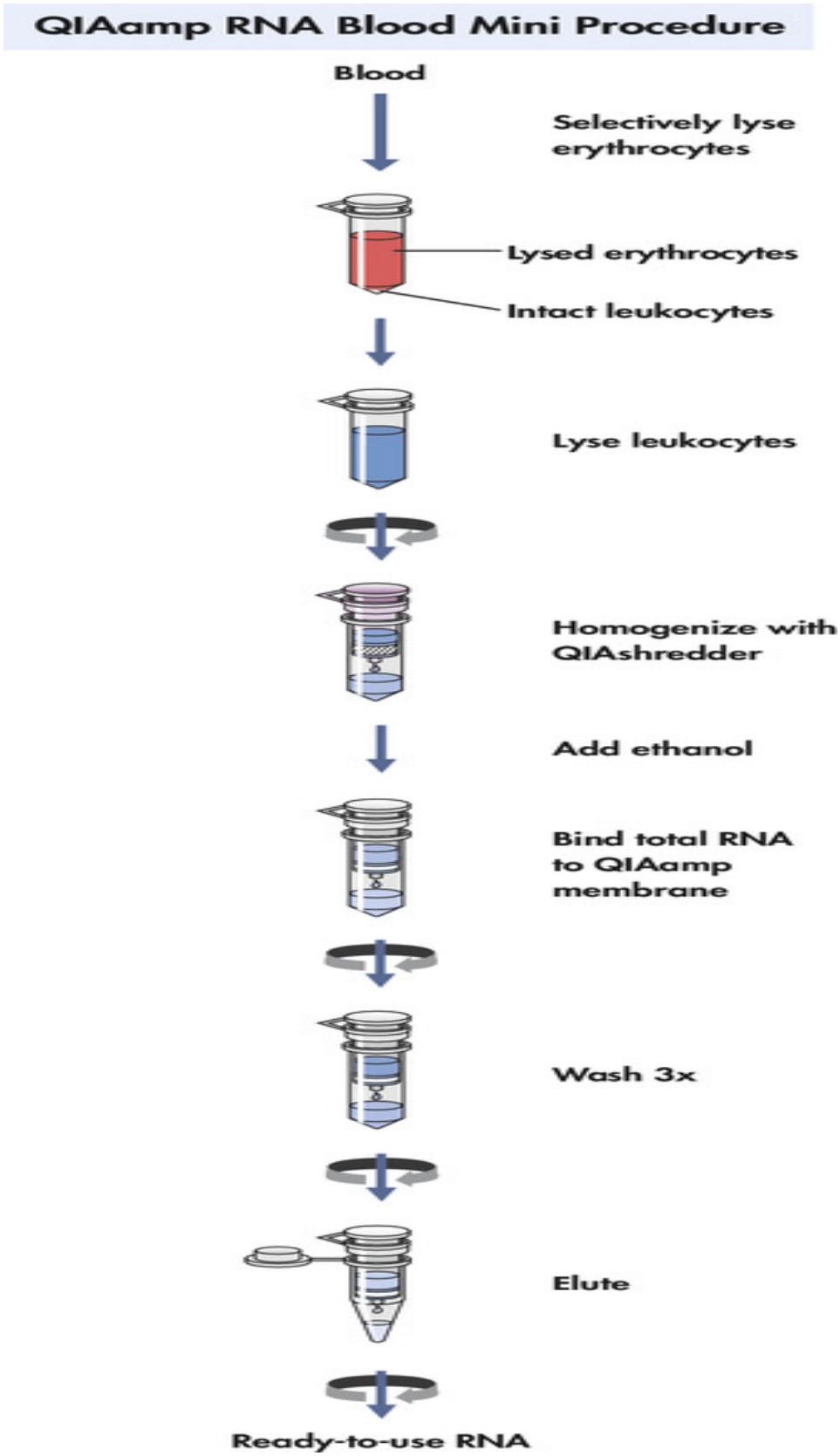


Fig 4.5 Schematic representation of RNA isolation from QIAamp RNA Blood Mini kit

4.6.2 Quality analysis of total RNA

Quality of isolated total RNA was analysed using 1.2% formaldehyde Agarose Gel Electrophoresis. The formaldehyde agarose (FA) gel electrophoresis gives enhanced sensitivity for gel and subsequent analysis (e.g., northern blotting).

1.2% FA gel preparation

Initially 1.2g agarose powder was added to 100ml of RNase-free water and stirred briefly and then 10ml 10x FA gel buffer was added. Further mixture was heated to melt the agarose. After heating, mixture was cooled at 65°C in a water bath. After cooling, 1.8 ml of 37% (12.3 M) formaldehyde and 1ul of a 10 mg/ml ethidium bromide was added. Further it was mixed thoroughly and poured onto gel casting tray. It was allowed to solidify the gel. Before running the gel, the gel was equilibrated in 1x FA gel running buffer for at least 30 minutes. 4ul of RNA sample was mixed with one volume of 5x RNA loading dye and loaded onto the equilibrated FA gel. Gel was electrophorised at 50V with 1x FA gel running buffer.

Composition of FA gel buffers

- **10x FA gel buffer**

  200 mM 3-(N-morpholino) propane-sulfonic acid, free acid (MOPS)

  50 mM sodium acetate

  10 mM EDTA

  pH to 7.0 with NaOH

- **1x FA gel running buffer**

  100 ml 10x FA gel buffer

  20 ml 37\% (12.3 M) formaldehyde

  880 ml RNase-free water

4.6.3 Quantity analysis of isolated total RNA

Concentration of the extracted total RNA sample was measured using Nanodrop UV spectrophotometer. It measures DNA, RNA and Protein concentration and sample purity also. The ratio 260/280 measures the purity of the samples. Pure

DNA sample gives 260/280 ratio ~1.8, for pure Protein 260/280 ratio is ~1.6 and for RNA 260/280 ratio is~2.

Procedure:

1. 1-2ul of deionized water was pipette out to cover the surface, clean the lever arm and optical surface with tissue paper (in between each measurement, it's important to wipe to prevent the sample carry over residue).
2. Required nucleic acid molecule was selected from nanodrop software 3000.
3. Blank measurement was carried out by loading 1ul of de-ionized water.
4. Once the blank reading was noted down, optical surface was carefully cleaned with a tissue.
5. Since the buffer does not contribute to absorbance at 260, it always makes sure that the instrument was set to zero.
6. Similarly, 1-2ul of the RNA sample to be analysed was loaded. The quantity of RNA was displayed on the screen and also the quality was measured using absorbance at 260/280.

4.6.4 Complementary DNA (cDNA) synthesis

Complementary DNA (cDNA) synthesis is the first step in gene expression profiling studies using real-time PCR. cDNA synthesis was catalysed by reverse transcriptase enzyme in presence of RNA template. Further cDNA can serve as template in gene expression.

First Strand of cDNA for targeted samples were synthesised by using following protocol

1. 10 µL of total RNA, 1 µL of Oligo (dT)18 primer and 1 µL Random Hexamer primer was added into a sterile, nuclease free tube on ice in the indicated order.

   Optional: If the targeted RNA template is GC-rich or contains secondary structures then mix gently, centrifuge briefly and incubate at 65 ^{0}C for 5 min. Chill on ice

2. The reaction mixture was mixed gently followed by the brief centrifuge and incubated the reaction mixture at 65°C for 5 min. After incubation, reaction was cooled on ice immediately.

3. After incubation 4ul 5X Reaction Buffer, 1ul RiboLock RNase Inhibitor (20 U/µL), 2ul 10 mM dNTP Mix and 1ul RevertAid M-MULV RT (200 IU/ mu L) were added in the indicated order. Mixed gently and centrifuged briefly.

4. The reaction was incubated for 5 min at 25°C followed by 60 min at 42°C incubation.

   Note. For GC-rich RNA templates the reaction temperature can be increased up to 45°C.

5. Reaction was terminated by heating at 70°C for 5 min.

6. Thus produced cDNA product can be directly used in QPCR applications or stored at -20°C/ -70°C.

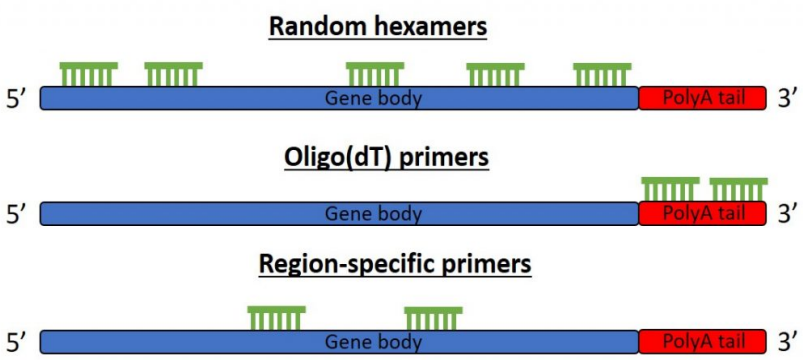


Fig.4.6 Representation of primers used in RT PCR reaction

Control reactions

Positive and negative control reactions were used to verify the results of the first strand cDNA synthesis steps.

- **Reverse transcriptase minus (RT-) negative control** is important in RT-PCR or RT-qPCR reactions to assess for genomic DNA contamination of the RNA sample. The control RT-reaction contains every reagent for the reverse transcription reaction except for the RT enzyme.

- **No template negative control (NTC)** is important to assess for reagent contamination. The NTC reaction contains every reagent for the reverse transcription reaction except for RNA template.
- **Positive control (PC)** is RNA template and gene-specific primers are supplied with the kit. The human GAPDH control RNA (1.3 kb) was produced by in vitro transcription. The GAPDH specific control PCR primers are designed to be complementary to human, mouse and rat GAPDH genes and generate 496bp RT-PCR product. The protocol for the positive control RT-PCR is provided below.

Control first Strand of cDNA was synthesised by using following protocol

1. Initially 10µL of Control GAPDH RNA, 1µL of Oligo (dT)18 primer and 1µL Random hexamer primers were added into a sterile, nuclease free tube on ice in the indicated order.
2. The reaction mixture was mixed gently followed by the brief centrifuge and incubated the reaction mixture at 65°C for 5 min. after incubation reaction was cooled on ice immediately.
3. After incubation, 4ul 5X Reaction Buffer, 1ul RiboLock RNase Inhibitor (20 U/µL), 2ul 10 mM dNTP Mix and 1ul RevertAid M-MULV RT (200 IU/ mu L) were added in the indicated order. Mixed gently and centrifuged briefly.
4. The reaction was incubated for 5 min at 25°C followed by 60 min at 42°C incubation.

   Note. For GC-rich RNA templates the reaction temperature can be increased up to 45°C
5. Reaction was terminated by heating at 70°C for 5 min.
6. Thus, produced cDNA product was directly used in QPCR applications or stored at -20°C/ -70°C.

4.6.4.1 Quality analysis of cDNA

Quality of synthesised cDNA was checked by using Agarose gel (1.5%) electrophoresis with standardised protocol. Further synthesised cDNA was used for qPCR analysis.

4.6.5 qPCR/ quantitative PCR analysis

A dsDNA binding dye named SYBR® Green directly interacts non-specifically into dsDNA and enables assessment of the amount of PCR product. When SYBR® Green is successfully incorporated with dsDNA, its fluorescence can rise up to 1,000-fold. As the amplification process continues, more DNA product is produced, which results in more SYBR® green molecules being integrated into DNA. SYBR® Green qPCR can be employed for relative DNA quantification because the increase in fluorescence increases in proportion to the amount of product produced.

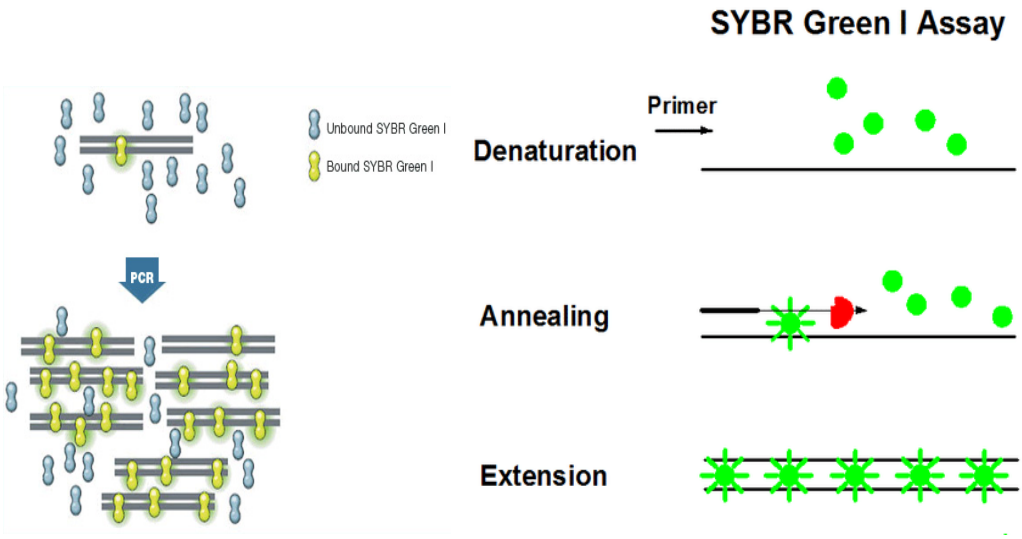


Fig.4.7 Representation of SYBR green during PCR cycle

In relative qPCR, the output is the quantification cycle (Cq) or threshold cycle (Ct), which is reached during the geometric phase of PCR. The linearity of the qPCR reaction is tracked by an increase in SYBR® Green fluorescence, since the dye's strong interaction is proportionate to the amount of product produced. The Cq value, is defined as the cycle number at a given fluorescence threshold set at a fluorescence level higher than the background, usually in the exponential point of the amplification curve.

The number of target copies in the sample correlates with the Cq values, which are inversely proportional to the amount of target nucleic acid in the sample. Higher concentrations of the target sequence are indicated by lower Cq values (usually below 28 cycles). Lower concentrations of your target nucleic acid are indicated by higher Cq values (above 34 cycles).

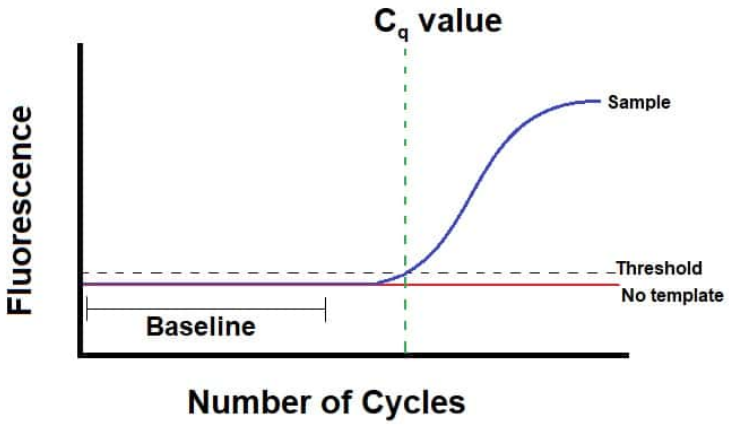


Fig.4.8 Representation of Cq values in qPCR/RT-PCR

Brief protocol

1. Prior to the preparation of reaction, all the RT PCR reagents were gently vortexed and briefly centrifuged after thawing.
2. Reaction master mix was prepared for gene of interest (*NLGN3, NLGN4X*) by adding the following components to sterile, nuclease free tube without template cDNA.

Table. 4.2 Master Mix composition for GOI

Components	**volume in µL (for 9µL)**
Nuclease free water	3.0 µL
SYBR Green master mix	4.0µL
Forward Primer of gene of interest	1.0µL
Reverse Primer of gene of interest	1.0 µL

3. Master Mix was mixed thoroughly and appropriate volumes were added into sterile, nuclease free PCR tubes.
4. Further 1 µL template cDNA was added to the individual PCR tubes containing the master mix.

   Note. For two-step RT-qPCR, the volume of the cDNA added from the RT reaction should not exceed 10% of the final PCR volume.
5. The reactions were mixed gently without creating bubbles (do not vortex). Centrifuge briefly if needed. Bubbles will interfere with fluorescence detection.
6. Program for the thermal cycler as recommended below.

Two-step cycling protocol

Table.4.3 qPCR cycling condition used for mRNA quantification

Step	Temperature, ^{O}C	Time	Number of cycles
Initial denaturation	95	10 min	1
Denaturation	95	10 sec	35
Annealing/ Extension	60	30 sec	

Data acquisition should be performed during the annealing/extension step of each cycle.

7. Samples were placed in the cycler and saved program was started.

Similarly, reaction master mix was prepared for internal control i.e. Housekeeping gene (GAPDH). Housekeeping genes are genes that are required for the maintenance of basal cellular functions that are essential for the existence of a cell, regardless of its specific role in the tissue or organism.

Table.4.4 Master Mix composition for Housekeeping gene

Components	**volume in µL (for 10µL)**
Nuclease free water	3.0 µL
SYBR master mix	4.0µL
Forward Primer of GAPDH	1.0 µL
Reverse Primer of GAPDH	1.0 µL
template cDNA	1.0 µL

Real-Time PCR studies commonly incorporate internal controls, which are essential for the accurate interpretation of the data. The MIQE guidelines state that although if the term "housekeeping genes" is commonly used, the correct term is "reference genes". Generally, internal controls and the target sequence are amplified independently during the same PCR run or within a same PCR reaction.

Positive controls are frequently used to ensure that the experiment's reagents (also known as the PCR master mix) are functioning correctly, that the primer annealing temperatures are appropriate, that the extension times are appropriate, and that there are no PCR inhibitors present in the experiment. Positive controls can be obtained from Different sources. Purchasing a commercially available positive control for a particular kind of experiment is the easiest method.

Table.4.5 Master Mix composition for positive control

Components	**volume in µL (for 10µL)**
Nuclease free water	3.0 µL
SYBR master mix	4.0µL
Forward Primer of interest	1.0 µL
Reverse Primer of interest	1.0 µL
Frog; human GAPDH control RNA	1.0 µL

Negative control (no template control or NTC) conventionally, contain the PCR reaction mixture and molecular grade water (7), which substitutes the volume of genetic material that is added to the other experiment wells or tubes. This kind of reaction checks whether there is any contamination within the reaction mix, and that there is no primer-dimers formation in the reaction.

Table.4.6 Master Mix composition for negative control

Components	**volume in µL (for 10µL)**
Nuclease free water	6.0 µL
SYBR master mix	4.0µL

Each reaction (Sample, control) for both gene of interest and housekeeping gene were analysed in triplicates with one positive control and one negative control and mean Cq value of triplicate was used for expression analysis.

4.6.5.1 Quality analysis of qPCR product

Amplified qPCR product was checked by using 1% Agarose gel electrophoresis with standardised protocol. A distinct proper base pair PCR product was visualised after ethidium bromide staining.

4.6.6 Expression calculation

After measuring Cq (quantification cycle), different methods can be used to evaluate the expression of gene of interest in the sample relative to calibrator gene/reference gene/housekeeping gene.

4.6.6.1 The 2-ΔΔCq (Livak) method

The Livak method is one of the most commonly used methods for relative gene expression. This method assumes that both target and reference genes are amplified

with efficiencies near 100% and within 5% of each other. The Livak method for calculating relative gene expression is valid only when the amplification efficiencies of the target and reference genes are similar.

- **Normalize the Cq of the target gene to that of the reference (ref) gene for both the test sample and the calibrator sample**

  ΔCq (test) = Cq(target, test) - Cq(ref, test)

  ΔCq (calibrator) = Cq(target, calibrator) - Cq(ref, calibrator)

- **Normalize the ΔCq of the test sample to the ΔCq of the calibrator**

  ΔΔCq = ΔCq(test) - ΔCq(calibrator)

- **Calculate the expression ratio**

$2^{-\Delta\Delta Cq}$ = normalized expression ratio

The result is the ratio of the target gene in the test sample to the calibrator sample, normalized to the expression of the reference gene. Normalizing the expression of the target gene to that of the reference gene compensates for any difference in the amount of sample tissue.

4.6.6.2 The Pfaffl method

PfaffI method is used calculate the relative expression of the target gene in different samples when amplification efficiencies of the target gene and the normalizing/housekeeping gene are different. To determine the expression ratio between the test sample and calibrator for a target normalized to a reference (ref), use the following equation:

$$\textbf{Ratio} = (E_{target})^{\Delta Cq,\ target\ (calibrator\ -\ test)} / (E_{ref})^{\Delta Cq,\ ref\ (calibrator\ -\ test)}$$

Where;

E_{target} - The amplification efficiency of the target gene.

E_{ref} - The amplification efficiency of the reference gene.

ΔCq, ref (calibrator -test)- is the Cq of the reference gene in the calibrator minus the Cq of the reference gene in the test sample.

ΔCq, target (calibrator-test)- is the Cq of the target gene in the calibrator minus the Cq of the target gene in the test sample.

4.6.6.3 ΔCq method

This method uses the difference between reference and target Cq values for each sample. In ΔCq Method the expression level of the reference gene is considered. The key difference in the results is that the expression value of the calibrator sample is not 1.0. If the resulting expression values obtained in this method are divided by the expression value of a chosen calibrator, the results of this calculation are exactly the same as those obtained with the Livak method.

Normalisation of expression

Relative expression = $2^{Cq(ref) - Cq(target)}$

For control- $2^{Cq(ref) - Cq(target)}$

For test sample- $2^{Cq(ref) - Cq(target)}$

Ratio of expression

For control expression: control /control

For sample expression**:** Target/control

The mathematical assumptions for this approach are the same as those for the Livak method.

4.7 Insilico analysis

The concept "in silico" refers to computational models that evaluate the experimental hypotheses using techniques such as databases, data mining, data analysis tools, homology models, machine learning, pharmacophores, quantitative structure-activity associations, and network analysis tools.

In the present study Functional analysis and Structural analysis of mutation in NLGN3, NLGN4X and NLGN4Y protein was predicted using bioinformatics tools.

4.7.1 Pathogenicity prediction: Pathogenic effects of the non-synonymous variants were analysed by using following bioinformatics tools;

4.7.1.1 PROVEAN- Protein Variation Effect Analyser

PROVEAN is a software tool which is used to predict whether an amino acid substitution or indel (insertion/deletion) has an impact on the biological functions of protein (1).

Web link: http://provean.jcvi.org/seqsubmit.php

Brief protocol

1. PROVEAN web interface was accessed at

   http://provean.jcvi.org/seqsubmit.php

2. The “Protein sequence in FASTA format” was entered in the empty text box
3. The position of the mutation in the protein sequence was assigned
4. Submit Query button was clicked
5. The corresponding view link was clicked to browse the PROVEAN prediction (deleterious/neutral) report for query of interest

4.7.1.2 POLYPHEN2- Polymorphism Phenotypic 2

PolyPhen2 is web-based tool to predict possible impact of an amino acid substitution on the structure and function of protein especially human protein using physical and comparative considerations. PolyPhen-2 is a recent version of the PolyPhen2 tool for annotating coding non-synonymous SNPs (2).

Web link: http://genetics.bwh.harvard.edu/pph2/index.shtml

Brief protocol

1. The PolyPhen-2 Web interface was accessed at

   http://genetics.bwh.harvard.edu/pph2/.

2. The “Protein sequence in FASTA format” was entered in the empty text box
3. The position of the substitution mutation in the protein sequence was assigned
4. Appropriate boxes for the wild-type (query sequence) amino acid residue AA1 and the substitution residue AA2 was selected in the web page
5. Optional: description about query sequence can be entered in the “Query description” text box:
6. Submit Query button was clicked
7. The corresponding view link was clicked to browse the PolyPhen-2 prediction report for query of interest

4.7.1.3 PANTHER

PANTHER estimates the likelihood of a coding single nucleotide polymorphism particularly non-synonymous variant's functional impact on the protein. It determines how long each amino acid has been present in the protein in its ancestors. The longer the preservation time, the greater the likelihood of functional impact (3).

Web link: http://www.pantherdb.org/

Brief protocol

1. PANTHER Web interface was accessed at http://www.pantherdb.org/
2. Protein sequence in FASTA format was entered in text box
3. Nucleotide substitution was entered on
4. Select the organism, to which protein of interest belongs to
5. Run prediction was clicked
6. Prediction results for mutations of interest were checked

4.7.1.4 SNAP2

SNAP2 is a tool used to predict functional effects of mutations over prot. SNAP2 is a well-developed classifier that uses a "neural network," a type of machine learning tool. It utilizes a number of sequence and variant properties to identify between effect variant and neutral variants/non-synonymous SNPs. The evolutionary data from a machine-generated multiple sequence alignment serves as the important input signal for prediction along with structural characteristics like projected secondary structure and solvent accessibility (4).

Web link: https://www.rostlab.org/services/snap/

Brief protocol

1. SNAP2 Web interface was accessed at https://www.rostlab.org/services/snap/
2. Protein sequence in FASTA format was entered in text box

   Optional: Enter the EMAIL address.
3. Run prediction was clicked
4. Prediction results for mutations of interest were checked

4.7.1.5 SNP & GO

SNPs &GO aggregate data from protein sequence, 3D structure, protein sequence profile, and protein function into a single framework. SNP & GO determines if a particular variation is disease-related or neutral by combining several pieces of data, such as those derived from the Gene Ontology annotation (5).

Web link: https://snps.biofold.org/snps-and-go/snps-and-go.html

Brief protocol

1. The SNP & GO Web interface was accessed at https://snps.biofold.org/snps-and-go/snps-and-go.html
2. Protein sequence in FASTA format or the file containing protein sequence of interest or swiss prot code was entered in a text box
3. The nucleotide substitution was entered

   Optional: Enter the EMAIL address
4. Clicked on run prediction
5. Prediction results for mutations of interest was checked

4.7.1.6 PHD SNP- Predictor of human Deleterious Single Nucleotide Polymorphisms

PHD-SNP is a web-based tool used to predict effect of single nucleotide polymorphism on human protein. PHD SNP Support Vector Machines based Predictor of human Deleterious Single Nucleotide Polymorphisms (6).

Web link: https://snps.biofold.org/phd-snp/phd-snp.html

Brief protocol

1. The PHD SNP web interface was accessed at https://snps.biofold.org/phd-snp/phd-snp.html
2. Protein sequence in FASTA format or the file containing protein sequence of interest or swiss prot code was entered in a text box
3. The nucleotide substitution was entered
4. Suitable prediction was selected viz sequence based or sequence and profile based

5. Then select multi SVM option

   Optional: Enter the EMAIL address

6. Clicked on run prediction
7. Prediction results for mutations of interest were checked
8. The output consisted of a table listing the number of the mutated position in the protein sequence, the wild-type residue, the new residue and if the related mutations were predicted as disease-related (Disease) or as neutral polymorphism (Neutral)

   The RI value (Reliability Index) was evaluated from the output of the support vector machine O as

   RI=20*abs(O-0.5)

4.7.2 Conservation analysis: The evolutionary conservation of variant residue over different species was investigated by using Clustal Omega and Consurf tool.

4.7.2.1 CLUSTAL OMEGA

Clustal Omega is a multiple sequence alignment program for aligning three or more nucleic acid or protein sequences together in a computationally efficient and accurate manner. It produces biologically meaningful multiple sequence alignments of divergent sequences. Evolutionary relationships can be seen via viewing Cladograms or Phylograms. Using HMM profile-profile algorithms and seeded guide trees, the multiple sequence alignment tool Clustal Omega creates alignments between three or more sequences. Pairwise sequence alignment tools will be used to align two sequences (7).

Note:

An * (asterisk) represent positions which have a single and fully conserved residue.

A: (colon) represent conservation between groups of strongly similar properties.

A. (period) represent conservation between groups of weakly similar properties.

Web link: https://www.ebi.ac.uk/Tools/msa/clustalo/

Brief protocol

1. Clustal omega tool was opened using following web link

   https://www.ebi.ac.uk/Tools/msa/clustalo/

2. Input sequences were selected as DNA/RNA/Protein.
3. Sequence of interest was uploaded in any supported format like FASTA, Genbank, and GCG etc…
4. Parameters of interest were selected as Clustal with character counts
5. Clicked on submit

4.7.2.2 CONSURF

The ConSurf is a web-based bioinformatics tool for predicting the evolutionary conservation of amino/nucleic acid positions in a protein/DNA/RNA molecule based on the phylogenetic relations between homologous sequences. It reveals the functional regions in protein/DNA by analysing the evolutionary dynamics of amino/nucleic acids substitutions among homologous sequences (8).

The degree to which an amino (or nucleic) acid position is evolutionarily conserved is strongly dependent on its structural and functional importance Thus, the importance of each position for the structure or function of the protein (or nucleic acid) can commonly be revealed through conservation analysis of positions among members of the same family. In ConSurf, the evolutionary rate is estimated based on the evolutionary relatedness between the protein (DNA/RNA) and its homologues and considering the similarity between amino (nucleic) acids as reflected in the substitutions matrix. One of the advantages of ConSurf in comparison to other methods is that, the accurate computation of the evolutionary rate by using either an empirical Bayesian method or a maximum likelihood (ML) method (9).

Web link: https://consurf.tau.ac.il/consurf_index.php

Brief protocol

1. Consurf server was opened using following web link

   https://consurf.tau.ac.il/consurf_index.php

2. Sequence of interest was selected and it was either a Nucleotides or Amino Acid sequences
3. Known protein structure for given sequence of interest was searched.
4. If yes, PDB ID was entered or own PDB file was uploaded
5. If no, Consurf will automatically make multiple sequence analysis
6. Protein sequence of interest was uploaded in FASTA format
7. Default parameters were kept to homolog search algorithm
8. Automatic Consurf analysis was selected
9. Clicked on submit

4.7.3 Protein stability prediction: Protein stability upon single point mutation was predicted using I-Mutant 3.0.

4.7.3.1 I-Mutant 2.0

I-Mutant 2.0 for predictor of effects of single point protein mutation. I-Mutant2.0 support vector machine (SVM)-based tool for the automatic prediction of protein stability changes upon single point mutation. Predictions are performed for stability change upon single site mutation starting either from the protein structure or, more importantly, from the protein sequence. When the three-dimensional structure is known then I-Mutant2.0 predicts 80% of the cases correctly whether the protein mutation stabilises or destabilises. In case, only the protein sequence is available, it predicts 77% accurately. The DDG value is calculated from the unfolding Gibbs free energy value of the mutated protein minus the unfolding Gibbs free energy value of the wild type (Kcal/mol) (9).

Web link: https://folding.biofold.org/i-mutant/i-mutant2.0.html

Brief protocol

1. The I-Mutant 2.0 Web interface was accessed at https://folding.biofold.org/i-mutant/i-mutant2.0.html
2. Protein structure (if available) or protein sequence option was selected
3. Clicked on enter to proceed
4. The protein sequence of interest was pasted in query box
5. The position of amino acid substitution was entered
6. New/mutated amino acid in one letter residue code was given
7. Condition such as temperature; 25 degree Celsius and PH;7 was kept as default
8. Prediction carried based on Free Energy change value (DDG)
9. Email address was entered for further proceeding
10. Clicked on submit and run the prediction
11. Prediction results for mutations of interest were checked

4.7.4 Protein structure prediction

Homology modelling of wild type and mutant protein was developed using the Swiss model (https://swissmodel.expasy.org/). SWISS model is web-based server used for automated comparative modelling of three dimensional (3D) structures of proteins. It started in 1993 and it became pioneer in the field of automated protein modelling and it is one of the most commonly used tool today. SWISS-MODEL offers different levels of user interaction. For example, in the "initial approach mode," simply the amino acid sequence of a protein is provided in order to create a 3D model. The server handles template selection, alignment, and model construction entirely automatically. In "alignment mode" the modelling is based on a user-defined target-template alignment. In "project mode" the integrated sequence-to-structure workbench DeepView (Swiss-PdbViewer) can handle complex modelling jobs. Each model is returned with a thorough modelling report through email (10).

Results were visualized and analysed using downloaded version of UCSF ChimeraX program. UCSF Chimera is an extensible molecular modelling program for

the interactive visualization and analysis of molecular structures and related data, including density maps, trajectories, and sequence alignments. (https://www.cgl.ucsf.edu/chimera/)

Brief protocol

1. The homepage of Swiss model was opened using

   https://swissmodel.expasy.org/ web link

2. Clicked on start modelling
3. Target protein sequence in FASTA format was uploaded (UniProt accession number also entered if protein structure is known)
4. Project title and email address was entered in a text box
5. Clicked on target-template alignment for proper template selection
6. Clicked on start structure for model building of protein of interest
7. Predicted protein model was evaluated for given protein of interest

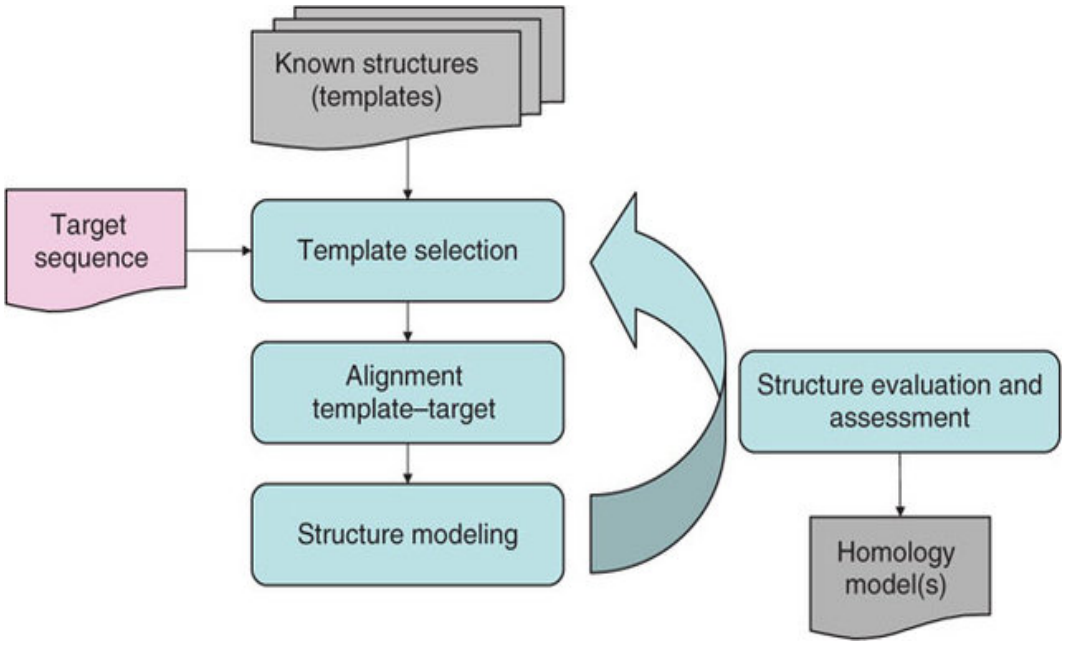


Fig.4.9 Brief protocol of Protein homology modelling using SWISS-MODEL

4.7.5 Protein-Protein interaction

Protein-protein and protein-DNA/RNA interactions play an important fundamental role in a majority of the biological processes. Molecular docking has played major role in determining the complex structures of these interactions. HDOCK, a novel web-based server with hybrid docking algorithm of template-based modelling and free docking, importantly cases with misleading templates can be rescued by this free docking protocol. The server supports protein-protein and protein-DNA/RNA docking and accepts both sequence and structure inputs for proteins.

4.7.5.1 Homology modelling and model validation

Homology models of the neuroligin proteins particularly *NLGN3, NLGN4X* and *NLGN4Y* were built to study their interaction with neurexin.

A co-crystal of wild type protein was obtained from Protein Data Bank. Wild type was used as template, and MODELLER v. 9.24 was employed to obtain the 3D structure. After running the simulation, 100 different structures with different model quality scores (molpdf, DOPE, GA341) were constructed. The homology models of the mutant and the wild type templates were structurally aligned to assay the root mean square deviation (RMSD) differences among the model and template structures through TM-Align, a protein structure alignment algorithm based on the TM score (11).

The homology models were evaluated with computational tools as SWISS-MODEL™, which calculates the Z-score, QMEAN, and Ramachandran plot (12). ProSA-Web, another Z-score for the overall model quality, enables to establish whether the z-score value of the model structure is located in the range of Z-scores exhibited by native proteins of similar size, with PDB as the reference database (13). All these computational tools enable to determine whether 3D models of NLGN3, NLGN4X and NLGN4Y are reliable models to employ in molecular docking analysis.

4.7.5.2 Molecular docking analysis

Protein-protein docking is a molecular modelling technique that uses computer algorithms and techniques to predict the mutual orientation and position of two molecules in a complex.

Molecular docking was performed on the HDOCK (http://hdock.phys.hust.edu.cn/) to determine the possibility of interaction between Neuroligin and Neurexin. Protein-protein docking is a molecular modelling technique

that predicts the mutual orientation and position of two molecules in a complex using computer algorithms and techniques. The HDOCK server differs from similar docking servers in that it accepts amino acid sequences as input and employs a hybrid docking strategy in which experimental data about the protein–protein binding site and small-angle X-ray scattering can be incorporated during the docking and post-docking processes.

The HDOCK score reflects the attempt to find the native site with the lowest free binding energy. The size of each protein, the weighted energy score of the cluster center (i.e., the structure with the most neighbouring structures in the cluster), and the energy score of the cluster's lowest energy structure (14).

4.7.6 Statistical analysis

All characteristics were summarized descriptively. For continuous variables, the summary statistics of mean± standard deviation (SD) were used. For categorical data, the number and percentage were used in the data summaries and diagrammatic presentation. Chi-square ($\chi 2$) test was used for association between two categorical variables. The difference of the means of analysis variables between two independent groups was tested by unpaired t test.

The difference of the means of analysis variables between more than two independent groups was tested by ANOVA test for testing of equality of Variance. Bivariate correlation analysis using Pearson's correlation coefficient (r) was used to test the strength and direction of relationships between the interval levels of variables.

If the p-value was < 0.05, then the results were considered to be statistically significant otherwise it was considered as not statistically significant. Data were analysed using SPSS software v.23.0 and Microsoft office 2007.

4.8 References

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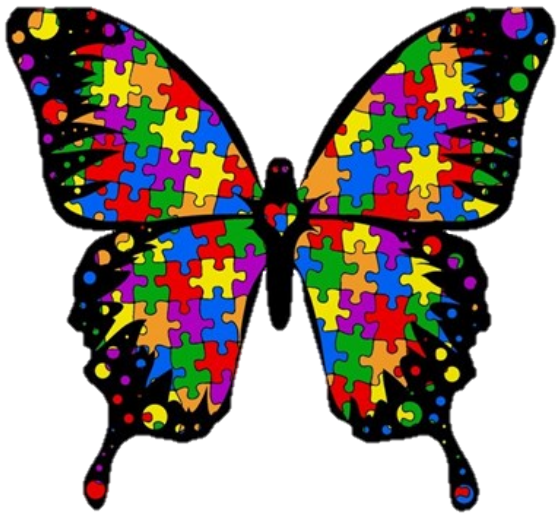
Chapter 5

RESULTS



5.1 Sample collection

Detailed complete screening of 42 Special schools during the year 2018-2020 in around North Karnataka regions of Karnataka, India recorded a total of 1878 special children. Among 1878 special children, 136 special children belong to autism spectrum disorder. The same are represented in table 5.1

Table 5.1 represents the total number of screened special children from North Karnataka

Sl. No	District	No's of screened special children	No's of Autistic Children	No's of non-Autistic children
1	Belgaum	385	45	340
2	Gadag	163	1	162
3	Dharwad	215	13	202
4	Kalburgi	40	1	39
5	Bagalkot	154	17	137
06	Bellary	390	21	369
07	Bidar	35	0	35
08	Raichur	25	8	17
09	Bijapur	40	10	30
10	Koppal	28	2	26
11	Haveri	363	4	359
12	Yadagiri	40	14	26

5.2 Epidemiological analysis

Detailed screening recorded total 1878 special children, in that total 136 were autistic children. This accounts for 7.24% autistic children among studied mentally ill children. Remaining 1742 children belong to other neurological conditions such as Attention Deficit Hyperactivity Disorder (ADHD), Epilepsy, Intellectual disability, Down's syndrome etc… the same are represented in fig.5.1

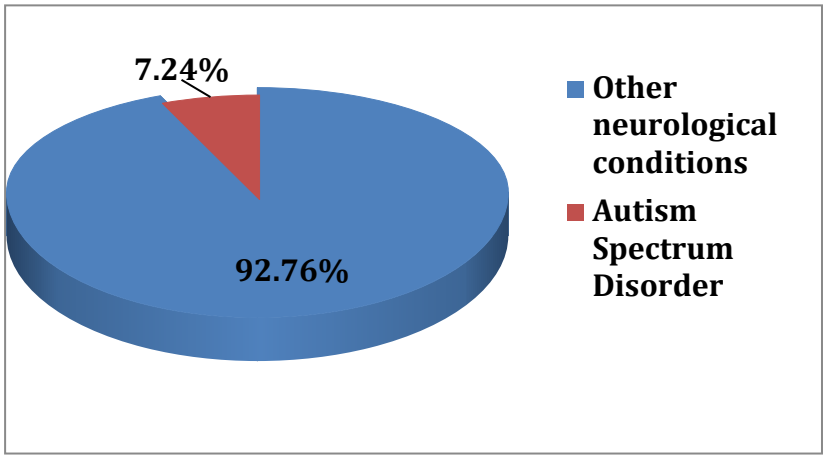


Fig 5.1 Distribution of ASD cases among other conditions

Age wise distribution analysis of autistic children from the present study cohort recorded 87 (64%) autistic children belonged to 11-18 years of age followed by 49 (36%) autistic cases belonged to <10 years of age. Mean age of the study group was 11.7±3.5 years. The same are represented in fig.5.2

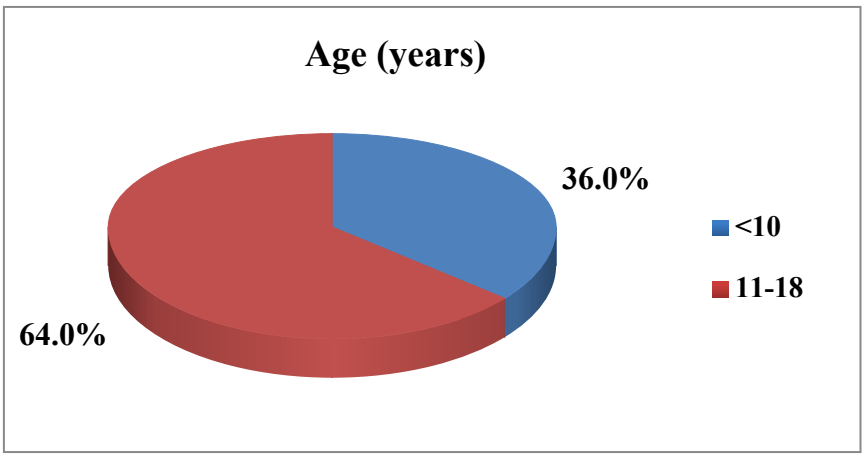


Fig 5.2 Distribution of cases according to age

Table 5.2 Distribution of cases according to age

Descriptive Statistics	Range	Mean	SD
Age (yrs)	5-18	11.7	3.5

Gender wise distribution analysis of autistic children in the present study cohort showed 79.4% were male children and 20.6% were female children. The global record of male to female ratio of autism is 3-4:1. But our study recorded 4:1 male to female ratio. Fig.5.3 represents the distribution of cases according to gender

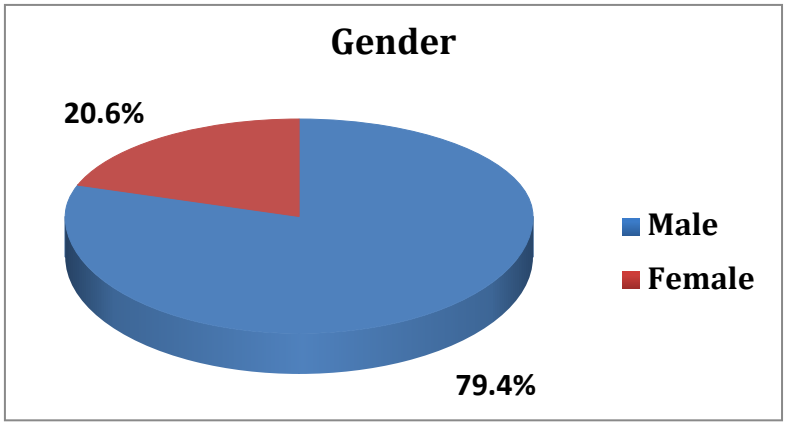


Fig.5.3 Distribution of cases according to gender

Age wise distribution of gender in the present study cohort showed 40 male children and 9 female children belonged to <10 age. 68 male children and 19 female children belonged to 11-18 years of age. But the chi-square test shows there is no significant association between age group and gender of autism (p value-0.74) in our study group. The same are represented in table.5.3 and fig.5.4

Table.5.3 Distribution of Cases according to age and gender

Age	Male	Female	P-value (Chi-square)
<10	40	9	0.74
11-18	68	19	
Total	108	28	

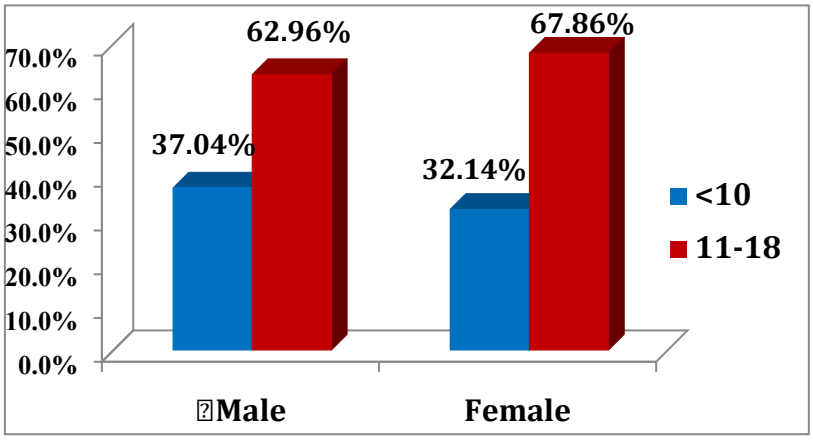


Fig.5.4 Distribution of cases according to age and gender

The severity of autism was measured using Childhood Autism Rating Scale (CARS). Total scores can range from a low of 15 to a high of 60; scores below 30 indicate that the individual is in the non-autistic range, scores between 30 and 36.5 indicate mild to moderate autism, and scores from 37 to 60 indicate severe autism. In the present study as per the CARS, out of 136 identified autistic children, 47 (34.6%) children were mild-moderate autism condition and 89 (65.4%) were severe autism. The CARS score in the present study ranges from 30-58 with a mean score of 43.0±8.6. The same are represented in fig.5.5 and table 5.4

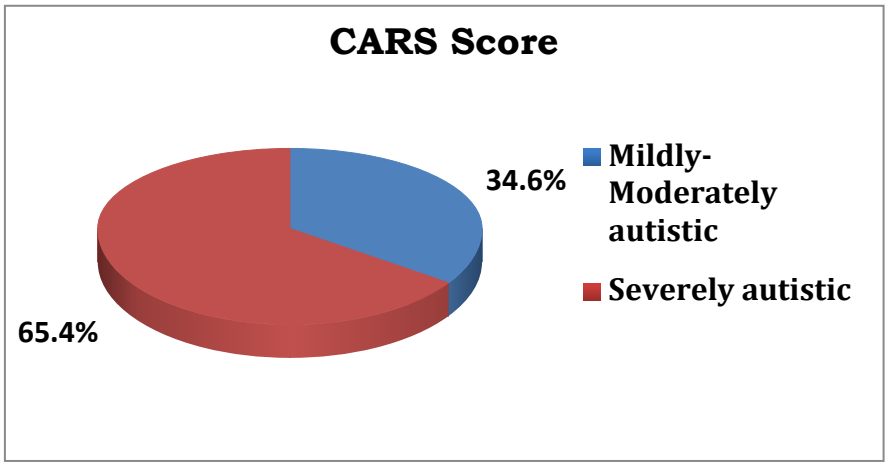


Fig.5.5 CARS Score according to the severity of autism

Table.5.4 Descriptive statistics of CARS score and degree of autism

Descriptive Statistics	Range	Mean	SD
CARS Score	30-58	43.0	8.6

Gender wise distribution of severity analysis based on CARS score showed 33 (30.6%) male children were mild-moderate autistic and 75 (69.4%) male children were severely autistic. 14 (50%) female children were mild- moderate autistic and 14 (50%) female children were severely autistic. The present study recorded majority of the male children belonged to the severely autistic group. The male and female groups showed a significant association for the severity of autism based on CARS rating (p value-0.054). Table 5.5 and fig. 5.6 shows distribution of cases according gender.

Table.5.5 CARS Score according to gender

CARS Score	Male		Female		p-value
	N	**Percent**	**N**	**Percent**	
Mildly- Moderately autistic	33	30.6%	14	50.0%	0.054
Severely autistic	75	69.4%	14	50.0%	
Total	108	100.0%	28	100.0%	

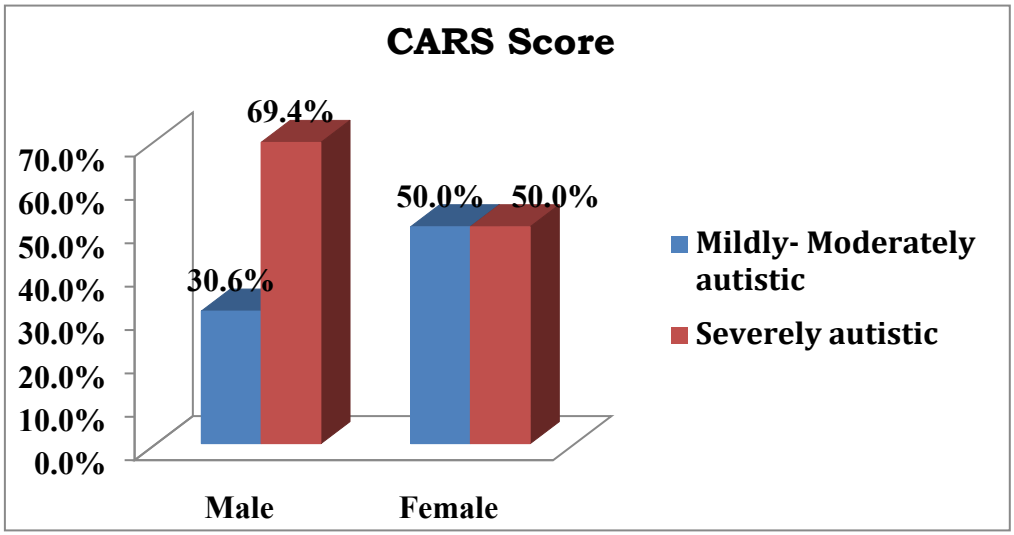


Fig.5.6 CARS Score according to gender

The severity of autism was also measured using DSM-V criteria. As per DSM-V criteria severity is classified into 3 types; Level 1-“Requires support”, Level 2- "Requiring substantial support” and Level 3- "Requiring very substantial support”. 15% of the children were categorised as Level-1, 26% of the children were categorised as Level-2 and 59% of the children were categorised as Level-3 autism. The same are represented in fig.5.7

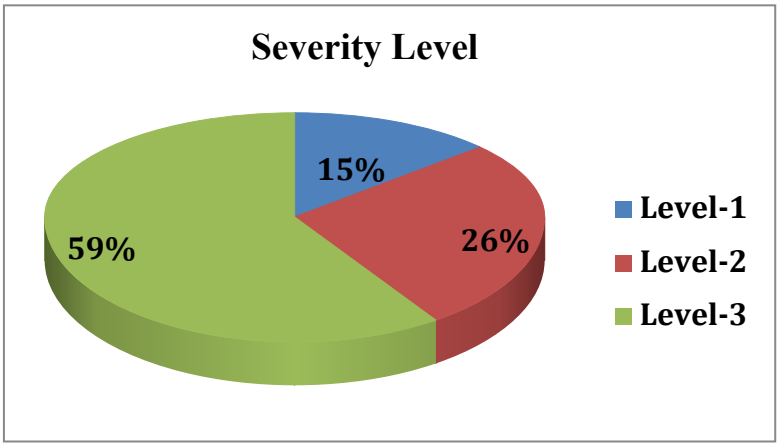


Fig.5.7 Severity levels of ASD cases according to DSM-V

Gender wise distribution of severity level of autism as per DSM-V showed 15.7% males and 10.7% females belong to the Level-1 group, 25% males and 28.6% females belong to the Level-2 group and 59.3% males and 59.3% males and 60.7% females belong to Level-3. In the present study cohort maximum autistic children belonged to severe autistic group.Fig.5.8 represents severity levels of ASD cases according to gender.

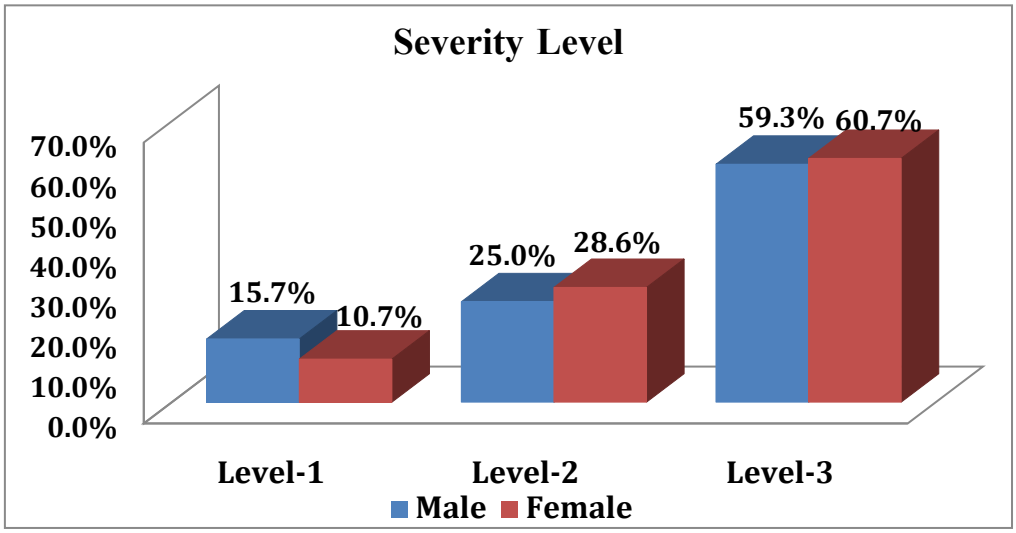


Fig.5.8 Severity levels of ASD cases according to gender

Analysis of parental age factors in autism showed 47 (34.6%) autistic children were born to father's age ranging from 21-30 years, 77 (56.6%) children with father's age ranging from 31-40 years and 12 (8.8%) children with father's age ranging from 41-50 years at the time of birth. Fig.5.9 shows the distribution of cases according to parental age.

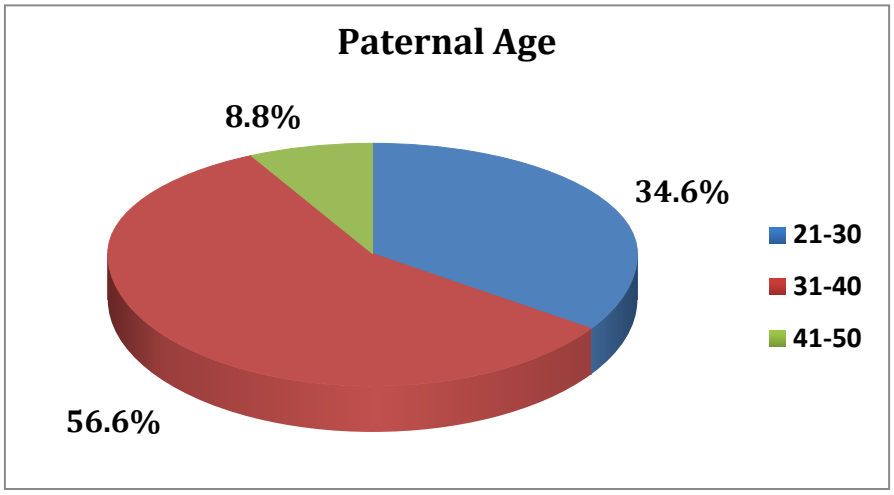


Fig. 5.9 Distribution of cases according to paternal age

34 (25%) autistic children were born to mother's with age ranging from between 21-30 years, 57 (41.9%) children with maternal age ranging from 31-40 years and 45 (33.1%) children with maternal age ranging from 41-50 years at the time of birth. the same are represented in fig.5.10

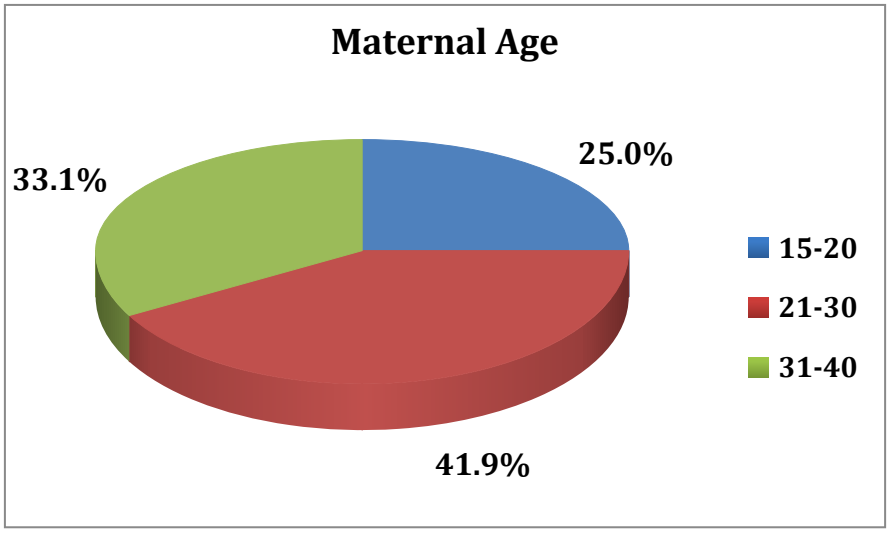


Fig.5.10 Distribution of cases according to maternal age

The age gap between the parents was also recorded in autistic children during the time of birth. 22 (16.2%) autistic children belonged to parents with 1-5 years of the age gap. 75 (55.1%) autistic children belong the parents with 5-10 years age gap. 39 (28.7%) autistic children belonged to parents with >10 years of age gap. The same are presented in fig.5.11

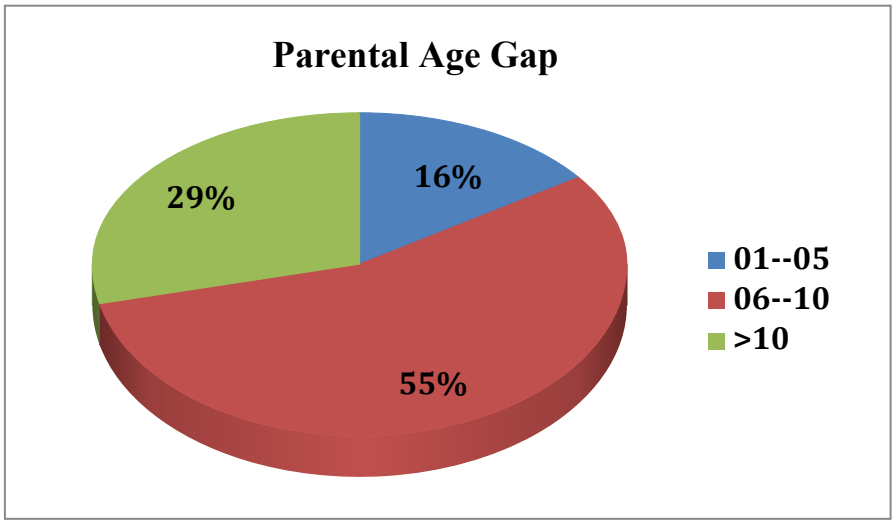


Fig.5.11 Distribution of cases according to parental age gap

In the present study, 89 (65.4%) autistic children had normal delivery during the time birth and 47 (34.6%) autistic children had a premature delivery. Fig.5.12 represents the distribution of ASD cases according to nature of delivery.

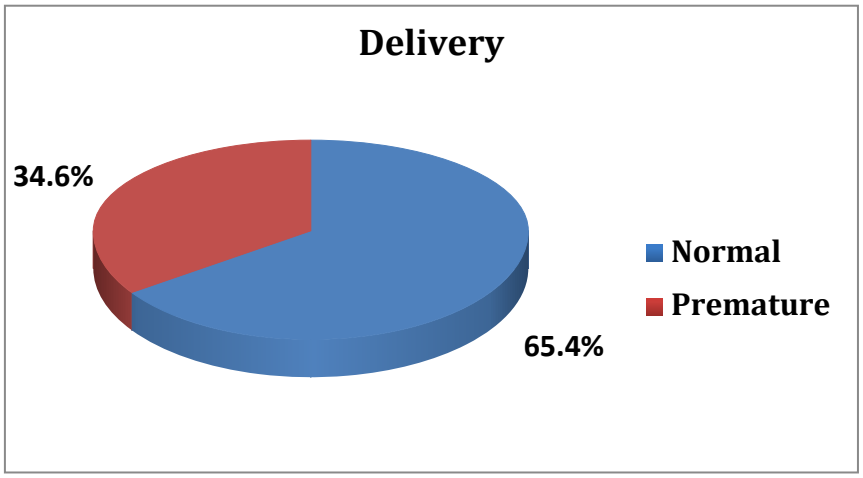


Fig.5.12 Distribution of cases according to delivery

Consanguineous marriage is most common in practice in the North Karnataka region of Karnataka, India. In the present study, 84 autistic children out of 136 (61.8%) had consanguineously married parents. The degree of consanguinity was analysed in the present study and the maximum number of autistic parents with consanguineous marriage were third degree relatives i.e. uncle-niece marriage (36.9%) followed by fourth degree relatives (29.8%) and sixth degree relatives (4.8%). Interestingly 28.6% of the autistic children parents had an undetermined degree of relation or unknown relationship between the couples. The same are represented in fig.5.13 and fig.5.14

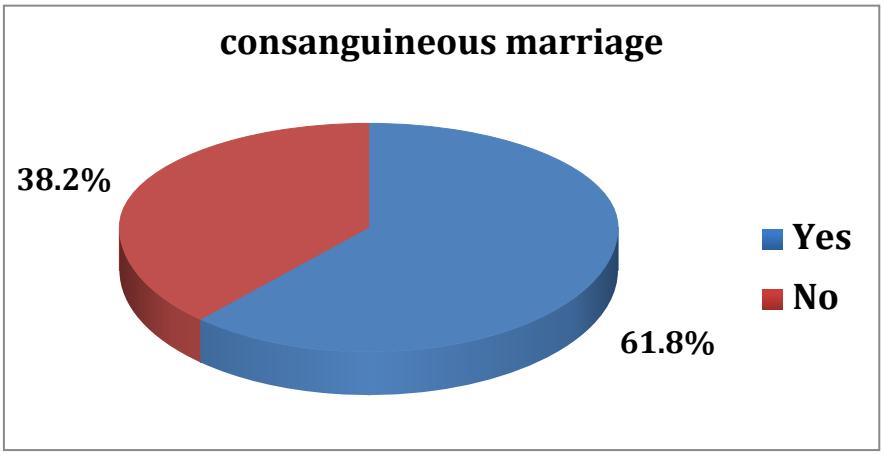


Fig.5.13 Distribution of Cases according to consanguineous marriage

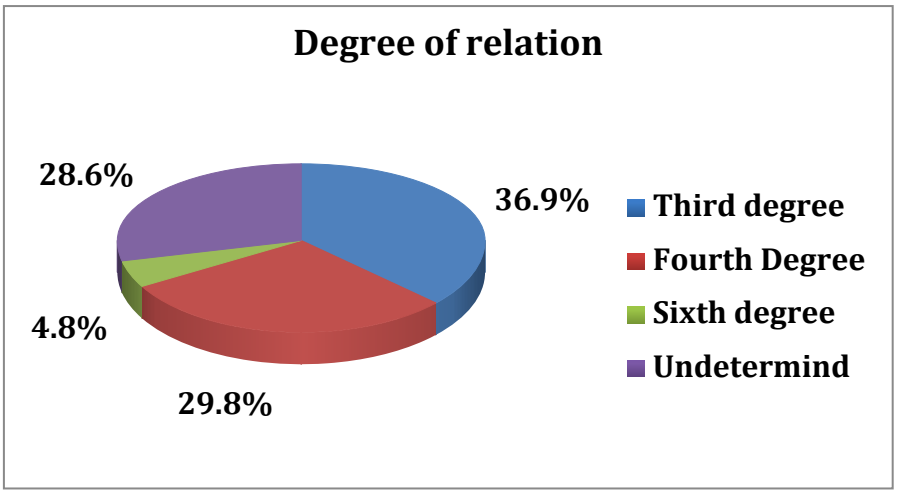


Fig.5.14 Distribution of Cases according to Degree of relation

The present study recorded prenatal factors in the study cohort. 5.1% of the autistic children mother has a birth complication, 3.7% of the autistic children mother has pregnancy complication, 3.7% of the autistic children mother showed high blood pressure, 2.2% of the autistic children mother has hyperthyroidism, 0.7% of the autistic children mother has a mental illness, 36% of the autistic children have more than 30 years aged father and 44.9% of children have more than 30 years aged mother during birth. Fig.5.15 represents the distribution of ASD cases according to prenatal factors.

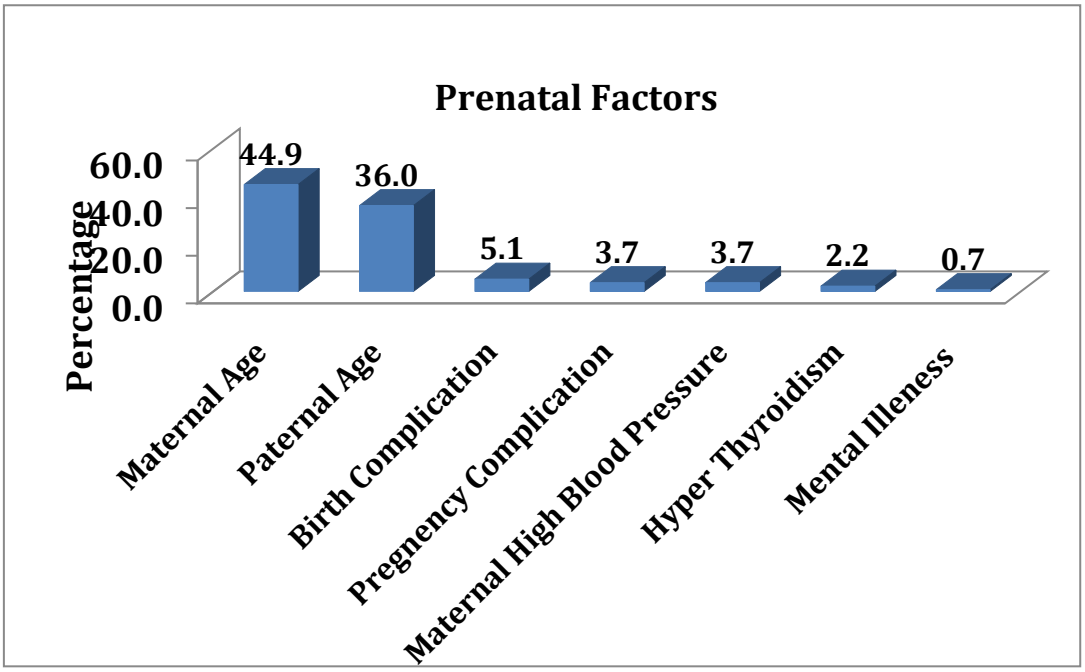


Fig.5.15 Distribution of cases according to prenatal factors

The present study also recorded postnatal factors in the study cohort. 8.8% of the autistic children had a gastrointestinal problem, 8.1% of the autistic children mother had birth asphyxia, 7.4% of the autistic children showed feeding problems, 6.6% of the autistic children has respiratory problems, 5.1% of the autistic children had developmental delay, 2.2% of the autistic children encountered high fever, 2.2% of children showed sleep dysfunction, 0.7% of the autistic children encountered jaundice, 0.7% of the autistic children had partial agenesis of the corpus callosum and 0.7% of the autistic children had congenital abnormalities. Fig.5.16 represents the distribution of ASD cases according to postnatal factors.

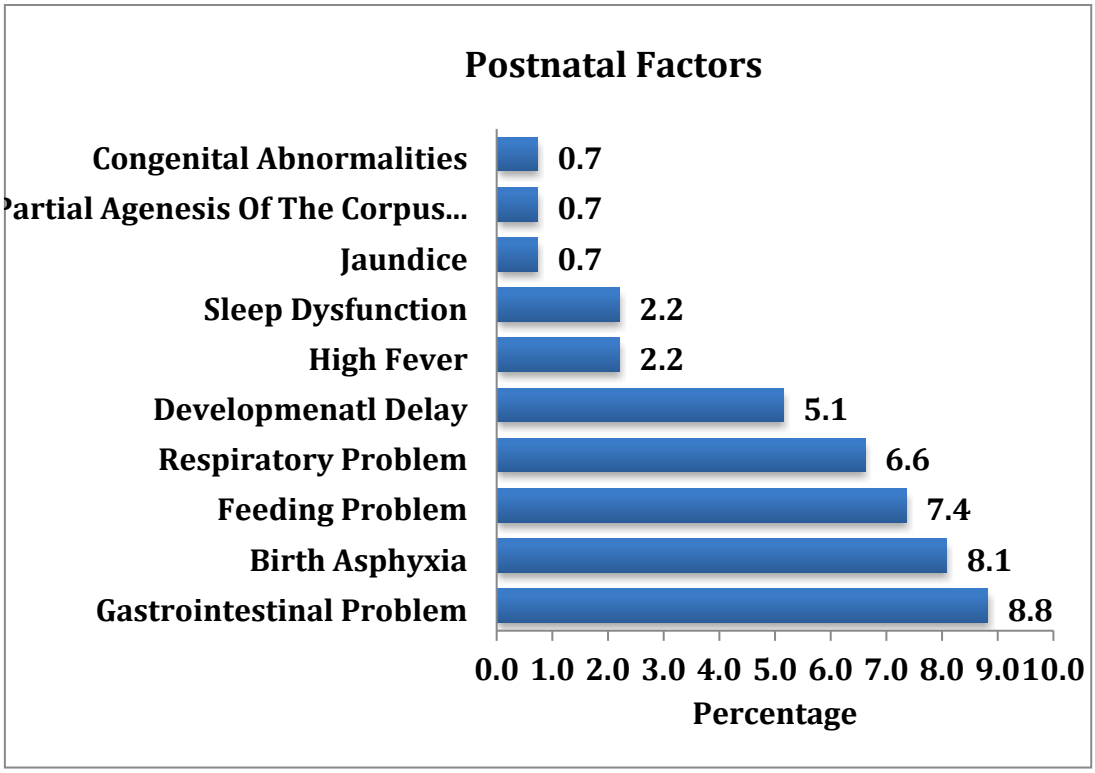


Fig.5.16 Distribution of cases according to postnatal factors

5.3 Molecular Analysis

5.3.1 DNA isolation: Agarose gel electrophoresis

Genomic DNA was successfully isolated from all 108 autistic samples using a blood and tissue kit (QIAgen, Germany) as per manufactures instruction. The quality of the genomic DNA was analysed on 0.8% agarose gel electrophoresis. Fig.5.17 shows a sample image of genomic DNA from 12 autistic samples.

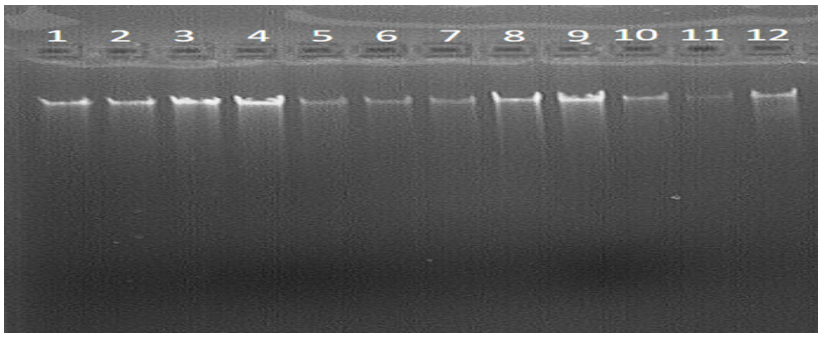


Fig.5.17 Agarose gel electrophoresis image of genomic DNA. Lane No. 1 – 12 Genomic DNA from 12 different DNA samples of autistic children

5.3.2 Nanodrop UV spectrophotometer

The quantity of the isolated genomic DNA samples was measured using a nanodrop UV spectrophotometer. Table.6 Shows sample results of genomic DNA from 12 autistic samples.

Table.5.6 Showing Nanodrop UV spectrophotometer reading of isolated genomic DNA of autistic children

Sl No	Sample ID	Sample Type	A260	A280	260/280	ng/µl
1	ASD- 1	dsDNA	0.257	0.123	2.09	12.8
2	ASD- 2	dsDNA	0.318	0.174	1.83	28.4
3	ASD- 3	dsDNA	0.203	0.105	1.94	15.1
4	ASD- 4	dsDNA	0.477	0.258	1.85	23.8
5	ASD- 5	dsDNA	0.236	0.127	1.85	11.8
6	ASD- 6	dsDNA	0.156	0.077	2.03	12.8
7	ASD- 7	dsDNA	0.196	0.10	1.96	12.8
8	ASD -8	dsDNA	0.183	0.095	1.92	17.9
9	ASD -9	dsDNA	0.442	0.211	2.09	22.1
10	ASD- 10	dsDNA	0.25	0.127	1.96	26.3
11	ASD- 11	dsDNA	0.257	0.123	2.09	15.8
12	ASD- 12	dsDNA	0.203	0.105	1.94	20.01

5.3.3 PCR amplification: Polymerase Chain Reaction (PCR) amplification for *NLGN3, NLGN4X* and *NLGN4Y* was carried out using a previously standardised protocol.

5.3.3.1 PCR standardisation of *NLGN3*

In the present study, a total of 13 sets of primers were designed for 7 exons of the *NLGN3* gene. Standardised annealing temperature and amplicon size for designed primers (Oligos) of *NLGN3* gene were summarised in Table.5.7

Table.5.7 Standardised annealing temperature and amplicon size for designed primers of *NLGN3* gene

Exon	Primer Id	Sequence	Product size	Tm
Exon 1	NL3X-EX1-F	CCAGGACTTGAGCCATCTCT	303bp	62.7
	NL3X-EX1-R	AGTGGAGGAGCCTGGGATT		
Exon 2	NL3X-EX2A-F	CCCCAAAGACCAACTCTGTT	451bp	55.0
	NL3X-EX2A-R	TCTCACTGGGCAGTGGTACTC		
	NL3X-EX2B-F	CAGCACCCACAGTCAACACT	409bp	54.9
	NL3X-EX2B-R	CATGCAAGCCACAAACACA		
Exon 3	NL3X-EX3-F	CTGCAGTCATGCTGTTTTTGA	248bp	55.0
	NL3X-EX3-R	AACACACCAACGGACAGACA		
Exon 4	NL3X-EX4-F	CACCTGGGATAGCTTTGCTG	359bp	55.3
	NL3X-EX4-R	AGCACCAGCTAGAGAAGCAAG		
Exon 5	NL3X-EX5-F	TCATCACCCAAATCCTCCAT	351bp	54.8
	NL3X-EX5-R	AGAAGAGAGCTGGCCGATTC		
Exon 6	NL3X-EX6A-F	CAGCCTCAGTGACAAAGGAA	520bp	55.2
	NL3X-EX6A-R	CAGGGTGTCCTTACCCTCAG		
	NL3X-EX6B-F	GACCCTGAGGATGGTGTCTC	489bp	56.3
	NL3X-EX6B-R	TGGGGTCTCAAAGAGGAAAA		
Exon 7	NL3X-EX7A-F	GGCCTTTTCCTCATCCAGA	493bp	54.3
	NL3X-EX7A-R	CGGCGATGGTGACACTTAAT		
	NL3X-EX7B-F	CCACTCCTGGTGGAGAACC	494BP	53.3
	NL3X-EX7B-R	CTGGAGATTGGCTGTGCTCT		
	NL3X-EX7C-F	CGCAGGGTTCAACAGTACC	496BP	57.0
	NL3X-EX7C-R	CCTGGGGAGGAAGAGCTATC		
	NL3X-EX7D-F	CTTCGGCCTCTCTTGGAACT	495BP	54.8
	NL3X-EX7D-R	GGGGCCCAATAGTGATGTG		
	NL3X-EX7E-F	GCCAGACCAGGTGACCTTAG	364BP	57.9
	NL3X-EX7E-R	ACCCTGTTCGGAGGTCTGTT		

Agarose gel electrophoresis of gradient PCR products of *NLGN3* gene was electrophoresed on 2-2.5% agarose gel with respect to amplicon size. The details are repersented in fig.5.18 to fig.5.30

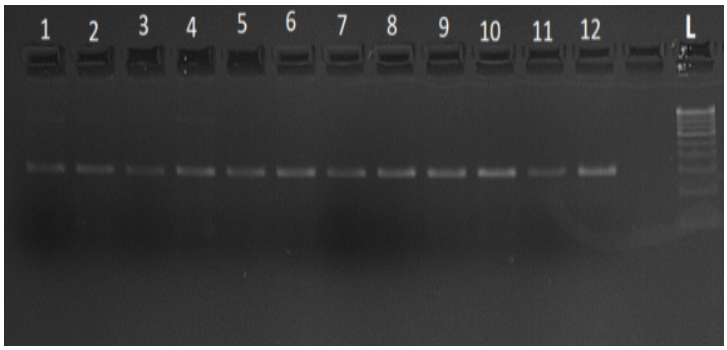


Fig.5.18 Agarose gel electrophoresis results of gradient PCR of EXON-1

L- 100bp Marker, Lane No. 1 – 12 PCR product from the different temperatures in degree Celsius.

T_m=61.0, G= ±4.0

Lane No.1- 59.0, Lane No.2-59.1, Lane No.3-59.3, Lane No.4- 59.7,
Lane No.5- 60.2, Lane No.6-60.8, Lane No.7-61.2, Lane No.8- 61.8,
Lane No.9- 62.3, **Lane No.10-62.7**, Lane No.11- 62.9, Lane No.12- 63.0

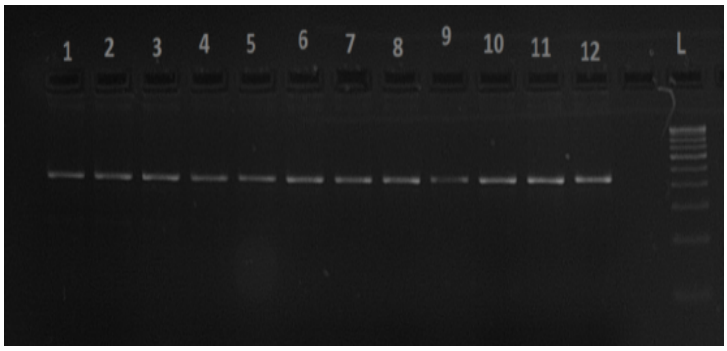


Fig.5.19 Agarose gel electrophoresis results of gradient PCR of EXON-2A

L- 100bp Marker, Lane No. 1 – 12 PCR product from the different temperatures in degree Celsius.

T_m=54.5.0, G= ±0.5

Lane No.1- 54.0, Lane No.2-54.0, Lane No.3-54.1, Lane No.4- 54.2,
Lane No.5- 54.3, Lane No.6-54.4, Lane No.7-54.6, Lane No.8- 54.7,
Lane No.9- 54.8, Lane No.10-54.9, **Lane No.11- 55.0,** Lane No.12- 55.0

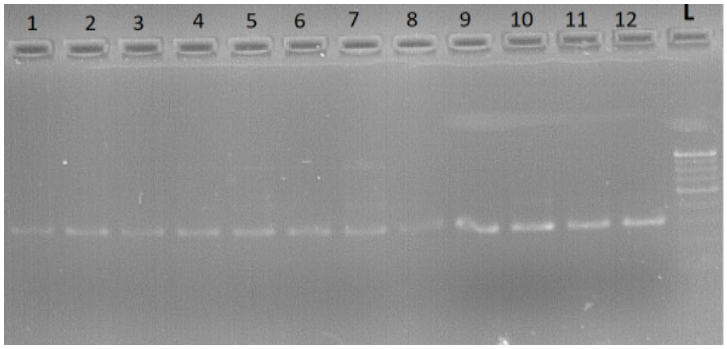


Fig.5.20 Agarose gel electrophoresis results of gradient PCR of Exon-2B

L- 100bp Marker, Lane No. 1 – 12 PCR product from the different temperatures in degree Celsius.

T_m=54.5.0,G= ±0.5

Lane No.1- 54.0,	Lane No.2-54.0,	Lane No.3-54.1,	Lane No.4- 54.2,
Lane No.5- 54.3,	Lane No.6-54.4,	Lane No.7-54.6,	Lane No.8- 54.7,
Lane No.9- 54.8,	**Lane No.10-54.9,**	Lane No.11- 55.0,	Lane No.12- 55.0

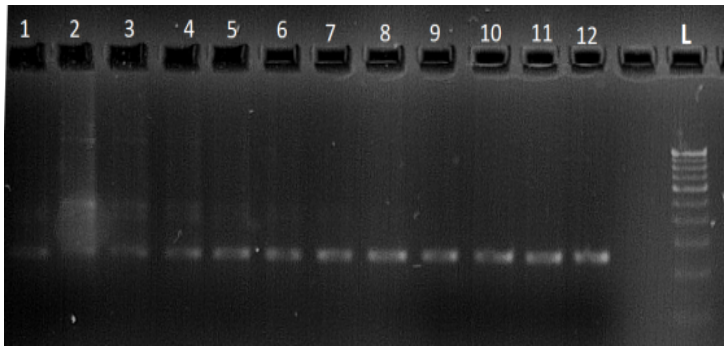


Fig.5.21 Agarose gel electrophoresis results of gradient PCR of EXON-3

L- 100bp Marker, Lane No. 1 – 12 PCR product from the different temperatures in degree Celsius.

T_m=54.5.0, G= ±0.5

Lane No.1- 54.0,	Lane No.2-54.0,	Lane No.3-54.1,	Lane No.4- 54.2,
Lane No.5- 54.3,	Lane No.6-54.4,	Lane No.7-54.6,	Lane No.8- 54.7,
Lane No.9- 54.8,	Lane No.10-54.9,	Lane No.11- 55.0,	**Lane No.12- 55.0**

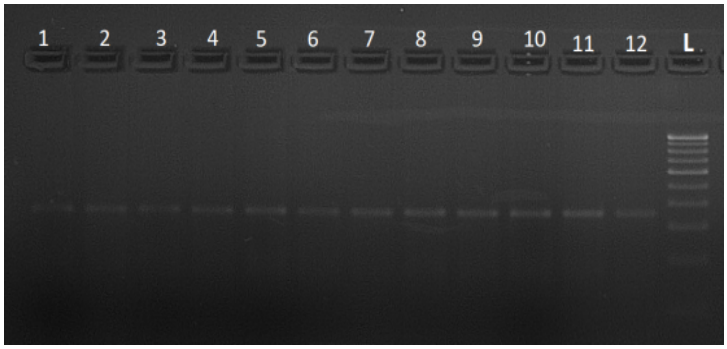


Fig.5.22 Agarose gel electrophoresis results of gradient PCR of EXON-4

L- 100bp Marker, Lane No. 1 – 12 PCR product from the different temperatures in degree Celsius.

T_m=54.8, G= ±2.0

Lane No.1- 54.2,	Lane No.2-54.3,	Lane No.3-54.4,	Lane No.4- 54.5,
Lane No.5- 54.7,	Lane No.6-54.8,	Lane No.7-55.0,	Lane No.8- 55.1,
Lane No.9- 55.3,	Lane No.10-55.4,	Lane No.11- 55.5,	Lane No.12- 55.6

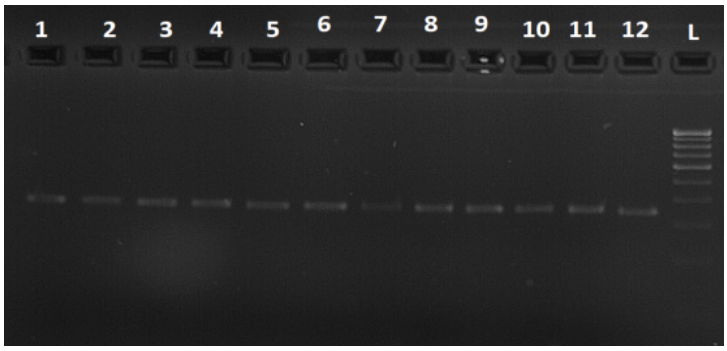


Fig.5.23 Agarose gel electrophoresis results of gradient PCR of EXON-5

L- 100bp Marker, Lane No. 1 – 12 PCR product from the different temperatures in degree Celsius.

T_m=53.5, G= ±2.0

Lane No.1- 51.5,	Lane No.2-51.6,	Lane No.3-51.8,	Lane No.4- 52.2,
Lane No.5- 52.7,	Lane No.6-53.3,	Lane No.7-53.7,	Lane No.8- 54.3,
Lane No.9- 54.8,	Lane No.10-55.2,	Lane No.11- 55.4,	Lane No.12- 55.5

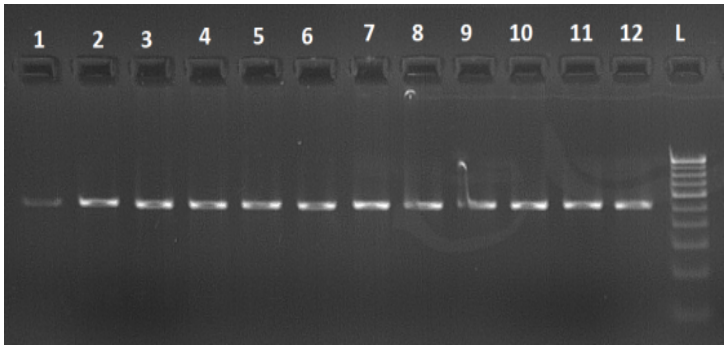


Fig.5.24 Agarose gel electrophoresis results of gradient PCR of EXON-6A

L- 100bp Marker, Lane No. 1 – 12 PCR product from the different temperatures in degree Celsius.

T_m=55.0, G= ±2.0

Lane No.1- 53.0,	Lane No.2-53.1,	Lane No.3-53.3,	Lane No.4- 53.7
Lane No.5- 54.2,	Lane No.6-54.8,	**Lane No.7-55.2,**	Lane No.8- 55.8,
Lane No.9- 56.3,	Lane No.10-56.7,	Lane No.11- 56.9,	Lane No.12- 57.0

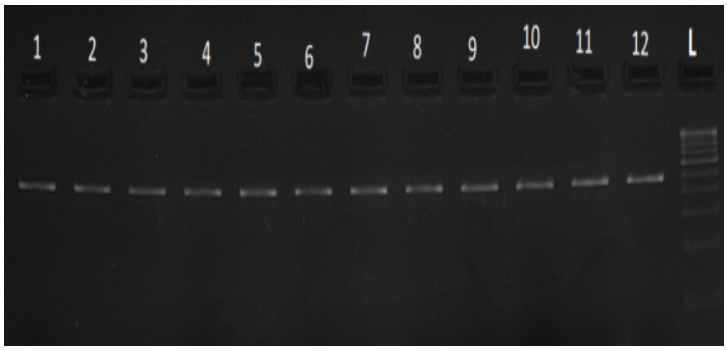


Fig.5.25 Agarose gel electrophoresis results of gradient PCR of Exon-6B

L- 100bp Marker, Lane No. 1 – 12 PCR product from the different temperatures in degree Celsius.

T_m=56.0, G= ±2.5

Lane No.1- 53.7,	Lane No.2-53.9,	Lane No.3-54.1,	Lane No.4- 54.6,
Lane No.5- 55.2,	Lane No.6-55.7,	**Lane No.7-56.3,**	Lane No.8- 56.8,
Lane No.9- 57.4,	Lane No.10-57.9,	Lane No.11- 58.1,	Lane No.12- 58.3

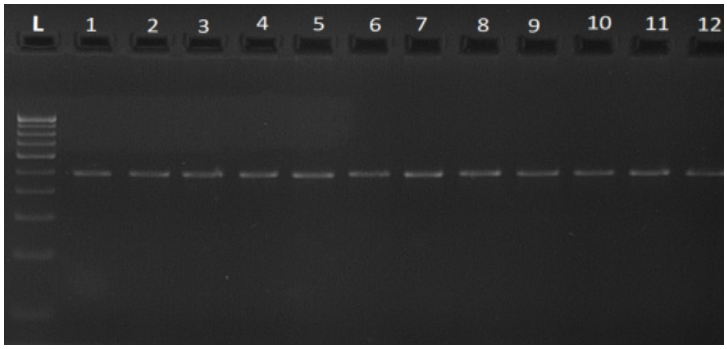


Fig.5.26 Agarose gel electrophoresis results of gradient PCR of EXON-7A

L- 100bp Marker, Lane No. 1 – 12 PCR product from the different temperatures in degree Celsius.

T_m=53.5, G= ±2.0

Lane No.1- 51.5, Lane No.2-51.6, Lane No.3-51.8, Lane No.4- 52.2,
Lane No.5- 52.7, Lane No.6-53.3, Lane No.7-53.7, **Lane No.8- 54.3,**
Lane No.9- 54.8, Lane No.10-55.2, Lane No.11- 55.4, Lane No.12- 55.5

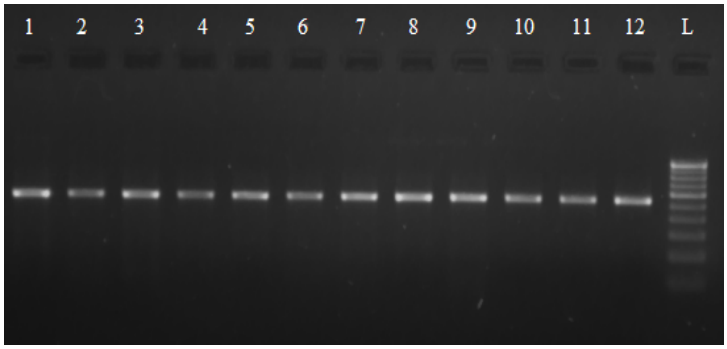


Fig.5.27 Agarose gel electrophoresis results of gradient PCR of EXON-7B

L- 100bp Marker, Lane No. 1 – 12 PCR product from the different temperatures in degree Celsius.

T_m=53.5, G= ±2.0

Lane No.1- 51.5, Lane No.2-51.6, Lane No.3-51.8, Lane No.4- 52.2,
Lane No.5- 52.7, **Lane No.6-53.3,** Lane No.7-53.7, Lane No.8- 54.3,
Lane No.9- 54.8, Lane No.10-55.2, Lane No.11- 55.4, Lane No.12- 55.5

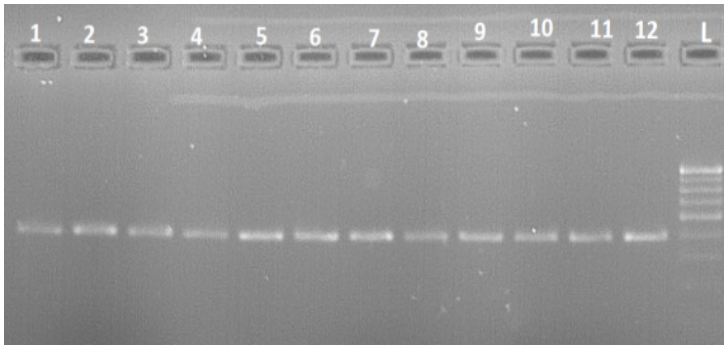


Fig.5.28 Agarose gel electrophoresis results of gradient PCR of EXON-7C

L- 100bp Marker, Lane No. 1 – 12 PCR product from the different temperatures in degree Celsius.

T_m=57.2, G= ±2.0

Lane No.1- 55.9,	Lane No.2-56.0,	Lane No.3-56.2,	Lane No.4- 56.4,
Lane No.5- 56.7,	**Lane No.6-57.0,**	Lane No.7-57.4,	Lane No.8- 57.7,
Lane No.9- 58.0,	Lane No.10-58.2,	Lane No.11- 58.4,	Lane No.12- 58.5

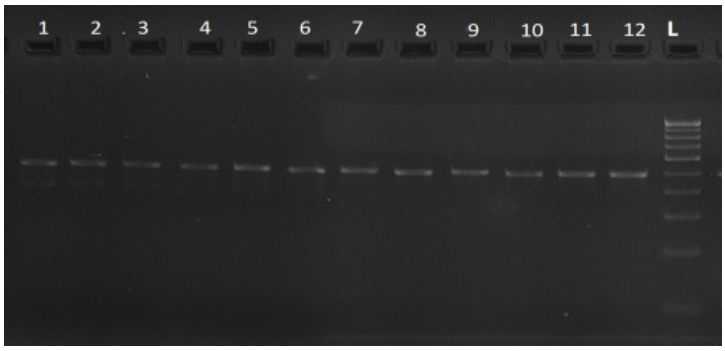


Fig.5.29 Agarose gel electrophoresis results of gradient PCR of EXON-7D

L- 100bp Marker, Lane No. 1 – 12 PCR product from the different temperatures in degree Celsius.

T_m=53.5, G= ±2.0

Lane No.1- 51.5,	Lane No.2-51.6,	Lane No.3-51.8,	Lane No.4- 52.2,
Lane No.5- 52.7,	Lane No.6-53.3,	Lane No.7-53.7,	Lane No.8- 54.3,
Lane No.9- 54.8,	Lane No.10-55.2,	Lane No.11- 55.4,	Lane No.12- 55.5

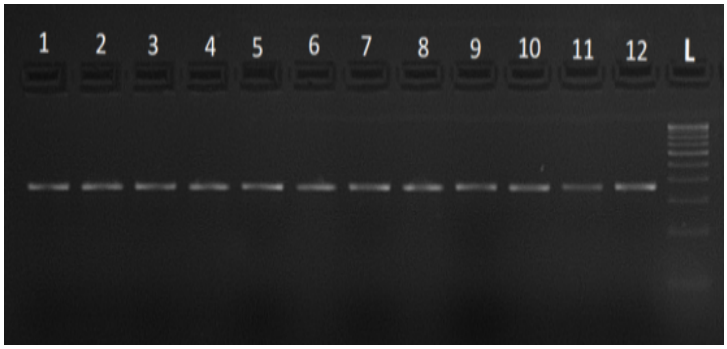


Fig.5.30 Agarose gel electrophoresis results of gradient PCR of EXON-7E

L- 100bp Marker, Lane No. 1 – 12 PCR product from the different temperatures in degree Celsius.

T_m=56.0, G= ±2.5

Lane No.1- 53.7,	Lane No.2-53.9,	Lane No.3-54.1,	Lane No.4- 54.6,
Lane No.5- 55.2,	Lane No.6-55.7,	Lane No.7-56.3,	Lane No.8- 56.8,
Lane No.9- 57.4,	**Lane No.10-57.9,**	Lane No.11- 58.1,	Lane No.12- 58.3

5.3.3.2 PCR standardisation of *NLGN4X*

In the present study, a total of 15 sets of primers were designed for 7 exons of the *NLGN4X* gene. Standardised annealing temperature and amplicon size for designed primers (Oligos) of *NLGN4X* gene were summarised in Table.5.8

Table 5.8 Standardised annealing temperature and amplicon size for designed primers of *NLGN4X* gene

Exon	Primer Id	Sequence	Product size	Tm
Exon1	NL4X-EX1-F	CCGACTCCGGAGATCTATTG	538bp	55.9
	NL4X-EX1-R	GTGAGGCTTTCCATCCTTTG		
Exon 2	NL4X-EX2A-F	GAGCCAGCCAGTGTTCTAGG	420BP	55.3
	NL4X-EX2A-R	GGGTGAACAACAAAGGAAGC		
	NL4X-EX2B-F	GGATGTGGATGCAGATTTGA	536BP	53.7
	NL4X-EX2B-R	TGCACAAGAGGTATTGTTTTCTG		
Exon 3	NL4X-EX3-F	TGCTTAATTCAAGTCAAAATAGGG	358BP	52.6
	NL4X-EX3-R	AACAGGATTCAAATCCACGAG		
Exon 4	NL4X-EX4-F	GAGAGAATGAAGAAGACAAGCTATGA	379BP	58.5
	NL4X-EX4-R	AACCAGGACATGCATCTGAG		
Exon 5	NL4X-EX5A-F	TCACAGCCTTCATTGCTCAG	520BP	51.8
	NL4X-EX5A-R	CGCAAAGTGTCTTTCCCTTC		
	NL4X-EX5B-F	GCCCAACGACTTTGACTTCT		54

	NL4X-EX5B-R	GGACACAAACAAGTGGCAAG	482BP	
Exon 6	NL4X-EX6A-F	CACGTCACATGTGGAAGAGT	528BP	56.5
	NL4X-EX6A-R	GCTAAGATGTTGAGGAAGAGGA		
	NL4X-EX6B-F	TCCACCGAATTAAGTGTCACC	550BP	55.9
	NL4X-EX6B-R	CATTCCTGGTCTGGAGACTTTC		
	NL4X-EX6C-F	TCCTATCCCTCTGCCCTACC	536BP	57.0
	NL4X-EX6C-R	ATTTCGCTGCACAGTCCATT		
	NL4X-EX6D-F	GCACATGGAGCTGTAATCCA	526BP	57.0
	NL4X-EX6D-R	AAGTTTTTCAAAACAGTGGTCTCC		
	NL4X-EX6E-F	CGGTCTGTGTGGCCTATTTC	557BP	54.9
	NL4X-EX6E-R	TGACCGGATACACAAATCCA		
	NL4X-EX6F-F	TTTTATATCATTTATGGGATCAAACAT	537BP	54.3
	NL4X-EX6F-R	TCACTATAGTTTGAGTGTAGGGATTCA		
	NL4X-EX6G-F	CAGCTGCCTGGCTCTTTT	543BP	53.2
	NL4X-EX6G-R	AAAGCTAAAGTTATTCACTTAACAGGA		
	NL4X-EX6H-F	AGTTGTCTCCTGCTAGCAATATGT	544BP	55.0
	NL4X-EX6H-R	CAAAGAAAAAGACATTCAAAGAACA		

Agarose gel electrophoresis of gradient PCR products of *NLGN4X* was electrophoresed on 2-2.5% agarose gel with respect to amplicon size. The details are repersented in fig.5.31 to fig.5.45

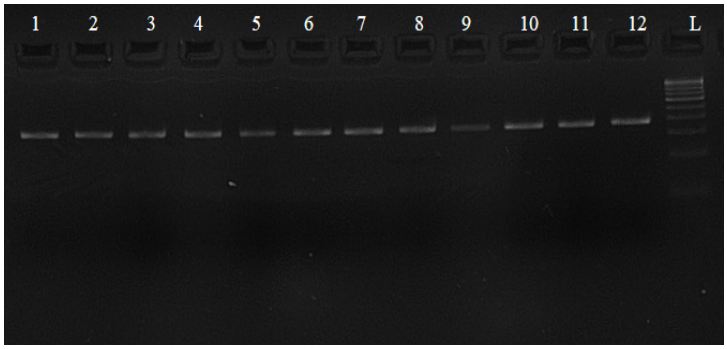


Fig.5.31 Agarose gel electrophoresis results of gradient PCR of EXON-1

L- 100bp Marker, Lane No. 1 – 12 PCR product from the different temperatures in degree Celsius.

T_m=54.0, G= ±2.0

Lane No.1- 52.0, Lane No.2-52.1, Lane No.3-52.3, Lane No.4- 52.7,
Lane No.5- 53.2, Lane No.6-53.8, Lane No.7-54.2, Lane No.8- 54.8,
Lane No.9- 55.3, Lane No.10-55.7, **Lane No.11- 55.9,** Lane No.12- 56.0

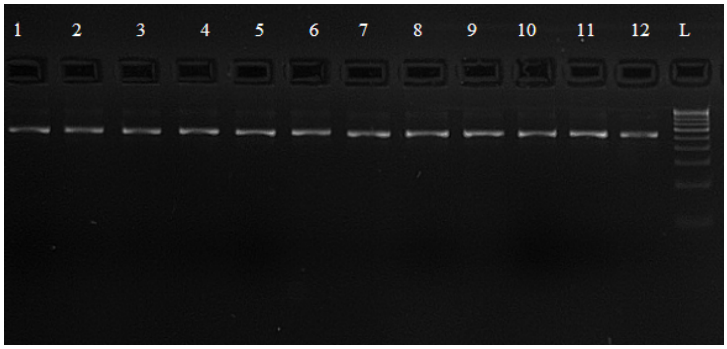


Fig.5.32 Agarose gel electrophoresis results of gradient PCR of EXON-2A

L- 100bp Marker, Lane No. 1 – 12 PCR product from the different temperatures in degree Celsius.

T_m=54.0, G= ±2.0

Lane No.1- 52.0, Lane No.2-52.1, Lane No.3-52.3, Lane No.4- 52.7,
Lane No.5- 53.2, Lane No.6-53.8, Lane No.7-54.2, Lane No.8- 54.8,
Lane No.9- 55.3, Lane No.10-55.7, Lane No.11- 55.9, Lane No.12- 56.0

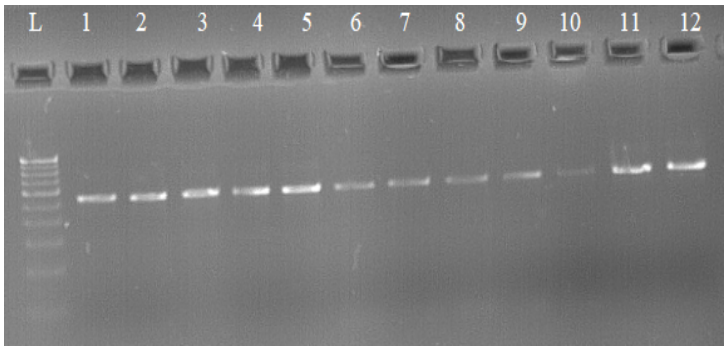


Fig.5.33 Agarose gel electrophoresis results of gradient PCR of EXON 2B

L- 100bp Marker, Lane No. 1 – 12 PCR product from the different temperatures in degree Celsius.

T_m=51.8, G= ±2.5

Lane No.1- 51.0, Lane No.2-51.0, Lane No.3-51.3, Lane No.4- 51.7,
Lane No.5- 52.2, Lane No.6-52.3, Lane No.7-53.2, **Lane No.8- 53.8,**
Lane No.9- 54.3, Lane No.10-54.7, Lane No.11- 54.9, Lane No.12- 55.0

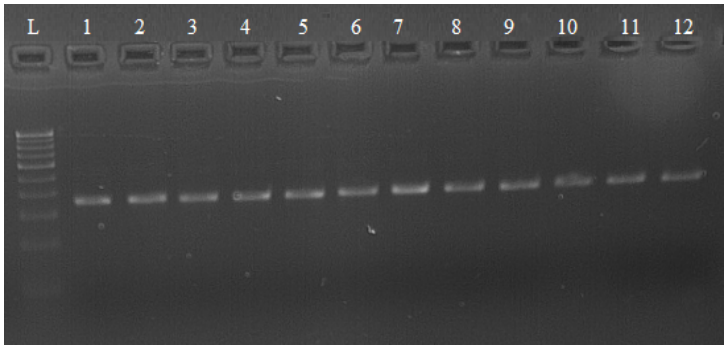


Fig.5.34 Agarose gel electrophoresis results of gradient PCR of EXON-3

L- 100bp Marker, Lane No. 1 – 12 PCR product from the different temperatures in degree Celsius.

T_m=51.8, G= ±2.0

Lane No.1- 49.5,	Lane No.2- 49.7,	Lane No.3-49.9,	Lane No.4- 50.4,
Lane No.5- 51.0,	Lane No.6-51.5,	Lane No.7-52.1,	**Lane No.8- 52.6,**
Lane No.9- 53.2,	Lane No.10-53.7,	Lane No.11- 53.9,	Lane No.12- 54.1

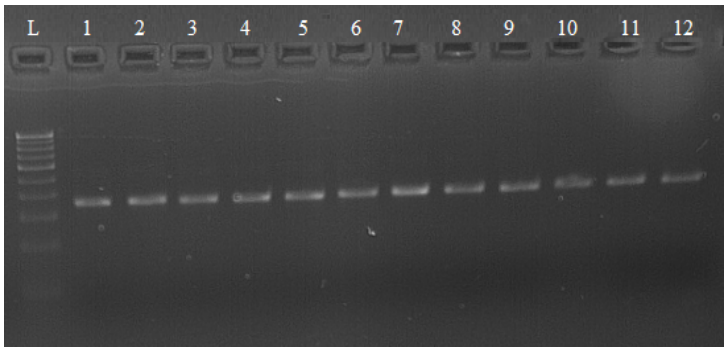


Fig.5.35 Agarose gel electrophoresis results of gradient PCR of EXON-4

L- 100bp Marker, Lane No. 1 – 12 PCR product from the different temperatures in degree Celsius.

T_m=58.2, G= ±2.0

Lane No.1- 56.9,	Lane No.2-57.2,	Lane No.3-57.4,	Lane No.4- 57.6,
Lane No.5- 57.7,	Lane No.6-58.0,	**Lane No.7-58.5,**	Lane No.8- 58.7,
Lane No.9- 59.0,	Lane No.10-59.2,	Lane No.11- 59.4,	Lane No.12- 59.5

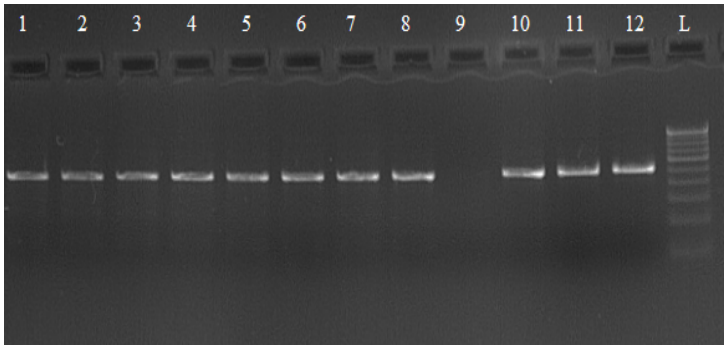


Fig.5.36 Agarose gel electrophoresis results of gradient PCR of EXON-5A

L- 100bp Marker, Lane No. 1 – 12 PCR product from the different temperatures in degree Celsius.

T_m=52.0, G= ±2.0

Lane No.1- 50.0,	Lane No.2-50.1,	Lane No.3-50.3,	Lane No.4- 50.7,
Lane No.5- 51.2,	**Lane No.6-51.8,**	Lane No.7-52.2,	Lane No.8- 52.8,
Lane No.9- 53.3,	Lane No.10-53.7,	Lane No.11- 53.9,	Lane No.12- 54.0

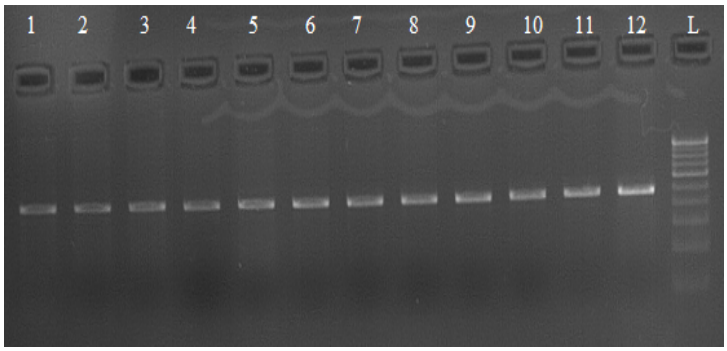


Fig.5.37 Agarose gel electrophoresis results of gradient PCR of EXON-5B

L- 100bp Marker, Lane No. 1 – 12 PCR product from the different temperatures in degree Celsius.

T_m=52.0, G= ±2.0

Lane No.1- 50.0,	Lane No.2-50.1,	Lane No.3-50.3,	Lane No.4- 50.7,
Lane No.5- 51.2,	**Lane No.6-51.8,**	Lane No.7-52.2,	Lane No.8- 52.8,
Lane No.9- 53.3,	Lane No.10-53.7,	Lane No.11- 53.9,	Lane No.12- 54.0

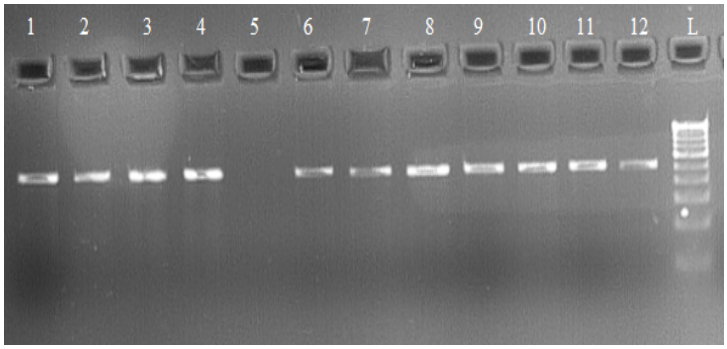


Fig.5.38 Agarose gel electrophoresis results of gradient PCR of EXON-6A

L- 100bp Marker, Lane No. 1 – 12 PCR product from the different temperatures in degree Celsius.

T_m=55.7, G= ±1.3

Lane No.1- 54.4, Lane No.2-54.5, Lane No.3-54.6, Lane No.4- 54.9,
Lane No.5- 55.2, Lane No.6-55.5, Lane No.7-55.9, Lane No.8- 56.2,
Lane No.9- 56.5, Lane No.10-56.8, Lane No.11- 56.9, Lane No.12- 57.0

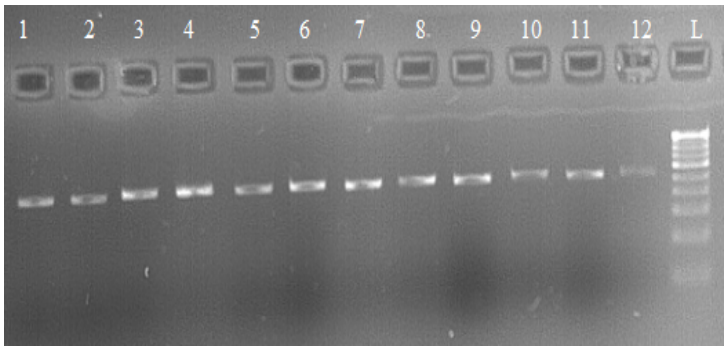


Fig.5.39 Agarose gel electrophoresis results of gradient PCR of EXON-6B

L- 100bp Marker, Lane No. 1 – 12 PCR product from the different temperatures in degree Celsius.

T_m=55.7, G= ±1.3

Lane No.1- 54.4, Lane No.2-54.5, Lane No.3-54.6, Lane No.4- 54.9,
Lane No.5- 55.2, Lane No.6-55.5, **Lane No.7-55.9**, Lane No.8- 56.2,
Lane No.9- 56.5, Lane No.10-56.8, Lane No.11- 56.9, Lane No.12- 57.0

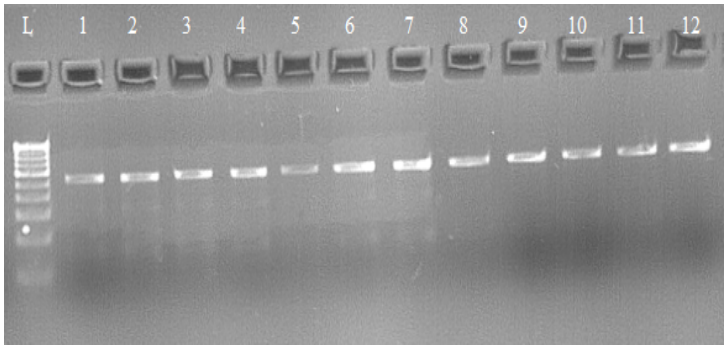


Fig.5.40 Agarose gel electrophoresis results of gradient PCR of EXON-6C

L- 100bp Marker, Lane No. 1 – 12 PCR product from the different temperatures in degree Celsius.

T_m=55.7, G= ±1.3

Lane No.1- 54.4,	Lane No.2-54.5,	Lane No.3-54.6,	Lane No.4- 54.9,
Lane No.5- 55.2,	Lane No.6-55.5,	Lane No.7-55.9,	Lane No.8- 56.2,
Lane No.9- 56.5,	Lane No.10-56.8,	Lane No.11- 56.9,	**Lane No.12- 57.0**

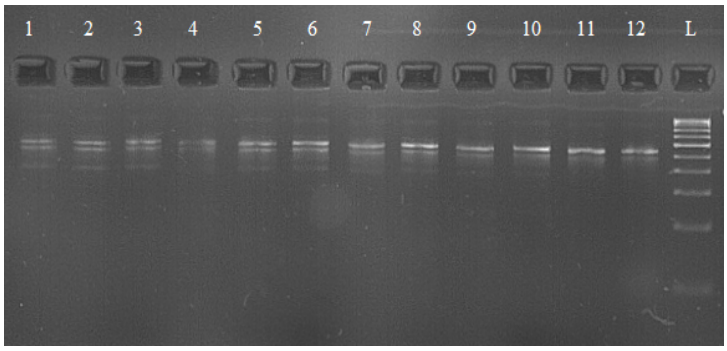


Fig.5.41 Agarose gel electrophoresis results of gradient PCR of EXON-6D

L- 100bp Marker, Lane No. 1 – 12 PCR product from the different temperatures in degree Celsius.

T_m=55.7, G= ±1.3

Lane No.1- 54.4,	Lane No.2-54.5,	Lane No.3-54.6,	Lane No.4- 54.9,
Lane No.5- 55.2,	Lane No.6-55.5,	Lane No.7-55.9,	Lane No.8- 56.2,
Lane No.9- 56.5,	Lane No.10-56.8,	Lane No.11- 56.9,	**Lane No.12- 57.0**

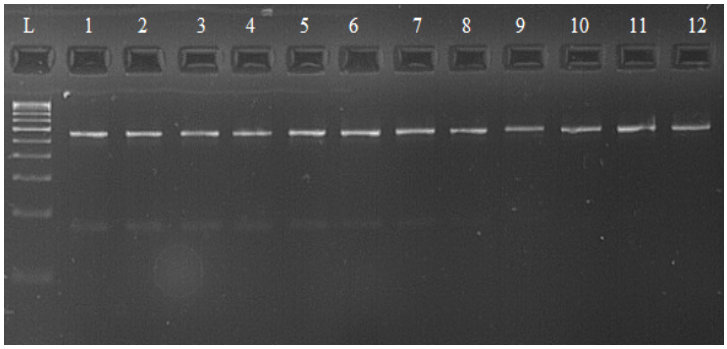


Fig.5.42 Agarose gel electrophoresis results of gradient PCR of EXON-6E

L- 100bp Marker, Lane No. 1 – 12 PCR product from the different temperatures in degree Celsius.

T_m=55.0, G= ±1.0

Lane No.1- 54.0,	Lane No.2-54.1,	Lane No.3-54.2,	Lane No.4- 54.4,
Lane No.5- 54.6,	**Lane No.6-54.9,**	Lane No.7-55.1,	Lane No.8- 55.4,
Lane No.9- 55.6,	Lane No.10-55.8,	Lane No.11- 55.9,	Lane No.12- 56.0

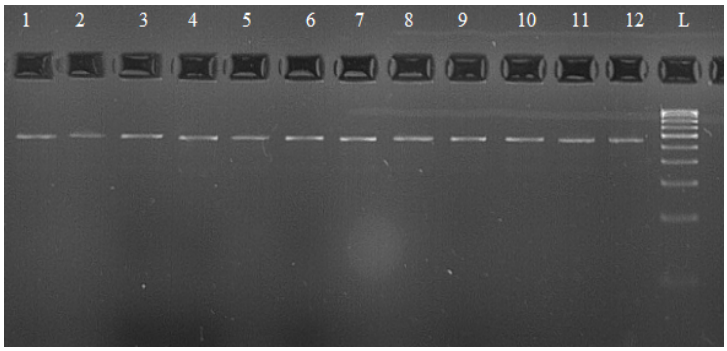


Fig.5.43 Agarose gel electrophoresis results of gradient PCR of EXON-6F

L- 100bp Marker, Lane No. 1 – 12 PCR product from the different temperatures in degree Celsius.

T_m=53.0, G= ±2.0

Lane No.1- 51.0,	Lane No.2-51.1,	Lane No.3-51.3,	Lane No.4- 51.7,
Lane No.5- 52.2,	Lane No.6-52.8,	Lane No.7-53.2,	Lane No.8- 53.8,
Lane No.9- 54.3,	Lane No.10-54.7,	Lane No.11- 54.9,	Lane No.12- 55.0

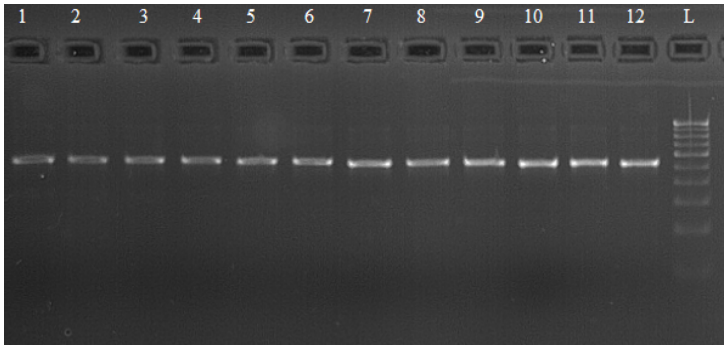


Fig.5.44 Agarose gel electrophoresis results of gradient PCR of EXON-6G

L- 100bp Marker, Lane No. 1 – 12 PCR product from the different temperatures in degree Celsius.

T_m=51.8, G= ±2.0

Lane No.1- 49.5,	Lane No.2- 49.7,	Lane No.3-49.9,	Lane No.4- 50.4,
Lane No.5- 51.0,	Lane No.6-51.5,	Lane No.7-52.1,	Lane No.8- 52.6,
Lane No.9- 53.2,	Lane No.10-53.7,	Lane No.11- 53.9,	Lane No.12- 54.1

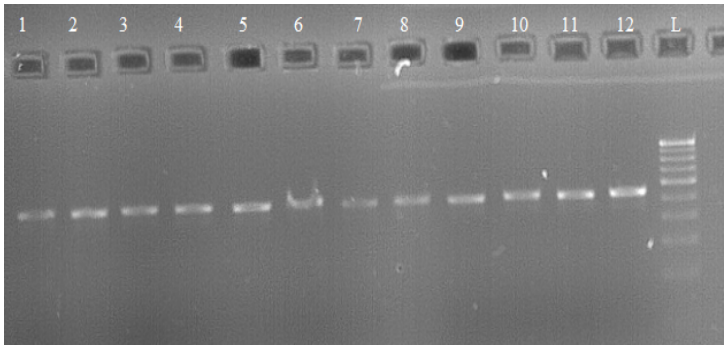


Fig.5.45 Agarose gel electrophoresis results of gradient PCR of EXON-6H

L- 100bp Marker, Lane No. 1 – 12 PCR product from the different temperatures in degree Celsius.

T_m=51.8, G= ±2.0

Lane No.1- 49.5,	Lane No.2- 49.7,	Lane No.3-49.9,	Lane No.4- 50.4,
Lane No.5- 51.0,	Lane No.6-51.5,	Lane No.7-52.1,	Lane No.8- 52.6,
Lane No.9- 53.2,	Lane No.10-53.7,	Lane No.11- 53.9,	Lane No.12- 54.1

5.3.3.3 *PCR* standardisation of *NLGN4Y*

In the present study, a total of 15 sets of primers were designed for 7 exons of the *NLGN4Y* gene. Standardised annealing temperature and amplicon size for designed primers (Oligos) of *NLGN4Y* gene were summarised in Table 5.9

Table.5.9 Standardised annealing temperature and amplicon size for designed primers of *NLGN4Y* gene

Exon	Primer Id	Sequence	Product size	Tm
Exon 1	NL4Y-EX1-F	GCAAACTGTGAGCATGGAAG	471BP	59.1
	NL4Y-EX1-R	TTCCGAAGAAGCAAAACCAC		
Exon 2	NL4Y-EX2A-F	TTGTGGATTTCATGGAAGAGC	404BP	54.1
	NL4Y-EX2A-R	TGGACCCAAGATCTCACTGG		
	NL4Y-EX2B-F	CAATGTTCTTCTGTGGATAACTGC	471BP	54.1
	NL4Y-EX2B-R	CCCTCCTGGTAAATCTCTCCA		
Exon 3	NL4Y-EX3-F	TTCAAGTCAAAATAAGGAAATCAGG	384BP	51.0
	NL4Y-EX3-R	CGTAAAGTGGAATTAACCAGGAA		
Exon 4	NL4Y-EX4-F	GGACAGGGTTGTGGAGAGAG	409BP	56.4
	NL4Y-EX4-R	CCACAGTGACTTCTGGCAAC		
Exon 5	NL4Y-EX5A-F	CCAGAAATTCACATGCTTGC	526BP	52.4
	NL4Y-EX5A-R	CAGGGTAGCCGTAAAGGTTG		
	NL4Y-EX5B-F	GCCCAACGACTTTGACTTCT	532BP	54.9
	NL4Y-EX5B-R	GCATGCATGTGTGCTCCT		
Exon 6	NL4Y-EX6A-F	TGAAGAGCAGATTGTAACTTCCTG	542BP	55.4
	NL4Y-EX6A-R	GACGGCAATGGTGACACTTA		
	NL4Y-EX6B-F	TCCTCATTGAAACCAAACGA	558BP	51.7
	NL4Y-EX6B-R	CATTCCTGGTTTGGAGATTTTC		
	NL4Y-EX6C-F	CCCCTACTGCTCAGCAATGT	549BP	57.2
	NL4Y-EX6C-R	TTCAGGCTGGCAGAATACCT		
	NL4Y-EX6D-F	GCGAATTGACTGTGCAGAAA	548BP	51.0
	NL4Y-EX6D-R	TTTCTCTGCTGATTCTTTGTGTG		
	NL4Y-EX6E-F	CACACACACACAGACACACACA	538BP	54.2
	NL4Y-EX6E-R	TCTCTGATCCCCAAGTAACCA		
	NL4Y-EX6F-F	TTCACTGACAAGACACTGAATGG	547BP	55.1
	NL4Y-EX6F-R	GAAATGTTGCTTCATGTGTGC		
	NL4Y-EX6G-F	CTGTTGCTTATGTTGGGTTAAAT	508BP	53.5
	NL4Y-EX6G-R	GCAAGTATATTTTTCTCATGGAACTC		
	NL4Y-EX6H-F	TCTTACATGATATCTCATTTCTACGTG	441BP	57.4
	NL4Y-EX6H-R	CCATTTGTAGCCTATGTGAGAGC		

Agarose gel electrophoresis of gradient PCR products of *NLGN4X* was electrophoresed on 2-2.5% agarose gel with respect to amplicon size. The details are repersented in fig.5.46 to fig.5.60

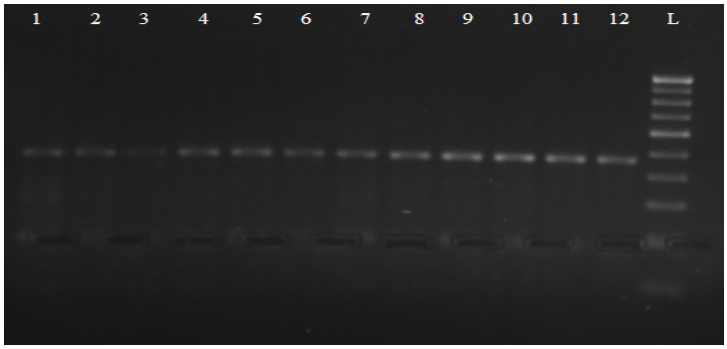


Fig.5.46 Agarose gel electrophoresis results of gradient PCR of EXON-1

L- 100bp Marker, Lane No. 1 – 12 PCR product from the different temperatures in degree Celsius.

T_m=59.0, G= ±1.1

Lane No.1- 57.9,	Lane No.2- 58.1,	Lane No.3-58.3,	Lane No.4- 58.5,
Lane No.5- 58.7,	Lane No.6-58.9,	**Lane No.7-59.1,**	Lane No.8- 59.3,
Lane No.9- 59.5,	Lane No.10-59.5,	Lane No.11- 59.9,	Lane No.12- 60.1

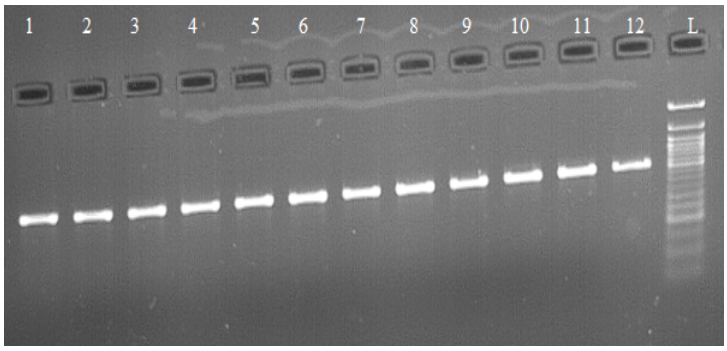


Fig.5.47 Agarose gel electrophoresis results of gradient PCR of EXON-2A

L- 50bp Marker, Lane No. 1 – 12 PCR product from the different temperatures.

T_m=53.0, G= ±2.0

Lane No.1- 51.0,	Lane No.2-51.1,	Lane No.3-51.3,	Lane No.4- 51.7,
Lane No.5- 52.2,	Lane No.6-52.8,	Lane No.7-53.2,	Lane No.8- 53.8,
Lane No.9- 54.1,	Lane No.10-54.5,	Lane No.11- 54.8,	Lane No.12- 55.0

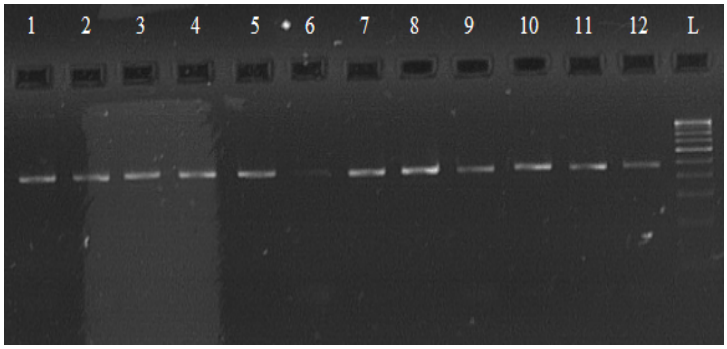


Fig.5.48 Agarose gel electrophoresis results of gradient PCR of EXON-2B

L- 100bp Marker, Lane No. 1 – 12 PCR product from the different temperatures.

T_m=53.0, G= ±2.0

Lane No.1- 52.0, Lane No.2-52.1, Lane No.3-52.3, Lane No.4- 52.5,
Lane No.5- 52.9, Lane No.6-53.3, Lane No.7-53.7 **Lane No.8- 54.1**,
Lane No.9- 54.5, Lane No.10-54.7, Lane No.11- 54.9, Lane No.12- 55.0

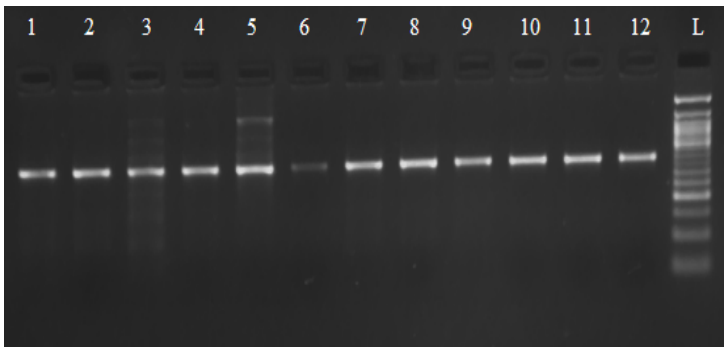


Fig.5.49 Agarose gel electrophoresis results of gradient PCR of EXON-3

L- 50bp Marker, Lane No. 1 – 12 PCR product from the different temperatures.

T_m=53.5, G= ±1.5

Lane No.1- 52.1, Lane No.2-52.2, Lane No.3-52.4, Lane No.4- 52.6,
Lane No.5- 53.0, Lane No.6-53.4, Lane No.7-53.8 Lane No.8- 54.2,
Lane No.9- 54.6, **Lane No.10-54.8,** Lane No.11- 55.0, Lane No.12- 55.1

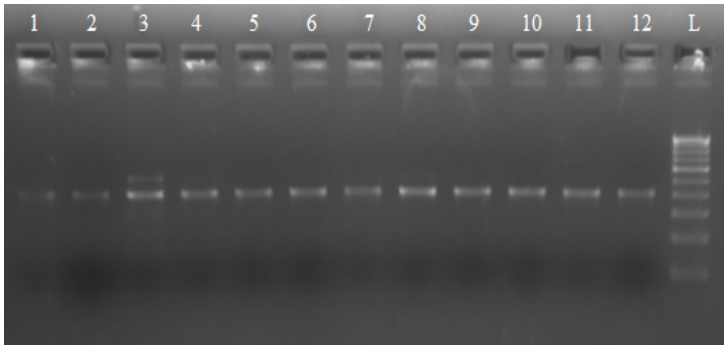


Fig.5.50 Agarose gel electrophoresis results of gradient PCR of EXON-4

L- 100bp Marker, Lane No. 1 – 12 PCR product from the different temperatures in degree Celsius.

T_m=55.7, G= ±1.3

Lane No.1- 54.4, Lane No.2-54.5, Lane No.3-54.6, Lane No.4- 54.9,
Lane No.5- 55.2, Lane No.6-55.5, Lane No.7-55.9, Lane No.8- 56.2,
Lane No.9- 56.4, Lane No.10-56.8, Lane No.11- 56.9, Lane No.12- 57.0

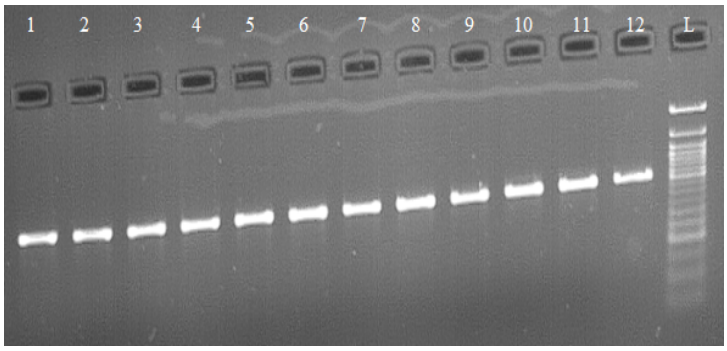


Fig.5.51Agarose gel electrophoresis results of gradient PCR of EXON-5A

L- 50bp Marker, Lane No.1–12 PCR product from the different temperatures in degree Celsius.

T_m=53.0, G= ±2.0

Lane No.1- 51.2, Lane No.2-51.4, Lane No.3-51.6, Lane No.4- 51.8,
Lane No.5- 52.0, Lane No.6-52.2, **Lane No.7-52.4,** Lane No.8- 52.6,
Lane No.9- 52.8, Lane No.10-53.0, Lane No.11- 53.2, Lane No.12- 53.4

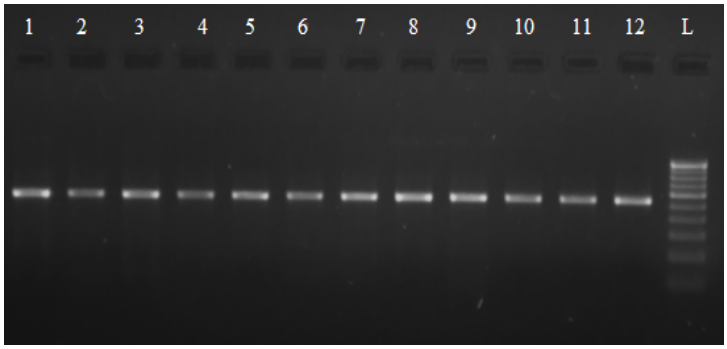


Fig.5.52 Agarose gel electrophoresis results of gradient PCR of EXON-5B

L- 100bp Marker, Lane No. 1 – 12 PCR product from the different temperatures in degree Celsius.

T_m=54.5, G= ±1.0

Lane No.1- 53.5, Lane No.2-53.7, Lane No.3-53.9, Lane No.4- 54.1,
Lane No.5- 54.3, Lane No.6-54.5, Lane No.7-54.7, **Lane No.8- 54.9,**
Lane No.9- 55.1, Lane No.10-55.3, Lane No.11- 55.5, Lane No.12- 55.7

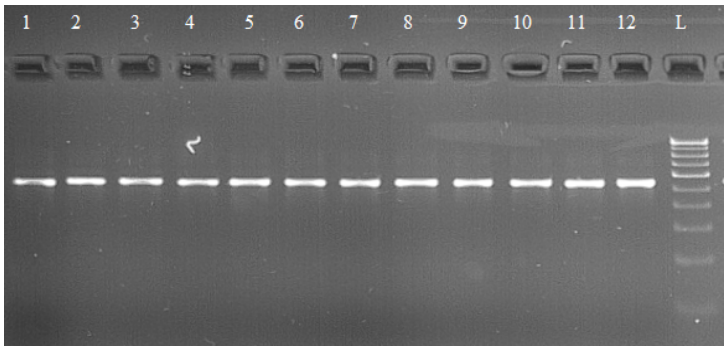


Fig.5.53 Agarose gel electrophoresis results of gradient PCR of EXON-6A

L- 100bp Marker, Lane No. 1 – 12 PCR product from the different temperatures in degree Celsius.

T_m=55.0, G= ±1.0

Lane No.1- 54.0, Lane No.2-54.1, Lane No.3-54.2, LaneNo.4- 54.4,
Lane No.5- 54.6, Lane No.6-54.9, Lane No.7-55.1, Lane No.8- 55.4,
Lane No.9- 55.6, Lane No.10-55.8, Lane No.11- 55.9, Lane No.12- 56.0

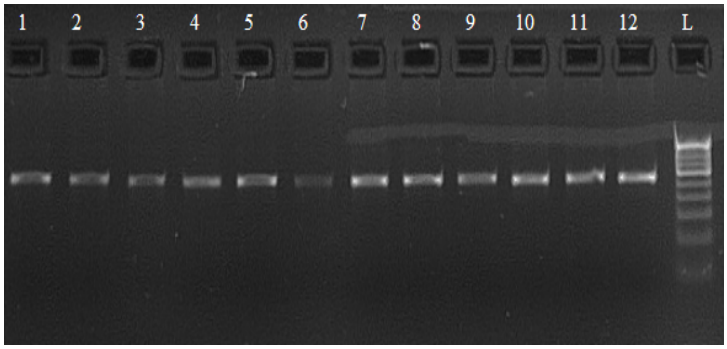


Fig.5.54 Agarose gel electrophoresis results of gradient PCR of EXON-6B

L- 100bp Marker, Lane No. 1 – 12 PCR product from the different temperatures in degree Celsius.

T_m=50.0, G= ±2.0

Lane No.1- 48.0,	Lane No.2- 48.1,	Lane No.3-48.3,	Lane No.4- 48.7,
Lane No.5- 49.2,	Lane No.6-49.8,	Lane No.7-50.2,	Lane No.8- 50.8,
Lane No.9- 51.3,	**Lane No.10-51.7,**	Lane No.11- 51.9,	Lane No.12- 52.0

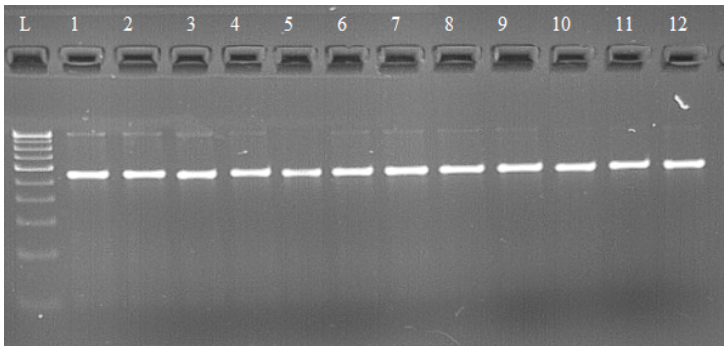


Fig.5.55 Agarose gel electrophoresis results of gradient PCR of EXON-6C

L- 100bp Marker, Lane No. 1 – 12 PCR product from the different temperatures in degree Celsius.

T_m=55.7, G= ±1.3

Lane No.1- 54.4,	Lane No.2-54.5,	Lane No.3-54.6,	Lane No.4- 54.9,
Lane No.5- 55.2,	Lane No.6-55.5,	Lane No.7-55.9,	Lane No.8- 56.2,
Lane No.9- 56.4,	Lane No.10-56.8,	Lane No.11- 56.9,	**Lane No.12- 57.2**

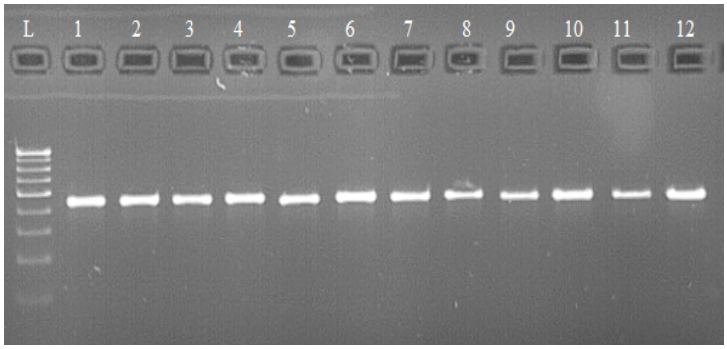


Fig.5.56 Agarose gel electrophoresis results of gradient PCR of EXON-6D

L- 100bp Marker, Lane No. 1 – 12 PCR product from the different temperatures in degree Celsius.

T_m=51.0, G= ±1.0

Lane No.1- 50.0,	Lane No.2- 50.2,	Lane No.3-50.4,	Lane No.4- 50.6,
Lane No.5- 50.8,	**Lane No.6-51.0,**	Lane No.7-51.2,	Lane No.8- 51.4,
Lane No.9- 51.6,	Lane No.10-51.8,	Lane No.11- 52.0,	Lane No.12- 52.0

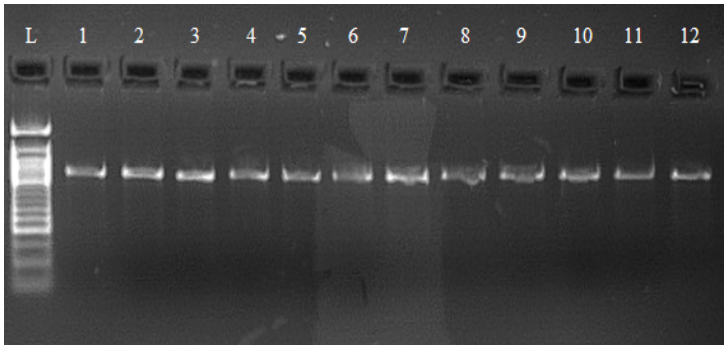


Fig.5.57 Agarose gel electrophoresis results of gradient PCR of EXON-6E

L- 50bp Marker, Lane No. 1 – 12 PCR product from the different temperatures in degree Celsius.

T_m=55.0, G= ±1.0

Lane No.1- 54.0,	Lane No.2-54.1,	**Lane No.3-54.2**,	Lane No.4- 54.4,
Lane No.5- 54.6,	Lane No.6-54.9,	Lane No.7-55.1,	Lane No.8- 55.4,
Lane No.9- 55.6,	Lane No.10-55.8,	Lane No.11- 55.9,	Lane No.12- 56.0

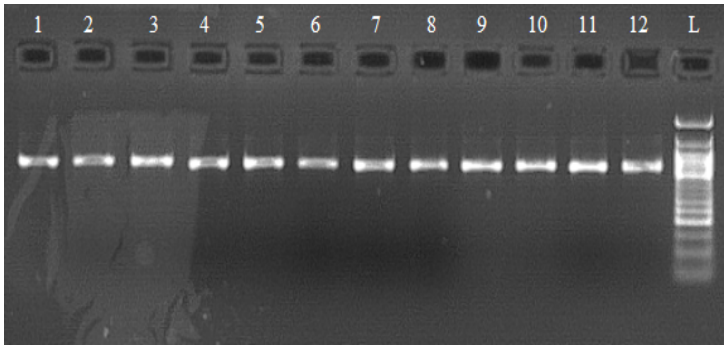


Fig.5.58 Agarose gel electrophoresis results of gradient PCR of EXON-6F

L- 50bp Marker, Lane No. 1 – 12 PCR product from the different temperatures.

T_m=54.0, G= ±1.2

Lane No.1- 54.0,	Lane No.2-54.1,	Lane No.3-54.2,	Lane No.4- 54.4,
Lane No.5- 54.6,	Lane No.6-54.9,	**Lane No.7-55.1,**	Lane No.8- 55.4,
Lane No.9- 55.6,	Lane No.10-55.8,	Lane No.11- 55.9,	Lane No.12- 56.0

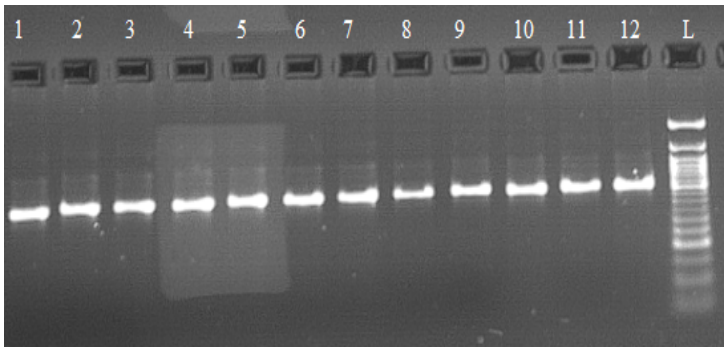


Fig.5.59 Agarose gel electrophoresis results of gradient PCR of EXON-6G

L- 100bp Marker, Lane No. 1 – 12 PCR product from the different temperatures in degree Celsius.

T_m=54.0, G= ±1.0

Lane No.1- 53.0,	Lane No.2-53.1,	Lane No.3-53.3,	**Lane No.4- 53.5,**
Lane No.5- 53.7,	Lane No.6-53.9,	Lane No.7-54.1,	Lane No.8- 54.5,
Lane No.9- 54.7,	Lane No.10-54.9,	Lane No.11- 55.1,	Lane No.12- 55.3

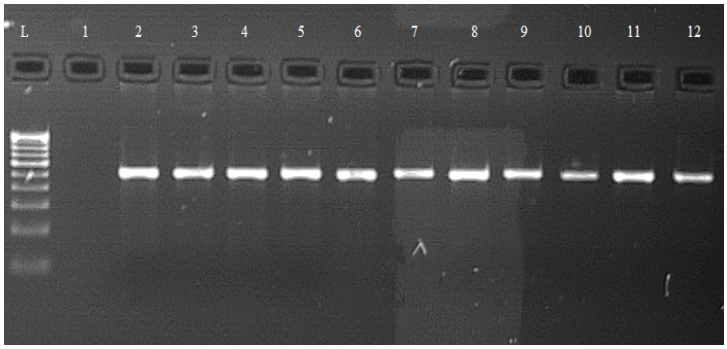


Fig.5.60 Agarose gel electrophoresis results of gradient PCR of EXON-6H

L- 100bp Marker, Lane No. 1 – 12 PCR product from the different temperatures in degree Celsius.

T_m=57.0, G= ±1.5

Lane No.1- 55.9,	Lane No.2-56.0,	Lane No.3-56.2,	Lane No.4- 56.4,
Lane No.5- 56.7,	Lane No.6-57.0,	**Lane No.7-57.4,**	Lane No.8- 57.7,
Lane No.9- 58.0,	Lane No.10-58.2,	Lane No.11- 58.4,	Lane No.12- 58.5

5.3.4 Mutation analysis: After epidemiological analysis, mutation analysis was performed in 108 autistic children.

5.3.4.1. Mutation analysis of *NLGN3*

Genetic analysis of all the exons of the *NLGN3* gene from 108 autistic children revealed, 80% of non-coding sequence variants which included 40% of 5'UTR variant & 3' UTR each followed by 20% of coding sequence variants (missense variant). The same are represented in fig.5.61.

One coding sequence variant and four non-coding sequence variants which included two 5'UTR variant & two 3' UTR variants were observed. The coding sequence variant c.551 T>C (p.V184A) was a missense variant recorded in 27 (25%) autistic children. The mapping of the mutations on the *NLGN3* gene is shown in fig.5.62

Only one autistic child (0.92%) recorded g.5040 C>W5' UTR variant and g.5041 T>A was found in five (4.6%) autistic children. 3'UTR variant g.30370 C>Y was recorded in 75 (69.4%) and g.30349-30350 InsAC was recorded in 21 (19.4%) autistic children. g.5040 C>W and g.30370 C>Y variants were heterozygous. In the present study, 3'UTR variants were recorded in high frequency compared to 5'UTR variants, particularly g.30370 C>Y followed by g.30349-30350InsAC. Interestingly

all the coding and non-coding variants recorded in the present study were novel mutations. Which were not recorded previously recorded in any in-house human SNP databases viz;

dbSNP (https://www.ncbi.nlm.nih.gov/snp/),
1000genomes (https://www.internationalgenome.org/home),
gnomAD (https://gnomad.broadinstitute.org/),
ClinVar (https://www.ncbi.nlm.nih.gov/clinvar/) etc…the same are represented in table.5.10

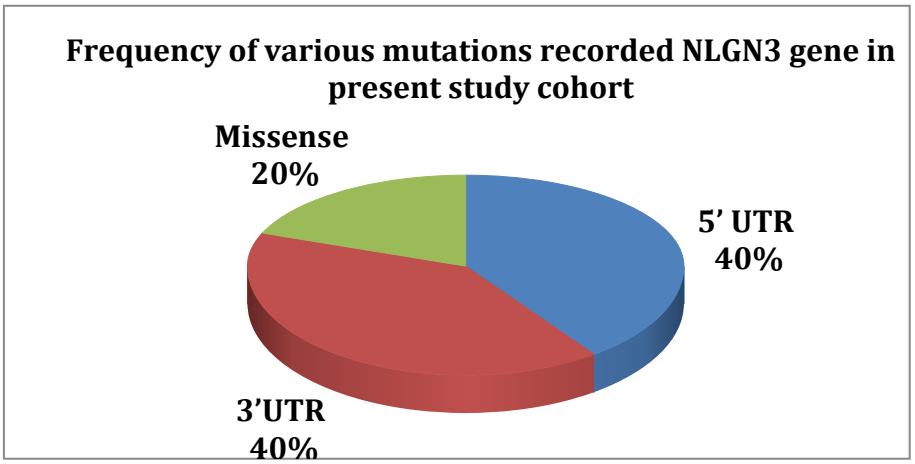


Fig.5.61 Frequency of various mutations recorded in *NLGN3* gene in the present study cohort

Table.5.10 List of variations recorded in *NLGN3* gene in autistic children of our population

Sl. No	Mutation Type	Nucleotide Change	A.A change	Exon	Frequency of autism
1.	5' UTR	g.5040 C>W	……	1	1 (0.92%)
2.	5' UTR	g.5041 T>A	…….	1	5 (4.6%)
3.	Missense	g.15417 (c.551 T>C)	p. V184A	4	27 (25%)
4.	3' UTR	g.30349-30350 Ins AC	…….	7	21 (19.4%)
5.	3' UTR	g.30370 C>Y	…….	7	75 (69.4%)

The NG_015874.1 reference sequence was used for genomic DNA variant nomenclature, the ENST00000374051.7 reference sequence was used for coding region variant nomenclature, and the ENSP00000363163.3 reference sequence was used for protein variant nomenclature. The nomenclature followed the Human Genome Variation Society (HGVS) guidelines.

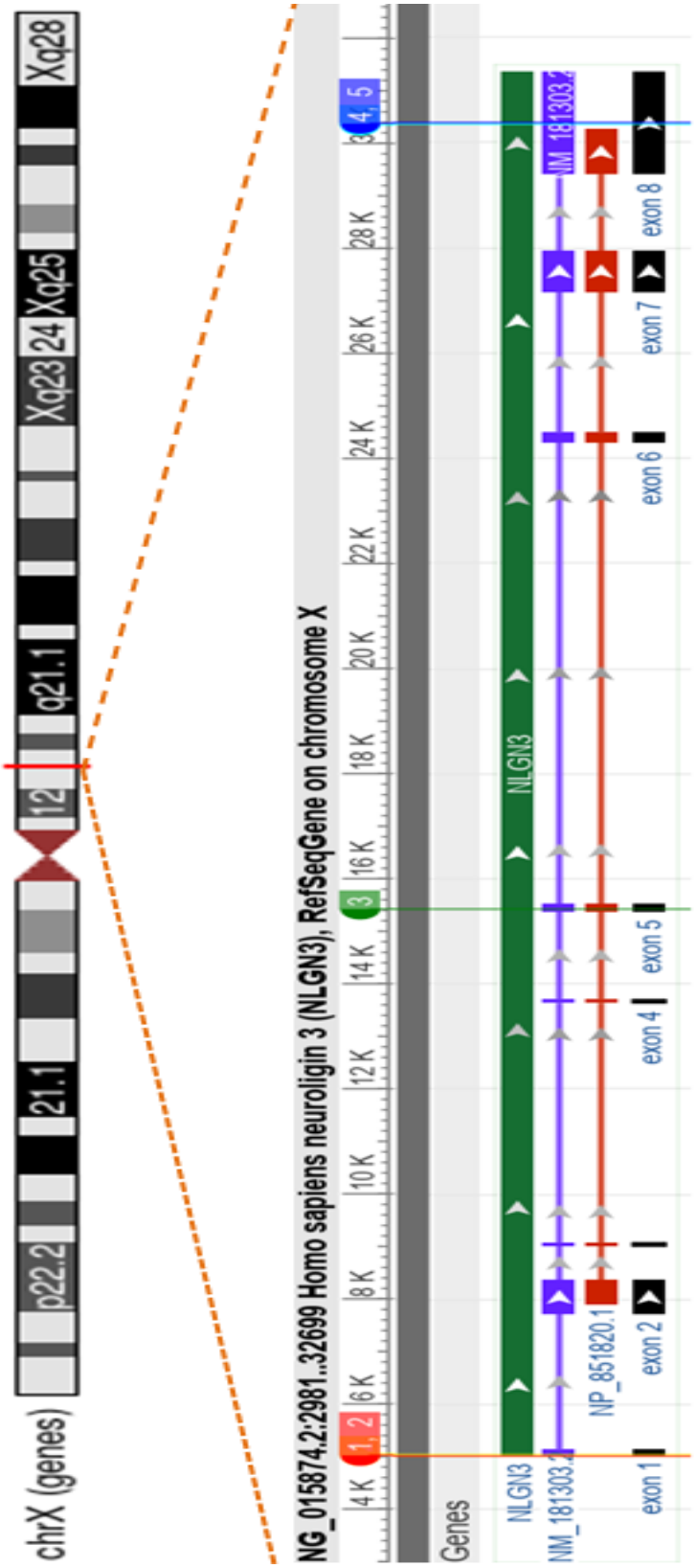


Fig.5.62 Graphical representation of mapping of the mutations on the *NLGN3* gene. The location of mutations is shown in numeric form and details of the respective numbers are explained in table.5.10

5.3.4.1.2 Pathogenicity prediction of nsSNPs

Insilco pathogenicity prediction of novel missense variant; c.551 T>C (p.V184A) was done to evaluate the deleterious/harmful effect on the functions of NLGN3 protein by PolyPhen2, PROVEAN, PANTHER, SNP&GO, PHD-SNP and SNAP2.

- PROVEAN prediction tool which provided the score of -3.094 indicates deleterious effect on the function of NLGN3 protein ("Deleterious" if the prediction score was </ -2.5, "Neutral" if the prediction score was >/-2.5).
- PHD-SNP tool provided a reliability index of 8, which indicates the status Disease for the function of NLGN3 protein (if the probability is >0.5 mutations are predicted "Disease" and if less than <0.5 mutations are predicted to be "Neutral").
- SNP&GO prediction tool also provided status of the Disease for the function of NLGN3 protein (Probability is >0.5 then it is predicted to be Disease causing).
- Polyphen-2 predicted as probably damaging for the function of NLGN3 protein with a score of 0.982 ("Probably damaging"-most disease-causing ability with a score near 1. "Possibly damaging"-less disease-causing ability with a score of 0.5–0.8. "Benign" which does not alter protein functions with a score closer to zero).
- SNAP2 prediction tool predicted as effect causing mutation on the function of NLGN3 protein with a score of 23 ("Neutral" if the score between 0 to -100. "Effect" if the score between 0 to +100).
- PANTHER prediction tool also predicted novel missense mutation as probably damaging to the function of NLGN3 protein. Pathogenicity results are represented in table.5.11.

Table.5.11 Pathogenicity prediction of novel missense mutation using insilico tools

PROVEAN	**PHD-SNP**	**SNP &GO**	**SNAP2**	**Polyphen-2**	**PANTHER**
Deleterious Score: -3.094	Disease Reliability index:8	Disease Probability: 0.682	Effect Score:23	Probably Damaging Score: 0.98	Probably damaging Pdel-0.85

5.3.4.1.3 Protein stability prediction

The effect of novel missense mutation on the stability of the NLGN3 protein was predicted using the I-Mutant 2.0v tool. Prediction results showed, the substitution of Valine to Alanine at 184 residue (p.V184A) decreases the protein stability largely with DDG Value: -1.72 Kcal/mol (DDG<-0.5: Large Decrease of Stability; DDG>0.5: Large Increase of Stability; -0.5<=DDG<=0.5: Neutral Stability). Fig.63 showed a stability plot of wild type over mutant protein. The red colour indicates the wild type protein stability and the green colour indicates the mutant protein stability. The same are represented in fig.5.63 and table.5.12.

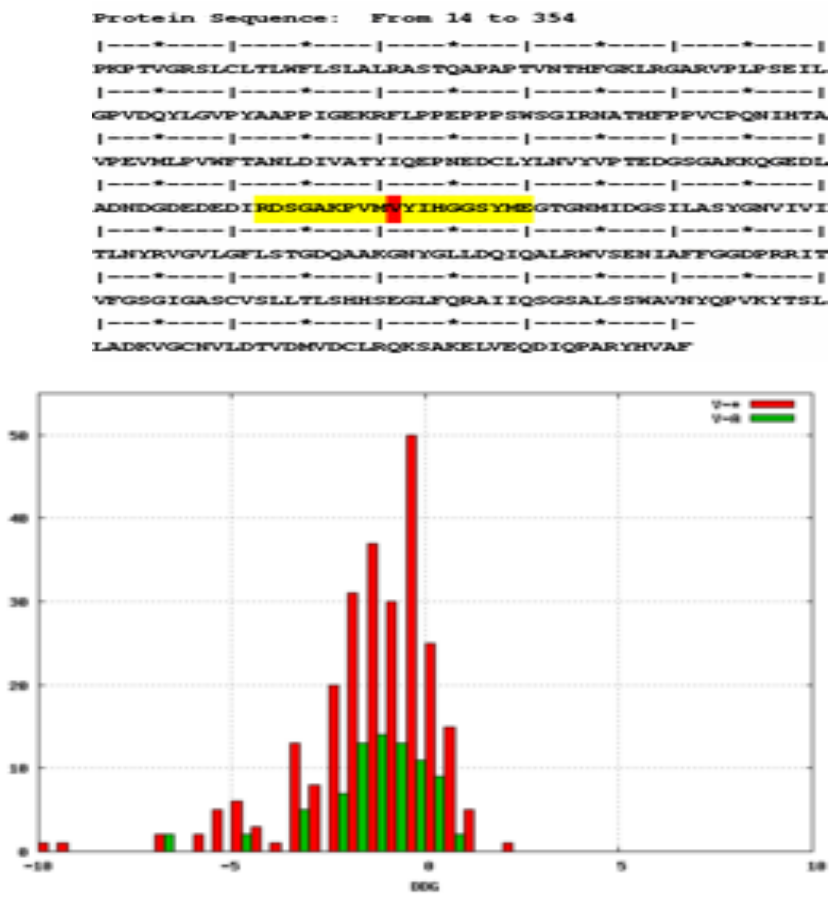


Fig.5.63 NLGN3 Protein stability prediction over p.V184A missense mutation

Table.5.12 NLGN3 Protein stability predictions over p.V184A missense mutation

Mutation	Position	WT	NEW	pH	Temp	SVM2 Prediction Effect	RI	DDG Value
p.V184A	184	V	A	7.0	25	Large Decrease	9	-1.72 Kcal/mol

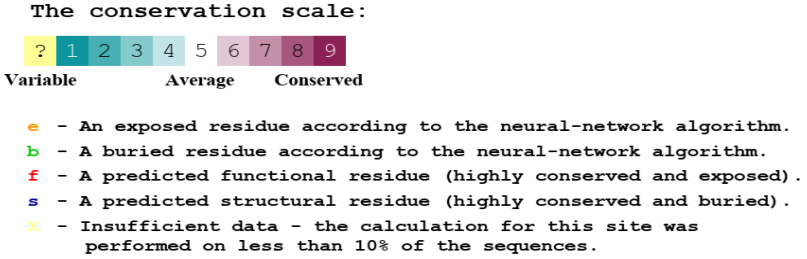
5.3.4.1.4 Conservation status analysis

Multiple sequence alignment of Neuroligin 3 protein sequence was carried out using Clustal omega multiple sequence alignment tool which indicates that the residue 184 Valine is fully conserved across different species ranging from Chicken to Human. Uniprot accession numbers of sequences used for analysis were Q9NZ94 (*Homo sapiens*), Q8BYM5 (*Musmusculus*), Q62889 (*Rattus*), F1Q3I9 (*Canis lupus*), G3MXP5 (*Bostaurus*), A0A2I2UDX2 (*Feliscatus*), E9KFA0 (*Gallus gallaus*), G3RBW3 (*Gorilla gorilla*) and G7NRV3 (*Macacamulatt*). The same are represented in fig.5.64.

Fig.5.64 Multiple sequence alignment of NLGN3 protein over different species showing conservation status of missense variant residue 184V.

Conservation analysis of 184 residue of NLGN3 protein by consurf analysis tool showed the residue 184valine is highly conserved and buried. The conservation score of V184 is 9. The fig.5.65 represents the conservation status of 163 residue.

Fig.5.65 Conservation status of p.N163K mutation, amino acid residue at 163 position shows that it is not conserved; it is variable and exposed residue



5.3.4.1.5 Protein structure prediction

The 3D models for wild type NLGN3 protein and NLGN3 mutated (p.184A) protein were generated using the homology modelling server SWISS-MODEL. The wild type protein has a QMEAN of -1.24 and the NLGN3 mutated protein has a QMEAN of -1.32. At residue V184, two H-bonds were observed with V264 in both wild types (V184) and mutant (A184) and the mutant residue was smaller than the wild-type residue which might lead to loss of interactions with other molecules. The 5.66 and 5.67 represents the protein structure of wild type and mutant protein.

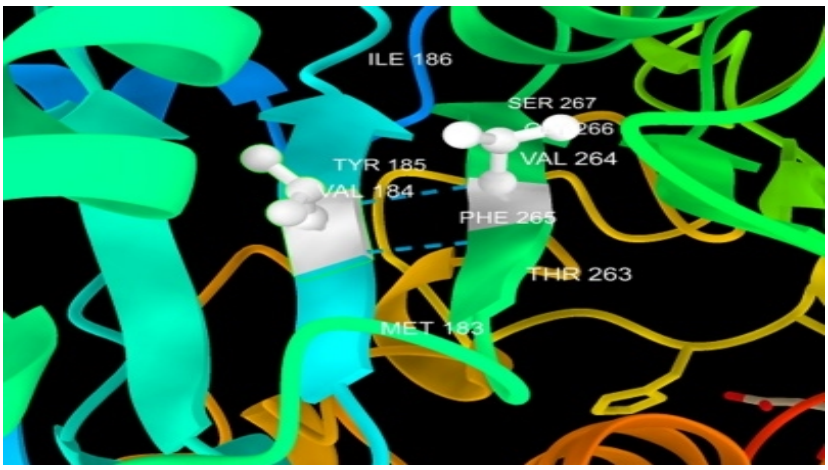


Fig.5.66 Showing the 3D structure of the wild type NLGN3 protein. Interaction of Valine at residue 184 with Valine at residue 264

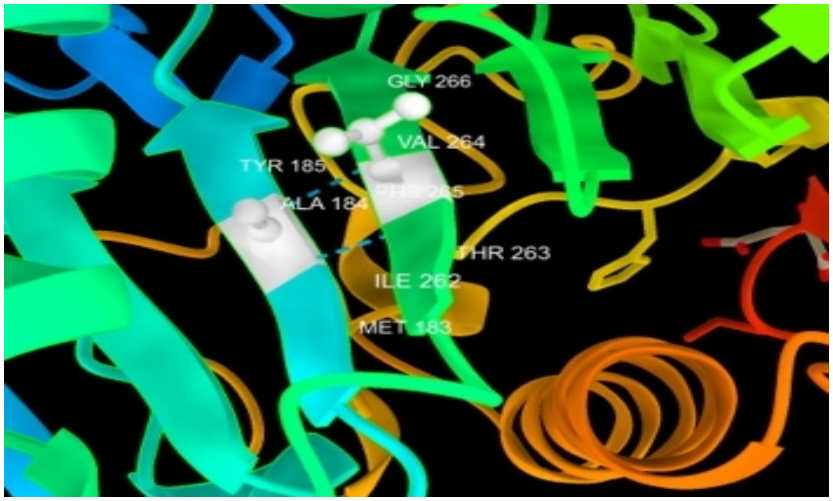


Fig.5.67 Showing the 3D structure of the mutant NLGN3 protein (p.V184A). Interaction of Alanine at residue 184 with Valine at residue 264

5.3.4.1.6 Protein-Protein interaction

The insilico protein-protein interaction analysis revealed that there was good protein interaction among wild type NRXN & wild type NLGN3 (Docking Score: -223.49, binding energy kcal/mol: -6.2) but the interaction between wild type NRXN & mutant type NLGN3 showed a decrease in binding energy (Docking Score:-423.42, Binding energy kcal/mol:-8.29) due to novel missense mutation. Neuroligin3 protein with novel missense mutation fails in binding to presynaptic neurexin during functional synaptogenesis leading to abnormal synapses. The same are represented in table.5.13 and fig.5.68, fig.5.69.

Table.5.13 Showing results of Protein-Protein interaction analysis

Molecules	**Docking Score**	**Binding energy kcal/mol**
Wild type NRXN & Wild type NLGN3	-223.49	-6.2
Wild type NRXN & mutant type NLGN3 (p.V184A)	-423.42	-8.29

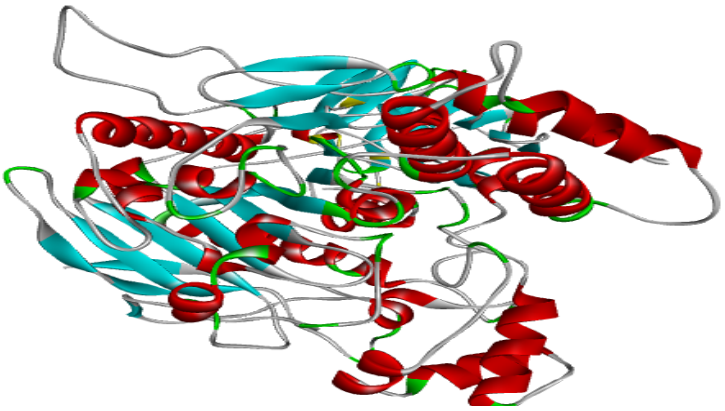


Fig.5.68 Interaction of wild type NRXN and wild type NLGN3

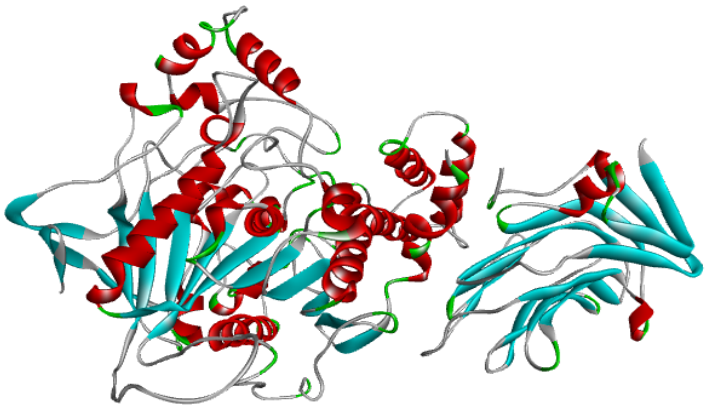


Fig.5.69 Interaction of Wild type NRXN & mutant type NLGN3 with p.V184A

To confirm the effect of novel missense mutation (p.V184A) on the expression of neuroligin3, NLGN3 mRNA expression profiling was carried out in 27 autistic children with age and ethnically matched healthy controls.

5.3.4.1.7 Expression profiling of *NLGN3*

The relative expression of the *NLGN3* gene in 27 autistic children with novel missense mutation; p.V184A and 27 healthy age matched control groups was calculated by normalising the ratio for *NLGN3* to *GAPDH*. The mean expression level of NLGN3 mRNA in autistic children with novel missense mutation and the control groups were 1.94 and 5.36 respectively. A significant decrease in the expression level of the *NLGN3* gene was observed in autistic children with novel missense mutation compared to control groups (p-value 0.001). The same are represented in fig.5.70 and fig.5.71.

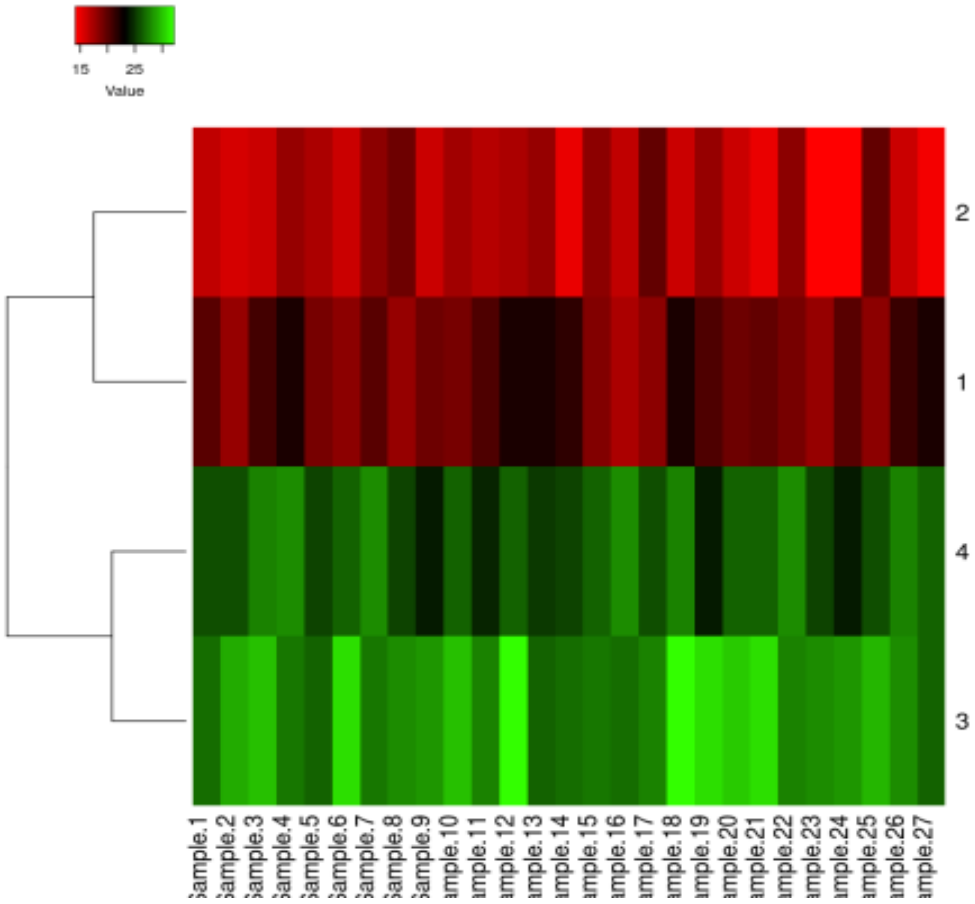


Fig.5.70 Heat map representation of expression of *GAPDH* and *NLGN3* gene in control and cases. 2-*GAPDH* (Control); 1-*GAPDH* (Case); 3- *NLGN4X* (Case); 4-*NLGN4X* (Control)

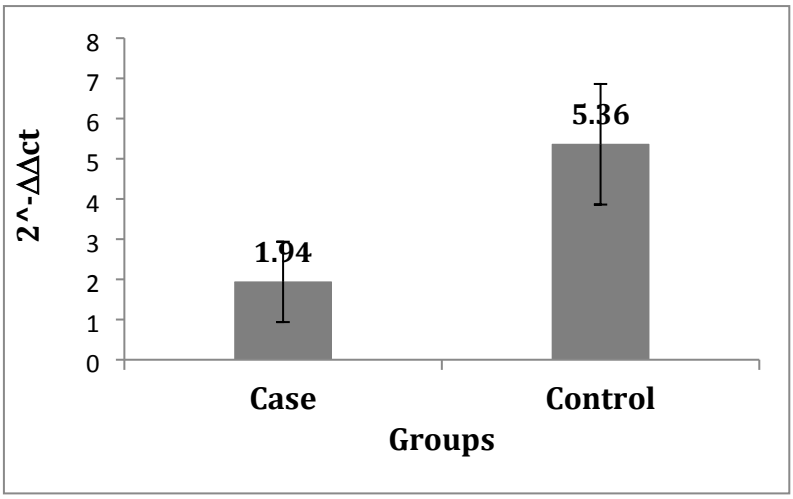


Fig.5.71 The mean relative expression level of NLGN3 mRNA in case and autistic children with the novel missense mutation

The novel missense mutation; c.551T>C was observed in 27 autistic children out of that 18 male and 09 female autistic children. Were there total 14 children had severe autism as per childhood autism rating scale followed by 13who were mild-moderate autistic.

Prenatal damage which includes preeclampsia, infection during pregnancy, hyperthyroidism, and maternal hypertension was observed in 13 autistic children. Postnatal damage which includes birth asphyxia, respiratory illness and delayed crying was observed in 12 autistic children. Total 14 autistic children had consanguineously married couple. Detailed clinical features of 27 autistic children with novel missense mutation have been elaborated in the following table.5.14.

Table.5.14 Clinical features of autism children with novel missense mutation; c.551T>C in *NLGN3* gene

Case	Sex and age	CARS Score and Severity	IQ	Age of mother*	Age of father **	Prenatal damage	Postnatal damage	CM*
Child 1	Male; 13	Severe; 51	20	32	36	None	birth asphyxia	Yes
Child 2	Female; 10	Severe; 50	25	25	30	Preeclampsia	respiratory illness	Yes
Child 3	Male; 14	Severe; 46	30	32	35	Infection during Pregnancy	None	No
Child 4	Male; 11	Severe; 48.5	30	26	32	Hyperthyroidism	None	Yes
Child 5	Female; 14	Severe; 47.5	20	28	33	None	birth asphyxia	Yes
Child 6	Male; 10	Severe; 52	30	32	37	None	delayed	Yes

							crying	
Child 7	Male; 09	Severe; 43.5	20	34	43	None	None	No
Child 8	Female; 08	Severe; 49.5	30	16	36	Preeclampsia	None	No
Child 9	Male; 10	mildly moderate; 37	35	22	30	None	None	Yes
Child 10	Male; 13	Severe; 51.5	30	33	38	maternal hypertension	None	Yes
Child 11	Male; 11	Severe; 42	20	22	30	None	delayed crying	Yes
Child 12	Female; 13	Severe; 40.5	20	28	35	None	None	No
Child 13	Female; 11	mildly moderate; 35	65	32	38	infection during Pregnancy	birth asphyxia	No
Child 14	Male; 10	mildly moderate; 30	65	25	32	maternal hypertension	None	No
Child 15	Male; 12	mildly moderate; 36	35	28	36	None	delayed crying	No
Child 16	Male; 14	Severe; 50	20	20	29	maternal hypertension	None	Yes
Child 17	Male; 09	mildly moderate; 32	20	33	39	preeclampsia	None	No
Child 18	Female; 08	Severe;50	20	30	36	hyperthyroidism	respiratory illness	No
Child 19	Male; 10	mildly moderate; 33	25	26	30	None	None	Yes
Child 20	Male; 11	mildly moderate; 36	40	19	29	None	None	Yes
Child 21	Male; 08	mildly moderate; 35	35	28	34	None	feeding problem	Yes
Child 22	Female; 10	Severe; 56	20	30	36	infection during Pregnancy	respiratory illness	No
Child 23	Female; 13	Severe; 49	25	35	45	None	delayed crying	Yes
Child 24	Male; 12	mildly moderate; 33	30	18	25	maternal hypertension	feeding problem	No
Child 25	Male; 09	Severe; 53	30	29	38	None	None	No
Child 26	Male; 11	mildly moderate; 32	20	36	43	None	None	Yes
Child 27	Female; 12	mildly moderate; 35	30	35	38	preeclampsia	None	Yes

* Age of mother at childbirth, ** Age of father at childbirth, CM* Consanguineous marriage

5.3.4.2. Mutation analysis of *NLGN4X*

Sanger sequencing analysis of the *Neuroligin 4X* gene showed total of 25 mutations which include eight 5′UTR variants (32%), four missense variants (16%), four synonymous variants (16%), one frameshift variant (4%) and eight 3′ UTR variants (32%). In that, 09(36%) were novel variants and 16 (64%) were previously recorded in in-house human SNP databases viz; dbSNP, 1000genomes, GnomAD, ClinVar etc… the same are represented in fig.5.72.

Previously reported 16 variants included three 5′ UTR, three missense, three synonymous and seven 3′UTR variants. Novel variants include five 5′UTR, one missense, one synonymous, one frameshift and one 3′ UTR variant. The novel frameshift mutation was found in 19 (17.5%) autistic children. The mapping of the mutations on the *NLGN4X* gene is shown in fig.5.73

All 5′ UTR variants recorded in our study cohort were found to be heterozygous. Two missense variants (g. 82723 A>R & g.82880 C>Y) and two synonymous variants (g. 82774 C>Y &g. 82872 C>Y) were heterozygous. Three 3'UTR variants (g.342537A>G, g.343053T>G & g.343063C>T) were recorded in both homozygous and heterozygous conditions. All the nomenclatures are made as per HGVS guidelines. All the coding and mom coding mutations were represented in table.5.15 and table.5.16.

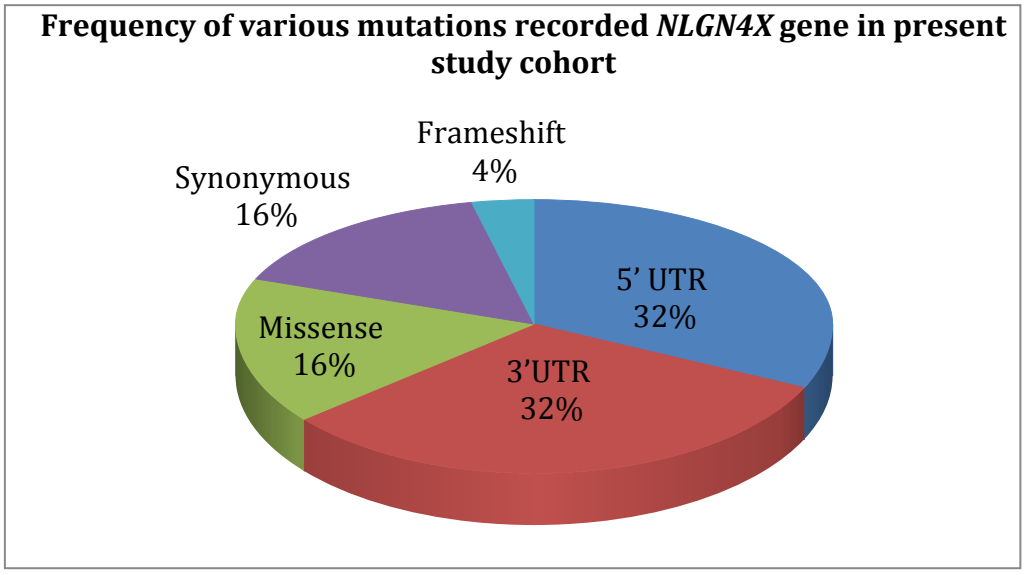


Fig.5.72 Frequency of various mutations recorded NLGN4X gene in the present study cohort

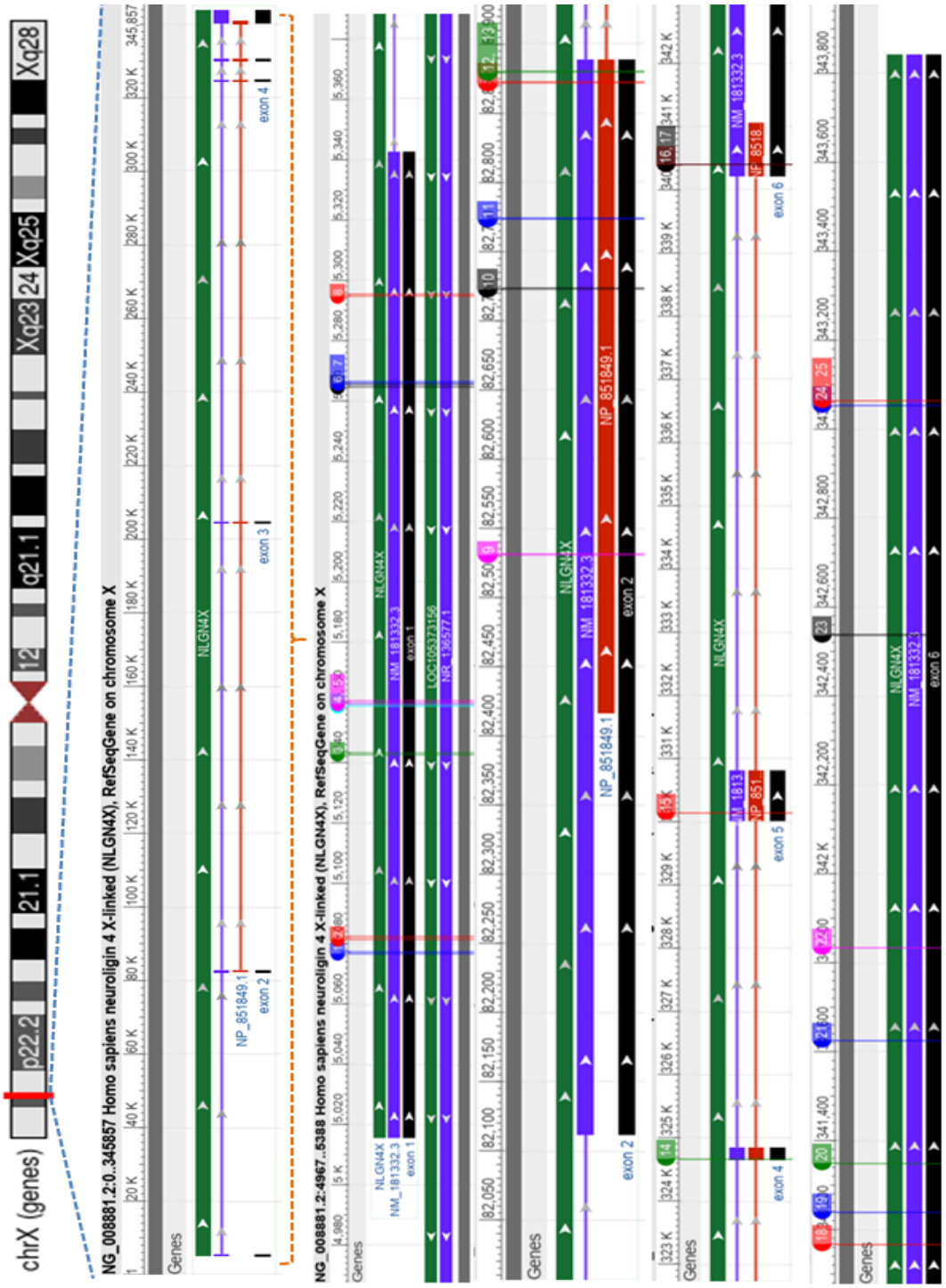


Fig.5.73 Graphical representation of mapping of the mutations on the *NLGN4X* gene. The location of mutations are shown in numeric form and details of the respective numbers are explained in table.5.10

Table.5.15. List of non-coding variations recorded in *NLGN4X* Gene in Autistic children from study population

Mutation No	Mutation type	Nucleotide position	Exon	SNP	Frequency of mutation in percentage
1	5' UTR	g. 5077 T>Y	1	rs149114901	3 (2.77%)
2	5' UTR	g. 5082 C>Y	1	Not reported.	2 (1.85%)
3	5' UTR	g. 5143 C>Y	1	rs755890454	1 (0.92%)
4	5' UTR	g. 5159 T>Y	1	Not reported.	1 (0.92%)
5	5' UTR	g.5160 A>M	1	Not reported	2 (1.85%)
6	5' UTR	g.5265 C>Y	1	rs971248204	5 (4.6%)
7	5' UTR	g.5266 T>K	1	Not reported.	3 (2.77%)
8	5' UTR	g.5295 C>Y	1	Not reported.	4 (3.7%)
18	3'UTR	g.341240C>A	6	rs3810687	2 (1.85%)
19	3'UTR	g.341168G>A	6	rs3810688	1 (0.92%)
20	3'UTR	g.341350G>A	6	rs3810686	2 (1.85%)
21	3'UTR	g.341625C>T	6	rs5916269	1 (0.92%)
22	3'UTR	g.341834T>C	6	rs3810685	3 (2.77%)
23	3'UTR	g.342537A>G	6	Not reported	6 homozygous (5.5%), 3 heterozygous (2.77%)
24	3'UTR	g.343053T>G	6	rs16983882	2 homozygous (1.85%), 2 heterozygous (1.85%)
25	3'UTR	g.343063C>T	6	rs1882260	2 homozygous (1.85%), 2 heterozygous (1.85%)

NG_008881.2 Reference sequence was used for Genomic DNA variant nomenclature, ENST00000381095.8 reference sequence was used for Coding region variant nomenclature and ENSP00000370485.3 reference sequence was used for protein variant nomenclature.

Table.5.16 List of coding variations recorded in *NLGN4X* Gene in autistic children from study population

M. No	Mutation type	Nucleotide position	cDNA position	A.A position	Exon	SNP	Frequency of mutation
9	Missense	g.82531A>G	c.115A>G	p.I39V	2	rs201534650	2 (1.85%)
10	Missense	g. 82723A>R	c.307A>R	p.T103A	2	not reported	8 (7.04%)
11	Synonymous	g. 82774C>Y	c.358C>Y	p.L120=	2	not reported	6 (5.5%)
12	Synonymous	g. 82872C>Y	c.456C>Y	p.Y152=	2	rs1460330547	11(10.2%)
13	Missense	g.82880 C>Y	c.464C>Y	p.T155M	2	rs770601703	7 (6.5%)
14	Frameshift	g.324659_324060 insG	c.641_642 insG	p.Gly214GlyfsTer2	4	Not reported	19(17.5%)
15	Synonymous	g.330138C>T	c.933C>T	p.T331=	5	rs7049300	2 (1.85%)
16	Missense	g.340392C>T	c.1777C>T	p.L593F	6	rs3747333	2 (1.85%)
17	Synonymous	g.340394C>G	c.1779C>T	p.L593=	6	rs3747334	1 (0.92%)

M.No- mutation number on the gene map, A.A position- amino acid position

NG_008881.2 Reference sequence was used for Genomic DNA variant nomenclature, ENST00000381095.8 Reference sequence was used for Coding region variant nomenclature and ENSP00000370485.3 Reference sequence was used for Protein variant nomenclature.

5.3.4.2.1 Pathogenicity prediction

Insilco pathogenicity prediction of missense variants was done to evaluate the deleterious/harmful effect on the functions of NLGN4X protein by PolyPhen2, PROVEAN, PANTHER, SNP&GO, PHD-SNP and SNAP2. The same are represented in table.5.17.

Table.5.17. Pathogenicity prediction of missense variants in NLGN4X gene

Variants	PROVEAN	SNP &GO	SNAP2	Polyphen-2	PHD-SNP	Panther
p.I39V	Neutral Score:0.27	Neutral P- 0.07	Neutral Score: -65	Benign 0.246	Neutral RI-8	PSD Pdel-0.5
p.T103A	Neutral Score: 2.39	Neutral P- 0.03	Neutral Score: -54	Benign 0.0	Neutral RI- 9	PBB Pdel-0.19
p.T155M	Deleterious Score: -3.07	Effect P- 0.17	Disease Score:15	Pd Score:0.87	Disease RI-4	PBD Pdel- 0.74
p.L593F	Deleterious Score: -2.69	Neutral P-0.39	Neutral Score: -57	Benign 0.346	Neutral RI- 1	PBD Pdel-0.74

PSB- possibly damaging, PDD-probably damaging, PBB- probably benign, Pdel-probability of the deleterious effect

- PROVEAN tool predicted p.I39V and p.T103A variants having a neutral effect on the function of NLGN4X protein with a score of 0.271 and 2.396 respectively. p.T155M and p. L593F missense variants were predicted to cause a deleterious effect on the NLGN4X protein with the score of -3.070 and -2.699 respectively ("Deleterious" if the prediction score was </ -2.5 and "Neutral" if the prediction score was >/-2.5).
- PHD-SNP pathogenicity prediction showed all the 3 missense variants having a neutral effect except the p.T155M missense variant on the function of NLGN4X protein.
- SNP&GO tool predicted, variants p.I39V, p.T103A and p.L593F having neutral effect and p.T155M had a pathogenic effect on the function of NLGN4X protein (Probability is >0.5 then it is predicted to be Disease causing nsSNP).

- Polyphen-2 predicted that only the p.T155M variant had a disease causing effect with a score of 0.871 and remaining variants such as p.I39V, p.T103A and p.L593F had a neutral effect on the function of NLGN4X protein (Probably damaging" is the most disease causing ability with a score near to 1. "Possibly damaging" is less disease-causing ability with a score of 0.5–0.8. "Benign" which does not alter protein functions with a score closer to zero).
- SNAP2 prediction tool also predicted that only the p.T155M variant had a disease causing effect with a score of 15 and reaming variants such as p.I39V, p.T103A and p.L593F had a neutral effect on the function of NLGN4X protein ("Neutral" if the score lays 0 to - 100. "Effect" if the score lays 0 to 100).
- PANTHER prediction tool predicted that p.I39V mutation has a possibly damaging role and p.T103A mutation has probably benign role but p.T155Mand p.Leu593 had probably damaging effect on the function of neuroligin 4X protein. ("probably damaging" (time > 450my, corresponding to a false positive rate of ~0.2 as tested on HumVar), "possibly damaging" (450my>time>200my, corresponding to a false positive rate of ~0.4) and "probably benign" (time< 200my).

5.3.4.2.2 Protein stability prediction

The effect of missense mutations on the stability of the NLGN4X protein was predicted using the I-Mutant 2.0v tool. Prediction results showed the following results;

- Substitution of Valine to Isoleucine at 39 residue (p.I39V) decreases the protein stability with DDG Value Prediction: -0.95 Kcal/mol
- Substitution of Threonine to Alanine at 103 residue (p.T103A) decreases the protein stability with DDG Value Prediction: -1.07 Kcal/mol
- Substitution of Threonine to Methionine at 153 residue (p.T155M) decreases the protein stability with DDG Value Prediction: -0.33Kcal/mol
- Substitution of Leucine to Phenylalanine at 593 residue (p.L593F) decreases the protein stability with DDG Value Prediction: -0.89 Kcal/mol. The same are represented in table.5.18

Table.5.18 NLGN4X Protein stability prediction of missense mutation recorded in the present study cohort.

Mutation	WT	New	pH	Temp	SVM2 Prediction	RI	DDG Value
p.I39V	I	V	7.0	25	Decrease	8	-0.95 Kcal/mol
p.T103A	T	A	7.0	25	Decrease	7	-1.07 Kcal/mol
p.T155M	T	M	7.0	25	Decrease	1	-0.33 Kcal/mol
p.L593F	L	F	7.0	25	Decrease	5	-0.89 Kcal/mol

DDG<-0.5: Large Decrease of Stability; DDG>0.5: Large Increase of Stability;-0.5<=DDG<=0.5: Neutral Stability.

Fig.5.74 to fig.5.77 shows the stability plot of wild type over different mutant proteins. The red colour indicates the wild type protein stability and the green colour indicates the mutant protein stability.

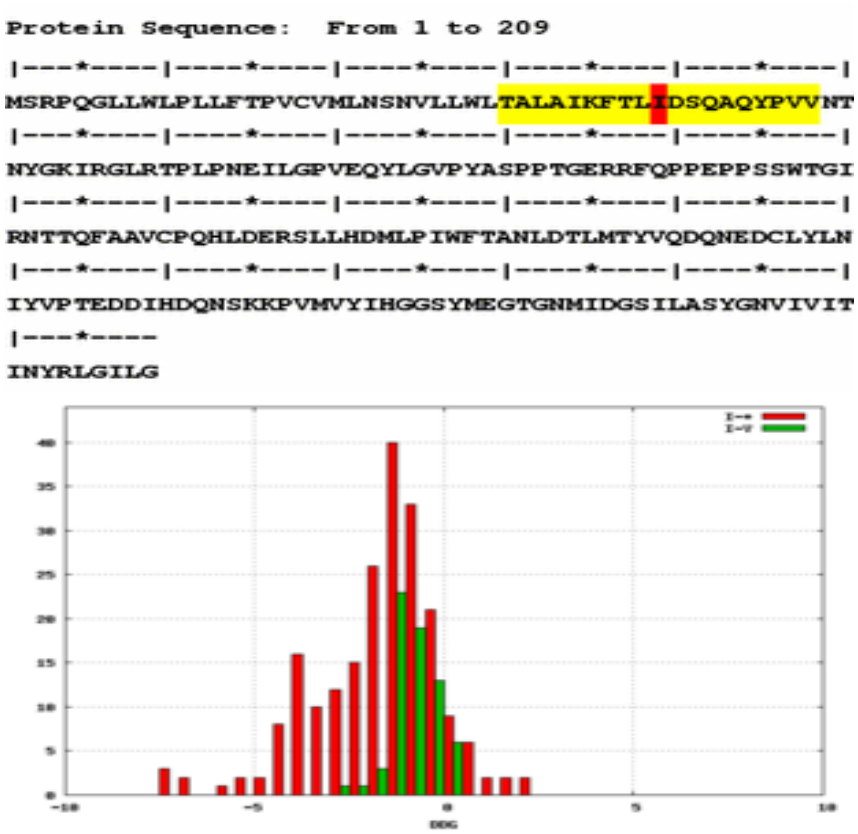


Fig.5.74 NLGN4X Protein stability prediction over p.I39V missense mutation

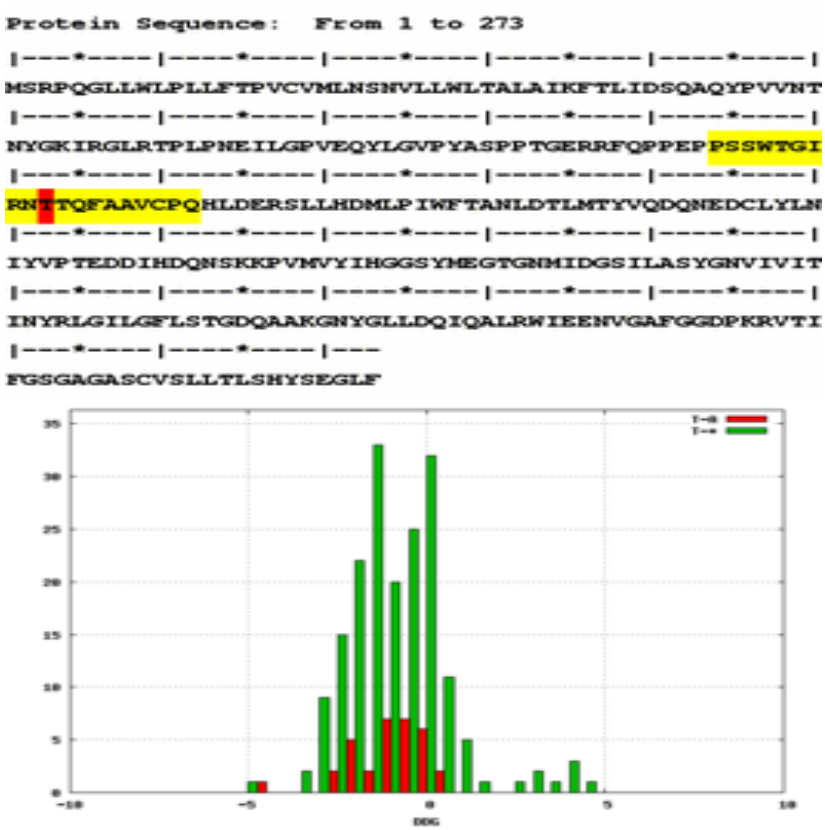


Fig.5.75 NLGN4X protein stability prediction over p.T103Amissense mutation

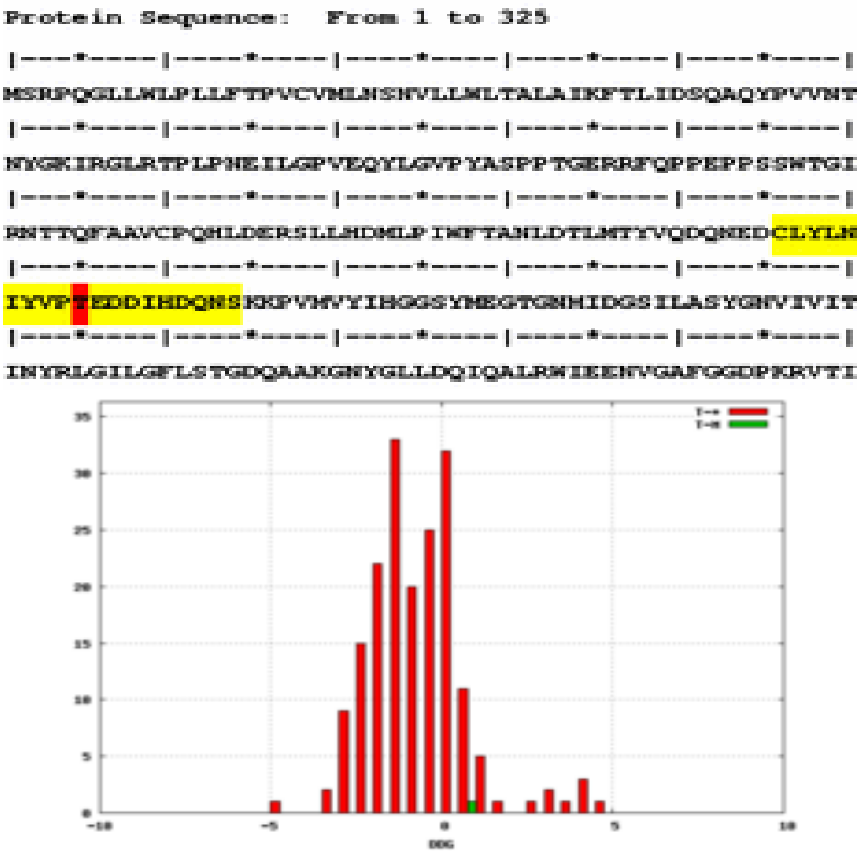


Fig.5.76 NLGN4X protein stability prediction over p.T155M missense mutation

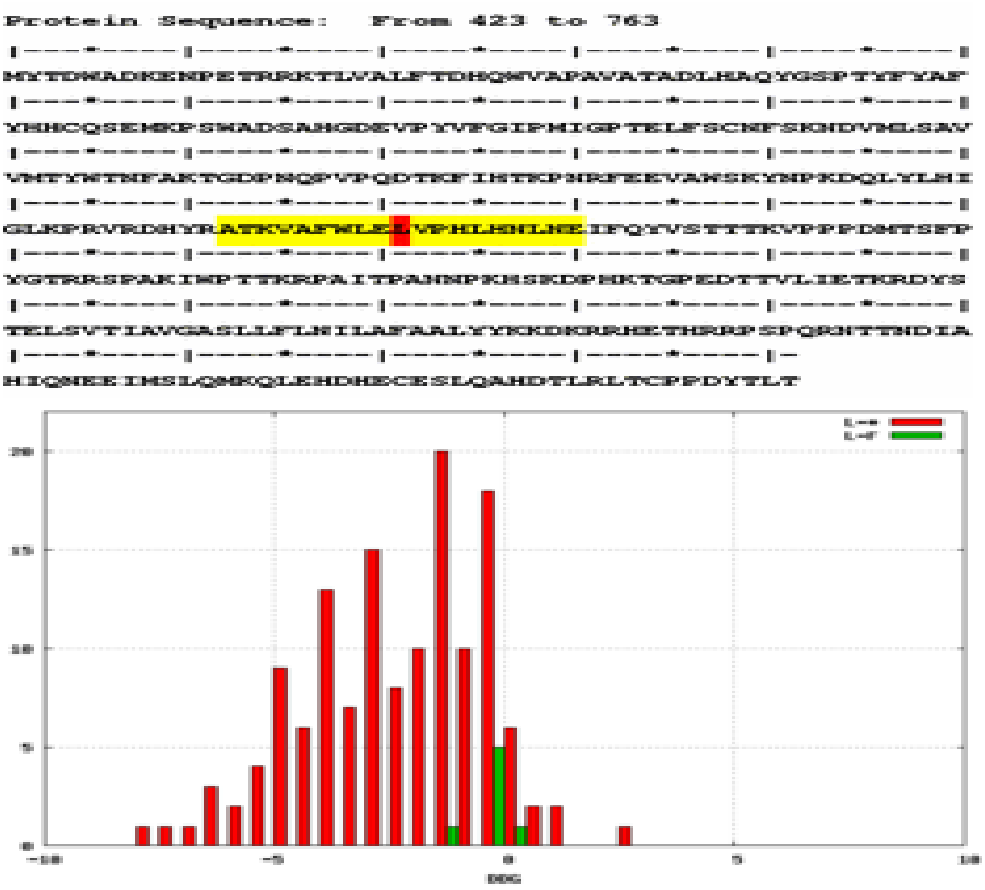


Fig.5.77 NLGN4X protein stability prediction over p.L593Fmissense mutation

5.3.4.2.3 Conservation status analysis

Multiple sequence alignment of NLGN4X protein showed p.T155M and p.L593F variant being located in the conserved region across the different species and showed high evolutionary functions but the remaining p.T103Aand p. I39V variants were conserved only in higher primates. Uniprot accession numbers of sequence used were as follows; D2X2K7- *Xenopustropicalis*, D2X2I0- *Anoliscarolinensis*, A0A2I3MYY3- *Papioanubis*, H2PUU5- *Pongo abelii*, Q8N0W4- *Homo sapiens*, A0A2K6P1V0-*Rhinopithecus roxellana*, H2R0H2- *Pan troglodytes*, A0A2I3HLL4-*Nomascus leucogenys*. The same are represented in fig.5.78 to fig.5.81.

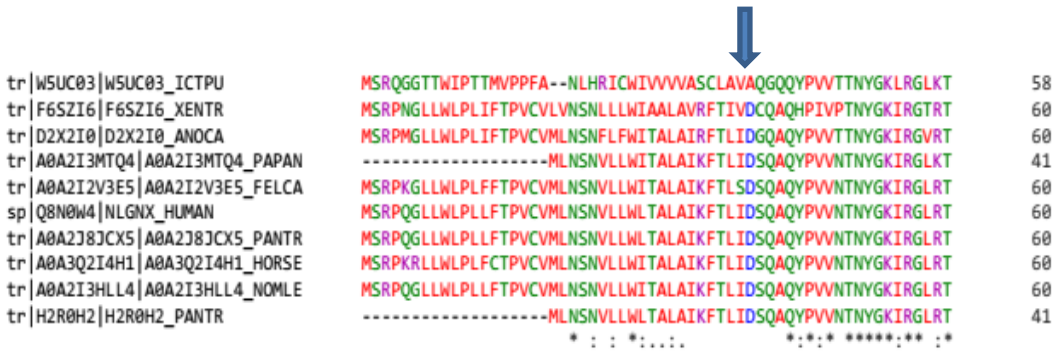


Fig5.78 Multiple sequence alignment of NLGN4X protein over different species showing conservation status of missense variant residue 39I. The arrow mark indicates the position of the variant residue.

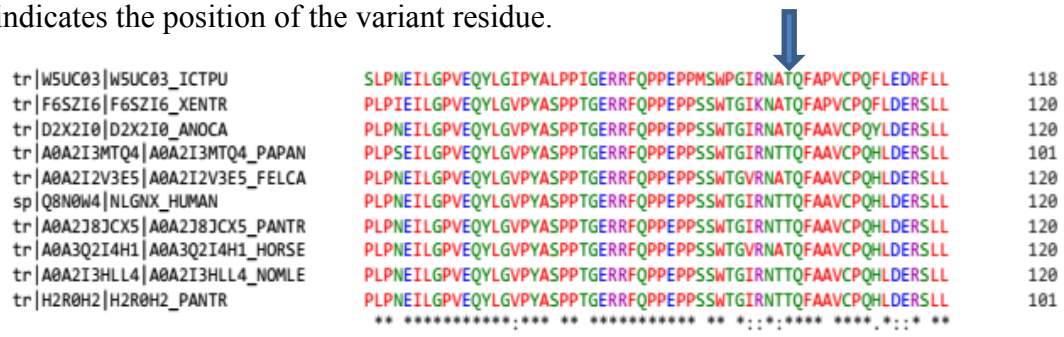


Fig.5.79 Multiple sequence alignment of NLGN4X protein over different species showing conservation status of missense variant residue 103T. The arrow mark indicates the position of the variant residue.

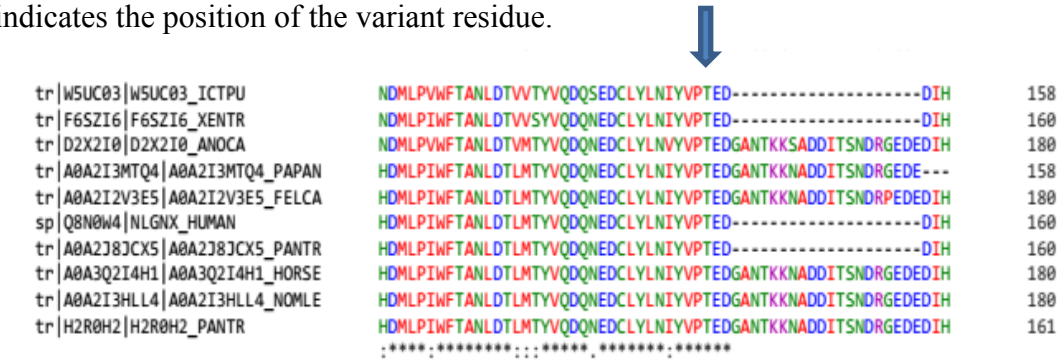


Fig.5.80 Multiple sequence alignment of NLGN4X protein over different species showing conservation status of missense variant residue 155T. The arrow mark indicates the position of the variant residue.

Fig.5.81 Multiple sequence alignment of NLGN4X protein over different species showing conservation status of missense variant residue 593L. The arrow mark indicates the position of the variant residue.

Conservation status of p.I39V, p.T103A, p.T155M and p.L593F mutation residue on NLGN4X protein is represented from fig.5.82 to fig.5.85.

Conservation analysis of 39 residue of NLGN4X protein by consurf analysis tool showed the residue 39 Isoleucine was not conserved and it was exposed according to neural network algorithm. The conservation score of I39 is 1.

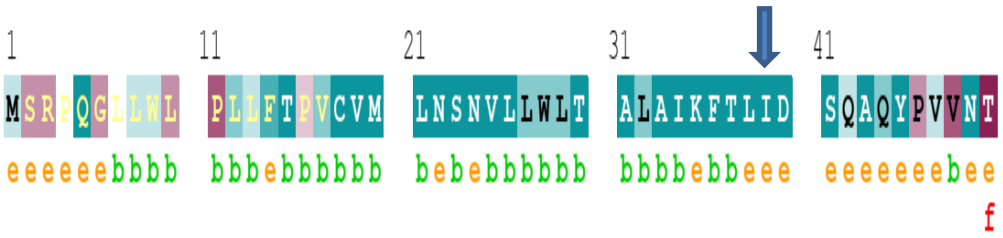


Fig.5.82 Conservation status of p.I39V mutation residue indicated using arrow mark in NLGN4X protein.

Conservation analysis of 103 residue of NLGN4X protein by consurf analysis tool showed the residue 103 Threonine was slightly conserved and it was buried according to neural network algorithm. The conservation score of T103 was 7.

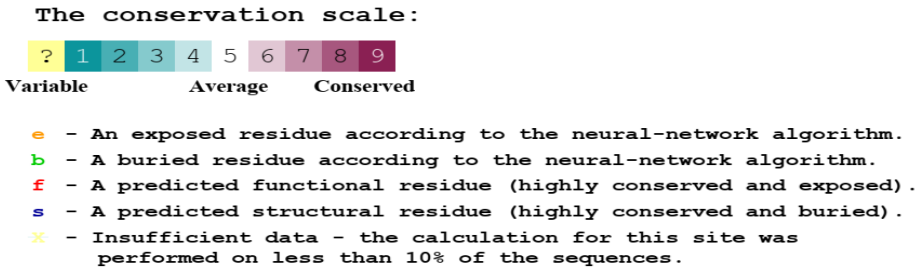
Fig.5.83 Conservation status of p.T103A mutation residue indicated using arrow mark in NLGN4X protein.

Conservation analysis of 155 residue of NLGN4X protein by consurf analysis tool showed the residue 155 Threonine was not conserved and it was exposed according to neural network algorithm. The conservation score of T155 was 4.

Fig.5.84 Conservation status of p.T155M mutation residue indicated using arrow mark in NLGN4X protein.

Conservation analysis of 593 residue of NLGN4X protein by consurf analysis tool showed the residue 593 Leucine was not conserved and it was exposed according to neural network algorithm. The conservation score of L593 was 7.

Fig.5.85 Conservation status of p.L593F mutation residue indicated using arrow mark in NLGN4X protein.



5.3.4.2.4 Protein structure prediction

The homology modelling of NLGN4X protein with two novel variants, p.T103A and p.Gly214GlyfsTer2 was generated using the SWISS-MODEL server and the structure was analysed using UFSC chimera software. At residue T103, two H-bonds were observed with L74 in both wild types (T103) and mutant (A103). The mutant residue was smaller than the wild-type residue and the mutant residue was more hydrophobic than the wild-type residue. The difference in hydrophobicity may affect hydrogen bond formation. This loss of hydrogen bond affects NLGN4X protein folding and thus decreases the stability of the protein. The same are represented in fig.5.85

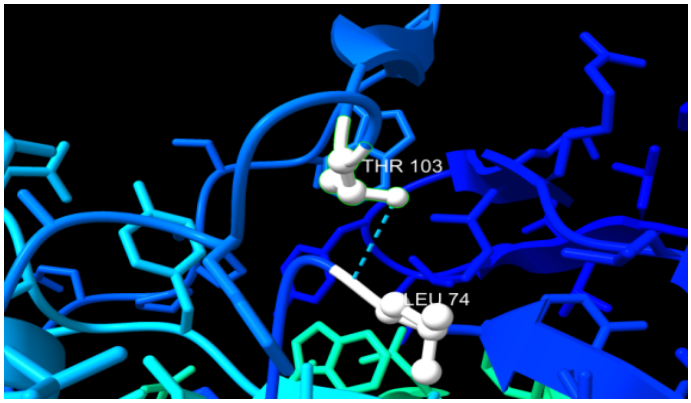


Fig.5.86 Showing the 3D structure of the wild-type NLGN4X protein. Interaction of Threonine at residue 103 with Leucine at residue 74.

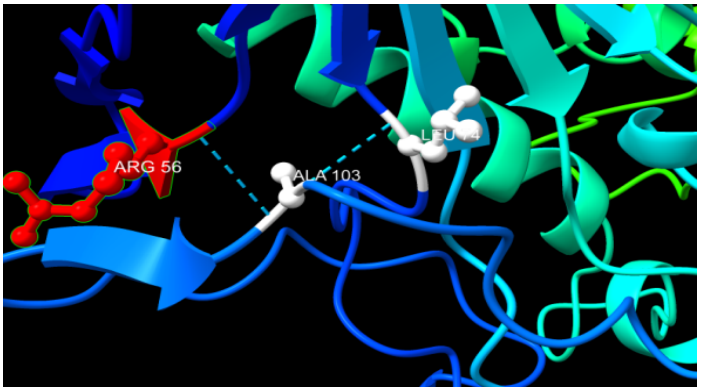


Fig.5.87 Showing the 3D structure of themutantNLGN4X protein with p.T103A substitution. Interaction of Threonine at residue 103 with Leucine at residue 74

The novel frameshift mutation p.Gly214GlyfsTer2 alters the reading frame further resulting in an immediate stop codon and forming truncated protein with 214 amino acids which causes loss of approximately 74% of the wild type protein. That was very much important for binding to presynaptic neurexin protein during functional synaptogenesis.

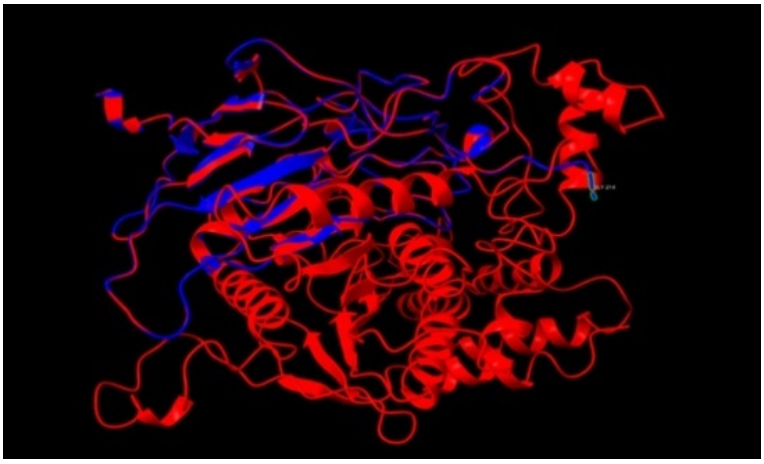


Fig.5.88 Superimposed model of NLGN4X wild type and truncated protein due to p.Gly214GlyfsTer2 mutation (blue- truncated protein, Red- wild typeNLGN4X protein)

To confirm the effect of novel frameshift mutation (p.Gly214GlyfsTer2) on the expression of neuroligin 4X gene, mRNA expression profiling was carried out in 19 autistic children with age matched control group.

5.3.4.2.5 Expression profiling of *NLGN4X*

The relative expression quantification of the *NLGN4X* gene in autistic children with a novel frameshift mutation (p.Gly214GlyfsTer2) and healthy age, sex and ethnically matched control group was analysed by normalising the ratio for *NLGN4X* to *GAPDH*. Expression profiling was carried out in 19 autistic children with novel frameshift mutation and 19 healthy age, sex and ethnically matched control children. Relative expression analysis was calculated using the $2^{\Delta\Delta ct}$ formula. The average cycle quantification (Cq) of positive control (*GAPDH*) in cases was16.61±2.7 and in the control group, it was15.39±1.3. The average cycle quantification (Cq) of the *NLGN4X* gene in the case was 27.50 ±1.3 and in the control, it was 26.51± 1.0. Cycle quantification (Cq) of both the control gene and targeted *NLGN4X* gene in both the autistic group and control groups were summarised and represented using a heat map. The mean relative expression level of NLGN4X mRNA in autistic children with a novel frameshift mutation (p.Gly214GlyfsTer2) and the control groups were 0.05 and 1.22 respectively. A significant large decrease in the expression level of the *NLGN4X* gene was observed in autistic children with novel frameshift mutation compared to control groups (p-value 0.00001).

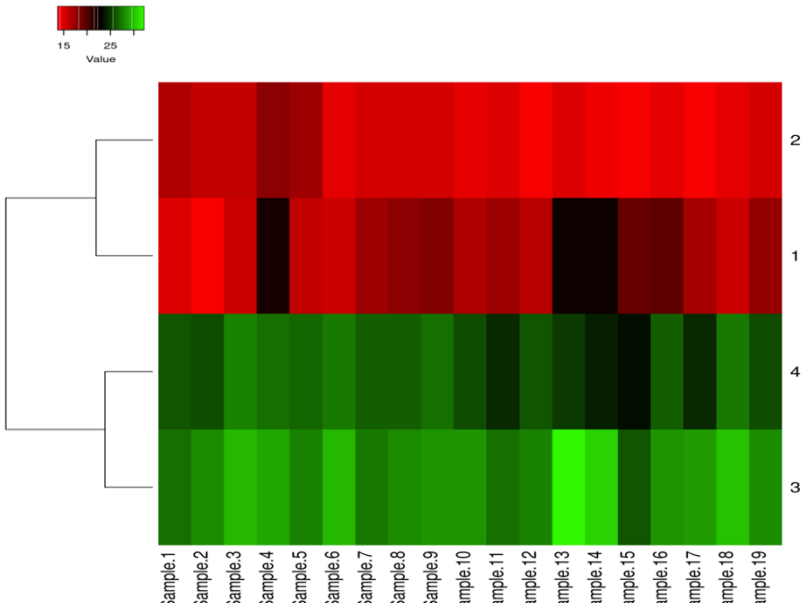


Fig.5.89 Heat map of expression of *GAPDH* and *NLGN4X* gene in control and autistic cases. 2-*GAPDH* (Control); 1-*GAPDH* (Case); 3- *NLGN4X* (Case); 4-*NLGN4X* (Control)

Electrophoresis of image cDNA of *NLGN4X* gene of control and autistic with Frameshift mutation and relative mRNA expression of *NLGN4X* gene are represented in fig.5.90 and fig.5.91 respectively

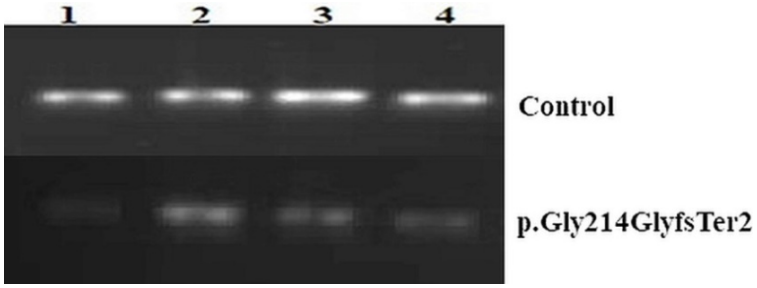


Fig.5.90 Agarose gel electrophoresis of amplified cDNA of *NLGN4X* gene. Control (top) and autistic with Frameshift mutation; p.Gly214GlyfsTer2 (below)

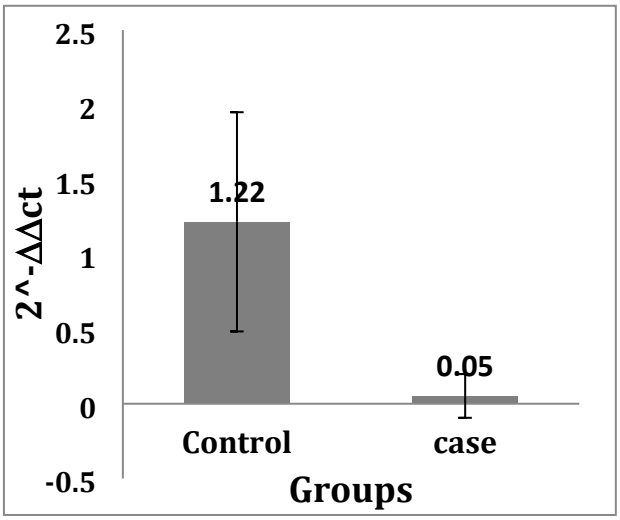


Fig.5.91 The mean relative expression level of NLGN4X mRNA in control groups and autistic children with a novel frameshift mutation

5.3.4.2.6 Protein-Protein interaction

The insilico protein-protein interaction analysis revealed a good protein interaction among wild type NRXN & wild type NLGN4X (Docking Score: -208.97, Binding energy kcal/mol: -4.84). While the interaction between wild type NRXN & mutant type NLGN4X showed no difference in binding energy (Docking Score: -210.10, Binding energy kcal/mol: -4.38) due to novel missense mutation. Neuroligin4X protein with novel missense mutation binds normally to presynaptic neurexin during functional synaptogenesis and it may not lead to abnormal synapses. The same are represented in table.5.19. The mutant and wild protein interaction are shown in fig.5.92 and fig.5.93.

Table.5.19 showing the results of Protein- Protein interaction analysis

Molecules	Docking Score	Binding energy kcal/mol
Wild type NRXN & Wild type NLGN4X	-208.97	-4.84
Wild type NRXN & mutant type NLGN4X (Novel missense mutation; p.T103A)	-210.10	-4.38

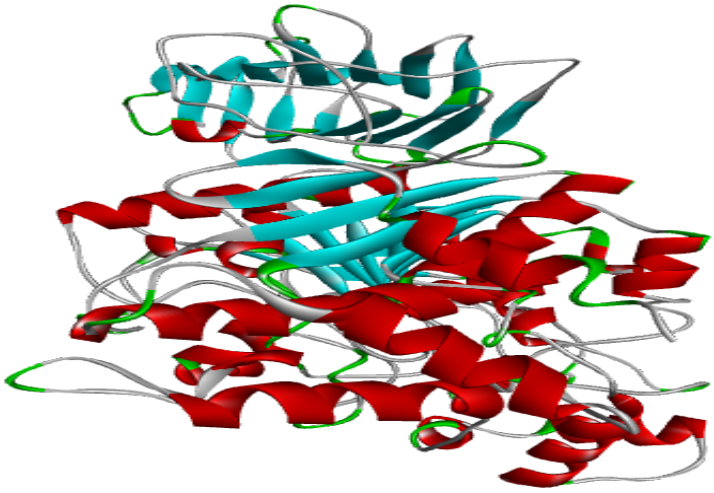


Fig.5.92 Interaction between Wild type NRXN and wild type NLGN4X protein

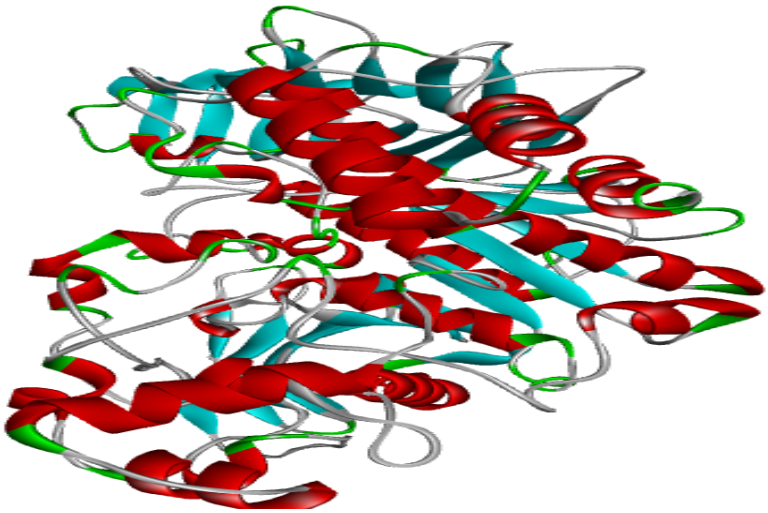


Fig.5.93 Interaction between Wild type NRXN and mutant NLGN4X protein with p.T103A

The novel frameshift mutation was observed in 19 autistic children in that there were 13 male and 06 female autistic children. Total 14 children had severe autism as per childhood autism rating scale followed by 05 who were mild-moderate autistic. Prenatal damage, which includes preeclampsia, infection during pregnancy, hyperthyroidism and maternal hypertension, was observed in 08 autistic children.

Postnatal damage, which includes birth asphyxia, respiratory illness and delayed crying, was observed in 07 autistic children. Total 08 autistic children had consanguineously married couple. Detailed clinical features of 19 autistic children with novel missense mutation have been elaborated in the following table.5.20**.**

Table.5.20 Clinical features of autistic children with novel frameshift mutation in *NLGN4X* gene

Sl.No	Sex and age	CARS Score and Severity	IQ	Age of mother*	Age of father **	Prenatal damage	Postnatal damage	CM
Child 1	Male; 12	Severe; 51	20	32	36	Infection during Pregnancy	birth asphyxia	Yes
Child 2	Female; 11	Severe; 50	25	34	30	Preeclampsia	respiratory illness	Yes
Child 3	Male; 09	Severe; 46	30	18	38	None	None	No
Child 4	Male; 11	Severe; 48.5	30	22	30	Hyperthyroidism	None	Yes
Child 5	Female;14	Severe; 47.5	20	33	33	None	None	Yes
Child 6	Male; 10	Severe; 52	30	22	37	None	None	Yes
Child 7	Male; 09	Severe; 43.5	20	32	43	None	None	No
Child 8	Female; 08	Severe; 49.5	30	25	36	Preeclampsia	None	No
Child 9	Male; 10	mildly moderate; 37	35	32	35	None	None	Yes
Child 10	Male; 13	Severe; 51.5	30	26	32	None	None	Yes
Child 11	Male; 11	Severe; 42	20	28	33	None	birth asphyxia	Yes
Child 12	Female; 13	Severe; 40.5	30	32	37	maternal hypertension	delayed crying	No
Child 13	Female; 11	mildly moderate; 35	65	30	36	None	birth asphyxia	No
Child 14	Male; 10	mildly moderate; 30	65	30	37	maternal hypertension	None	No
Child 15	Male; 12	mildly moderate; 36	35	28	36	None	delayed crying	No
Child 16	Male; 14	Severe; 50	20	20	29	None	None	Yes
Child 17	Male; 09	mildly moderate; 32	20	33	39	preeclampsia	None	No
Child 18	Female; 08	Severe;50	20	30	36	hyperthyroidism	respiratory illness	No
Child 19	Male; 10	Severe; 44	31	18	36	None	None	No

CM* Consanguineous marriage

5.3.4.3 Mutation analysis of *NLGN4Y*

NLGN4Y gene is located on the Y chromosome and Y chromosome is absent in female hence sequencing analysis for *NLGN4Y* gene was carried only in males. Sanger sequence analysis of neuroligin 4Y gene from 85 male autistic children revealed nine variants, which include one missense and eight synonymous variants. Among 8 variants, four variants which were recorded in the present study cohort were not previously recorded in any in house human SNP databases viz; dbSNP,1000genomes, gnomAD, ClinVar etc…Four novel variations included one missense mutation (p.N163K) which was recorded in 3.5% of the studied population. Only novel missense mutation was recorded in exon3 of *NLGN4Y* gene. Variants, g.312652 T>C and g.312787 A>G were observed in both the homozygous and heterozygous conditions. All the mutations recorded in *NLGN4Y* gene were in low frequency. All the synonymous mutations recorded are located in exon5 of the *NLGN4Y* gene. The list of recorded variants is represented in Fig. 5.94 and table.5.21

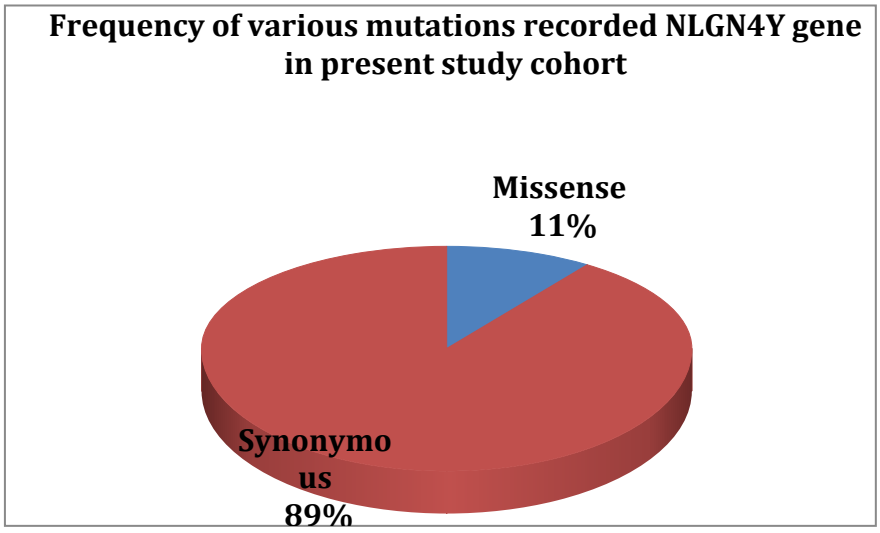


Fig.5.94 Frequency of various mutations recorded *NLGN4Y* gene in the present study cohort

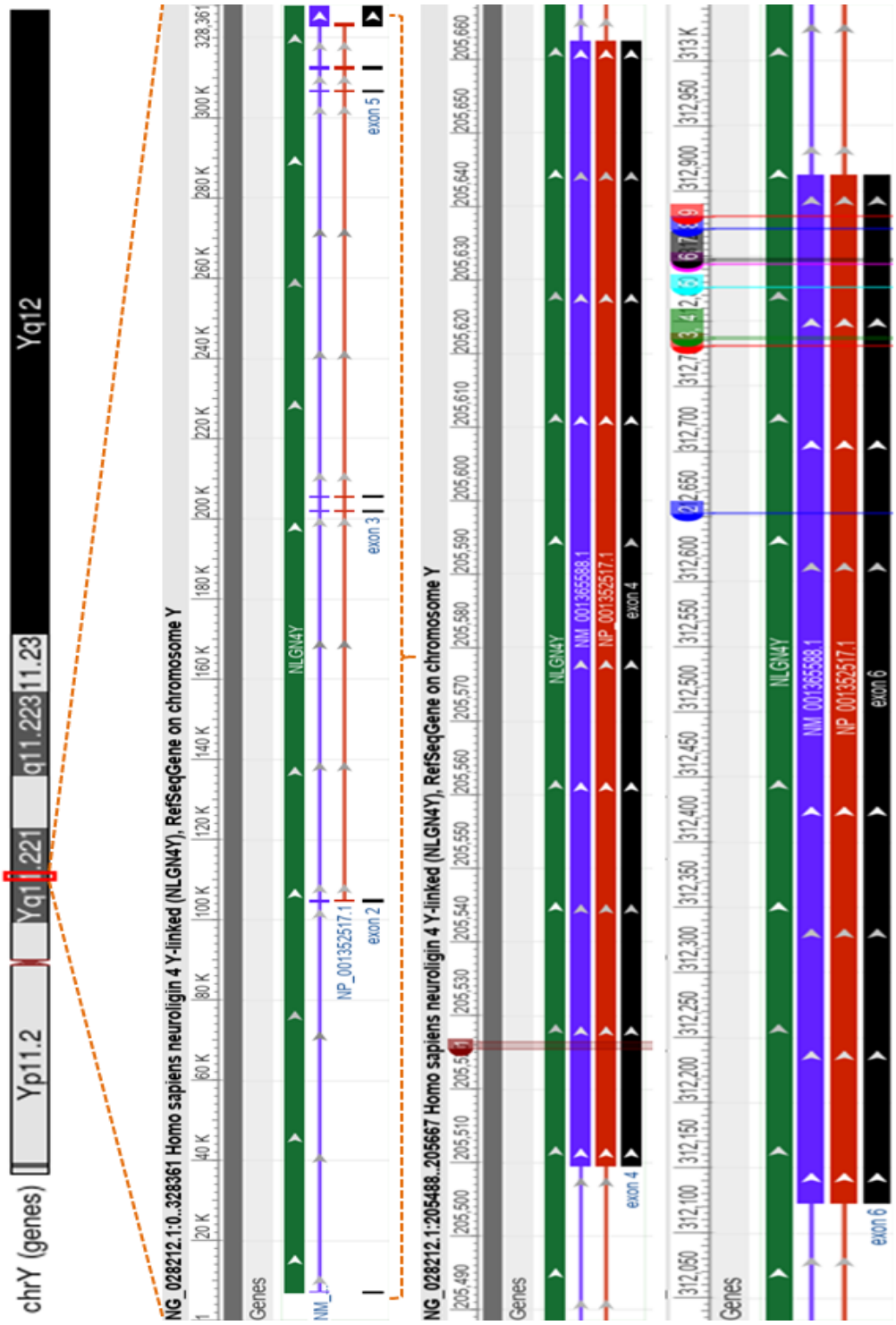


Fig.5.95 Graphical representation of mapping of mutations on the *NLGN4Y* gene and the location of mutations are shown in numeric form and details of the respective numbers are explained in table.5.21

Table.5.21 List of variations recorded in *NLGN4Y* gene in autistic children of present population

M. No	Variation	Nucleotide change	A.A Change	Status	Frequency of mutation
1.	Missense	g. 205526C>A	p.N163K	Not recorded	3 (3.5%)
2.	Synonymous	g.312652T>C	p. H447=	rs777234513	4 homozygous (4.7%) 2 heterozygous (2.3%)
3.	Synonymous	g.312781C>T	p.G490=	rs767447455	3 (3.5%)
4.	synonymous	g.312787A>G	p.E492=	rs750273940	3 homozygous (3.5%) 2 heterozygous (2.3%)
5.	synonymous	g.312826A>C	p.T505=	Not recorded	1 (1.2%)
6.	synonymous	g.312844T>C	p.N512=	Not recorded	2 (2.3%)
7.	synonymous	g.312847C>T	p.F513=	Not recorded	1 (1.2%)
8.	synonymous	g.312871T>C	p.S520=	rs1423308667	1 (1.2%)
9.	synonymous	g.312880G>C	p.V523=	rs753006927	3 (3.5%)

M. No- Mutation number, A.A change- Amino acid change

5.3.4.3.1 Pathogenicity prediction

Insilco pathogenicity prediction of novel missense variant; p.N163K was done to evaluate the deleterious effects on the functions of NLGN4Y protein by PolyPhen2, PROVEAN, PANTHER, SNP&GO, PHD-SNP and SNAP2. The same are shown in table 5.22

- PROVEAN prediction tool provided the score of -0.992 which indicated neutral effect on the function of NLGN4Y protein ("Deleterious" if the prediction score was </-2.5, "Neutral" if the prediction score was >/-2.5).
- PHD-SNP tool provided a reliability index of 0, which indicated the status neutral effect on the function of NLGN4Y protein (if the probability is >0.5 mutations is predicted "Disease" and if less than <0.5 mutations are predicted to be "Neutral").

- SNP&GO prediction tool also provided a status Neutral effect on the function of NLGN4Y protein with a score of 0.381 (Probability is >0.5 then it is predicted to be Disease causing nsSNPs).
- Polyphen-2 was predicted as benign with a score of 0.023("Probably damaging" is the most disease-causing ability with a score near 1. "Possibly damaging" is less disease-causing ability with a score of 0.5–0.8. "Benign" which does not alter protein functions with a score closer to zero).
- SNAP2 prediction tool predicted a neutral effect on the function of NLGN4Y protein with a score of -79 ("Neutral" if the score between 0 to -100. "Effect" if the score between 0 to +100).

Table.5.22 Prediction results of NLGN4Y Protein stability over p.V184A missense mutation

Variant	PROVEAN	SNP&GO	PolyPhen2	SNAP2	PHD-SNP
p.N163K	Neutral Score: -0.992	Neutral 0.381	Benign Score: 0.023	Neutral Score: -79	Neutral RI:00

5.3.4.3.2 Protein stability prediction

The effect of novel missense mutation on the stability of the NLGN4Y protein in male autistic children was predicted using the I-Mutant 2.0v tool. Prediction results showed substitution of Asparagine to Lysine at 163 residue (p.N163K) decreases the protein stability with DDG Value Prediction: -0.05 Kcal/mol. The same are shown in table.5.23

Table.5.23 Protein stability prediction of p.N163K mutation of NLGN4Y gene

Mutation	WT	NEW	pH	Temp	SVM2 Prediction Effect	RI	DDG Value
p.N163K	N	K	7.0	25	Decrease	2	-0.05 Kcal/mol

(DDG<-0.5: Large Decrease of Stability; DDG>0.5: Large Increase of Stability; -0.5<=DDG<=0.5: Neutral Stability).

Fig.5.96 showed a stability plot of wild type over mutant protein. The red colour indicates the wild type protein stability and the green colour indicates the mutant protein stability.

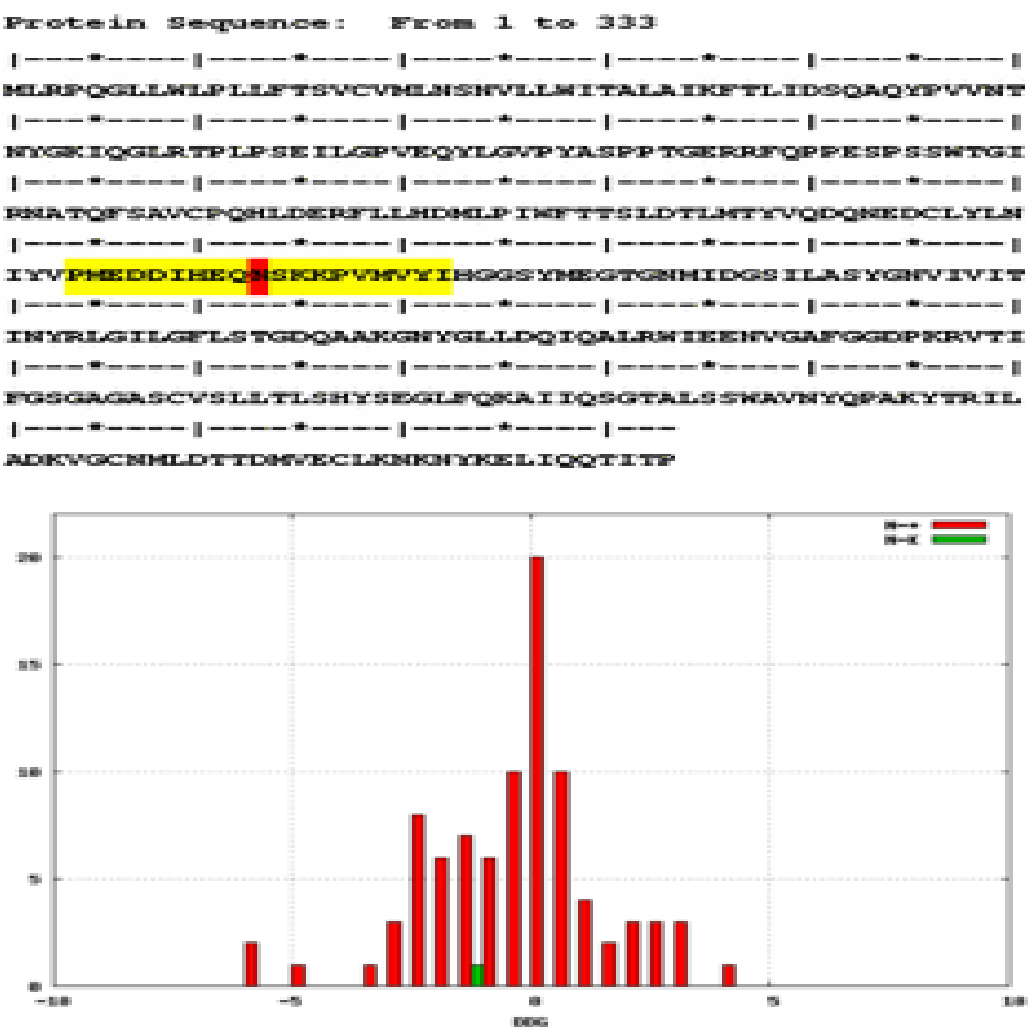


Fig.5.96 NLGN4Y protein stability prediction over p.N163K missense mutation

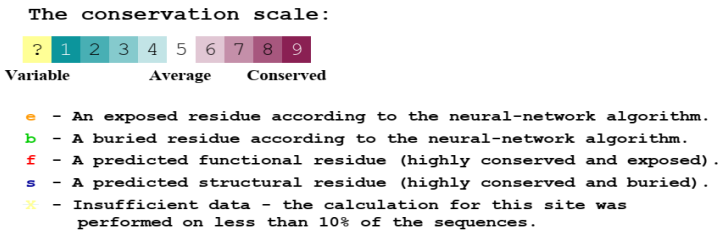
5.3.4.3.3 Conservation status analysis

Multiple sequence alignment of NLGN4Y protein shows p.N163K variant being located in the conserved region only in higher eukaryotes. Uniprot accession numbers of sequence used were as follows; *Gallus gallus* (D3WGL4), *Pan troglodytes* (H2R3R1), *Homo sapiens* (Q8NFZ3), *Macacamulatta* (B8YE04) and*Gorilla gorilla* (C3UJQ3). the same are shown in fig.5.98.

Fig.5.97 Conservation status of p.N163K mutation, the amino acid residue at 163 position is not conserved; it is variable and exposed residue.

Conservation status analysis of NLGN4Y protein sequences showed that amino acid residue at 163 position is not conserved; it is variable and exposed residue according to the neural network algorithm. The same are shown in fig.5.99.

Fig.5.98 Conservation status of p.N163K mutation residue indicated using arrow mark in NLGN4Y protein.



5.3.4.4.4 Protein structure prediction

Three-dimensional protein modelling analysis of NLGN 4Y protein revealed that mutant residue was bigger than wild type residue and it possesses a positive charge whereas wild type protein possesses a neutral charge. The wild type of residue was predicted to be located in its preferred secondary structure, a turn but the mutant residue prefers to be in another secondary structure; therefore, the local conformation was slightly destabilized. The mutated residue was located in a domain that is important for the binding of other molecules. Mutation of the residue might disturb this function of the NLGN4Y protein. the same are represented in fig.5.99 and fig.5.100.

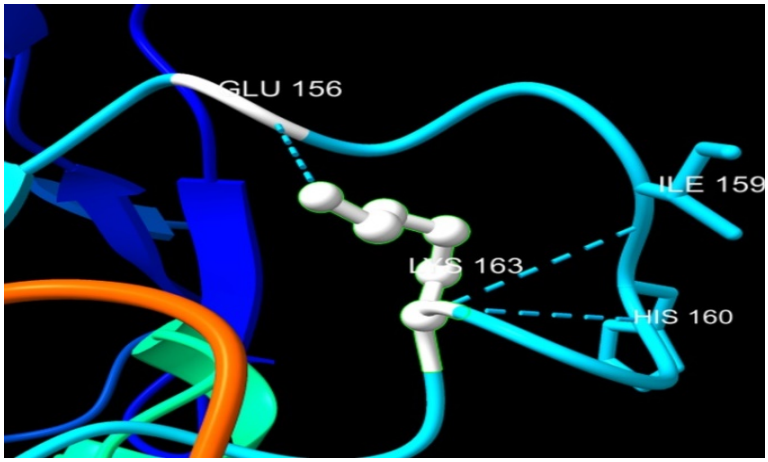


Fig.5.99 Showing the 3D structure of wild-type NLGN4Y protein. Interaction of Asparagine at residue 163 with Glutamate at residue 156, Histidine at residue 160 and Isoleucine at residue 15

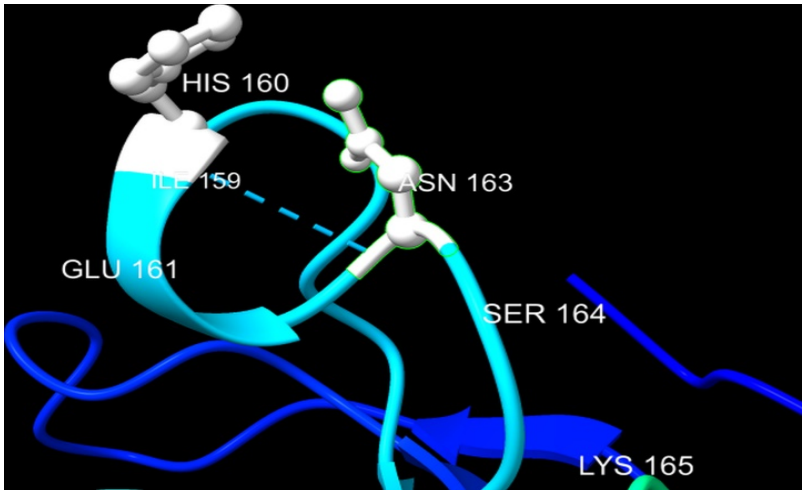


Fig.5.100 Showing the 3D structure of mutant NLGN4Y protein (p.N163K). Interaction of Asparagine at residue 163 with Isoleucine at residue 159

5.3.4.4.5 Protein-Protein interaction

The insilico protein-protein interaction analysis revealed a good protein interaction among wild type NRXN & wild type NLGNY (Docking Score: -223.74, Binding energy kcal/mol: -6.8). Whereas interaction between wild type NRXN & mutant type NLGN4Y showed no difference in binding energy (Docking Score: -236.83, Binding energy kcal/mol: -6.4) due to a novel missense mutation (p.N163K) when compared to wild type interactions. The interaction results are represented in table.5.24 and fig.5.101 and fig.5.102.

Table.5.24 showing results of Protein-Protein interaction analysis.

Molecules	**Docking Score**	**Binding energy kcal/mol**
Wild type NRXN & Wild type NLGN4X	-223.74	-6.8
Wild type NRXN & mutant type NLGN4X (p.N163K)	-236.83	-6.4

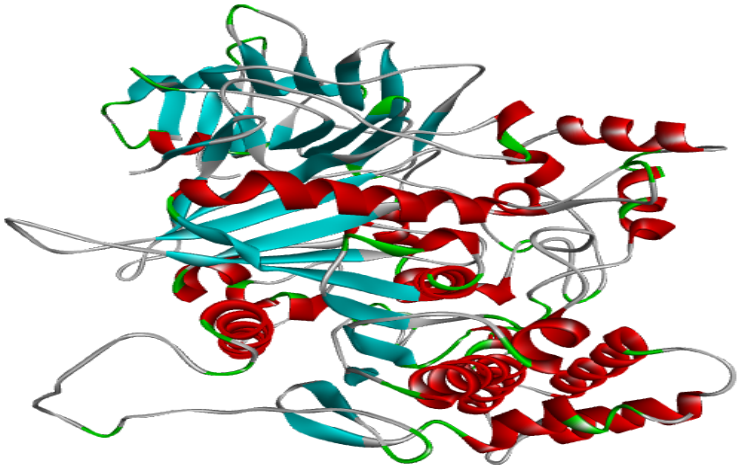


Fig.5.101 Interaction between Wild type NRXN and wild type NLGN4Yprotein

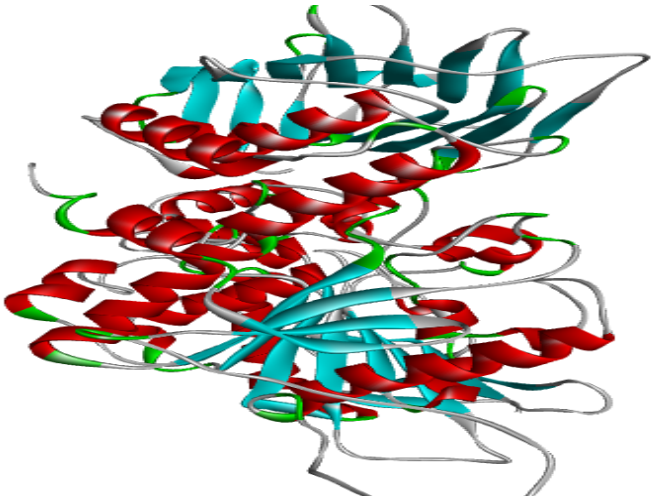


Fig.5.102 Interaction between Wild type NRXN and mutant NLGN4Yprotein with p.N163K

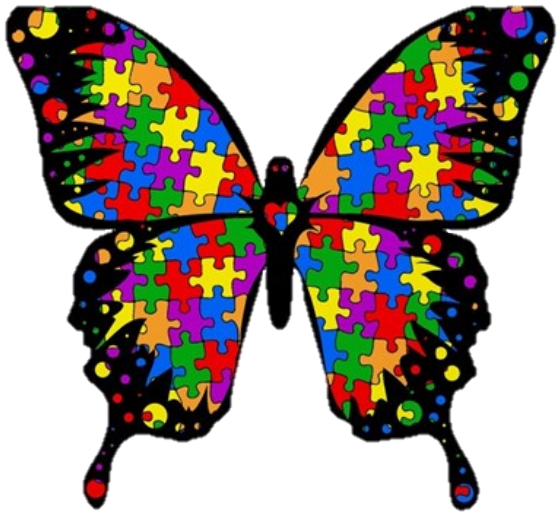
The novel missense mutation p.N163K was observed in 3 children and all are males. All belonged to severe autistic group with mean CARS score of 45.3 and mean IQ was 27.6. Child-3 suffered from birth asphyxia remaining 2 children did not suffer from any postnatal damages. Consanguineous marriage was observed in parents of child-2 and remaining children had non-consanguineously married couple.

Table.5.25 Clinical features of autism children with missense mutation, p.N163K of *NLGN4Y* gene

Demographic character	**Child 1**	**Child 2**	**Child 3**
Ethnic Origin	Indian	Indian	Indian
Sex	Male	Male	Male
Age of father at child's birth	38	29	35
Age of mother at child's birth	33	19	34
Consanguineous marriage	No	yes	no
Prenatal damage	preeclampsia	none	none
Postnatal damage	None	none	birth asphyxia
IQ	25	30	28
CARS Score and Severity	40; Severe	44; Severe	52; Severe
Co-morbid condition	None	None	None

Chapter 6

DISCUSSION



Autism (MIM 209850) is a heterogeneous neurological disorder manifesting before the age of three (1, 2). Autism results in impaired social interaction, impaired communication and abnormal behaviour (2, 3). Autism spectrum disorder is the leading cause of disability among all other mental disorders in the below 5-year age group. Autism persists for a lifetime. The biology of autism spectrum disorder and scientific interventions are limited to the western countries, where only 10% of the world's children live (4). India is the world's second most populated nation, with a population of 1.4 billion people. With 3.2 million km of geographical area, it makes up around 2.4% of the world's surface (5). In India, autism is still under the shed. No sufficient experimental data is available on its clinical aspects. Since last few years, several attempts have been carried to understand the autism in Indian population. But all these attempts to give an overview of the disorder were only able to highlight some of the aspects and have not provided an in-depth knowledge of the disease.

Suravi Patra & Sujita Kumar Kar in 2021 reviewed 159 research articles. The research category includes clinical profile, biomarkers, intervention, social, psychological, risk factors and epidemiological. The majority of the publications were from south Indian states followed by North Indian states and East Indian states. (6.) They recommended that there is a need for nationwide studies to understand the biology of autism in India. The prevalence of autism spectrum disorder has largely varied and increased significantly in recent years. The global prevalence is about 1 in 64 to 1 in 150. As noted above, the varied prevalence may be due to age group, diagnostic criteria, geographic location and culture of the studied population (7).

Still, there is no exact estimation of the prevalence of autism in India because the epidemiological studies that have been conducted so far have only included clinic-based case series and local community-based research. To our knowledge, the present study is the first of its kind research study on autism spectrum disorder in children aged between 1-18 years in the North Karnataka population of Karnataka, India. Our study recorded 136 (7.4%) autistic children among 1828 studied mentally ill children in the North Karnataka population of Karnataka, India. A similar school-based study which was conducted by Sharda V *et al.,* in 2012 on 500 mentally ill children recorded 14.8% of autistic children among the studied subjects (8). This increasing frequency may be due to a low sample size compared to the present study cohort or it might be due to DSM-IV criteria. In present study, the diagnosis of the children was according to recently updated DSM-V criteria. Few studies from northwest and south

India recorded a prevalence of autism to be 0.15% and 0.23%, respectively (9). However, several recent epidemiological studies in urban and rural areas of India recorded an overall prevalence of autism in the Indian population ranging from 0.15% to 1.01% depending upon the geographical area and screening method of the study conducted (9, 10). Variability in methodology leads to inconsistency and unreliable prevalence estimation in the Indian population. The low and high prevalence of autism reported in different Indian studies may be due to restrictions on case definition and the instruments available for autism screening and diagnosis.

Autism spectrum disorder was recorded in high frequency in males than in females. The global record of male to female ratio of autism is 3-4:1 (11). It is debatable whether this arises from a sex difference in the frequency of autism spectrum disorders or whether females are underdiagnosed. The present study cohort recorded 79.4% of male children and 20.6% of female children among studied 136 autistic children. Our study recorded a 4:1 male to female ratio which is similar to the study by Sharda V *et al.,* 2012 i.e. 3.8:1 in Mangalore autistic population (8). Several other Indian studies from different states also recorded greater male cases compare to female ASD cases (12, 13). In contradiction to these studies, few studies recorded no significant difference in male predominance in autism (7, 14, 15). This difference may be due to the sample size of the studied groups and the ethnicity of the study population.

Globally clinical evaluation of autism spectrum disorder was carried out by the majority of professional psychiatrists and paediatricians using ICD or DSM criteria (must use the recent version at the time of diagnosis). The present study used the latest version of the Diagnostic and Statistical Manual of Mental Disorder-V (DSM-V) criteria and the International Classification of Diseases-10 (ICD-10) criteria. DSM-V has categorised the severity of autism spectrum disorder into 3 different levels. Level-1: Requires Support, Level-2: Requires Substantial Support and Level-3: Requires Very Substantial Support. As per the DSM-V criteria in our study 15% of the autistic children were categorized as Level-1, 26% of the autistic children were categorized as Level-2 and 59% of the autistic children were categorized as Level-3 autism (16).

The Childhood Autism Rating Scale (CARS) is a widely used assessment for diagnosing and evaluating the severity of autism. A score of 30 or above shows the

existence of autism, 30–36.5: mild/moderate autism, and 37–60: severe autism. CARS is commonly used in the Indian community for evaluating the severity of autism due to its accuracy, reliability, and validity. The Indian Scale for Assessment of Autism (ISAA) was created by the National Institute for Mentally Handicapped (NIMH) in 2009 to diagnose and assess the severity of autism in the Indian autistic population. (17). ISAA was developed based on the childhood autism rating scale and has 40 items divided into six categories; social relationship and reciprocity, emotional responsiveness, speech, language and communication, behaviour patterns, sensory aspects and cognitive component. In the present study, we decided to evaluate the severity of autism based on CARS compared to ISAA due to better sensitivity and reliability.

In the present study as per the CARS, out of 136 identified autistic children, 47 (34.6%) children were having Mild-Moderate conditions and 89 (65.4%) were having severe autism. Gender wise distribution of severity analysis based on CARS score showed 33 (30.6%) male children were Mild-Moderate autistic and 75 (69.4%) male children were severely autistic. 14 (50%) female children were mild- moderate autistic and 14 (50%) female children were severely autistic. In the present study majority of the male autistic children belonged to the severely autistic group compared to females.

Advanced paternal and maternal age is a potential risk factor for ASD in the offspring **(**18). The risk of autism according to the highest vs. the lowest parental age category is unknown (19, 20). There is a hypothesis that advanced paternal age may increase the risk of autism more than advanced maternal age. This might be a result of genetic imprinting or sporadic denovo variants emerging in the spermatogonia with increased paternal age or confounding environmental and social factors (21). Several Human and animal investigations supported the hypothesis that de novo variants contribute to the connection between advanced paternal age and the risk of ASD (19, 22, 23). The present study recorded the maximum number of autistic children born to fathers belonging to the 31-40 years age group followed by the 21-30 years age group and 41-50 years age group. In detail 34.6% of autistic children were born to a father age ranging from 21-30 years, 56.6%children with a father's age ranging from 31-40 years and 18.8% of children with a father's age ranging from 41-50 years at the time of birth. The study by Ravi S *et al.,* 2016 in the Indian autistic population showed increased maternal age as a significant risk factor for ASD (7) which was supported

by earlier studies by Gardener H, *et al.,* in 2009 and El-Baz, *et al*, in 2011 (15, 21). In the case of maternal age, the maximum number of autistic mothers belongs to 31-40 years of age followed by 41-50 years and 21-30 years at the time of birth. In detail 25% of autistic children were born to mothers with ages ranging from 21-30 years, 41.9% of children with maternal ages ranging from 31-40 years and 33.1% of children with maternal ages ranging from 41-50 years at the time of birth. Some studies explained advanced maternal age has been associated with a high risk of obstetrical complications, while it is not known which, of these complications may increase the risk of autism (15, 21, 24). According to Wu *et al*. (2017), an increase in maternal and paternal age was associated with a 41% and 55% higher risk of autism, respectively (18). According to studies, there was a 20% and 30% increase in ASD for each 10-year increase in maternal and paternal age respectively (26). Andoy Galvan JJ *et al.,* in 2020 studied the mode of delivery, order of birth and parental age gap in Malaysian autistic children and they found that there was a significant association between advanced maternal age and risk of autism, but no significant association with advanced paternal age and risk of autism (26). But mechanisms related to advanced maternal and paternal age on ASD risk have not been frequently studied.

Recently, few studies investigated the parental age gap which showed that moderate to large (10 years or more) differences between the parental ages resulted in an increased risk of ASD (24). In the present study cohort, 55.1% of autistic children belonged to parents with a 5-10 years age gap. 28.7% belonged to parents with >10 years of the age gap. A population study by Croen *et al*., in 2007 found no significant association between ASD and the age gap between the parents. However, only a few studies have worked on parental age gaps and the risk of ASD. This highlights the need for future investigations on the risk of ASD and the parental age gap (27). There was a joint effect of maternal and paternal age with increased risk of ASD for couples with increasing differences in parental ages.

ASD risk can also be significantly influenced by preterm birth and other early environmental exposures. But even though the mechanisms have not yet been defined, they might involve altered inflammatory pathways (28). Elevated pro inflammatory cytokine levels such as interleukin 1, interleukin 6, and tumour necrosis factor α, have been linked with uterine activation and the timing and beginning of preterm birth. (29). It has been observed that pro inflammatory cytokine overexpression in preterm

new-borns persists throughout the process of childhood development (30). Increased inflammatory markers have also been recorded in the brain and CSF fluid of individuals with autism (31). According to a recent meta-analysis, children born preterm (gestational age <37 weeks) have a 30% higher risk of developing autism spectrum disorder (ASD) than that born in term (32). The majority of the studies did not examine the sex specific risk of pre-term birth on autism but few studies hypothesised that prematurely delivered girl babies are more prone to autism than boys (33). Mamidala *et al.,* 2015 showed preterm birth in the Indian population was a significant risk contributor to ASD (34). In contrast to this study, our study recorded a maximum number of autistic children with normal delivery (65.4%) followed by premature delivery (34.6%).

Worldwide, there are about a billion individuals who live in communities where consanguineous marriages are preferable over non-consanguineous marriages (35). The consanguinity rates in India are around 14%, however, there are variations based on regional and religious preferences, with rates as high as 54.9% in Pondichery and South India and 77.1% in some parts of Pakistan (35). There is a lot of research that suggests that consanguinity increases the likelihood of developing autosomal recessive illnesses, as well as other complications like birth defects, stillbirths, and intellectual disability (35). Autism spectrum disorder is thought to have a genetic component that may be caused by an autosomal recessive transmission (36). Consanguinity is reported to have serious effects on fetal growth and development and increases the risk of congenital malformations and neurological abnormalities (34). Consanguineous marriage is most common in practice in the North Karnataka region of Karnataka, India. In the present study, 61.8% of autistic children have consanguineously married parents.

The degree of consanguinity was analysed in the present study and the maximum number of autistic parents with consanguineous marriage were second degree relative's i.e uncle-niece marriage (36.9%) followed by fourth degree relatives (29.8%) and sixth degree relatives (4.8%). Interestingly 28.6% of the autistic children's parents had an undetermined degree of relation or unknown relationship between the couples. There were no large studies in the Indian autistic population to correlate the association between the risk of autism and degree of consanguinity. One major study by Mamidala et al., in 2015, observed that consanguinity was an

independent potential risk factor for the development of Autism. First cousins, second cousins, uncle-niece, and double first cousins were the four consanguinity categories included in the study and the study included 500 children with ASD. When compared to controls, the ASD cases had significantly higher frequencies of consanguinity, with an overall risk factor of 3.22% (34). Recent Meta review by Roy et al., 2020 also supported the statement that consanguinity increases the risk for ASD (35). Similarly, Guisso et al. (2018) investigated the association between pregnancy/birth difficulties and autistic spectrum disorder in a population of Lebanese people who were known to have a high rate of consanguinity in the community and supported the previously established positive association of risk of autism by consanguineous marriage (37).

The development and growth of the foetus is rapid during the prenatal period, and entirely dependent on the gestational environment of the mother. Previous research has shown that there is an elevated risk for ASD in individuals with advanced maternal/paternal age, short gestational age, gestational hypertension, threatened abortion, cesarian delivery, prematurity, low birth-weight (LBW), and low Apgar scores (34, 38, 39). Zhang et al, in 2010 in their case control study found delayed crying at birth and neonatal complications to be significantly associated with an increased risk of ASD. However, more comprehensive research is still required to determine the precise mechanism underlying the major factors that contribute to ASD, and each factor must be examined independently. Although there is inadequate evidence to link any prenatal factor to the aetiology of autism, studies using prenatal optimisation scales do offer some evidence that exposure to pregnancy complications, in general, may raise the likelihood of autism (14).

Numerous perinatal and neonatal issues also represent what was going on throughout the pregnancy, so it's possible that prenatal environment compromises that manifest as difficulties with labour, delivery and neonatal health are etiologically important. The present study recorded prenatal factors in the study cohort. 5.1% of the autistic children mother had faced a birth complication during childbirth, 3.7% of the autistic children mother has pregnancy complication, 3.7% of the autistic children mother showed high blood pressure, 2.2% of the autistic children's mother had hyperthyroidism, 0.7% of the autistic children mother had a mental illness, 36% of the autistic children had more than 30 years aged father and 44.9% of children have more than 30 years aged mother during birth.

The present study also recorded postnatal factors in the study cohort. 8.8% of the autistic children had a gastrointestinal problem, 8.1% of the autistic children's mothers had birth asphyxia, 7.4% of the autistic children showed feeding problems, 6.6% of the autistic children had respiratory problems, 5.1% of the autistic children had developmental delay, 2.2% of the autistic children encountered high fever, 2.2% of children showed sleep dysfunction, 0.7% of the autistic children encountered jaundice, 0.7% of the autistic children had partial agenesis of the corpus callosum and 0.7% of the autistic children had congenital abnormalities.

ASD is highly genetically heterogeneous with contributions from Alleles of varying frequencies (i.e., common, rare, very rare), Inheritance patterns (i.e. dominant, recessive, X-linked, de novo), Variant types (i.e., large chromosomal rearrangements, copy number variants (CNV), small insertions/deletions (indels), and single-nucleotide variants (SNVs)) (40). The majority of the genes associated with ASD are involved in the development and functions of the brain. This includes chromatin remodelling, regulation of protein synthesis and degradation, or synaptic plasticity. Many synaptic protein genes are linked to the pathogenesis of ASD. Variants in genes like *NRXN*, NLGN, *SHANK*, TSC1/2, FMR1 and *MECP2* converge on common cellular pathways that intersect at synapses. The proposed candidate genes for ASD in the present study were Neuroligins. Neuroligins are a family of postsynaptic cell adhesion molecules that have emerged as important factors regulating neuronal development and synaptic transmission. Genetic research on autism has made great progress in the past few years. Neuroligin is a postsynaptic transmembrane protein involved in synaptogenesis which is predicted to be a promising candidate gene for autism and other neurological disorders (41-43).

The majority of the variant recorded in the neuroligin family genes associated with autism are coding sequence variants, particularly missense variants. The present study also records the maximum number of missense variants compared to other coding sequence variants. Neuroligin shows diverse genetic modifications in autism spectrum disorder. No sufficient experimental data particularly genetic background on autism spectrum disorder in Indian ethnicity is available for clinical aspects. As per our knowledge, this is the first of its kind study in the Indian autistic population evaluating the association of the neuroligin gene in autism spectrum disorder. Neuroligin is one of the most targeted genes for neurological studies due to its crucial role in the formation and maturation of synaptogenesis. Various neuroligin variants

are associated with different neuropsychiatric conditions like Tourette syndrome, Attention Deficit Hyperactivity Disorder (ADHD), Autism, anxiety, depression, and intellectual disability (2). Two X-linked neuroligin genes, neuroligin 3 (*NLGN3*) and neuroligin 4X *NLGN4X*) were therefore considered as well functional and positional candidates for predisposition to ASDs and have been screened for variants in multiple studies (44). After the initial finding of the association of neuroligin gene, especially *NLGN3*and *NLGN4X* in the aetiology of autism in 2003 and 2004 (45, 46) several studies in the subsequent year from different ethnic populations around the globe have been conducted to evaluate the same. The studies in 2004 and 2005 from the American, UK and Canadian populations failed to record any variant in *NLGN3* and *NLGN4X* genes (47-49). The few studies which tried to establish the link between neuroligin and autism failed and concluded that coding sequence variants of the neuroligin gene in autism were rare (48-51). Several deletion variants successfully showed the pathogenic effect on the function of NLGN3 and NLGN4X protein in ASD (45, 46, 52). Not only does frameshift deletion variant have an effect on the *NLGN4X* protein however Single nucleotide variations in *NLGN4X* can cause ASD by a loss-of-function mechanism (36).

Chromosomal abnormalities have a substantial role in autism spectrum disorder (53, 54). The large genome wide study strongly supported the involvement of synaptic genes (*NLGN, NRXN AND SHANK*) along with novel loci (DPP6-DPP10-PCDH9 (synapse complex), *ANKRD11, DPYD, PTCHD1*, 15q24) in the causation of autism spectrum disorder. Later they also hypothesised that most structural variations found on the X chromosome are inherited from parents and that X-linked CNVs are maternally transmitted to males may help in understanding gender prevalence differences and the high degree of heritability in ASD (53).

As whole genome sequencing becomes more economical and a realistic experimental approach, it is expected that more genetic variants that contribute to complex conditions like autism would be discovered (55). According to exome and whole-genome sequencing (WGS) studies, ASD risk genes may be involved in dysregulation in different molecular processes, such as chromatin modifications, synaptogenesis, RNA splicing, signalling pathways, gene expression regulation, neuronal communication, cytoskeletal organisation, and cell cycling (56, 57). The study of ASD genetic risk factors is now being accelerated by big genomics data,

bioinformatics, and experimental innovation, particularly the common variations and those variants in non-coding and regulatory regions.

It is suggested that variants in the *NLGN3* and *NLGN4* genes could give rise to aberrant development of the nervous system and abnormal neural connectivity (58, 59). All the previous studies strongly supported the heterogenous nature of neuroligin in the pathophysiology of autism (47-51). In the present study, we studied *NLGN3*, *NLGN4* and *NLGN4Y* gene association in autistic children under the age of 18 years and free from neurological disorders. *NLGN3* and *NLGN4X* gene was studied in 108 autistic children and the *NLGN4Y* gene was studied only in 85 male autistic children.

In our study, we recorded 5 variants which included one coding sequence variant (missense variant) and four non-coding sequence variants (two 5'UTR variants & two 3' UTR variants) in the *NLGN3* gene, 25 variants which included eight 5′UTR variants, four missense variants, four synonymous variants, one Frameshift variant and eight 3′ UTR variants in *NLGN4X* gene and 09 variants, which included one missense and eight synonymous variants in *NLGN4Y* gene in our study cohort. In the *NLGN3* gene all the variants (one missense, two 5'UTR and two 3'UTR), in *NLGN4X* gene 09 variants (five 5′UTR, one missense, one synonymous, one frameshift and one 3′ UTR) and *NLGN4Y* gene 4 variants (one missense and 3synonymous) were novel variants which were not recorded in any in house SNP databases. Synonymous substitutions could affect the protein function through transcription or translational impairment (61-63). In the present study, no non synonymous variant was observed in *NLGN3*and the *NLGN4X* gene recorded the maximum number of synonymous variants followed by the *NLGN4Y* gene. In contrast, several studies failed to identify any non-synonymous variants in *NLGN3* and *NLGN4X* in samples of individuals with ASD (47-49, 64). The majority of the variants were recorded in the *NLGN4X* gene compared to *NLGN3* and *NLGN4Y* genes. *NLGN4Y* gene harbors a synonymous variant in high frequency. In the present study, we carried the detailed Insilico and functional analysis only for synonymous and frameshift variants, particularly novel variants.

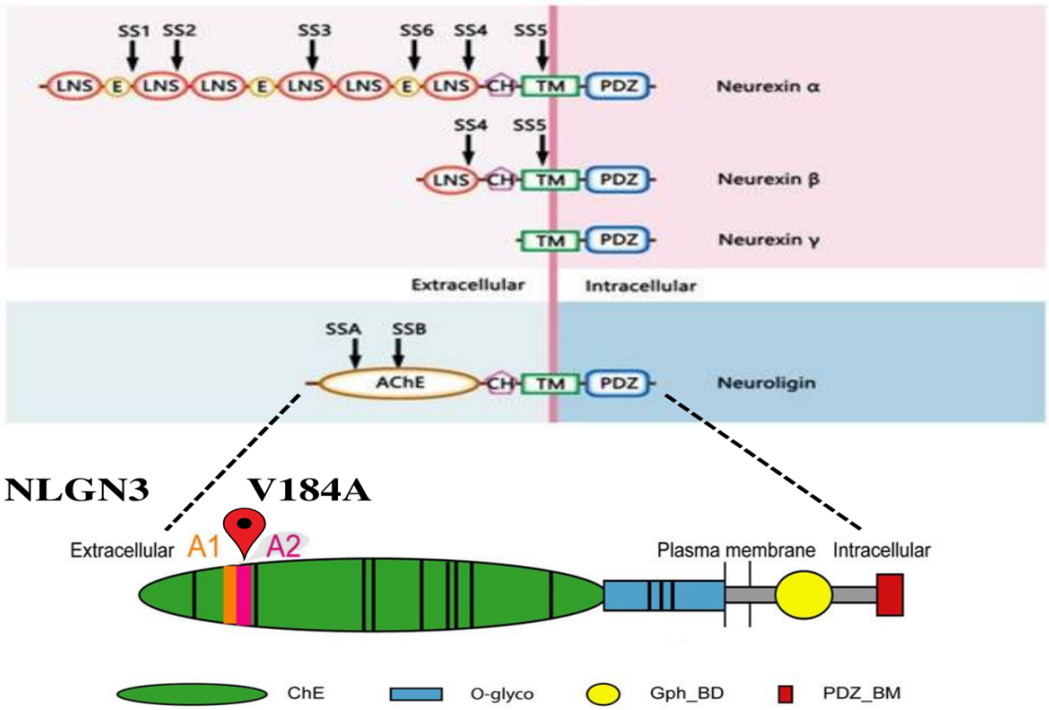


Fig.6.1 Representation of binding of neurexin and Neuroligin3 protein with a novel missense variant (p.V184A) in the present study cohort

The only coding sequence variant recorded was a missense variant in *NLGN3*gene, which was a novel variant. Novel missense variant c.551T>C in the *NLGN3* gene was not recorded in any inhouse SNP databases. Hence, we successfully submitted our novel variant to the ClinVar database National Center for Biotechnology Information. ClinVar; accession No: VCV001710287.1 (https://www.ncbi.nlm.nih.gov/clinvar/Variation/VCV001710287.1). Thymine to cytosine substitution at c.551 position (GTC-GCC) results in Alanine in place of Valine at 184 positions of NLGN3 protein. A novel missense variant was observed in 27 (25%) autistic children including 18 male and 09 female children. A novel missense variant was recorded in the extracellular domain of the NLGN 3 protein (Fig.6.1). Similar to our study, the majority of the previous studies also recorded pathogenic missense variants in the extracellular domain of the NLGN3 protein Such as R451C, G426S, P514S and R597W etc... (41, 45, 65).

PROVEAN, PolyPhen2, PANTHER, PHD-SNP, SNPs & GO and SNAP2 pathogenicity prediction tools showed c.551T>C variant was deleterious/disease causing. The Valine residue at variant site 184 was highly conserved and highly

involved in the evolutionary relationship over different species. Protein stability prediction also showed a large decrease in the stability of mutant protein over wild type protein. Protein homology modelling and structural analysis showed mutant residue was smaller than wild type residue and also variation in structural integrity and thus reducing the stability of mutant protein over the wild type. Protein-protein docking analysis showed decreased binding energy in the interaction of mutant NLGN3 protein with presynaptic neurexin protein compared to wild type NLGN3 and neurexin protein. This might have led to a loss of interaction with synaptic molecules because it is located in the extracellular region of the neuroligin3 protein. Similar to our study, previously recorded variants in the extracellular domain affect *NLGN3* protein function, such as p.G426S variant influences the binding of neuroligin and neurexin via abnormal Ca2+ concentration (66). The R451C variant is found in the EF-hand domain. All known neuroligins, including *Drosophila melanogaster*, have predicted conserved EF-hand domains. These domains are known to provide structural integrity and Ca2+-dependent functional features. Therefore, R451C might alter the binding of neuroligins to neurexin.

Certain previous studies showed that functional analysis of missense variant is very important to predict the risk of causing autism via defeated synaptogenesis (66). Hence, we performed the expression profiling of the *NLGN3* gene with a novel missense variant. A significant decrease in the expression of the *NLGN3* gene was observed in autistic children with novel missense variants compared to the control group (1.94 vs 5.36, p-value 0.001). Expression analysis and Insilco analysis showed abnormal structure and function of neuroligin3 which might cause abnormal synaptic homeostasis and altered *NRXN* binding which leads to abnormal excitatory and/or inhibitory synaptogenesis (fig.6.2).

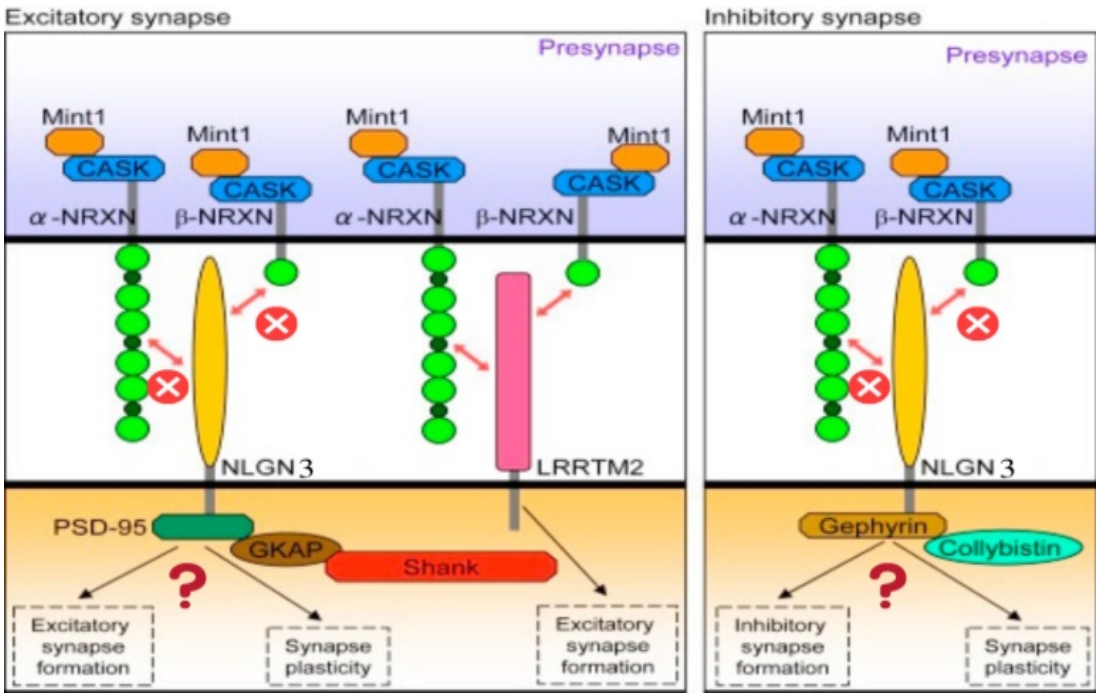


Fig.6.2 Representation of synaptogenesis pathway with NLGN3 and NRXN binding in both excitatory and inhibitory synapses. Cross marks represent abnormal binding of NLGN3 protein due to Novel missense variant in the extracellular domain with alpha and beta neurexin which further causes abnormal excitatory and inhibitory synapse formation and synaptic plasticity

Analysis of the *NLGN4X* gene recorded a novel missense variant and a frameshift variant in the extracellular domain of the NLGN4X protein (Fig.12). Adenine to guanine substitution at c.307 position (ACT-GCT) resulted in Alanine in place of Threonine at 103 position of NLGN4X protein. This variant was recorded in 7.04% of autistic children. Similar to our study, majority of the previous studies also recorded pathogenic missense variants in the extracellular domain of the NLGN4X protein. Such as G84R, Q162 K, A283T, G99, K378 and V403 are located in the esterase domain and R704 is located in the cytoplasmic domain (41, 66, 67).

Pathogenicity prediction of novel p.Thr103Ala variant was shown to be harmless/neutral on the function of NLGN4X protein by PROVEAN, PolyPhen2, PANTHER, PHD-SNP, SNPs & GO and SNAP2 and this variant residue was not conserved and buried, this was conserved only in higher primates.

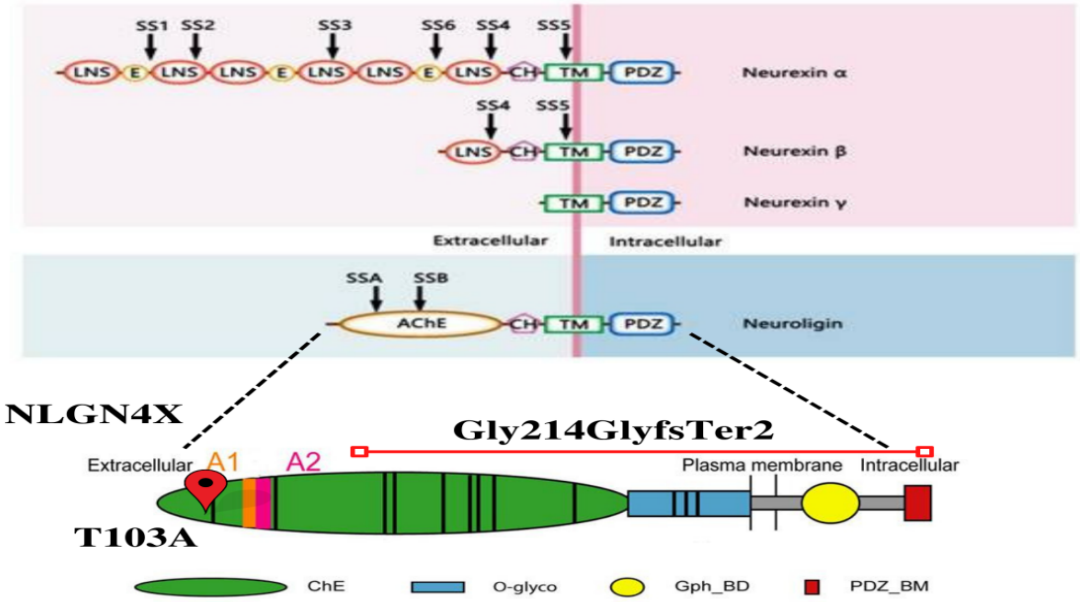


Fig.6.3 Representation of binding of Neurexin and Neuroligin4X protein with a novel missense variant (p.T103A) and novel frameshift variant p.Gly214GlyfsTer2 in the present study cohort

Protein stability analysis also showed a slight decrease in the function of NLGN4X protein due to novel missense variant. This was further supported by protein homology modelling and structural analysis which showed mutant residue was smaller than the wild-type residue and the mutant residue was more hydrophobic than the wild-type residue. The difference in hydrophobicity may or may not affect hydrogen bond formation. This loss of hydrogen bond affects NLGN4X protein folding and thus decreases the stability of the protein. Further docking analysis showed no difference in binding energy in the interaction of mutant NLGN4X protein with presynaptic neurexin protein compared to wild type NLGN4X and neurexin interaction (-4.38 vs-4.84). Neuroligin4X protein with novel missense variant binds normally to presynaptic neurexin during functional synaptogenesis and it may not lead to abnormal synapses. Due to insufficient insilico evidence to prove the pathogenic effect of the p.T103A variant, we did not perform the expression profiling of the *NLGN4X* gene with this variant. Along with novel missense variant, other missense variants were also studied for pathogenicity prediction, protein stability and conservation status analysis.

In contrast to our results, the previously recorded variants in the extracellular domain affect the *NLGN4X* protein function. p.G84R, p.Q162 K and p.A283T alter the polarization of the amino acid and which may influence the protein's structure.

R87W variant disrupted *NLGN4*Xs glycosylation process, altered its location on the cell surface, and rendered *NLGN4X* incapable of forming synapses (67).

The second novel variant recorded in the *NLGN4X* gene was frameshift variant; p. Gly214GlyfsTer2.This variant occurred due to the insertion of guanine (G) at g.324659_324060 position. This insertion of G altered the reading frame resulting in an immediate stop codon. Protein homology modelling and structural analysis showed truncated protein with only 214 amino acids due to this stop codon. Approximately 74% of the original protein was truncated due to p.Gly214GlyfsTer2 novel variant. This variant was recorded in 27 autistic children. Our study recorded similar results obtained by previous studies. The first study by Jamain *et al.,* in 2003 on the *NLGN4X* gene recorded a de novo frameshift variant 1186insT. This variant produces a stop codon at the 396 position, which causes the protein to be prematurely terminated before the transmembrane domain. Another study by Laumonnier F *et al.,* in 2004 recorded that 2bp deletion in the *NLGN4X* gene produces a stop codon at 429 position. The C-terminal transmembrane region and approximately 50% of the normal amino acid sequence were expected to be deleted in the resulting mutant protein (D429X). Both the frameshift variants resulted in the deletion of the AchE-homologous domain, which is important for the oligomerization and synapse-promoting function of neuroligins (45, 46). Compared to these two studies our study recorded approximately 74% loss of wild type *NLGN4X* protein (Fig.6.3).

To evaluate the severity of the frameshift variant we further carried out expression analysis. Relative expression analysis of *NLGN4X* showed a large significant decrease in autistic children with a frameshift variant compared to the control group i.e 0.05 and 1.22 respectively (p-value 0.00001). A large reduction in the relative expression of the *NLGN4X* gene in autistic children with novel frameshift variant strongly supports the insilco pathogenicity prediction result. So neuroligin 4X proteins with p.Gly214GlyfsTer2 variant failed in binding to presynaptic Neurexin during functional synaptogenesis leading to abnormal synapses (Fig.6.4). The concentration of the functional NL4GNX protein on the cell surface is a basic mechanism through which variants of the NLGN4X protein produce psychiatric disorders, although several molecular pathways are believed to be involved (68). Chih B *et al.,* in 2005 recorded electrophysiological studies on neuroligins genes having deletions in the cytoplasmic tail or the esterase homology domain which resulted

abnormal synaptogenesis because these regions play a crucial role in maintaining a functional balance between excitatory and inhibitory synapses in hippocampal neurons (69).

Interestingly, p.Gly214GlyfsTer2 variant was recorded in 16 male and 3 female autistic children, which supports the role of the *NLGN4X* gene in male predominance. So as in several previous studies coding sequence variants in the *NLGN4X* gene might be harmless or rare but these rare variants have strong significant effects on functions of the Neuroligin 4X gene in autism.

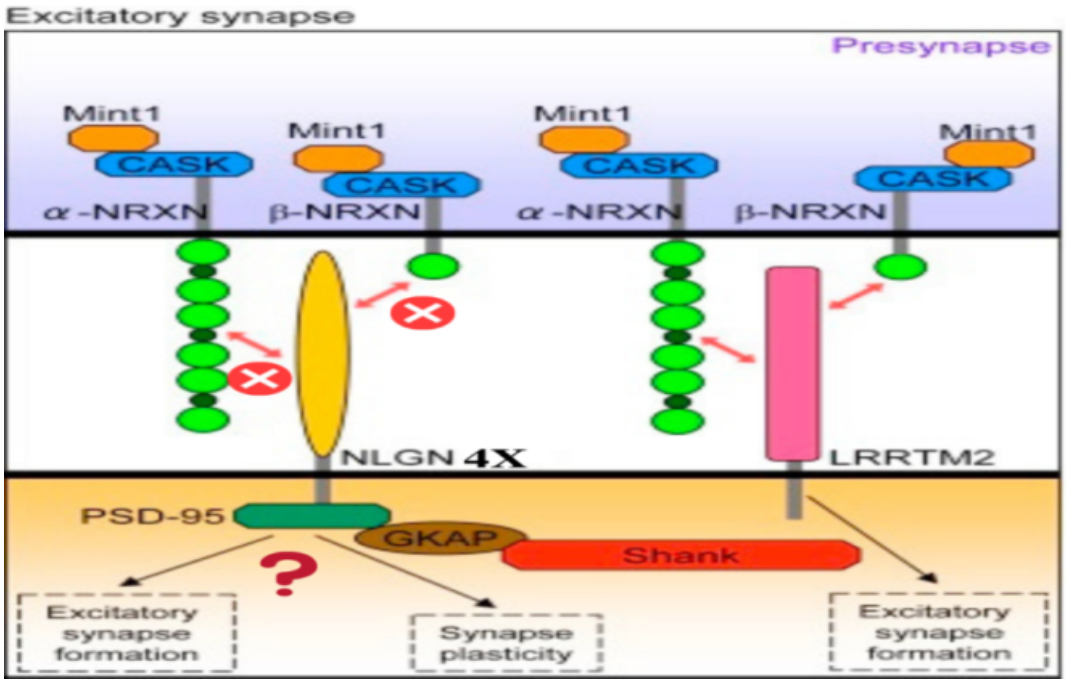


Fig.6.4 Representation of synaptogenesis pathway with NLGN4X and NRXN binding in the excitatory synapse. Cross marks represent a loss of binding of NLGN4X protein due to Novel Frameshift variant in the extracellular domain with alpha and beta neurexin which further causes abnormal excitatory synapse formation and synaptic plasticity.

Sequencing analysis of the *NLGN4Y* gene recorded 09 variants. One was novel missense variant and reaming 08 (88.9%) were synonymous variants. Cytosine to adenine substitution at p.163 position (AAC-AAA) results in Isoleucine in place of Asparagine at 163 position of NLGN4Y protein. A novel missense variant was observed only in 3 male children. Insilco pathogenicity prediction of a novel missense variant, p.N163K using PROVEAN, POLYPHEN2, SNAP2, SNP&GO and CADD showed harmless/non-pathogenic effects on the functions of neuroligin 4Y protein. Protein stability analysis showed a slight decrease in the function of NLGN4Y protein

but structural analysis of p.N163K mutant protein over wild type protein showed slight destabilisation in the local configuration by positioning on different secondary structures, a turn and it might cause bumps in protein structure due to differences in the size of amino acid. Meanwhile, the variant introduces a charge; this can cause the repulsion of ligands or other residues with the same charge. Structural and functional prediction of novel missense variant indicates slight changes only in concern to the structure of neuroligin 4Y protein, not on functions of the protein.

Further docking analysis showed no difference in binding energy in the interaction of mutant NLGN4Y protein with presynaptic neurexin protein compared to wild type NLGN4Y and neurexin interaction (-6.4 vs-6.8). Neuroligin4Y protein with novel missense variant binds normally to presynaptic neurexin during functional synaptogenesis and it may not lead to abnormal synapses. Due to insufficient Insilico evidence to prove the pathogenic effect of p.N163K variant, we did not perform the expression profiling of the *NLGN4Y* gene with p.N163K variant

Identification of coding sequence variants is not sufficient to disclose the exact role of the gene in disease, which is having complex genetic architecture. Not only missense variants or deletion variants but intronic and non-synonymous variants also affect the regulatory region such as an enhancer, and promoter, associated with histone modification sites NAS & TFBs are modest contributions to ASD pathogenesis. Other forms of genetic alterations including splice variants and non-coding sequence variants may also lead to potentially abnormal function of Neuroligin in autism (50, 70). Moreover, we also recorded novel non-coding sequence variants in *NLGN3* and *NLGN4X* genes but no non coding variant was observed in the *NLGN4Y* gene from our study cohort. Among *NLGN3* and *NLGN4X* genes, the maximum number of non-coding variants was recorded in the *NLGN4X* gene. These non-coding variants may also have a role in posttranslational modification.

Neuroligins are crucial components in the synaptogenesis mechanism. They interact with presynaptic b-neurexin, postsynaptic density proteins PSD-95 and S-SCAM, and are located post-synaptically in glutamatergic synapses. Synaptic vesicles and the exocytotic apparatus create functional presynaptic structures as a result of the neuroligin-b-neurexin interaction. In vitro and in vivo experiments have indicated that autism-related neuroligin variants may affect synapse maturation and function.

Studies using animal models revealed that mice with the *NLGN3* R451C variant (71) or the *NLGN4X* deletion (72) increased the inhibitory synaptic activity and displayed behavioural abnormalities typical of ASDs, suggesting a potential role for excessive inhibitory neurotransmission in ASDs. The *NLGN4* knockout mice displayed highly selective deficits in reciprocal social interactions and communications which are the key symptoms of ASD in humans (73).

Our study is preliminary basic research. Further in vivo and in vitro studies of novel variants in the Neuroligin pathway will provide a better understanding of the involvement of *NLGN3, NLGN4X* and *NLGN4Y* genes in autism spectrum disorder in the Indian population. Novel frameshift variants and missense variants of *NLGN4X* and *NLGN3* provide an important pathophysiologic and prognostic insight that post synaptic Neuroligin protein with novel frameshift variant failed in binding to presynaptic Neurexin during functional synaptogenesis leading to abnormal synapses. This causes abnormal verbal & nonverbal communication and deficient social interaction in autistic children.

In the present investigation, as compared to *NLGN3* and *NLGN4Y* genes, the *NLGN4X* gene recorded more variants in coding regions, non-coding regions and common polymorphisms associated with ASD. Neuroligin4X and Neuroligin3 genes are the probable candidate gene for future molecular investigation and functional analysis in the Indian autistic population because the components involved in the synaptogenesis and synaptic structures remain excellent functional candidates for future molecular genetic studies of autism and related neurodevelopmental disorders. With the advancement of each technology with greater precision (cytogenetics, microarrays, whole genome sequencing), there has been a tremendous advancement in the discovery of undiscovered ASD risk alleles. The discovery of genetic variants by WGS and extensive bioinformatics analysis will be the most powerful tool. In addition to improving our understanding of the genetics and genomics of the disorder, future research should focus on integrating the complex relationships between various genetic sources, biological pathways, and brain connectomes to identify potential biomarkers and treatments for ASD patients.

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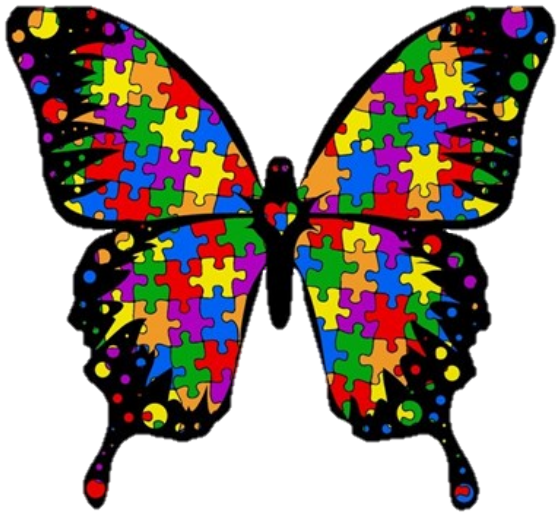
Chapter 7

SUMMARY AND CONCLUSION



Autism Spectrum Disorder (ASD) or Autism (MIM 209850) is a complex heterogeneous neurodevelopmental disorder. Individuals of all genders, races, ethnicities, and economic backgrounds can be diagnosed with ASD. Although ASD can be a lifelong disorder, treatments and services can improve a person's symptoms and daily functioning. The biology of autism spectrum disorder and scientific interventions are limited to the western countries. In India, autism is still under the shed. No sufficient experimental data is available on its clinical aspects. The exact prevalence and incidence of autism in India is still unknown.

The present study is preliminary basic research from the autistic population of North Karnataka, India. Our study screened 1878 mentally ill children from all the 12 districts of North Karnataka during 2018-2020 and identified 136 autistic children. The present study population recorded 7.44% of autistic children among the mentally ill children population of North Karnataka, India.

Genetic analysis of Neuroligin3, Neuroligin 4X and Neurolgin4Y genes among the autistic children revealed both previously recorded and novel pathogenic mutations in targeted genes. Mutation analysis of the NLGN3 gene recorded total 5 mutations. Novel pathogenic missense variant c.551T>C (p.V184A) was recorded in 27 (25%) autistic children among them 18 male and 9 female autistic children. Insilco structural and functional analysis and expression profiling showed abnormal structure and function of Neuroligin 3 protein which leads abnormal interaction of post synaptic NLGN3 and pre synaptic NRXN protein.

Mutation analysis of NLGN4X gene recorded total 25 mutations and majority of mutations were non coding mutations (3'UTR and 5'UTR). One Novel missense variant (p.Thr103Ala) and one novel frameshift mutation (p.Gly214GlyfsTer2) were also recorded. Insilco analysis of p.Thr103Ala showed no structural and functional effect on NLGN4X protein. Novel frameshift mutation p.Gly214GlyfsTer2 was recorded in 19 (17.5%) autistic children. Expression analysis and Insilco structural and functional analysis showed the abnormal function of truncated neuroligin 4X protein which causes post synaptic NLGN4X and pre synaptic NRXN biding defects leading to abnormal synaptogenesis.

Mutation analysis of the NLGN4Y gene recorded novel missense variant p.N163K in 3 (3.5%) autistic children. Insilco analysis showed no structural and

functional effect on NLGN4Y protein. Mutation in NLGN4Y may be an uncommon cause of autism in the Indian autistic population.

The present study also provides the first genetic mutation data from the Indian autistic population concerning neuroligin family genes.

Clinical significance

Novel frameshift mutation and missense mutation of NLGN4X and NLGN3 provide an important pathophysiologic and prognostic insight that post synaptic Neuroligin protein with novel frameshift mutation fails in binding to presynaptic Neurexin during functional synaptogenesis leading to abnormal synapses, which causes abnormal verbal & nonverbal communication and deficient social interaction in autistic children.

Neuroligin4X and Neuroligin3 are the probable candidate genes for future molecular investigation and functional analysis in the Indian autistic population, because the components involved in the synaptogenesis and synaptic structures remain excellent functional candidates for future molecular genetic studies of autism and related neurodevelopmental disorders.

Management strategies

India is multi ethnic population country. It is hoped and expected that, instead of using current western influenced general drugs, it is important to develop patient specific/population specific new treatments after genetic screening for markers for more effective therapy. This happens only when we have our population specific genetic database.

With the advancement of each technology with greater precision (cytogenetics, microarrays, whole genome sequencing), there has been a tremendous advancement in the discovery of undiscovered ASD risk alleles. The discovery of genetic variants by WGS and extensive bioinformatics analysis will be the most powerful tool. In addition to improving our understanding of the genetics and genomics of the disorder, future research should focus on integrating the complex relationships between various genetic sources, biological pathways, and brain connectomes to identify potential biomarkers and treatments for ASD patients.

Informed Consent Form & Data Sheet

Karnataka Institute for DNA Research, Dharwad
and
BLDE (Deemed to be University), Vijayapura

Informed consent form-cum-Patient details for "Genetic and Molecular Profiling of Autism Spectrum Disorder".

a) I am giving the sample with my own knowing fully well about the purpose of collection of the sample.
b) I consent to the test (s), which I understand will be based on DNA/RNA.
c) I agree to the request to use the blood sample for genetic studies, which may lead to the discovery of new technique or improving the existing one. Furthermore, I also investigators of Karnataka Institute for DNA Research to use the blood samples for research purpose that may facilitate the better understanding of the human genome and diseases provided confidentiality of the identity of the sample is maintained.
d) I also allow investigators to publish the data obtained from aforementioned studies.
e) I agree to have no financial claims out of the study.

1. Name of the subject :
2. Hospital case NO. :
3. Clinical diagnosis :
4. Date of birth :
5. Gender :
6. Blood group of the patient:
7. Permanent Address:
   Street: Taluk:
   Dist: Pincode :
8. Phone number:
   a) Mobile:
   b) Landline no. with code:

Signature or Thumb impression of Parent/ Guardian of the respective subject (Autistic child)

.....

- Name of the Hospital :
- Blood collected by :
- Blood sample relates to:

Data Sheet for **"Genetic and Molecular Profiling of Autism Spectrum Disorder"**

The data obtained from the patient will be kept as a secret; the data will be coded and utilized for our work.

Name:

Present Address: -

Street: Taluk:

Dist: Pincode:

Permanent Address: -

Street: Taluk:

Dist: Pincode:

Age: Sex:

Occupation: Height:

Weight: Religion:

Socio economic status:

Low…… Middle…… High……

Co-morbid History:

a. Mental retardation: Yes ( ) / No ( )

b. ADHD: Yes ( ) / No ( ) EEG:

c. Epilepsy: Yes ( ) / No ( ) seizures:

d. Others

Criteria:

1. Social interaction:

a. sharing emotions /interest: Yes ( ) /No ( )

b. social response: Yes ( ) /No ( )

c. Friendship with same age children: Yes ( ) /No ( )

Others:

2. Verbal & Non-verbal communication:

a. eye to eye contact : Yes ( ) / No( )

b. body language and gestures:

c. facial expression: Yes ( ) / No ( )

d. Name recognition: Yes ( ) / No ( )

Others:

3. Behavioural problems:

Hand flapping: Spinning in a circle:

Finger flicking: Staring at lights:

Tapping ears: Lining up toys:

Spinning objects: Self-harm:

Repeating words or noises: Watching-moving object

Others:

4. Treatment/Therapies:

a) Anger management: b) Family therapy:

c) Behaviour analysis: d) sensory processing:

e) Animal assisted therapy:

Others:

Conditions:

Mild: Moderate: Severe:

Food Habit:

Veg/Non veg/ Both: Frequency:

Other:

Sleep dysfunction: Anxiety:

Pica: Gastrointestinal problems:

Disorders (If any)**:**

Family history of subject:

Pedigree chart:

Institutional Ethical Clearance Certificate

BLDE (DEEMED TO BE UNIVERSITY)

[Declared as Deemed-to-be-University u/s 3 of UGC Act, 1956 vide Government of India Notification No.F.9-37/2007-U.3(A)]

The Constituent College

SHRI. B. M. PATIL MEDICAL COLLEGE, HOSPITAL & RESEARCH CENTRE, VIJAYAPURA

BLDE (DU)/IEC/337/2018-19 21-12-2018

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The ethical Committee of this University met on21st December 2018 at 11 a.m.to scrutinizes the Synopsis/ Research Projects of Post Graduate Student / Under Graduate Student Faculty members of this University / College from ethical clearance point of view. After scrutiny, the following original/ corrected and revised version Synopsys of the thesis/ research projects has been accorded ethical clearance.

Title. "Genetic and MolecularProfiling of Neuroligin3, Neuroligin4X, Neuroligin4y Genes in AutismSpectrum Disorder among Population of North Karn ataka"

Name of the Faculty member /PhD/PG/UG student.. Mr. Rajat Hegde,

Name of the Guide; Prof. Kusal Das.Prof,Dept of Physiology

Dr. Sharada Metgud
Chair person
IEC, BLDE (DU),
VIJAYAPURA

Dr.G.V.Kulkarni
Member Secretary
IEC, BLDE (DU),
VIJAYAPURA

MEMBER SECRETARY
Institutional Ethics Committee
BLDE (Deemed to be University)
Vijayapura-586103, Karnataka

Note:-Kindly send Quarterly progress report to the Member Secretary

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- Copy of Synopsis/Research Projects
- Copy of inform consent form
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2. Title of the Thesis: **Genetic and Molecular Profiling of Neuroligin3, Neuroligin4X, Neuroligin4Y Genes in Autism Spectrum Disorder among the Population of North Karnataka.**
3. Department: **PHYSIOLOGY**
4. Name of the Guide & Designation: **Prof. Kusal K. Das**, Ph.D, Distinguished Chair Professor, Department of Physiology, BLDE (Deemed to be University), Vijayapura
5. Name of the Co Guide & Designation: **Prof. Pramod B Gai**, Director, Karnataka Institute for DNA Research [KIDNAR], Dharwad

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Novel frameshift mutation in Indian autistic population causes neuroligin and neurexin binding defect

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ABSTRACT

Autism is a complex neurodevelopmental condition which is clinically, etiologically and genetically heterogeneous. The predisposition of genes and genetic loci has been under study over the last few decades. Autism is increasing dramatically over the last few years in India. Mutations in two x-linked neuroligin genes viz. *NLGN3* and *NLGN4X* have been recently implicated in autism pathogenesis. In the current study, for the first time in India, we screened the genetic variations in transmembrane neuroligin 4× gene in the Indian autistic population using sequencing technology. 1828 mentally ill children were screened around the North Karnataka region of India and 136 autistic children were identified, out of which 108 autistic children who were fulfilling the inclusion criteria were taken for the study. DNA was isolated from the clinical samples and exonic regions were amplified. Sanger sequencing was carried to screen the genetic variations and expression profiling was performed using Real time PCR. Sequencing results revealed 25 different variants (eight 5′UTR variants, four missense variants, four synonymous variants, one frameshift variant and eight 3′ UTR variants). One novel frameshift variation (p.Gly214GlyfsTer2) was recorded in 19 (17.5%) autistic individuals. This novel frameshift mutation leads to a premature stop codon. Relative expression of the NLGN4X gene in autistic children with novel frameshift mutation was largely reduced compared to the control group [0.052 and 1.217 respectively, *p*-value 0.00001]. The present study shows novel frameshift mutation causes abnormal NLGN4× protein that leads to loss of binding to presynaptic neurexin during synapses. Therefore neuroligin 4× is the potential target gene in the Indian autistic population for future functional analysis.

1. Introduction

Autism (MIM 209850) is a complex neurodevelopmental disorder that results due to impaired social communication, impaired verbal communication as well as repeated stereotyped behaviours (Spence, 2004; Nguyen et al., 2020a). An autism diagnosis cannot be made on a single specific symptom but it requires a wide spectrum of impairments in verbal and nonverbal communication, social interaction, and behavioural problems according to the Diagnostic and statistical manual of mental disorders V (DSM-V, American Psychiatric Association, 2000) (https://www.psychiatry.org/psychiatrists/practice/dsm) and/or International Classification of Diseases-10 (ICD-10, WHO) (https://www.who.int/classifications/icd/icdonlineversions/en/). It manifests before the age of three. Epidemiological studies reported that autism affects 01 in 54 children in the United States (Nguyen et al., 2020a). No convincing data is available from India to provide an Indian specific estimate of the prevalence, but recent studies on the epidemiological survey in urban and rural areas of India showed an overall prevalence of autism in India

Abbreviations: ASD, Autism Spectrum Disorder; NLGN, Neuroligin; NRXN, Neurexin; UTR, untranslated region; CNV, copy number variations; SNP, single nucleotide polymorphism; DNA, deoxyribonucleic acid; PROVEAN, Protein Variation Effect Analyser; PANTHER, Protein Analysis through Evolutionary Relationship; PolyPhen2, Polymorphism Phenotypic-2 and PHD-SNP.

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ranges from 0.15% to 1.01% depending upon the screening method and geographical area of the study conducted (Raina et al., 2017; Chauhan et al., 2019). But from last few years prevalence of autism is increasing drastically over the globe. Etiology of autism is very complex. Genetics and environmental factors are playing role in etiology of autism. There is strong evidence of interactions of multiple genetic factors involved in autism. Concordance rates are 30–99%, 0–65%, and 3–30% respectively, among monozygotic twins, dizygotic twins, and siblings, with an estimated total heritability of 0.7–0.8 (Ramaswami and Geschwind, 2018). The genetic framework of autism is extremely diverse with contributions from alleles of varying frequencies (i.e., common, rare, very rare), patterns of inheritance (i.e. dominant, recessive, X-linked, de novo), and types of variants (i.e., large chromosomal rearrangements, copy number variants (CNV), small insertions/deletions (indels), and single-nucleotide variants (SNVs)) (Ramaswami and Geschwind, 2018; De Rubeis and Buxbaum, 2015; De La Torre-Ubieta et al., 2016). Autism is more common in males than female; male to female ratio is 3–4:1 but this strong sex bias is still unclear. Significantly, a set of genes associated with autism are found on X-chromosomes suggesting that the Sex chromosomes play a part in at least some of the gender differences in autism (Nguyen et al., 2020b).

The neurexin family consists of three members, *NRXN1, NRXN2* and *NRXN3*. Each member with a long alpha and short beta forms. Four members, *NLGN1, NLGN2, NLGN3* and *NLGN4* of the neuroligin family were found in mice. While in humans five members have been identified *NLGN1* (3q26), *NLGN2* (17p13), *NLGN3* (Xq13), *NLGN4*× (Xp22.3) and *NLGN4Y* (Yq11.2) (Lawson-Yuen et al., 2008). Neuroligins consist of an extended extracellular N-terminal domain composed of a broad esterase homology domain, a short O-glycosylated region attached to a common transmembrane domain, and a short intracellular C-terminal domain (Al-Ayadhi et al., 2020). In the present study, for the first time in India, we screened all the exonic regions of the *NLGN4*× gene in Indian autistic children by using a sequencing technique to record the molecular changes.

2. Methods and material

2.1. Clinical sample collection

Large population screening of 1828 mentally ill children from North Karnataka region of India and identified 136 autistic children out of that 108 autistic individuals [$n_{male} = 85$, $n_{female} = 23$, age range = 5–18 years and mean age 11.7 ± 3.5] below 18 years of age free of any neurological, psychiatric disorders were included in the study. Children were screened as per Diagnostic and statistical manual of mental disorders V [DSM-V, American Psychiatric Association, 2000] (https://www.psychiatry.org/psychiatrists/practice/dsm) and International Classification of Diseases-10 (ICD-10, WHO) (https://www.who.int/classifications/icd/icdonlineversions/en/). Informed consent was obtained from parents/guardians before the collection of blood samples. Ethical approval for the study was obtained from the Institutional ethical committee of Shri B.M Patil Medical College, Hospital and Research Centre, BLDE (Deemed to be University), Vijayapura [Ref No: BLDE (DU)/IEC/337/2018-19].

2.2. PCR amplification and sequencing

Genomic DNA extraction was performed using Blood and Tissue DNA extraction kit (QIAGEN, Germany) as per manufacturer guidelines. The quality and quantity of extracted DNA were checked. All 6 exons of the *NLGN4X* gene were amplified using PCR master mix components (New England Biolab, USA) and designed oligomers. Amplified products were verified by using agarose gel electrophoresis. Cycle sequencing was carried out by using Big Dye terminator kit v3.1 (Applied Biosystem, USA) as per manufacturer guidelines. The product was run on ABI 3500 Sanger sequencing platform and results were analysed on DNA Sequencing Analysis Software v5.4 (Applied Biosystem, USA).

2.3. Bioinformatics analysis

Pathogenic effects of the Non synonymous variants were analysed by using various bioinformatics tools such as PROVEAN- Protein Variation Effect Analyser (http://provean.jcvi.org/seq_ submit.php), PolyPhen2-Polymorphism Phenotypic 2 (http://genetics.bwh.harvard.edu/pph2/index.shtml), PHD SNP (https://snps.biofold.org/phd-snp/phd-snp.html), SNP & GO (https://snps.biofold.org/snps-and-go/snps-and-go.html). SNAP2 (https://www.rostlab.org/services/snap/) & PANTHER (http://www.pantherdb.org/). The evolutionary conservation of variants over different species was investigated by using Clustal Omega program (https://www.ebi.ac.uk/Tools/msa/clustalo/). Homology modelling of wild type and mutant NLGN4X protein was developed using the Swiss model (https://swissmodel.expasy.org/) and results were visualized and analysed using UCSF Chimera program.

2.4. Gene expression analysis

19 autistic children with novel frameshift mutation and 19 age matched healthy children were included as a control group for expression analysis of the NLGN4X gene. Total RNA was isolated from peripheral blood using QIAamp RNA blood mini kit [QIAGEN, Germany] as per the manufacturer's instructions. Yield and purity were quantified by using a nanodrop spectrophotometer [QUAELL, Q3000UV Spectrophotometer]. The integrity of total RNA was analysed using 1.5% agarose gel electrophoresis. cDNA was synthesised by using RevertAid First Strand cDNA Synthesis Kit [Thermo Scientific, Lithuania] as per the manufacturer's instruction and the quality of cDNA was analysed using 2.0% agarose gel electrophoresis. Amplification was carried using maxima SYBER green/ROX qPCR Master mix [Thermo Scientific, Lithuania] in Bio Rad CFX 100 real time instrument. Glyceraldehyde 3-phosphate dehydrogenase [GAPDH] gene was used as a control gene.

Statistical analysis was performed using SPSS 17.0. The student *t*-Test was performed to compare the relative levels of mRNA of autistic children with novel frameshift mutation and controls. The p-value <0.05 was considered statistically significant.

3. Results

Genomic DNA samples from all 108 autistic individuals underwent Sanger sequencing to determine the variants within the *NLGN4X* gene for autism pathogenicity. Sequencing of all 6 exons revealed the presence of 25 different variants (eight 5′UTR variants, four missense variants, four synonymous variants, one Frameshift variant and eight 3′ UTR variants). 09(36%) novel variants and 16 (64%) previously recorded variants were recorded in our study population. All 5′ UTR variants were found to be heterozygous. Two missense and two synonymous variants were also heterozygous. All variants are summarised in the following Tables 1, 2 and 3. [NG_008881.2 Reference sequence was used for Genomic DNA variant nomenclature, ENST00000381095.8 reference sequence was used for Coding region variant nomenclature and

Table 1
Showing 5′ UTR variations of NLGN4× gene.

Mutation type	Nucleotide position	Exon	SNP	Frequency of mutation in percentage
5′ UTR	g. 5077 T > Y	1	rs149114901	3 (2.77%)
5′ UTR	g. 5082C > Y	1	Not reported.	2 (1.85%)
5′ UTR	g. 5143C > Y	1	rs755890454	1 (0.92%)
5′ UTR	g. 5159 T > Y	1	Not reported.	1 (0.92%)
5′ UTR	g.5160 A > M	1	Not reported	2 (1.85%)
5′ UTR	g.5265C > Y	1	rs971248204	5 (4.6%)
5′ UTR	g.5266 T > K	1	Not reported.	3 (2.77%)
5′ UTR	g.5295C > Y	1	Not reported.	4 (3.7%)

Table 2
Showing coding sequence variations of NLGN4X gene.

Mutation type	Nucleotide position	c.DNA position	A.A position	Exon	SNP	Frequency of mutation (%)
Missense	g.82531 A > G	c.115 A > G	p.Ile39Val	2	rs201534650	2 (1.85%)
Missense	g. 82,723 A > R	c. 307A > R	p.Thr103Ala	2	not reported	8 (7.04%)
Synonymous	g. 82,774C > Y	c.358C > Y	p.Leu120=	2	not reported	6 (5.5%)
Synonymous	g. 82,872C > Y	c.456C > Y	p.Tyr152=	2	rs1460330547	11 (10.2%)
Missense	g.82880C > Y	c.464C > Y	p.Thr155met	2	rs770601703	7 (6.5%)
Frameshift	g.324659_324060 insG	c.641_642 insG	p.Gly214GlyfsTer2	4	Not reported	19 (17.5%)
Synonymous	g.330138C > T	c.933C > T	p.Thr331=	5	rs7049300	2 (1.85%)
Missense	g.340392C > T	c.1777C > T	p.Leu593Phe	6	rs3747333	2 (1.85%)
Synonymous	g.340394C > G	c.1779C > T	p.Leu593=	6	rs3747334	1 (0.92%)

Table 3
Showing 3′ UTR variations of NLGN4X gene.

Mutation type	Nucleotide position	Exon	SNP	Frequency of mutation in percentage
3′UTR	g.341240C > A	6	rs3810687	2 (1.85%)
3′UTR	g.341168 G > A	6	rs3810688	1 (0.92%)
3′UTR	g.341350G > A	6	rs3810686	2 (1.85%)
3′UTR	g.341625C > T	6	rs5916269	1 (0.92%)
3′UTR	g.341834 T > C	6	rs3810685	3 (2.77%)
3′UTR	g.342537A > G	6	Not reported.	6 homozygous (5.5%), 3 heterozygous (2.77%)
3′UTR	g.343053 T > G	6	rs16983882	2 homozygous (1.85%), 2 heterozygous (1.85%)
3′UTR	g.343063C > T	6	rs1882260	2 homozygous (1.85%), 2 heterozygous (1.85%)

ENSP00000370485.3 reference sequence was used for protein variant nomenclature. All the nomenclatures are made as per HGVS guidelines].

3.1. Reported variants

Previously reported 16 variants to include Three 5′ UTR, Three missense, Three synonymous and seven 3′UTR variants were observed. Two 3′UTR variants g.343053 T > G (rs16983882) and g.343063C > T (rs1882260) were found in both heterozygous as well as homozygous condition.

3.2. Novel variants

Novel variants include five 5′UTR, one missense, one synonymous, one frameshift and one 3′ UTR variants. All novel variants were cross-checked in in-house human SNP databases. None of the variants was reported previously in dbSNP, 1000genomes, ExAc, ClinVar. The novel frameshift mutation was found in 19 (17.5%) autistic children.

3.3. In silico analysis

Pathogenicity of all missense variants were predicted using different bioinformatics tools. The scores of PROVEAN, PANTHER, SNP&GO, SNAP2, PolyPhen2 and PHD-SNP predicted that novel missense variant p.Thr103Ala was harmless but p.Thr155met variant score found to be deleterious effects on the functions of the NLGN4X protein (Table 4).

Multiple sequence alignment of NLGN4X protein shows p. Thr155Met variant was located in the conserved region across the different species and which shows high evolutionary functions but p. Thr103Ala variants were conserved only in higher primates (Fig. 1). Uniprot accession numbers of sequence used were as follows; **D2X2K7**- *Xenopus tropicalis*, **D2X2I0**- *Anolis carolinensis*, **A0A2I3MYY3**- *Papio anubis*, **H2PUU5**- *Pongo abelii*, **Q8N0W4**- *Homosapiens*, **A0A2K6P1V0**- *Rhinopithecus roxellana*, **H2R0H2**- *Pan troglodytes*, **A0A2I3HLL4**- *Nomascus leucogenys*.

The homology modelling of p.Thr155Met and p.Gly214GlyfsTer2 mutant protein was generated using the SWISS-MODEL server and structure was analysed on UFSC Chimera software. The 3D model of wild type protein showed two H-bonds at residue Thr155 with Pro69 but mutant protein (Met155) lost one hydrogen bond with Pro69 (Fig. 2). This loss of hydrogen bond affects NLGN4X protein folding and thus decreases the stability of the protein. Frameshift mutation produces a truncated protein of 214 amino acids which leads to loss of approximately 74% of the total NLGN4X protein which is very much important for binding to presynaptic neurexin protein (Fig. 3).

3.4. Relative expression quantification of NLGN4X

The relative expression quantification of the NLGN4X gene in autistic children with novel frameshift mutation and healthy age matched control group was calculated by normalising the ratio for NLGN4X to GAPDH. Amplified cDNA products were electrophoresed on 2% agarose gel [Fig. 4]. The mean expression level of NLGN4× mRNA in autistic children with novel frameshift mutation and the control groups were 0.052 and 1.217 respectively [Fig. 5]. A significant large decrease in the expression level of the NLGN4× gene was observed in autistic children with novel frameshift mutation compare to control groups (p-value 0.00001).

Table 4
In silico prediction of pathogenic effect Missense variants.

Variants	PROVEAN	SNP &GO	SNAP2	Polyphen-2	PHD-SNP	PANTHER
p.Thr103Ala	Neutral	Neutral	Neutral	Benign	Neutral	PB
	Score: 2.396	P- 0.030	Score: −54	0.0	RI- 9	
p.Thr155met	Deleterious	Effect	Disease	PD	Disease	PD
	Score: −3.070	P- 0.173	Score:15	Score:0.871	RI-4	

P- Probability. PD- Probably Damaging. PSD- Possibly Damaging. PB- Probably benign. RI-Reliability index. *PROVEAN: "Deleterious" if the prediction score was </−2.5, "Neutral" if the prediction score was >/−2.5. * SNP & GO: Probability is >0.5 then it is predicted to be Disease causing nsSNP. *SNAP2: "Neutral" if the score lays 0 to −100. "Effect" if the score lays 0 to 100. *PolyPhen2: "Probably damaging" is the most disease causing ability with a score near to 1. "Possibly damaging" is less disease causing ability with a score of 0.5–0.8. "Benign" which does not alter protein functions with a score closer to zero.*PHD-SNP: if the probability is >0.5 mutation is predicted "Disease" and if less than <0.5 mutation is predicted to be "Neutral".

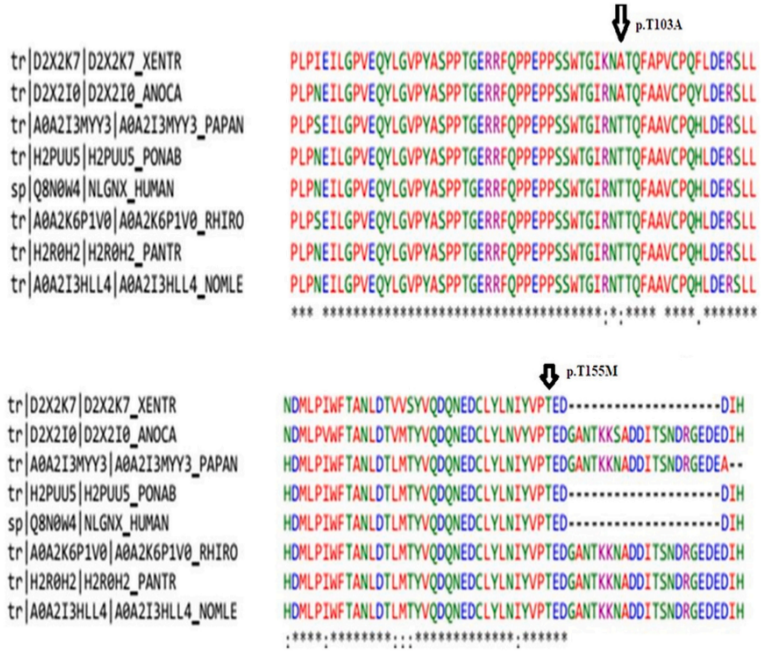

Fig. 1. Multiple sequence alignment of NLGN4X protein over different species showing conservation status of missense mutations.

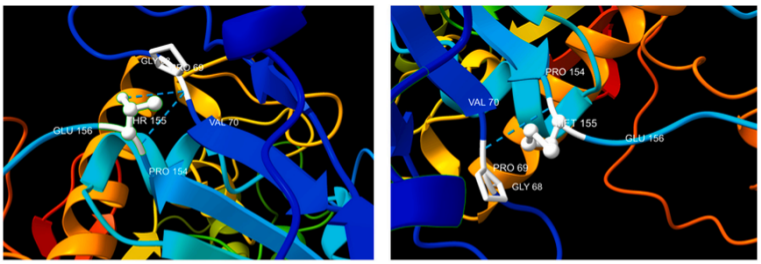

Fig. 2. Sowing 3Dstructure of Thr155Met variation using chimera software. The wild type residue (left) and the mutant residue (right).

4. Discussion

Autism [MIM 209850] is a neurodevelopmental disorder, characterised by the triad of symptoms viz., abnormal communication, social interaction and repetitive behaviours. The term was first used by Leo Kenner in 1943 (Kanner, 1971). The prevalence of autism is increased over the past few years dramatically. In India etiology and prevalence of autism is still unclear. Neuroligin is the most targeted gene for neuropsychiatric studies due to its active role in synaptogenesis. Neuroligin variants have been associated with various neuropsychiatric conditions like Tourette syndrome, Attention Deficit Hyperactivity Disorder (ADHD), Autism, anxiety, depression, intellectual disability (Lawson-Yuen et al., 2008; Yan et al., 2005; Kent and Simonoff, 2017; Volaki et al., 2013).

Initial mutation screening associated with Neuroligin 3 and Neuroligin 4× was missense and frameshift mutation respectively in Swedish families with autism and frameshift mutation appeared as denovo variant (Jamain et al., 2003). Later Subsequent investigation showed various findings state that Neuroligin 4× is probably a candidate gene with heterogeneous nature in association with autism. Deletion mutations; 2 bp deletion (1253 delAG) causing premature deletion, deletion of exon 4 in the transcript of female and deletion of exon 4,5,6 of NLGN4X gene in a family was reported in 2004, 2006 and 2008 respectively (Lawson-Yuen et al., 2008; Laumonnier et al., 2004; Talebizadeh et al., 2006). Interestingly *NLGN4X* showed a variety of variations in association with severity of autism, p.G99S in Female proband with severe autism and MR with carrier mother has Learning Disorder. p.K378R in a male proband with mild autism and carrier mother was healthy. p.V403M in a male proband with PDD-NOS, carrier brother has autism and carrier mother was healthy. p.R704C in a male proband with autism and healthy carrier sister and mother (Yan et al., 2005). Study in 2009 reported the same variant p.K378R in a male proband with mild autism and healthy carrier mother (Pampanos et al., 2009).

Chinese autism population study recorded 4 novel mutations in *NLGN4X*, p.G426S and p.Q162 K were de novo mutations, while p.G84R and p.A283T were inherited from their asymptomatic mothers (Xu et al., 2014). Whole genome studies identify *NLGN4X* as the candidate gene for association study for autism (Yu et al., 2013; Yuen et al., 2017). Several

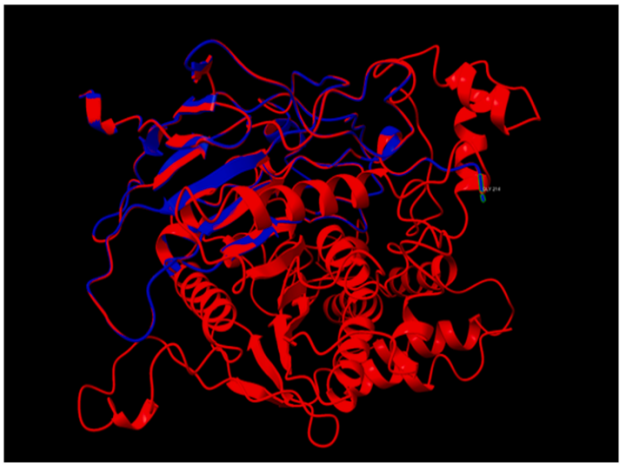

Fig. 3. Superimposed model of NLGN4× wild type and truncated protein due to p.Gly214GlyfsTer2 mutation (blue - truncated protein, Red - wild type-NLGN4× protein).

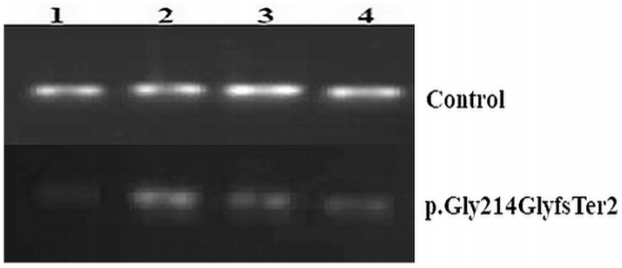

Fig. 4. Agarose gel electrophoresis of amplified cDNA of *NLGN4*× gene. Control (top) and autistic with Frameshift mutation; p.Gly214GlyfsTer2 (below).

denovo variations of *NGN4X* are associated with autism and contribute to the etiology of autism (Zhang et al., 2009; Daoud et al., 2009; Wang et al., 2018; Martínez et al., 2017). Subsequently, few studies in different autism population failed to find the variants to prove the involvement of the *NLGN4X* gene in autism so maybe *NLGN4X* variants account the only a small proportion of autism (Wermter et al., 2008; Vincent et al., 2004; Gauthier et al., 2005; Blasi et al., 2006) But Neuroligin is dynamically regulated through post translational modification. Similar to *NLGN1*and *NLGN2*, posttranslational modification has an important role in regulating NLGN4X function (Jeong et al., 2017). Hence neuroligin plays a heterogenous role in the causation of autism.

In our study, we sequenced all 6 exonic regions of the *NLGN4X* gene in 108 autistic children and we recorded 25 variants viz., eight 5′UTR variants, four missense variants, four synonymous variants, one Frameshift variant and eight 3′ UTR variants. 09 variants were novel variants which were not recorded in any of the human SNP databases. In silico prediction of missense variants showed that p.Thr155Met variant was found to be harmful by PROVEAN, PolyPhen-2, PHD SNP, SNP & GO, SNAP2 and PANTHER and this residue is evolutionarily conserved over different species. This variant leads to loss of one hydrogen bond in mutant protein and loss of hydrogen bond affects the folding of NLGN4X protein, thus decreases the stability of the protein p.Thr103Ala variant has a neutral effect on functions of NLGN4X protein and these variant residues do not have evolutionary conservation, they were conserved only in higher primates. 19(17.5%) autistic children showed novel frameshift mutation p.Gly214GlyfsTer2 this alters the reading frame that results in immediate stop codon and forms truncated protein with 214 amino acid (loss of approximately 74% of original protein) (Fig. 3). Relative expression analysis of NLGN4X shows a large significant reduction in autistic children with a frameshift mutation compared to the control group i.e. 0.052 and 1.217 respectively [*p*-value 0.00001]. Reduction in the relative expression of the NLGN4× gene in autistic children with novel frameshift mutation strongly supports the in silico pathogenicity prediction. So neuroligin protein with novel frameshift mutation fails in binding to presynaptic Neurexin during functional synaptogenesis leading to abnormal synapses.

In ongoing study, along with *NLGN4*× gene we have also studied NLGN3 and NLGN4Y gene in the present study population. In *NLGN3* gene, one novel missense variant and four 3′ UTR variants was observed and in silico prediction showed a novel missense mutation was predicted to be pathogenic on both structure and functions of neuroligin 3 protein. In *NLGN4Y* gene, 9 variants were observed [one missense and eight synonymous variants]. Novel missense variant prediction showed to be harmless. Over all novel frameshift mutation of *NLGN4*× was observed in high frequency compared to novel missense mutation of *NLGN3* gene in our population.

Interestingly, p.Gly214GlyfsTer2 variant was found in 16 males and 3 females that support the involvement of the *NLGN4*× gene in male predominance. So as like previous studies coding sequence variants in the NLGN4× gene might be harmless or rare but these rare mutations have strong effects on functions of the Neuroligin 4× gene in autism. Along with the coding sequence variant we also recorded non coding sequence variants which may have the posttranslational effect on neuroligin 4× protein. It only revealed after functional analysis of the gene,

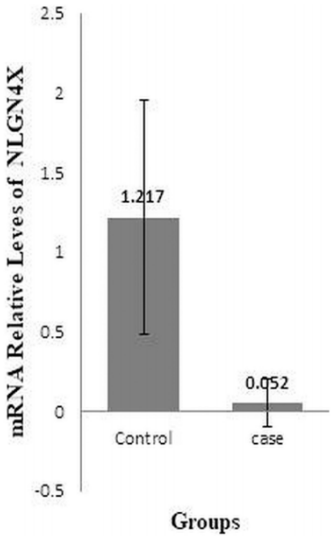

Fig. 5. The mean relative expression level of NLGN4X mRNA in control groups and autistic children with novel frameshift mutation.

which was the limitation of our study.

5. Conclusion

Mutations of the neuroligin 4× gene are highly heterogeneous ranging from the types of mutation to the position of the mutation. Our population showed a novel frameshift mutation causing abnormal neuroligin 4× functions during functional synaptogenesis. So neuroligin 4× is one of the promising candidate genes for functional studies on autism in the Indian autistic population.

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CRediT authorship contribution statement

Rajat Hegde and **Smita Hegde** were involved in Sample collection, genetic analysis and data analysis. **Suyamindra S Kulkarni** helped in genetic analysis and over all data analysis and interpretation. **Aditya Pandurangi** helped in identification, screening, counselling and sample collection. **Kusal K Das** and **Pramod B Gai** were involved in overall work design and valuation on results.

Declaration of competing interest

The authors declare that there are no conflicts of interests.

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Original Article

Genetic Analysis of Neuroligin 4Y Gene in Autism Population of India

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Abstract

Background Autism is one of the most complex, heterogeneous neurological disorders. It is characterized mainly by abnormal communication, impaired social interaction, and restricted behaviors. Prevalence of autism is not clear in Indian population.

Aim The present study hypothesizes that Y chromosome plays role in sex bias of autism in Indian autistic population. To investigate our hypothesis, we underwent genetic analysis of neuroligin 4Y [*NLGN4Y*] gene by sequencing 85 male autistic children after screening large population of 1,870 mentally ill children from North Karnataka region of India.

Result Detailed sequencing of the single targeted gene revealed nine variants including, one novel missense mutation and eight synonymous variants; this accounts for 88.9% of synonymous variants. A single novel missense mutation is predicted to be nonpathogenic on the functions of neuroligin4Y protein but it slightly affects the local configuration by altering the original structure of a protein by changing charge and size of amino acid.

Conclusion Probably *NLGN4Y* gene may not be the risk factor for autism in male children in Indian autistic population. Functional analysis was an important limitation of our study. Therefore, detailed functional analysis is necessary to determine the exact role of novel missense mutation of neuroligin 4Y [*NLGN4Y*] gene especially in the male predominance of autism in Indian autistic population.

Keywords
- autism
- neuroligin 4Y
- India
- novel missense mutation
- male predominance

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Introduction

Autism [ASD] [MIM 299850] is a heterogeneous neurodevelopmental disorder. Autism is not characterized based on a single symptom. It is usually characterized by the triad of symptoms viz lack of social interaction, abnormal verbal and nonverbal communications and stereotyped or repetitive behaviors.[1] Autism is classified as syndromic and non-syndromic autism. Syndromic autism is one in which patients who have pre-existing neurological disorders, example, a subset of patients with fragile x syndrome, tubers sclerosis, Rett syndrome displays phenotypes which are attributed to ASD. Non-syndromic autism accounts for autism cases that are not linked to any neurological disorders.[2] Neuroligin is trans-synaptic cell adhesion molecule present post-synaptically and plays a very important role in synaptogenesis with presynaptic neurexin.[3] Humans have neuroligin 4X [*NLGN4X*] on the X chromosome and neuroligin 4Y [*NLGN4Y*] on the Y chromosome. *NLGN4X* and *NLGN4Y* genes share 97% sequence identify.[4] The male bias seen from *NLGN4X* mutations is unclear since *NLGN4Y* plays a function similar to *NLGN4X* and should be sufficient to reimburse for *NLGN4X* ASD-related mutations. This lack of compensation in males suggested that *NLGN4Y* may have an uncharacterized distinct function that needs to be explored. Several studies are reported that synaptic cell adhesion molecules have been strongly involved in autism. Neuroligin has an important role in the maturation and functions of synapses.[5,6] The mechanism of Y chromosome contribution on to neurodevelopmental disorders is still not known very well. Originally, it was thought that Y chromosome contains only a few genes that are primarily involved in sex determination and testicular functions but now it is known to contain numerous genes with diverse functions.[7] Several shreds of evidence strongly suggested that *NLGN4X* deficiencies can cause autism and still there is no clear understanding of sex bias in autism.

We hypothesize that male individuals have both X and Y chromosome so analysis of sequence variants in *NLGN4Y* gene may be associated with sex bias in male autistic individuals. To address this objective, we sequenced all the exonic regions of *NLGN4Y* gene in 85 male autistic children from north Karnataka region of India.

Methods

Sample Collection

One-hundred fifty autistic children were identified after screening a large mentally ill population of 1,870 children from the entire North Karnataka region of India ($n_{male} = 117$, $n_{female} = 33$ mean age $= \pm 11.5$]. Eighty-five male autistic children were included for the genetic analysis of NLGN4Y gene. Screening of autistic children was performed using Diagnostic and Statistical Manual of Mental Disorders-V (DSM-V, American Psychiatric Association, 2000) (https://www.psychiatry.org/psychiatrists/practice/dsm) and/or International Classification of Diseases-10 (ICD-10, WHO) (https://www.who.int/ classifications/ icd/ icdonlineversions/en/). Child with associated neurological disorders and other comorbid diseases was excluded from the study. Around 1 to 2 mL of peripheral blood was collected in EDTA-coated vacutainers and stored in −20°C until further analysis.

DNA Isolation and PCR Amplification

Genomic DNA was isolated from peripheral blood using blood and tissue DNA isolation kit (QIAGEN, Germany) as per manufactures guidelines. Quality and quantity of isolated genomic DNA were checked using agarose gel electrophoresis and nanodrop UV spectrophotometer (Quawell, Q3000 UV spectrophotometer). Amplification of *NLGN4Y* gene was carried with designed primers using standard PCR reagents (New England Bio Labs, United States). Quality and quantity of PCR product were analyzed.

Sequencing

PCR products of *NLGN4Y* gene was sequenced using Sanger sequencing kit v3.1 on ABI 3500 Sanger sequencer platform. Sequence data were analyzed with ABI sequence analysis Software v5.4 (Applied Biosystem, United States).

Bioinformatics Analysis

Pathogenic effect of missense mutation was analyzed using Insilco tools like PROVEAN, SNAP2, polyphen2, SNP&GO, and CADD. Conservation status of amino acid residues of NLGN4Y protein at 163 position was checked using the ConSurf Server (https://consurf.tau.ac.il/).[8] Three-dimensional structure of wild type and mutant protein was developed using Swiss-model and structures were visualized and analyzed using UCSF Chimera tool.

Results

Detailed screening of 1,870 mentally ill children below 18 years of age from North Karnataka population of India revealed 150 autistic children [$n_{male} = 117$, $n_{female} = 33$ mean age $= \pm 11.5$] which accounts for 8.02% of autism in North Karnataka region of India. Sanger Sequence analysis of neuroligin 4Y gene from 85 male autistic children revealed the nine variants, which include one missense and eight synonymous variants. Four variants which were recorded in our study cohort are not previously recorded in any in house human SNP databases viz dbSNP, 1000 genomes, ExAc and ClinVar shown in ►**Table 1**. Novel missense, p.N163K mutation was recorded in three autistic children and clinical features of those autistic children with missense mutation are shown in ►**Table 2**. Pathogenicity prediction of missense variants was analyzed using Insilco tools viz PROVEAN, POLYPHEN2, SNAP2, SNP&GO, and CADD. Only Missense variant, p.N163K was found to be harmless on the functions of *NLGN4Y* protein by PROVEAN, POLYPHEN2, SNAP2, SNP&GO, and CADD shown in ►**Table 3**. Conservation status analysis of NLGN4Y protein sequences shows that amino acid residue at 163 position is not conserved; it is variable and exposed residue according to the neural network algorithm shown in ►**Fig. 1**.

Three-dimensional protein modeling analysis of NLGN 4Y protein revealed that mutant residue is bigger than wild type

Table 1 Showing list of mutations recorded in our study cohort

Variation	N. change	A.A Change	SNP id	Frequency of mutation
Missense	g. 205526 C > A	p.N163K	Not recorded	3 (3.5%)
Synonymous	g.312652 T > C	p. H447=	rs777234513	4 homozygous (4.7%) 2 heterozygous (2.3%)
Synonymous	g.312781C > T	p.G490=	rs767447455	3 (3.5%)
Synonymous	g.312787 A > G	p.E492=	rs750273940	3 homozygous (3.5%) 2 heterozygous (2.3%)
Synonymous	g.312826 A > C	p.T505=	Not recorded	1 (1.2%)
Synonymous	g.312844 T > C	p.N512=	Not recorded	2 (2.3%)
Synonymous	g. 312847 C > T	p.F513=	Not recorded	1 (1.2%)
Synonymous	g. 312871 T > C	p.S520=	rs1423308667	1 (1.2%)
Synonymous	g. 312880 G > C	p.V523=	rs753006927	3 (3.5%)

Table 2 Clinical features of autism children with missense mutation, p.N163K of NLGN4Y gene

Demographic character	Child 1	Child 2	Child 3
Ethnic origin	Indian	Indian	Indian
Sex	Male	Male	Male
Age of father at child's birth	38	29	35
Age of mother at child's birth	33	19	34
Consanguineous marriage	No	Yes	No
Prenatal damage	preeclampsia	None	None
Postnatal damage	None	None	Birth asphyxia
IQ	25	30	28
CARS Score and Severity	40; Severe	44; Severe	52 ; Severe
Co-morbid condition	None	None	None

Table 3 Showing pathogenicity prediction of a missense variant

Variant	PROVEAN	SNP&GO	PolyPhen2	SNAP2	CADD score
p.N163K	Neutral Score: −0.992	Neutral 0.381	Benign Score: 0.023	Neutral Score: −79	19.34 [Raw score 2.010689]

residue and it possesses a positive charge whereas wild type protein possesses neutral charge. The wild type of residue is predicted to be located in its preferred secondary structure, a turn but the mutant residue prefers to be in another secondary structure; therefore, the local conformation will be slightly destabilized shown in ►**Fig. 2A** and ►**Fig. 2B**. The mutated residue is located in a domain that is important for binding of other molecules. Mutation of the residue might disturb this function of NLGN4Y protein.

Discussion

Autism [MIM 299850] is a complex neurological condition which is characterized by abnormal social interaction, verbal and nonverbal communication and impaired behaviors. "Autism," the term was first used by Ukrainian-Austrian-American psychiatrist, Leo Kenner in 1943.[9] Rates of autism cases are increasing globally over the period of time when it comes to the Indian perspectives, cases are increasing dramatically, and it may be due to increased scientific knowledge and awareness or it may be an improper diagnosis. In recent days, neuroligin gene is the most targeted gene for the molecular studies on neurological disorders like autism, anxiety, attention deficit hyperactivity disorder and intellectual disability due to its active role in synaptogenesis.[10–13]

The Simons Foundation Autism Research Initiative (SFARI) [geneSFARI.org] lists four genes of Y chromosome associated with autism viz *NLGN4Y*, *ASMT*, *USP9Y*, and *SHOX*.[14] Only a few studies have been undertaken till now to study the role of *NLGN4Y* gene in autism. Studies conducted in 2005 and 2006, failed to identify the variants in the *NLGN4Y* gene in autistic patients.[15,16] But later several studies, record polymorphisms

Fig. 1 *Conservation status of p.N163K mutation*, amino acid residue at 163 position is not conserved; it is variable and exposed residue.

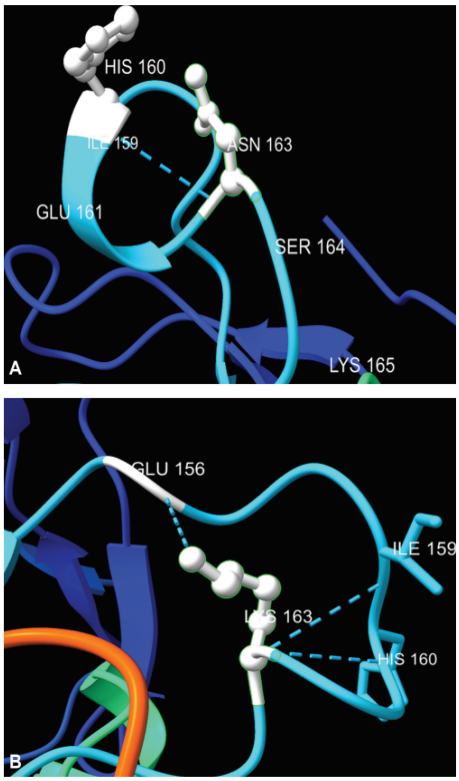

Fig. 2 (**A**) *Three-dimensional model of wild type protein*; wild type amino acid residue is small and neutrally charged. (**B**) *Three-dimensional model of mutant, p.N163K protein*. Mutant residue is bigger than wild type residue and it is positively charged.

of *NLGN4Y* gene involved in neurodevelopmental disorder and synaptic functions have been associated with autism.[17,18] Peripheral blood *NLGN4Y* gene expression showed an increased risk of autism in children with XYY symptoms.[7]

In the present study, for the first time in Indian autistic population, we analyzed a cohort of 85 male autistic children under the age of 18 years after screening large neurological disorders population. Our study population records 7.4% of autism over different neurological disorders. Sequencing analysis of the entire exonic region revealed 09 mutations. One is novel missense mutation and reaming 08 (88.9%) are synonymous variants. Out of eight synonymous variants, five (62.5%) were already reported in in-house human SNP databases. Insilco functional effect prediction of a novel missense mutation, p.N163K by PROVEAN, POLYPHEN2, SNAP2, SNP&GO, and CADD shows nonpathogenic effects on the functions of neuroligin 4Y protein. But structural analysis of p.N163K mutant protein shows slight destabilization in the local configuration by positioning on different secondary structure, a turn and it might cause bumps in protein structure due to differences in the size of amino acid. Meanwhile, the mutation introduces a charge; this can cause repulsion of ligands or other residues with the same charge.

Structural and functional prediction of novel missense mutation indicates slight changes related only to the structure of neuroligin 4Y protein, and not to the functions of the protein. Absence of functional analysis of gene was the important limitation of our study.

Conclusion

Mutation in *NLGN4Y* may be an uncommon cause of autism in Indian autistic population. But further detailed functional investigation of neuroligin 4Y gene in autism is important to understand the male predominance of autism and increased rate of autism in males in India autistic population.

Ethical Approval

Ethical approval for the study was taken from Institutional Ethical Committee of Shri B.M. Patil Medical College, Hospital and Research Centre, BLDE (Deemed to be University), Vijayapura, [Ref No: BLDE (DU) IEC/337–2018–19]. Informed written consent was obtained from parents/guardians before the collection of blood samples.

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Conflict of Interest

None declared.

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Genetic analysis of the postsynaptic transmembrane X-linked neuroligin 3 gene in autism

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Autism is a complex neurodevelopmental disorder, the prevalence of which has increased drastically in India in recent years. Neuroligin is a type I transmembrane protein that plays a crucial role in synaptogenesis. Alterations in synaptic genes are most commonly implicated in autism and other cognitive disorders. The present study investigated the neuroligin 3 gene in the Indian autistic population by sequencing and in silico pathogenicity prediction of molecular changes. In total, 108 clinically described individuals with autism were included from the North Karnataka region of India, along with 150 age-, sex-, and ethnicity-matched healthy controls. Genomic DNA was extracted from peripheral blood, and exonic regions were sequenced. The functional and structural effects of variants of the neuroligin 3 protein were predicted. One coding sequence variant (a missense variant) and four non-coding variants (two 5'-untranslated region [UTR] variants and two 3'-UTR variants) were recorded. The novel missense variant was found in 25% of the autistic population. The C/C genotype of c.551T>C was significantly more common in autistic children than in controls ($p = 0.001$), and a significantly increased risk of autism (24.7-fold) was associated with this genotype ($p = 0.001$). The missense variant showed pathogenic effects and high evolutionary conservation over the functions of the neuroligin 3 protein. In the present study, we reported a novel missense variant, V184A, which causes abnormal neuroligin 3 and was found with high frequency in the Indian autistic population. Therefore, neuroligin is a candidate gene for future molecular investigations and functional analysis in the Indian autistic population.

Keywords: autism, India, missense variant, neuroligin 3

Introduction

Autism (MIM 209850) is a complex neurodevelopmental disorder that is characterised by impaired verbal and nonverbal communication and social interaction, accompanied by restricted and stereotyped behavior [1,2]. The American child psychiatrist Leo Kanner was the first to clearly define the condition and to use the term "autism" [3,4]. The aetiology of autism is largely unknown, but many studies have shown that genetic factors play a major

role, along with environmental factors. The genetic architecture of autism is complex. Autism shows diverse forms of genetic variation, differing in frequency (i.e., very rare, rare, and common variations), the pattern of inheritance (i.e., autosomal, X-linked, and *de novo* variations), the type of variation (i.e., structural—including aneuploidy, copy number variations, indel mutations, and single-nucleotide variations), and mode of action (additive, recessive, dominant, and hemizygous) [5,6]. The causes of autism may be heritable, de novo, or both. Some family and twin studies have shown that autism is highly heritable. The concordance rate of autism is roughly 45% for monozygotic twins and 16% for dizygotic twins [7,8]. The co-occurrence rate of autism in siblings is approximately 45 times greater than in the general population. The male to female ratio is 3-4:1 [4,9]. The reason for the male predominance is still unknown.

Various studies from Asia, Europe, and North America have identified the average prevalence of autism spectrum disorder (ASD) to be 1% to 2% [10]. In India, the prevalence of autism has increased drastically over time, and the true reason for the cause of this change remains unclear. In a systematic review of four studies, one study included both rural and urban populations, while the remaining three included only urban populations. The study from the rural cohort showed a pooled prevalence of 0.11% (95% confidence interval [CI], 0.01% to 0.20%) in children of 1–18 years. A study conducted in the urban cohort showed a pooled prevalence of 0.09% (95% CI, 0.02% to 0.16%) in children aged 0–15 years [11]. A study conducted in 2017 amongst children from rural, urban, and tribal populations of children aged 1–10 years showed a prevalence of 0.15% (95% CI, 0.15% to 0.25%) [10].

Autism has diverse pathophysiological mechanisms, of which synaptic cell adhesion and associated molecules are currently amongst the most studied. Neuroligin is a type I transmembrane protein whose extracellular segments contain a globular domain homologous to acetylcholine esterase and a stalk rich in O-linked carbohydrates [12]. In the human genome, five genes have been identified that code for neuroligin: *NLGN1* (3q26), *NLGN2* (17p13), *NLGN3* (Xq13), *NLGN4X* (Xp22.3), and *NLGN4Y* (Yq11.2). The protein products of NLGN1 are located at excitatory synapses, those of *NLGN2* are found at inhibitory synapses, those of *NLGN3* are at both excitatory and inhibitory synapses, and those of *NLGN4X* are found at excitatory synapses. *NLGN4X* and *NLGN4Y* have almost identical sequences, and are therefore assumed to have the same function [13,14]. In the present study, we recorded the prevalence of autism in the North Karnataka region of India for the first time, investigated the genetic profile of neuroligin 3 gene in the Indian autistic population by DNA sequencing, and reported novel molecular-level changes.

Methods

Subjects

In the North Karnataka region of India, 1870 mentally ill children below 18 years of age were diagnosed using the Diagnostic and Statistical Manual of Mental Disorders (https://www.psychiatry.org/psychiatrists/practice/dsm) and the International Classification of Diseases, 10th revision (https://www.who.int/classifications/icd/icdonlineversions/en/). From these children, 150 autistic children were identified, of whom 108 children were included in this study (n_{male} = 85, n_{female} = 23; age range, 5 to 18 years, and mean age, 11.7 ± 3.5 years). Children with associated medical conditions, including fragile X syndrome, chromosomal abnormalities, and metabolic disorders were excluded from the study. Furthermore, 150 age-, sex-, and ethnicity-matched healthy control children were also included in the study (n_{male} = 100, n_{female} = 50; age range, 5 to 18 years, and mean age, 11.0 ± 2.0 years; p = 0.04). Shri B.M Patil Medical College, Hospital and Research Centre, BLDE (Deemed to be University), Vijayapura (Ref No: BLDE (DU) IEC/337-2018-19) approved the study. Clinical samples were obtained after receiving informed consent from the parents/guardian of the respective child.

DNA sequencing

Genomic DNA was extracted from peripheral blood using a DNA extraction kit (DNeasy Blood and Tissue Kit, Qiagen, Hilden, Germany) as per the manufacturer's instructions. All seven exonic regions of the NLGN3 gene were amplified by polymerase chain reaction (PCR) using PCR reaction kits (New England Biolabs, Ipswich, MA, USA) with specifically designed primers. Sequencing of the amplified product was performed on an ABI 3500 DNA analyzer using a Big Dye terminator version 3.1 cycle sequencing kit (Applied Biosystems, Waltham, MA, USA). Sequencing results were analyzed on DNA Sequencing Analysis Software v5.4 (Applied Biosystems).

Bioinformatics analysis

The pathogenic effects of non-synonymous variants were analyzed using the following tools:

- PROVEAN (Protein Variation Effect Analyser, http://provean.jcvi.org/seq_submit.php), which predicts the impact of amino acid substitutions on the biological function of a protein.
- PolyPhen-2 (Polymorphism Phenotypic-2, http://genetics.bwh.harvard.edu/pph2/index.shtml), which is a tool that predicts the

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impact of amino acid substitutions on the structure and function of human proteins using straightforward physical comparative considerations.
- PHD SNP (https://snps.biofold.org/phd-snp/phd-snp.html), a predictor of human deleterious SNPs.
- SNP & GO (https://snps.biofold.org/snps-and-go/snps-and-go.html), which predicts disease-associated variants using Gene Ontology terms.
- PANTHER (Protein Analysis through Evolutionary Relationships; http://www.pantherdb.org), which uses evolutionary relationships to infer gene function.
- SNAP2 (https://www.rostlab.org/services/snap/), which predicts the functional effects of sequence variants. The evolutionary conservation of the missense variant was investigated by Clustal Omega (https://www.ebi.ac.uk/Tools/msa/clustalo/) using *NLGN3* sequences from different species. Homology modelling of wild-type and mutant NLGN3 proteins was conducted using a fully automated protein structure homology-modelling server (Swiss Model; https:// swissmodel.expasy.org), and the results were visualized and analyzed using the UCSF Chimera program.

The Uniprot accession numbers were Q9NZ94 (*Homo sapiens*), Q8BYM5 (*Mus musculus*), Q62889 (*Rattus*), F1Q3I9 (*Canis lupus*), G3MXP5 (*Bos taurus*), A0A2I2UDX2 (*Felis catus*), E9KFA0 (*Gallus gallus*), G3RBW3 (*Gorilla gorilla*), and G7NRV3 (*Macaca mulatta*).

Statistical analysis

The obtained data were tabulated and analyzed via SPSS version 15.0 (SPSS Inc., Chicago, IL, USA). Quantitative statistical analysis was performed using the two-tailed Student t-test. Data are presented as mean ± SD. The chi-square test was used to calculate the genotype frequencies of cases and controls. The frequency of the variant was estimated using the Hardy-Weinberg equilibrium (HWE). The risk association between the novel missense mutation and autism was calculated by analyzing odds ratios along with their 95% CIs through allelic, dominant, and recessive genetic models by logistic regression. A p-value < 0.05 was considered to indicate statistical significance.

Results

In the present study, all seven exonic regions of the *NLGN3* gene were analyzed in 108 individuals. One coding sequence variant and four non-coding sequence variants (two 5′-untranslated region [UTR] variants and two 3′-UTR variants) were observed. The coding sequence variant c.551 T > C (p.V184A) was a missense variant observed in 27 autistic children (25%). The 5′-UTR variant g.5040 C > W was found in one (0.92%) and g.5041 T > A was found in five (4.6%) autistic children, while the 3′-UTR variant g.30370 C > Y was observed in 75 (69.4%) and g.30349-30350 Ins AC was observed in 21 (19.4%) autistic children. The g.5040 C > W and g.30370 C > Y variants were heterozygous (Table 1, Fig. 1). All the variants are novel and have not been reported previously in any of the in-house human SNP databases such as dbSNP, 1000genomes, ExAc, ClinVar, HapMap, and gnomAD. The clinical features of all the 27 autistic children in whom the novel missense mutation p.V184A was recorded are summarised in Table 2.

The novel missense variant c.551 T > C (p.V184A) was predicted to have a deleterious effect on the function of the NLGN3 protein by PolyPhen2, PROVEAN, PANTHER, SNP&GO, PHD-SNP, and SNAP2 (Table 3). Multiple sequence alignment of NLGN3 was carried out using Clustal Omega, which indicated that the valine at residue 184 is fully conserved across different species ranging from chickens to humans (Fig. 2). Three-dimensional models for the wild-type NLGN3

Table 1. List of variants and frequency of variants in our study population

Gene	Mutation type	Nucleotide change	Amino acid change	Exon	Frequency of mutation in autism group (%)	Frequency of mutation in control group (%)
NLGN3	5′ UTR	g.5040 C > W	-	1	1 (0.92)	0
	5′ UTR	g.5041 T > A	-	1	5 (4.6)	0
	Missense	g.15417 [c.551 T > C]	p.V184A	4	27 (25)	2 (1.3)
	3′ UTR	g.30349-30350 Ins AC	-	7	21 (19.4)	1 (0.7)
	3′ UTR	g.30370 C > Y	-	7	75 (69.4)	6 (4)

Autistic individuals (n = 108), n_{male} = 85, n_{female} = 23; age range, 5 to 18 years, and mean age, 11.7 ± 3.5, p = 0.03. Furthermore, 150 age-, sex-, and ethnicity-matched control children were also included in the study (n_{male} = 100, n_{female} = 50; age range, 5 to 18 years, and mean age, 11.0 ± 2.0, p = 0.04).
The NG_015874.1 reference sequence was used for genomic DNA variant nomenclature, the ENST00000374051.7 reference sequence was used for coding region variant nomenclature, and the ENSP00000363163.3 reference sequence was used for protein variant nomenclature. The nomenclature followed the Human Genome Variation Society guidelines.
UTR, untranslated region.

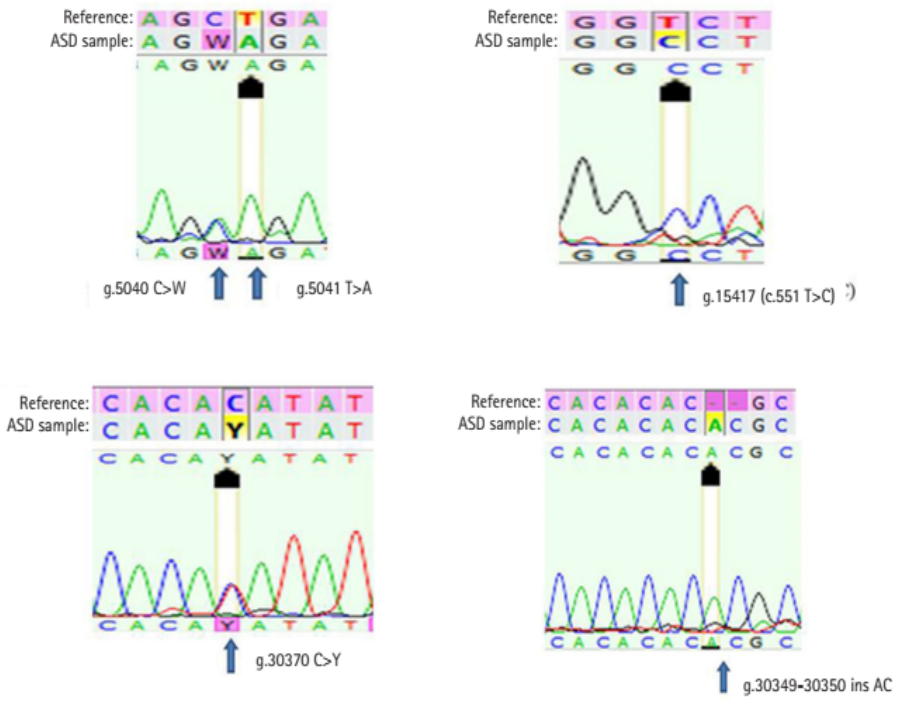

Fig. 1. All the four variants of the *NLGN3* gene recorded in the autistic population. ASD, autism spectrum disorder.

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                                                 ⇩
Gallus          KGGASAKKQGEDLADNDGDEDEDIRDSGAKPVMVYIHGGSYMEGTGNMIDGSVLASYGNV
Felis           KGGSGAKKQGEDLADNDGDEDEDIRDSGAKPVMVYIHGGSYMEGTGNMIDGSVLASYGNV
RAT             KGGSGAKKQGEDLADNDGDEDEDIRDSGAKPVMVYIHGGSYMEGTGNMIDGSVLASYGNV
MOUSE           --GSGAKKQGEDLADNDGDEDEDIRDSGAKPVMVYIHGGSYMEGTGNMIDGSVLASYGNV
Canis           KGGSGAKKQGEDLADNDGDEDEDIRDSGAKPVMVYIHGGSYMEGTGNMIDGSVLASYGNV
Bos             KGGSGAKKQGEDLADNDGDEDEDIRDSGAKPVMVYIHGGSYMEGTGNMIDGSVLASYGNV
ControlHUMAN    KGGSGAKKQGEDLADNDGDEDEDIRDSGAKPVMVYIHGGSYMEGTGNMIDGSILASYGNV
AffectedHUMAN   KGGSGAKKQGEDLADNDGDEDEDIRDSGAKPVMAYIHGGSYMEGTGNMIDGSILASYGNV
Gorilla         KGGSGAKKQGEDLADNDGDEDEDIRDSGAKPVMVYIHGGSYMEGTGNMIDGSVLASYGNV
Macaca          KGGSGAKKQGEDLADNDGDEDEDIRDSGAKPVMVYIHGGSYMEGTGNMIDGSVLASYGNV
                  *:.***************************.*****************:*******
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Fig. 2. Multiple sequence alignment of neuroligin 3 protein sequences. The amino acid residue at 184 is arrowed.

protein and the mutated NLGN3 (p.184A) protein were generated using the homology-modelling server Swiss Model. The wild-type protein had a Q_{mean} of −1.24 and the mutated NLGN3 protein had a Q_{mean} of −1.32. At residue V184, two H-bonds were observed with V264 in both wild-type (V184) and mutant (A184) proteins (Figs. 3 and 4), and the mutant residue was smaller than the wild-type residue, which might lead to a loss of interactions with other molecules.

Basic information containing allelic frequency, minor allele fre-

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Table 2. Clinical features of autistic children with the novel missense mutation c.551T>C in the *NLGN3* gene

No.	Sex/ Age (y)	CARS score and severity	IQ	Age of mother[a]	Age of father[b]	Prenatal damage	Postnatal damage	Consanguineous marriage	Co-morbid condition
Child 1	M/13	Severe, 51	20	32	36	None	Birth asphyxia	Yes	None
Child 2	F/10	Severe, 50	25	25	30	Preeclampsia	Respiratory illness	Yes	None
Child 3	M/14	Severe, 46	30	32	35	Infection during Pregnancy	None	No	None
Child 4	M/11	Severe, 48.5	30	26	32	Hyperthyroidism	None	Yes	None
Child 5	F/14	Severe, 47.5	20	28	33	None	Birth asphyxia	Yes	None
Child 6	M/10	Severe, 52	30	32	37	None	Delayed crying	Yes	None
Child 7	M/9	Severe, 43.5	20	34	43	None	None	No	None
Child 8	F/8	Severe, 49.5	30	16	36	Preeclampsia	None	No	None
Child 9	M/10	Mildly moderate, 37	35	22	30	None	None	Yes	None
Child 10	M/13	Severe, 51.5	30	33	38	Maternal hypertension	None	Yes	None
Child 11	M/11	Severe, 42	20	22	30	None	Delayed crying	Yes	None
Child 12	F/13	Severe, 40.5	20	28	35	None	None	No	None
Child 13	F/11	Mildly moderate, 35	65	32	38	infection during pregnancy	Birth asphyxia	No	None
Child 14	M/10	Mildly moderate, 30	65	25	32	Maternal hypertension	None	No	None
Child 15	M/12	Mildly moderate, 36	35	28	36	None	Delayed crying	No	None
Child 16	M/14	Severe, 50	20	20	29	Maternal hypertension	None	Yes	None
Child 17	M/9	Mildly moderate, 32	20	33	39	Preeclampsia	None	No	None
Child 18	F/8	Severe, 50	20	30	36	Hyperthyroidism	Respiratory illness	No	None
Child 19	M/10	Mildly moderate, 33	25	26	30	None	None	Yes	None
Child 20	M/11	Mildly moderate, 36	40	19	29	None	None	Yes	None
Child 21	M/8	Mildly moderate, 35	35	28	34	None	Feeding problem	Yes	None
Child 22	F/10	Severe, 56	20	30	36	Infection during pregnancy	Respiratory illness	No	None
Child 23	F/13	Severe, 49	25	35	45	None	Delayed crying	Yes	None
Child 24	M/12	Mildly moderate, 33	30	18	25	Maternal hypertension	Feeding problem	No	None
Child 25	M/9	Severe, 53	30	29	38	None	None	No	None
Child 26	M/11	Mildly moderate, 32	20	36	43	None	None	Yes	None
Child 27	F/12	Mildly moderate, 35	30	35	38	Preeclampsia	None	Yes	None

CARS, Childhood Autism Rating Scale.
[a]Age of mother at childbirth.
[b]Age of father at childbirth.

Table 3. Pathogenicity of the missense variant as identified by five *in silico* tools

PROVEAN	PHD-SNP	SNP & GO	SNAP2	Polyphen-2	PANTHER
Deleterious score: -3.094	Disease reliability index: 8	Disease probability: 0.682	Effect score: 23	Probably damaging score: 0.982	Probably damaging

NLGN3 mutation: missense, nucleotide change: c.551 T>C, amino acid change: p.V184A.
PROVEAN: "deleterious" if the prediction score is </-2.5, "neutral" if the prediction score is >/-2.5. SNP & GO: If the probability is >0.5, then it is predicted to be a disease-causing nsSNP. SNAP2: "neutral" if the score is from 0 to -100, "effect" if the score is from 0 to 100. PolyPhen-2: "probably damaging" indicates the strongest disease-causing ability with a score close to 1; "possibly damaging" refers to less disease-causing ability with a score of 0.5-0.8; and "benign" corresponds to no alteration of protein function, with a score closer to 0. PHD-SNP: if the probability is >0.5, the mutation is predicted as "disease" and if it is less than <0.5, the mutation is predicted to be "neutral."

quency distribution, HWE p-value, odds ratios (ORs), and 95% CIs of c.551T > C is shown in Table 4. c.551T > C was in HWE in the control group ($p > 0.05$). The frequency of the C allele of c.551T > C was significantly higher in cases than in controls (2.5% vs. 1%) which suggests that the C allele of the novel frameshift mutation c.551T > C was associated with an increased risk of autism (OR, 24.7; 95% CI, 5.7 to 106.4; $p = 0.001$) (relative risk, 18.9; 95% CI, 4.5 to 77.2; $p = 0.002$). Furthermore, we assumed that the

Table 4. Basic information on the novel missense mutation c.551T>C and MAF between cases and controls

Variation type	Position	Minor allele	MAF		HWE-p	Odds ratio (95% CI)	p-value	Relative risk (95% CI)	p-value
			Case	Control					
Missense	c.551	C	0.25	0.01	0.99	24.7 (5.7-106.4)	0.001*	18.9 (4.5-77.2)	0.002*

HWE p-values were calculated using the 2-sided chi-square test.
MAF, minor allele frequency; HWE, Hardy-Weinberg equilibrium; CI, confidence interval.
*p < 0.05 indicates statistical significant.

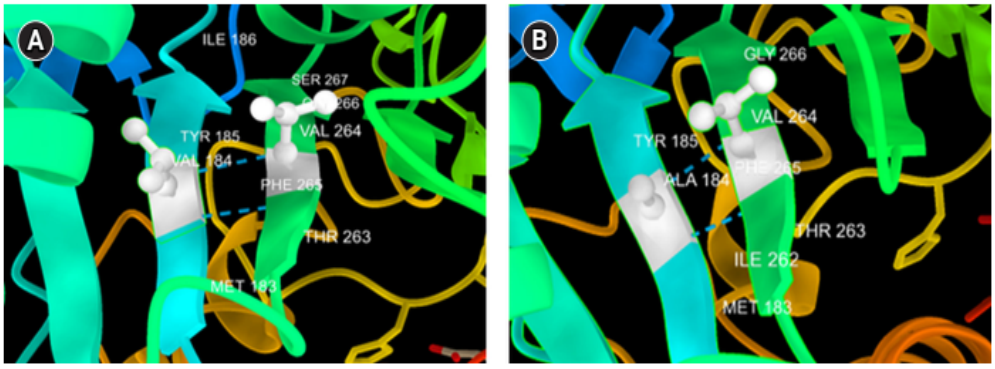

Fig. 3. 3D structure of V184A substitution using Chimera software. The wild-type residue (A) and the mutant residue (B).

Fig. 4. Amino acid structure of valine to alanine.

minor allele of c.551T > C was a risk factor compared with the wild-type allele. Three genetic models (codominant, dominant, and recessive) were applied to analyze the significance of the association between the novel mutation and autism risk using logistic regression. A statistically significant increase in the risk of autism was observed for the C/C genotype compared to the wild-type T/T genotype (OR, 96.68; 95% CI, 5.8 to 1,607.1; p = 0.001), which corresponds to a 96.68-fold increased risk of autism under the codominant model. The combined genotype (T/C + C/C) also significantly increased the autism risk under the dominant model (OR, 24.7; 95% CI, 5.7 to 106.4; p = 0.001) (Table 5). We implemented a stratification analysis by sex to evaluate the sex association between the novel mutation allele and autism. We found that the C/C genotype was associated with an increased risk for autism in males (OR, 55.1; 95% CI, 3.3 to 929.7; p = 0.005) under the codominant model, as was the combined genotype (T/C + C/C) under the dominant model (OR, 26.6; 95% CI, 3.5 to 204.1; p = 0.002). The C/C genotype was also associated with an increased risk for autism in females (OR, 55.4; 95% CI, 3.0 to 1,015.2; p = 0.006) under the codominant model, as was the combined geno-

Table 5. Association between the novel missense mutation c.551T>C and the risk of autism in genetic models

Models	Genotype	No. (%)		OR (95% CI)	p-value
		Case	Control		
Codominant	T/T	81	148	1 (ref.)	-
	T/C	1	2	0.69 (0.1-7.7)	0.38
	C/C	26	0	96.68 (5.8-1,607.1)	0.001*
Dominant	T/T	81	148	1 (ref.)	-
	T/C-C/C	27	2	24.7 (5.7-106.4)	0.001*
Recessive	T/T-T/C	81	150	1 (ref.)	-
	C/C	26	0	96.68 (5.8-1,607.1)	0.001*

OR, odds ratio; CI, confidence interval.
*p < 0.05 indicates statistical significant.

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type (T/C + C/C) under the dominant model (OR, 31.5; 95% CI, 3.7 to 270.3; p = 0.001) under the dominant model (Table 6).

Discussion

Autism (MIM 209850) is a heterogeneous neurological disorder manifesting before the age of three [1,3]. Autism results in impaired social interactions, impaired communication, and abnormal behavior [1,13]. Genetic research on autism has made great progress in the past few years. Neuroligin, a postsynaptic transmembrane protein involved in synaptogenesis, has been predicted to be a promising candidate gene for autism and other neurological disorders [15-18]. Neuroligin 3 shows diverse genetic alterations in autism. In India, autism is still understudied, and insufficient experimental data are available on its clinical aspects. To our knowledge, this is the first study from India on the role of the *NLGN3* gene in autism. The first-ever study to show the role of *NLGN3* in autism recorded a missense variant (R451C) that alters the binding of neuroligin to neurexin, resulting in the abnormal formation, stabilization, and recognition of specific synapses essential for communication process that are defective in autism, was conducted in 2003 [9]. Later studies showed that neuroligin gene mutations were implicated in rare cases of autism. Despite the rarity of its involvement, the components involved in the synaptogenesis and synaptic structures remain excellent functional candidates for future molecular genetic studies of autism and related disorders [19]. Subsequent studies reported different mutations in different regions of the *NLGN3* gene across the world. Mutated versions of the NLGN3 protein with the missense variants Pro514Ser and Arg597Trp do not reach the plasma membrane in the cell, preventing it from interacting with the neurexin protein in the human brain [20]. Certain missense variants may influence males' susceptibility to ASD [21]. Hence, *NLGN3* may be a candidate gene for the male predominance of autism. Several missense variants in *NLGN3* account for non-syndromic forms of intellectual disability associated with autism [20]. Moreover, missense variants with pathogenic effects are possible etiological factors for autism [22]. Coding sequence variations in *NLGN3* and *NLGN4* are rare, but contribute to the aetiology of autism [23]. In addition to missense variants, but intronic and non-synonymous variants also affect the regulatory region such as enhancers and promoters associated with histone modification sites (nuclease-accessible sites and transcription factor binding sites), making modest contributions to the pathogenesis of ASD [23-25]. Other forms of genetic alterations, including splice variants and non-coding sequence variants, may also lead to the potentially abnormal function of neuroligin in autism [26,27].

All previous studies strongly suggest that neuroligin plays a heterogeneous role in the aetiology of autism. No molecular study of neuroligin on autism in India has been conducted to date. We studied 108 autistic individuals and found the novel missense variant c.551T > C in the *NLGN3* gene, which was not recorded previously in any autistic population around the globe. In our study, the C/C genotype of c.551T > C was significantly more common in the autistic children than in the controls (p = 0.001). In particular, there was a significantly 96.68-fold increased risk of autism (p = 0.001) associated with this genotype. However, the combined T/C+C/C genotype showed a significantly 24.7-fold increased risk of autism (p = 0.001), implying that the T allele may be a protective factor and people who carry this allele may be less likely to develop autism. In both male and female children, the C/C genotype of

Table 6. Analysis of novel missense mutation c.515T>C genotype and autism risk in males and females based on logistic tests

Model	Genotype	Male		OR (95% CI)	p-value	Female		OR (95% CI)	p-value
		Case	Control			Case	Control		
Codominant	T/T	76	99	1 (ref.)	-	14	49	1 (ref.)	-
	T/C	0	1	0.39 (0.02-9.6)	0.56	1	1	2.2 (0.13-37.2)	0.57
	C/C	18	0	55.1 (3.3-929.7)	0.005*	8	0	55.4 (3.0-1015.2)	0.006*
Dominant	T/T	67	99	1 (ref.)	-	14	49	1 (ref.)	-
	T/C-C/C	18	1	26.6 (3.5-204.1)	0.002*	9	1	31.5 (3.7-270.3)	0.001*
Recessive	T/T-T/C	67	100	1 (ref.)	-	15	50	1 (ref.)	-
	C/C	18	0	55.1 (3.3-929.7)	0.005*	8	0	55.4 (3.0-1015.2)	0.006*

OR, odds ratio; CI, confidence interval.
*p < 0.05 indicates statistical significant.

c.551T > C showed a significantly similar risk ratio (55.1-fold vs. 55.4-fold). The c.551T > C variant was predicted to be a pathogenic variant of NLGN3 by the PROVEAN, PolyPhen2, PANTHER, PHD-SNP, SNPs & GO, and SNAP2 prediction tools. The V184 residue constitutes a highly conserved amino acid that shows a strong evolutionary relationship over different species. A structural analysis of the wild-type and mutant protein showed variation in structural integrity and a reduction of the stability of the mutant protein relative to the wild-type; the mutant residue was smaller than the wild-type residue. This might lead to a loss of interactions with other molecules because it is located in the extracellular region of the NLGN3 protein. However, as a limitation of this study, the identification of a coding sequence variant is not sufficient to disclose the exact role of the gene in a disease that has a complex genetic architecture. Moreover, we also recorded four novel non-coding sequence variants (g.5040 C > W, g.5041 T > A, g.30349-30350 Ins AC, and g.30370 C > Y). These non-coding variants may also play a role in posttranslational modification. Our study is only preliminary basic research, and further functional analysis of novel mutations in the neuroligin pathway will provide a better understanding of the involvement of the *NLGN3* gene in autism in the Indian population.

The findings of our study suggest that the novel missense variant c.551T > C (p.V184A) causes abnormalities in the NLGN 3 protein, which may lead to deficits in synaptogenesis, in the Indian autistic population. Neuroligin is probably a candidate gene for future molecular investigation and functional analyses in the Indian autistic population.

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Authors' Contribution

Conceptualization: RH, SH, AP. Data curation: RH, SH, AP. Formal analysis: RH, SH, SSK, AP Methodology: RH, SH, SSK, AP. Writing - original draft: RH, SH. Writing - review & editing: RH, SH, SSK, PBG, KKD.

Conflicts of Interest

No potential conflict of interest relevant to this article was reported.

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Human homeostatic iron regulator gene polymorphism in autistic population of India; a case-control study

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ABSTRACT

Background: Autism is a heterogeneous neurodevelopmental disorder. Human homeostatic iron regulator (HFE) codes for HFE protein. HFE protein is very essential for inhibitory regulation of the endocytosis of iron.
Objective: Present study aims to screen C282Y and H63D polymorphism of the HFE gene in autistic children.
Method: 30 autistic children and 30 healthy age-matched control children were included in the study. TXRF analysis was performed for quantification of Iron in plasma. Genomic DNA was extracted using peripheral blood samples and targeted SNPs were screened using Restriction fragment length polymorphism. Genotype, allelic frequencies and risk ratio were calculated using the statistical method.
Results: TXRF analysis shows a significantly very low concentration of iron in autistic children compared to the control group [1039.6 ± 28 μg/L vs 2372.2 ± 35 μg/L, *p*-value 0.001]. Genetic Study shows that all the 30 controls and 28 autistic cases showed homozygote C/C allele. Two heterozygote C/Y alleles and no homozygous Y/Y allele were observed for C282Y polymorphism for autistic cases. 29 control and 23 autistic cases showed a homozygote H/H allele. 01 control and 07 autistic cases showed heterozygote H/D allele for H63D polymorphism. C282Y and H63D polymorphisms of HFE gene for heterozygous condition showed non-significant evidence of risk for causing autism OR = 5.35, 95%CI = 0.25–116.3, *P*-value-0.29 and OR = 8.8, 95%CI = 1.0–76.9, P-value = 0.05 respectively.
Conclusions: Present study found that C282Y and H63D were not found to be the risk factor for autism in the targeted study cohort.

1. Introduction

Autism is a complex heterogeneous neurodevelopmental disorder by the influence of genetic and environmental factors. It is characterized by impaired social interactions, deficient communication, restricted interests and stereotyped activity (Hegde et al., 2021a). It predisposes at the early onset of the age of three. In the general population, it occurs approximately 0.6 to 1.2% (Sudarshan et al., 2016). The male to female ratio for autism is estimated at 4:1 (Laumonnier et al., 2004). Different studies on twins, high risk infant siblings, population, families have estimated concordance rates of 60–70% in monozygous twins and 5–30% in siblings (Yoo, 2015). No prevalence data for autism is available from India for the general population. Oxidative stress plays a major role in the aetiology of several neurological diseases and neurodevelopmental disorders such as Alzheimer's disease, Parkinson's disease, Schizophrenia, Autism and ADHD (Goldani et al., 2014). Increased oxidation stress caused by either increased production or decreased elimination of oxygen free radicals was associated with cell damage (Gebril and Meguid, 2011). Recently many studies have shown trace elements like zinc, copper, iron and heavy metals like mercury, cadmium is implicated as potential risk factors for autism. Iron is one of the most studied trace elements in autism that maintains the integrity and

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functioning of the central nervous system (Baj et al., 2021).

Hereditary hemochromatosis is an autosomal recessive condition characterized by an excess of iron in different organs, particularly the liver. The mutations, C282Y and H63D in the histone family E1 [HFE] gene on chromosome 6p21.3 are the most common causes of hemochromatosis (Lakshmiprabha et al., 2019). HFE has an extracellular peptide binding region [alpha 1 and alpha 2], an Ig-like transmembrane domain [Alpha3], as well as a short cytoplasmic tail. The alpha3 domain's functional three-dimensional structure is maintained by disputed bridges between cysteine residues (Mikhaïlova et al., 2003).

The human homeostatic iron regulator is a major histocompatibility complex (MHC) class l-like gene that codes for HFE protein combine with b2-microglobulin (ß2M) to compete with transferrin (Tf) for binding to the transferrin receptor (TfR). Normal HFE protein is very essential for inhibitory regulation of the endocytosis of iron (Enns, 2006; Bennett et al., 2000). Deregulation of iron metabolism leads to Hemochromatosis. The two most common SNPs in the HFE gene that cause Hemochromatosis are C282Y (rs1800562) and H63D (rs1799945). The C282Y polymorphism inhibits HFE from reading the cell surface, blocking interactions with hepcidin and TFRs. Unlike C282Y, H63D do not disrupt the binding with B2M. H63D may disrupt iron hemochromatosis and causes iron accumulation when it occurs simultaneously with C282Y (Katsarou et al., 2016).

In the present study, we analysed C282Y (rs1800562) and H63D (rs1799945) polymorphisms in autistic children to investigate the association of these polymorphisms with autism because iron metabolism has its known biological effect on autism and no previous attempt was made to study these two SPNs in Indian autistic population.

2. Materials and methods

The present case-control study included 30 autistic children [mean age: 11.5 ± 3.1, n_{male} = 20, n_{female} = 10] and 30 healthy age-matched children as control group [mean age: 12 ± 2.5, n_{male} = 20, n_{female} = 10]. All the autistic children were diagnosed and included as per the Diagnostic and Statistical Manual of Mental Disorders [DSM–5] [https://www.psychiatry.org/psychiatrists/practice/dsm] and International Classification of Diseases-10[ICD-10] [https://www.who.int/classifications/icd/icdonlineversions/en/] criteria. Associated medical conditions such as fragile-X syndrome, metabolic disorders and chromosomal aberrations were excluded from the study. Written informed consent was obtained from parents/guardians. Ethical approval for the study was obtained from Karnataka Institute for DNA Research, Dharwad.

2.1. Fe element analysis

Minimal sample preparation was required for total reflection x-ray fluorescence for the analysis of Fe elements in plasma. To 495 µl of plasma and 5 µl of Internal Standard [IS] gallium [Ga] solution [500 mg/L] was added to get concentration 5 mg/Sample was then vertex slightly and 10 µl of the sample was deposited as a single drop over a quartz glass sample carrier. Samples were allowed to dry for 5 min. After drying, the sample carrier was inserted into the TXRF instrument [Bruker S2 Picofox TXRF, Bruker AXS GmbH, Germany] and analysis was carried with an integration time of 1000s for each sample with 50 kV voltages and 698 µA Current. Each sample was prepared and analysed in triplicate.

2.2. Genotyping

1 ml of peripheral blood was drawn into EDTA coated vacutainer (BD, Vacutainer) for genomic DNA isolation. Genomic DNA was isolated using a Blood and tissue DNA isolation kit (QIAgen, Germany) as per the manufacturer's instruction. PCR amplification for C282Y SNP was carried out using 95 °C for 0.5 min followed by the 35 cycles of 95 °C for 0.5 min, 62.2 °C for 01 min, 68 °C for 01 min, and final extension of 68 °C for 05 min, hold at 04 °C and the same PCR condition was used for H63D SNP except annealing temperature being 64.4 °C. Forward Primer sequence for C282Y was 5'-TGGCAAGGGTAAACAGATCC-3′ and reverse primer sequence was 5'-CTCAGGCACTCCTCTCAACC-3′. For H63D forward primer sequence was 5'-ACATGGTTAAGGCCTGTTGC-3′ and the reverse primer sequence was 5'-GCCACATCTGGCTTGAAATT-3′ (Ogouma-Aworet et al., 2020; Leão et al., 2014). Amplified products were electrophoresed on 2% agarose gel and the amplified product was visualised.

The Candidate SNPs were identified using standardised restriction endonuclease mixtures *Rsa*I (New England Bio Labs, USA) for C282Y and MobI (New England bio labs, USA) for H63D. The digested products were electrophoresed on 3% agarose gel and visualised after staining with ethidium bromide by using a gel documentation unit (Cleaver Scientific, UK).

2.3. Statistical analysis

Descriptive statistics were used to calculate the mean and standard deviation (±SD) for numerical data. The student's *t*-test was used to assess the statistical significance of the difference between two study group means. The chi-square test was used to determine whether the allelic and genotype frequencies were distributed in accordance with Hardy-Weinberg equilibrium (HWE) or not. *P*-value <0.05 is considered statistically significant. Statistical analysis was carried by using SPSS software version 15.

3. Results

Present study population included 30 autistic children [mean age: 11.5 ± 3.1, n_{male} = 20, n_{female} = 10] and 30 healthy age-matched children as control group [mean age: 12 ± 2.5, n_{male} = 20, n_{female} = 10]. Demographical characters of autistic children were tabulated in table no.1 (Table 1). Results from Statistical tests showed that the children with ASD showed a significantly lower concentration of Iron [Fe] compared to the control group (1039.6 ± 28 µg/L vs 2372.2 ± 35 µg/L,

Table 1
Demographic characteristics of autistic children and healthy control children.

Demographic character	Autistic children	Healthy children
No of children	30	30
Sex		
• Male	20	20
• Female	10	10
Mean age	11.5 ± 3.1	12.0 ± 2.5
Ethnic origin	Indian	Indian
The average age of the father at child's birth	38 ± 2.0	31 ± 2.3
The average age of mother at child's birth	33 ± 1.5	28 ± 1.2
Consanguineous marriage		
• Yes	18	06
• No	12	24
Prenatal factors		
• Preeclampsia	04	01
• Maternal Hyper thyroidism	01	01
• Hypertension	01	00
• Gestational problem	02	00
Postnatal factor		
• Labour complication	06	02
• Forceps mediated delivery	01	01
• Birth asphyxia	02	00
• Feeding problem	01	01
• Delayed crying	01	00
Intelligent quotient [IQ]	30 ± 10	60 ± 5
Severity		
• Mild to moderate	18	00
• Severe	12	00
Co-morbid condition	None	None
Family history of the neurodevelopmental condition	None	None

p-value 0.001) [Table 2]. Iron showed a significantly slight higher concentration in ASD Females compared to ASD males (1043.2 ± 6.0 μg/L vs 1030.2 ± 10.5 μg/L) [Table 3].

3.1. C282Y genotyping

The PCR products (390 bp) of C282Y SNP were used for genotyping. The SNP analysis for allele C282Y was determined after restriction digestion with RasI. The C allele generates two fragments of 250 and 140 bp while the Y allele generates three fragments of 250, 111 and 29 bp (Fig. 1). In our study, 30 controls and 28 autistic cases showed homozygote C/C allele, two heterozygote C/Y allele and no homozygote Y/Y allele was detected in any of the autism cases (Table 4).

The Chi-Square test analysis did not show any significant difference between genotype frequencies and allelic frequencies of C282Y polymorphism of the HFE gene among cases ($p > 0.05$). The percentage of C/C and C/Y genotypes among autism cases was 93.33% and 6.67% respectively. The Control group shows 100% of C/C genotypes. The allelic frequencies of C and Y alleles were 96.77% and 3.33% respectively in cases. Among controls, 100% were C alleles (Table 4). 3 genetic models [codominant, dominant, and recessive] significant were applied to analyse the association between C282Y (rs1800562) and autism risk using logistic regression. A statistically non-significant risk of autism was observed for Y/Y genotype compared to wild-type C/C genotype [OR = 1.0, 95% CI = 0.02–52.1, P-value = 1.0] under codominant model. Genotype C/Y-Y/Y was also non-significant for the risk of autism risk under the dominant model [OR = 5.35, 95% CI = 0.25–116.3, P-value = 0.29] [Table 5].

3.2. H63D genotyping

The PCR products (208 bp) of H63D SNP were used for genotyping. The SNP analysis for allele H63D was determined after restriction digestion with MobI. H allele generates 2 fragments of 138 bp and 70 bp. D allele does not generate any fragments (no cutting sites are available for MobI restriction) (Fig. 2). In our study, 29 control and 23 autistic cases showed a homozygote H/H allele. 07 autistic cases and 01 control showed heterozygote H/D allele and no cases showed homozygote D/D allele (Table 4).

The Chi-Square test analysis did not show any significant difference between genotype and allelic frequencies of H63D polymorphism of the HFE gene among cases ($p > 0.05$) and control ($p > 0.05$). The percentage of H/H and H/D genotypes among autism cases was 76.67% and 23.33% respectively. The Control group shows 96.67% H/H genotypes and 3.33% of H/D genotypes. The allelic frequencies of H and D alleles were 88.33% and 11.67% respectively. H and D alleles were found in 98.33% and 1.67% of control groups, respectively (Table 4). under the codominant model, the D/D genotype was found to have a statistically non-significant risk of autism when compared to the wild-type H/H genotype [OR = 1.0, 95% CI = 0.02–52.0, P-value = 1.0]. Under the dominant model, genotype H/D-D/D was also non-significant for the risk of autism [OR = 8.8, 95% CI = 1.0–76.9, P = 0.05] [Table 5].

4. Discussion

The dramatic increase in the prevalence of autism throughout the globe in recent years may be due to an increase in the knowledge and awareness of autism or it may be due to improved diagnostic methods that are being developed during the last decade. Multiple studies throughout the globe showed that more than hundreds of genes are actively involved in autism and these genes are classified into multiple functioning pathways (Gupta and State, 2006). The ferrous ionic form of Iron catalyses the formation of toxic hydroxyl radicals and thus it initiates and exaggerates lipid peroxidation and ultimately leads to brain cell destruction (McCord and Day, 1978). Superoxide dismutase (SOD), ceruloplasm (copper-binding protein), transferrin (iron-binding protein) are the factors that neutralise the oxidative load with a potent role being implicated in autism (Gonzalez-Cuyar et al., 2008; Galaris and Pantopoulos, 2008). Several shreds of evidence have shown that HFE protein plays a major role in iron metabolism (Bennett et al., 2000). Iron deficiency might explain behavioural problems common to people with ASD and is also associated with ASD symptoms and particularly correlates with severity of emotional and behavioural problems and with developmental delay in autism (McCann and Ames, 2007; Saghazadeh et al., 2017). The present study supports this statement by recording a significantly very low concentration of iron in autistic children compared to the control group [1039.6 ± 28 μg/L vs 2372.2 ± 35 μg/L, *p*-value 0.001]. ASD Males showed a low concentration of plasma iron compared to ASD females [Table 2].

In the present study, we examined the two most common potential polymorphisms in the HFE gene (C282Y and H63D) in the autistic population of India for the first time. The idea to include this gene concerning autism came out from many recent studies that showed an excess of iron intake and disturbed iron metabolism as risk factors for autism (Gebril and Meguid, 2011; Padhye, 2003).

In the western population, the C282Y mutation in the HFE gene is a prominent cause of hereditary hemochromatosis (Sumi et al., 2020; Thakkar et al., 2018). In the general population of India, the frequency of C282Y mutation in the HFE gene that causes hereditary hemochromatosis is rare. C282Y is either missing or has a low frequency in non-Caucasian populations, such as Africans, Asians, people from the South Pacific, and Australians (Dhillon et al., 2012). The status of HFE mutations has not been explained well among Indians and currently, no exact incidence and prevalence for autism are available from India for the general population (Poddar, 2006; Hegde et al., 2021b).

The first studied SNP C282Y was very low in our targeted autistic samples and completely absent in controls. Non-significant difference between genotype frequencies of C282Y polymorphism of HFE gene among cases ($p > 0.05$) was observed. Logistic regression analysis of association between C282Y (rs1800562) and autism risk showed statistically non-significant non association for Y/Y genotype compared to wild-type C/C genotype [OR = 1.0, 95% CI = 0.02–52.1, $P = 1.0$]. This result is very similar to a study done by O.H. Gebril et al., 2011 on Egyptian autistic children (Gebril and Meguid, 2011). But heterozygous condition C/Y showed non-significant evidence of association for risk of causing autism OR = 5.35, 95%CI = 0.25–116.3, *P*-value-0.29 in our study population.

In the general population, the frequency of the second common mutation H63D ranges from 9.1% to 13.9%. HH is most common in the Caucasian population [2.6–14%] (Katsarou et al., 2016). H63D SNP was

Table 2
Plasma Iron [Fe] element level (μg/L) in children with autism and controls.

Element (μg/L)	ASD	Control	*P* value
	Mean ± SD	Mean ± SD	
Iron, Fe	1027.6 ± 25	2371.2 ± 30	0.001*

Data presented as Mean ± SD.
* Significant difference as compared to the control values at $p < 0.05$.

Table 3
Sex wise distribution of plasma iron level (μg/L) in children with ASD and control.

Element μg/L	Male		P-value	Female		P-value
	ASD *n* = 20	Control n = 20		ASD *n* = 10	Control n = 10	
Iron, Fe	1030.2 ± 10.5	2381 ± 12.2	0.001*	1043.2 ± 6.0	2355.1 ± 7.8	0.008*

Data presented as Mean ± SD.
* Significant difference as compared to the control values at $p < 0.05$.

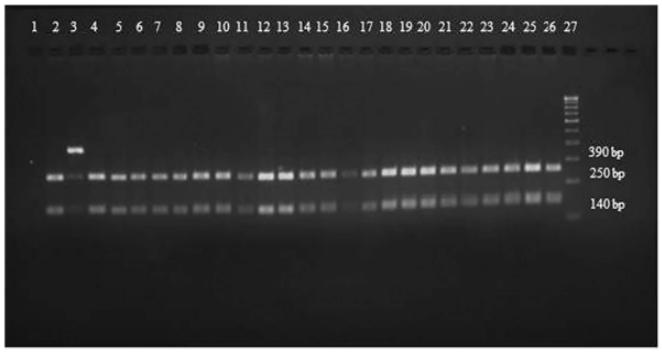

Fig. 1. Restriction analysis of C282Y SNP after digestion with RasI. Lane no:1 Negative control, Lane no: 2–26 autistic samples (Lane no:2 and 4–26 was homozygote C/C allele, Lane no:3 was undigested product), Lane no:27 marker 100 bp.

Table 4
Genotypic and allelic frequency distribution in autistic and control groups for C282Y and H63D polymorphisms of HFE gene.

Group	Genotype frequency (%)			Allelic frequency		Chi-square	p-value
	CC	CY	YY	C	Y		
Cases (*N* = 30)	28 (93.33%)	02 (6.67%)	00	0.97	0.03	0.04	0.98**
Control (N = 30)	30 (100%)	00	00	01	00	–	–

Group	Genotype frequency (%)			Allelic frequency		Chi-square	P-value
	HH	HD	DD	H	D		
Cases (N = 30)	23 (76.67%)	07 (23.33%)	00	0.88	0.12	0.52	0.77**
Control (N = 30)	29 (96.67%)	01 (3.33%)	00	0.98	0.02	0.01	0.99**

N = Number, CC = Homozygous Wild, YY = Homozygous Mutant, CY = Heterozygous (for C282Y polymorphism). HH = Homozygous Wild, DD = Homozygous Mutant, HD = Heterozygous (for H63D polymorphism). *Significant difference as compared to the control values at $p < 0.05$, **non significant difference as compared to the control values at $p > 0.05$.

Table 5
Association between SNPs and the risk of autism under genetic models.

Models	Genotype	Case	Control	OR (95% CI)	P value
C282Y (rs1800562)					
Co-dominant	C/C	28	30	1 (ref.)	–
	C/Y	02	00	5.35 (0.25–116.3)	0.29**
	Y/Y	00	00	1.0 (0.02–52.1)	1.0**
H63D (rs1799945)					
Co-dominant	H/H	23	29	1 (ref.)	–
	H/D	07	01	8.8 (1.0–76.9)	0.05*
	D/D	00	00	1.0 (0.02–52.0)	1.0**

OR = odds ratio, CI = confidence interval. *Significant difference as compared to the control values at $p < 0.05$, **non Significant difference as compared to the control values at $p > 0.05$.

Fig. 2. Restriction analysis of H63D SNP after digestion with MobI. Lane no: 1 Negative control, Lane no: 2–7 autistic samples (Lane no: 2,3,5,6 was homozygote H/H allele. Lane no: 4, 7 was heterozygote H/D allele), Lane no: 8 marker 100 bp.

shown in a few samples but heterozygotic form. No homozygotic mutant form was recorded. This result was slightly high when compared to a study done by O.H. Gebril et al. (2011)). A non-significant difference between genotype frequencies and allelic frequencies of H63D polymorphism of the HFE gene among cases ($p > 0.05$) was observed. Logistic regression analysis of association between H63D (rs1799945) and autism risk showed statistically non-significant non association for D/D genotype compared to wild-type H/H genotype [OR = 1.0, 95% CI = 0.02–52.0, $P = 1.0$]. But heterozygous condition H/D showed significant evidence of association for risk of causing autism OR = 8.8, 95%CI = 1.0–76.9, *P*-value = 0.05 in our study population.

The only pilot study at present on HFE gene polymorphism in autism by Gebril OH et al., 2011 in Egyptian autistic children included 26 patients with autism and their ages ranged between 5 and 15 years (mean age of 6.6 years ±4.4 and 24 males, 2 females). A Control sample consisting of normal healthy children (25 samples) within the same age range was studied. They failed to prove the role of HFE polymorphisms as risk factors for autism (Gebril and Meguid, 2011). Our study provides a similar result in Indian autistic children and supports their findings.

C282Y polymorphism and H63D polymorphism of the HFE gene does not show any evidence of risk for causing autism in our study cohort. Our study is only a preliminary basic research further functional analysis of SNPs is required and also more samples are required to evaluate the prevalence of SNPs in our population that was the limitation of the study.

5. Conclusion

This study is an attempt for the first time in India, to look into the association between HFE gene polymorphisms and autism. It was found that C28Y polymorphism and H63D polymorphism were not found to be the risk factors for autism in our study cohort with a negative association.

CRediT authorship contribution statement

Rajat Hegde and **Smita Hegde** were involved in Sample collection, genetic analysis and data analysis. **Suyamindra S Kulkarni** helped in genetic analysis and over all data analysis and interpretation. **Aditya Pandurangi** helped in identification, screening, counselling and sample collection. **Kusal K Das** and **Pramod B Gai** were involved in overall work design and valuation on results.

Declaration of competing interest

The authors declared no conflicts of interest concerning the research, authorship, funding and publication of this article.

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Total Reflection X-ray Fluorescence Analysis of Plasma Elements in Autistic Children from India

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Abstract
Trace elements are essential for the human body's various physiological processes but if they are present in higher concentration, these elements turn to be toxic and cause adverse effect on physiological processes. Similarly, deficiency of these essential elements also affects physiological processes and leads to abnormal metabolic activities. There is a lot of interest in recent years to know the mystery behind the involvement of trace elements in the metabolic activities of autistic children suspecting that it may be a risk factor in the aetiology of autism. The present study aims to analyse the plasma trace elements in autistic children using the total reflection X-ray fluorescence (TXRF) technique. Plasma samples from 70 autistic children (mean age: 11.5 ± 3.1) were analysed with 70 age- and sex-matched healthy children as controls (mean age: 12 ± 2.5). TXRF analysis revealed the higher concentration of copper (1227.8 ± 17.8), chromium (7.1 ± 2.5), bromine (2695.1 ± 24) and arsenic (126.3 ± 10) and lower concentration of potassium (440.1 ± 25), iron (1039.6 ± 28), zinc (635.7 ± 21), selenium (52.3 ± 8.5), rubidium (1528.9 ± 28) and molybdenum (162,800.8 ± 14) elements in the plasma of autistic children in comparison to healthy controls. Findings of the first study from India suggest these altered concentrations in elements in autistic children over normal healthy children affect the physiological processes and metabolism. Further studies are needed to clarify the association between the altered element concentration and physiology of autism in the North Karnataka population in India.

Keywords Autism · Total reflection X-ray fluorescence (TXRF) · Element analysis · Plasma · North Karnataka · India

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Introduction

Autism or autism spectrum disorder (ASD) [MIM 299850] is a neurodevelopmental disorder that usually appears before the age of 3 and is characterised by the altered social response, communication (verbal and/or non-verbal) and repetitive behaviour (https://www.psychiatry.org/psychiatrists/practice/dsm; https://www.Who.int/classifications/icd/icd/onlineversions/en/). Genetic factors might be largely responsible for the occurrence of ASD that alone or in a combination with specific environmental factors trigger the development of ASD [1]. The human body requires a sufficient supply of essential elements like iron, copper, zinc, cobalt, manganese and selenium, for proper functioning, especially for brain function. These elements play an important role in regulating the immune system and antioxidant system [2]. Neurochemical and neurophysiological evidence indicates that trace elements remarkably affect the functionaing of neurotransmitters [3]. An altered profile of trace elements has been observed among different medical

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conditions including neurological disorders and psychological disorders [2].

Very little is known about how trace elements contribute to the molecular mechanism of autism. Several studies suggest that dysfunction in excitatory and inhibitory synapses is a causative factor for autism like symptoms and trace elements influence synaptic functions in autism [2, 3]. Particularly, zinc is required for scaffolding of ProSAP/Shank proteins related to excitatory synapses. It is, thus, expected that altered zinc profile to be associated with different brain diseases and disorders. Higher zinc values might contribute to epileptogenesis [2], whereas lower zinc levels have been implicated in depression and ASD [4, 5]. Magnesium is a regulatory cation that modulates gamma-aminobutyric acid (GABA) signalling; thus, an altered magnesium profile might contribute to ASD. Iron is an essential element that plays important role in regulating brain function. Several studies have recorded that impaired iron haemostasis in neurodegenerative disorders [6].

Different habits in dietary consumption and environmental circumstances cause changes in these necessary nutrients. Several researches consider various biomarkers for autism diagnosis, but it is still difficult to explain the link between ASD and trace elements because of inconsistencies across studies. The purpose of this study is to examine the trace elements in the blood plasma of 70 autistic children and compare them to unaffected healthy children using total reflection X-ray fluorescence (TXRF) technology, as well as to look into the relationship between trace element concentration in male and female children and autism severity.

Materials and Methods

Participants

In total, 1870 mentally ill children below 18 years of age were diagnosed using DSM-V (https://www.psychiatry.org/psychiatrists/practice/dsm) and ICD-10 (https://www.Who.int/classifications/icd/icd/onlineversions/en/) criteria from the North Karnataka region of India. A total of 150 autistic children were identified and 70 autistic children ($N_{male} = 50$ and $N_{female} = 20$, mean age $= 11.5 \pm 3.1$) included in the study as a case group. A control group was selected, which included 70 age- and gender-matched healthy children. These control group children were unrelated to the cases. Consent was taken from parents/guardians and ethical approval for the study was obtained from the Institutional ethical committee of Shri B.M Patil Medical College, Hospital and Research Centre, BLDE (Deemed to be University), Vijayapura (Ref No: BLDE (DU) IEC/337–2018-19). If the children were suffering from any progressive neurological disorder, liver or kidney disease, anaemia or under any treatment or medication were excluded from the study. The severity of autism was measured using Childhood Autism Rating Scale (CARS). Total scores can range from a low of 15 to a high of 60; scores below 30 indicate that the individual is in the non-autistic range, scores between 30 and 36.5 indicate mild to moderate autism, and scores from 37 to 60 indicate severe autism [7].

Sample Collection

Two-millilitre peripheral blood samples were collected in EDTA vacutainer and plasma was separated immediately by centrifuging at 10,000 rpm for 10 min and separated plasma was stored in sterile microcentrifuge tube at − 80 °C until further analysis.

Sample Preparation and Element Analysis

Minimal sample preparation was required for total reflection X-ray fluorescence for the analysis of elements in plasma. To 495µL of plasma and 5µL of internal standard (IS) gallium (Ga) solution (500 mg/L) were added to get concentration 5 mg. Sample was then vertexed slightly and 10µL of sample was deposited as a single drop over a quartz glass sample carrier. Samples were allowed to dry for 5 min. After drying, the sample carrier was inserted into the TXRF instrument (Bruker S2 Picofox TXRF, Bruker AXS GmbH, Germany) and analysis was carried with an integration time of 1000 s for each sample with 50-kV voltages and 698-µA Current. Each sample was prepared and analysed in triplicate.

Statistical Analysis

Collected data was revised, coded, tabulated and subjected to Statistical Package for Social Science (SPSS 15.0.1) analysis. Descriptive statistics was used to calculate the mean and standard deviation (± SD) for numerical data. Analytical statistics and Student's *t*-test were used to assess the statistical significance of the difference between two study group means, and ANOVA test was used to assess the statistical significance of the difference between more than two study group means. The chi-square test was used to examine the relationship between two qualitative variables. *p*-value of 0.05 was considered significant.

Results

Present study population included 70 autistic children (mean age: 11.5 ± 3.1, $n_{male} = 50$, $n_{female} = 20$) and 70 healthy age-matched children as control group (mean age: 12 ± 2.5, $n_{male} = 50$, $n_{female} = 20$). There was a significant difference

between these groups regarding age and sex. The demographic characters are illustrated in Table 1.

Results from statistical tests showed that, the children with ASD showed a higher concentration of copper, Cu (children with ASD 1227.8 µg/L, control group 811.9 µg/L); chromium, Cr (children with ASD 7.1 µg/L, control group 2.8 µg/L); bromine, Br (children with ASD 2695.1 µg/L, control group 2157.8 µg/L) and arsenic, As (children with ASD 126.3 µg/L, control group 28.6 µg/L).

While the element such as potassium, K (children with ASD 440.1 µg/L, control group 525.0 µg/L); iron, Fe (children with ASD 1039.6 µg/L, control group 2372.2 µg/L); zinc, Zn (children with ASD 635.7 µg/L, control group 1024.1 µg/L); selenium, Se (children with ASD 52.3 µg/L, control group 69.6 µg/L); rubidium, Rb (children with ASD 1528.9 µg/L, control group 1725.9 µg/L) and molybdenum, Mo (children with ASD 162,800.8 µg/L, control group 187,684.3 µg/L) showed lower concentration in ASD, compared to control group, manganese and rubidium showed statistically non-significant higher concentration in autism ($p > 0.05$) (Table 2, Fig. 1).

In males, elements like manganese, bromine and rubidium showed non-significant difference in their concentration (p-value 0.09, 0.08 and 0.4 respectively) compared to the control group (Fig. 2). In females, elements like potassium, manganese, chromium, rubidium and molybdenum showed statistically non-significant difference in concentration (p-value 0.8, 0.25, 0.09, 0.12 and 0.34 respectively) compared to respective control group (Fig. 3). There was no larger difference in concentration between the ASD male group and ASD female group and the difference among ASD males and ASD females was non-significant in all the elements except potassium (Table 3). Potassium, iron, zinc, manganese and selenium showed slightly higher concentration in males and copper, chromium, bromium, rubidium, molybdenum and arsenic showed higher concentration in females (Fig. 4).

The present study included 50 autistic male and 20 autistic female children. In 50 autistic male children, 34 (68%) children showed mild-moderate autism (CARS score 30–36.5) whereas 14 (32%) children showed severe autism (CARS score 37–60). In 20 autistic female children, 14 (70%) children showed mild-moderate autism and 6 (30%) children showed severe autism. Concentration of copper, chromium, bromine and arsenic was observed to be higher in ASD and this concentration increased as the severity of autism increased from mild to severe. Elements, such as potassium, iron, zinc, selenium, rubidium and molybdenum, showed lower concentration and this

Table 1 Demographic characteristics of autistic children

Demographic character	Autistic children	Healthy children (control)
No of children	70	70
Mean age	11.5 ± 3.1	12 ± 2.5
Ethnic origin	Indian	Indian
Average age of father at child's birth	39 ± 2.3	30 ± 2.7
Average age of mother at child's birth	34 ± 2.1	29 ± 1.8
Consanguineous marriage		
• Yes	48	11
• No	22	57
Prenatal factors		
• Preeclampsia	16	03
• Maternal hyperthyroidism	04	04
• Hypertension	03	01
• Gestational problem	07	00
Postnatal factor		
• Labour complication	12	06
• Forceps-mediated delivery	08	01
• Birth asphyxia	10	02
• Feeding problem	04	02
• Delayed crying	09	01
Intelligent quotient (IQ)	30 ± 10	60 ± 5
Severity		
• Mild to moderate	48	00
• Severe	22	00
Comorbid condition	None	none
Family history of the neurodevelopmental condition		
• Autism	00	00
• ADHD	01	00
• Intellectual disability	10	02
• Learning disability	03	00
• Speech problem	08	01

Table 2 Plasma element levels (µg/L) in children with ASD and controls

Element (µg/L)	ASD Mean ± SD	Control Mean ± SD	p-value
Potassium, K	440.1 ± 25	525.0 ± 20	0.005*
Iron, Fe	1039.6 ± 28	2372.2 ± 35	0.001*
Zinc, Zn	635.7 ± 21	1024.1 ± 15	0.001*
Copper, Cu	1227.8 ± 17.8	811.9 ± 14.5	0.001*
Manganese, Mn	95.5 ± 14	117.6 ± 12	0.7**
Selenium, Se	52.3 ± 8.5	69.6 ± 10	0.001*
Chromium, Cr	7.1 ± 2.5	2.8 ± 1.1	0.001*
Bromine, Br	2695.1 ± 24	2157.8 ± 18.4	0.018*
Rubidium, Rb	1528.9 ± 28	1725.9 ± 34.6	0.114**
Molybdenum, Mo	162,800.8 ± 14	187,684.3 ± 17.2	0.001*
Arsenic, As	126.3 ± 10	28.6 ± 5.9	0.001*

Data presented as Mean ± SD; *significant difference as compared to the control values at $p < 0.05$; **non-significant difference as compared to the control values at $p > 0.05$

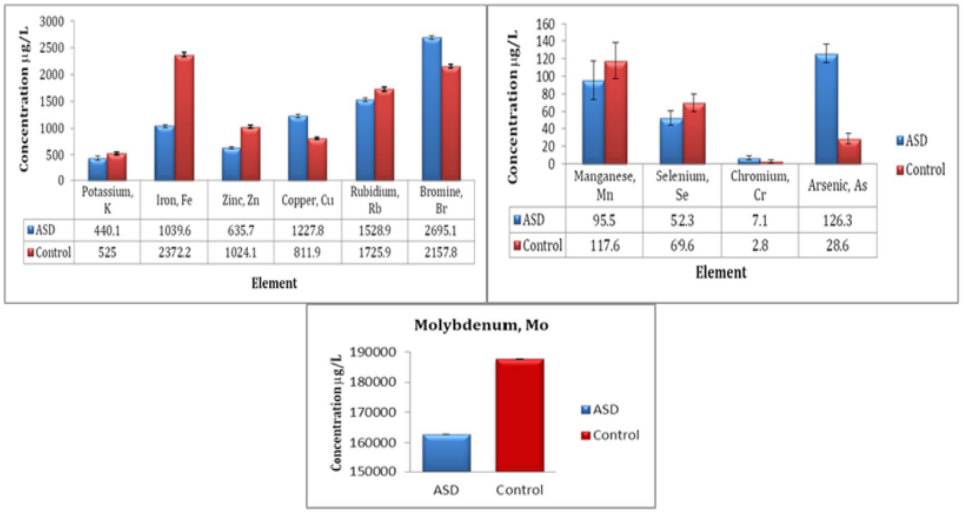

Fig. 1 Plasma element levels (µg/L) in children with ASD and controls

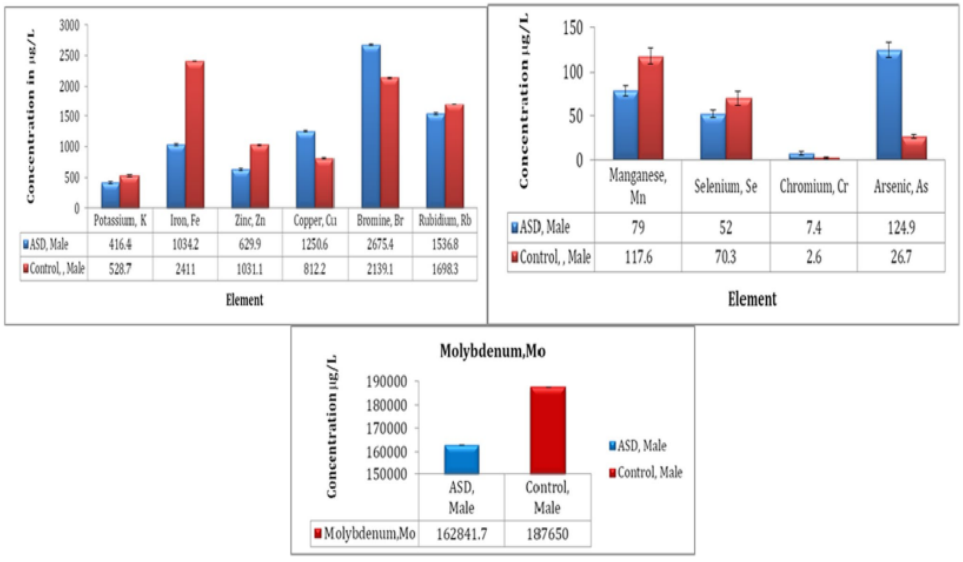

Fig. 2 Distribution of plasma element levels (µg/L) in male children with ASD and control group

plasma concentration decreased as the severity of autism increased from mild to severe (Table 4) (Figs. 5, 6 and 7). Manganese and rubidium showed non-significant difference among both male and female ASD groups.

Discussion

Autism [MIM 299850] is a complex neurological condition that is characterised by abnormal social interaction,

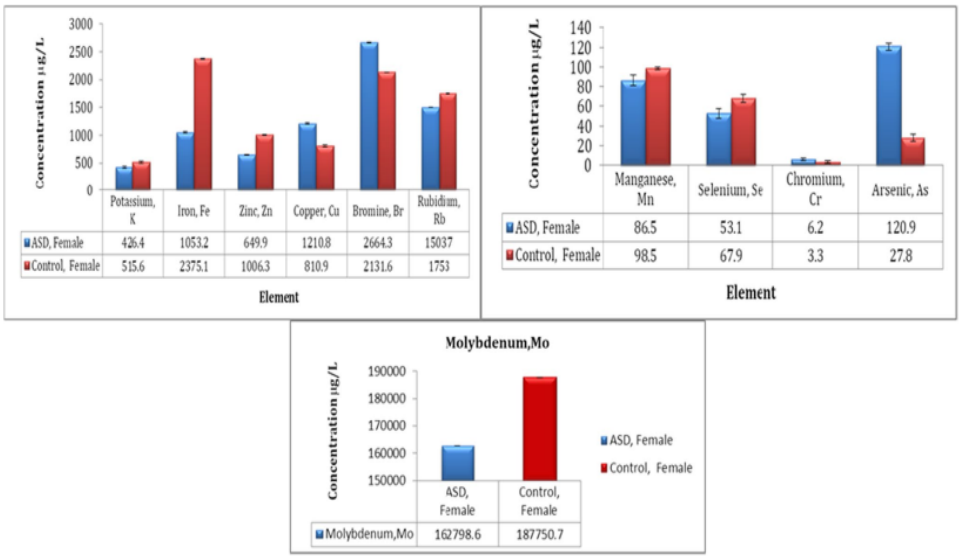

Fig. 3 Distribution of plasma element levels (µg/L) in female children with ASD and control group

Table 3 Sex-wise distribution of plasma element levels (µg/L) in children with ASD and control

Element µg/L	Male		*p*-value	Female		*p*-value	Male v/s female *p*-value
	ASD $n=50$	Control $n=50$		ASD $n=20$	Control $n=20$		
Potassium, K	416.4 ± 13.6	528.7 ± 15.9	0.013*	426.4 ± 21.0	515.6 ± 15.6	0.8**	0 .025*
Iron, Fe	1034.2 ± 16.5	2411 ± 2.2S	0.001*	1053.2 ± 8.0	2375.1 ± 8.8	0.008*	0.63**
Zinc, Zn	629.9 ± 14.8	1031.1 ± 11.3	0.001*	649.9 ± 6	1006.3 ± 6.2	0.001*	0.55**
Copper, Cu	1250.6 ± 10.6	812.2 ± 14.2	0.001*	1210.8 ± 12.7	810.9 ± 17.1	0.005*	0.98**
Manganese, Mn	79 ± 5.8	117.6 ± 9.0	0.09**	86.5 ± 5.1	98.5 ± 1.3	0.25**	0.10**
Selenium, Se	52 ± 3.8	70.3 ± 8.3	0.001*	53.1 ± 5.0	67.9 ± 4.3	0.03*	0.83**
Chromium, Cr	7.4 ± 2.1	2.6 ± 1.0	0.001*	6.2 ± 1.4	3.3 ± 1.1	0.09**	0.49**
Bromine, Br	2675.4 ± 13.2	2139.1 ± 10.3	0.08**	2664.3 ± 8.2	2131.6 ± 3.2	0.03*	0.14**
Rubidium, Rb	1536.8 ± 14.5	1698.3 ± 6.3	0.4**	1503.7 ± 3.0	1753.0 ± 10.0	0.12**	0.35**
Molybdenum,Mo	162,841.7 ± 30	187,650 ± 16	0.001*	162,798.6 ± 45	187,750.7 ± 15.6	0.34**	0.20**
Arsenic, As	124.9 ± 8.9	26.7 ± 2.3	0.001*	120.9 ± 3.5	27.8 ± 3.7	0.001*	0.72**

Data presented as Mean ± SD; *significant difference as compared to the control values at $p<0.05$; **non-significant difference as compared to the control values at $p>0.05$

verbal and non-verbal communication and impaired behaviours (https://www.psychiatry.org/psychiatrists/practice/dsm; https://www.Who.int/classifications/icd/icd/onlineversions/en/). There is a lot of interest in recent days to know about the involvement of elements in the metabolism in autistic children in order to see whether these elements are risk factor in the aetiology of autism. Certain essential trace elements are required for the bodies' various physiological processes but if they are present in higher concentration, these trace elements result in toxicity and cause adverse effect on physiological processes. Similarly, deficiency of these essential elements also affects physiological processes and leads to abnormal metabolic activities [2]. So it is very important to determine the trace elemental concentration in the neurological condition in affected individuals to monitor and assess their impact on health. Many studies recently have revealed that autistic children have multiple elemental variations in their body

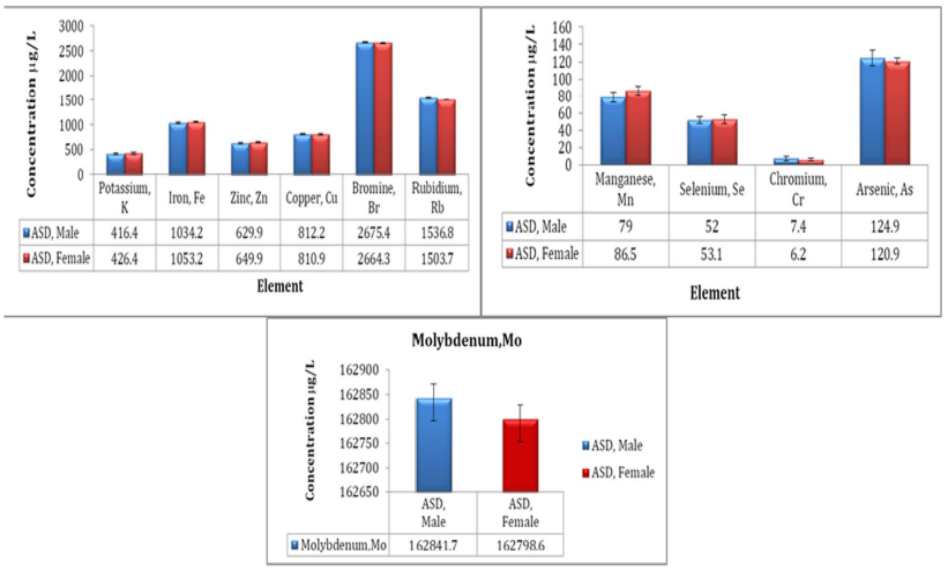

Fig. 4 Distribution of plasma element levels (µg/L) in male children with ASD and female children with ASD

Table 4 Plasma element levels (µg/L) in children with mild-moderate ASD and children with severe ASD

Element µg/L	Sex	Control	Mild to moderate autism (score 30–36.5)	Severe autism (score 37–60)	*p*-value
Potassium, K	Male	528.7 ± 15.9	426.5 ± 3.5	406.8 ± 2.1	0.006*
	Female	515.6 ± 15.6	439.6 ± 5.0	412.1 ± 2.0	0.07**
Iron, Fe	Male	2411 ± 2.2S	1045.5 ± 3.7	1021.5 ± 2.0	0.001*
	Female	2375.1 ± 8.8	1060.2 ± 1.0	1044.0 ± 0.8	0.004*
Zinc, Zn	Male	1031.1 ± 11.3	635.8 ± 5.0	619.0 ± 2.0	0.001*
	Female	1006.3 ± 6.2	654.4 ± 1.5	644.4 ± 0.5	0.001*
Copper, Cu	Male	804.2 ± 2.1	819.2 ± 3.6	1250.6 ± 10.6	0.001*
	Female	823.0 ± 3.1	824.2 ± 1.1	1210.8 ± 12.7	0.001*
Manganese, Mn	Male	117.6 ± 9.0	83.2 ± 1.5	76.6 ± 1.2	0.062**
	Female	98.5 ± 1.3	89.2 ± 1.1	82.5 ± 0.6	0.132**
Selenium, Se	Male	70.3 ± 8.3	52.3 ± 2	50.5 ± 0.8	0.001*
	Female	67.9 ± 4.3	56.1 ± 2.0	49.1 ± 0.7	0.001*
Chromium, Cr	Male	2.6 ± 1.0	6.5 ± 1.8	7.8 ± 1.2	0.001*
	Female	3.3 ± 1.1	5.6 ± 1.0	7.0 ± 0.4	0.001*
Bromine, Br	Male	2139.1 ± 10.3	2668.4 ± 3.0	2685.8 ± 1.2	0.416**
	Female	2131.6 ± 3.2	2660.6 ± 0.9	2670.6 ± 1.2	0.001*
Rubidium, Rb	Male	1698.3 ± 6.3	1545.0 ± 2.8	1547.0 ± 2	0 .498**
	Female	1753.0 ± 10.0	1504.7 ± 0.6	1505.7 ± 0.2	0.296**
Molybdenum, Mo	Male	187,650 ± 16.0	162,832.9 ± 3.5	162,866.0 ± 2.9	0.04*
	Female	187,750.7 ± 15.6	162,805.6 ± 10	162,830.6 ± 15	0.042*
Arsenic, As	Male	26.7 ± 2.3	118.9 ± 3.5	127.0 ± 3.8	0.001*
	Female	27.8 ± 3.7	118.0 ± 1.5	122.1 ± 0.5	0.001*

Data presented as Mean ± SD; *significant difference as compared to the control values at $p < 0.05$; **insignificant difference as compared to the control values at $p < 0.05$

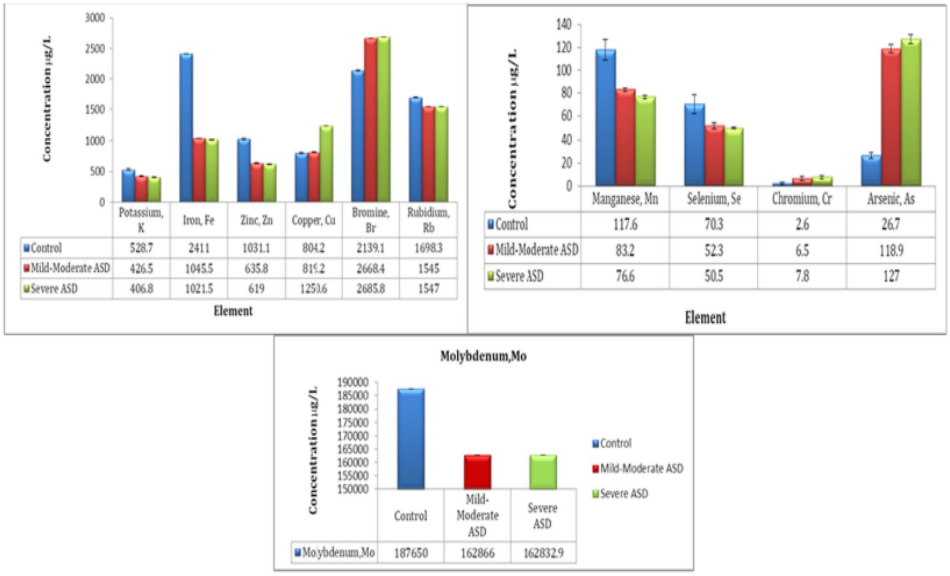

Fig. 5 Plasma element levels (µg/L) in male children with mild-moderate ASD and male children

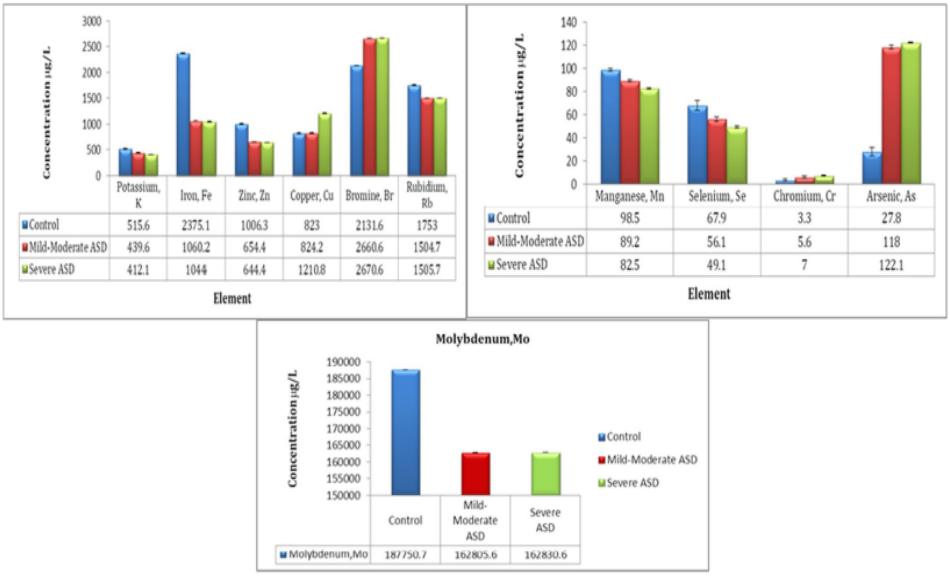

Fig. 6 Plasma element levels (µg/L) in female children with mild-moderate ASD and female children with severe ASD

which leads to abnormal metabolism [1, 2]. In the present study, we have analysed the trace elements in plasma of 70 autistic children including 50 male and 20 female children with a mean age of 11.5 ± 3.1 using the total reflection X-ray fluorescence (TXRF) technique.

Potassium is one of the most important elements for the functioning of the human body. It helps in the regulation of fluid balance, muscle contractions and nerve signals. Potassium allows brain cells to communicate with each other and also with cells that are farther away. Potassium deficiency

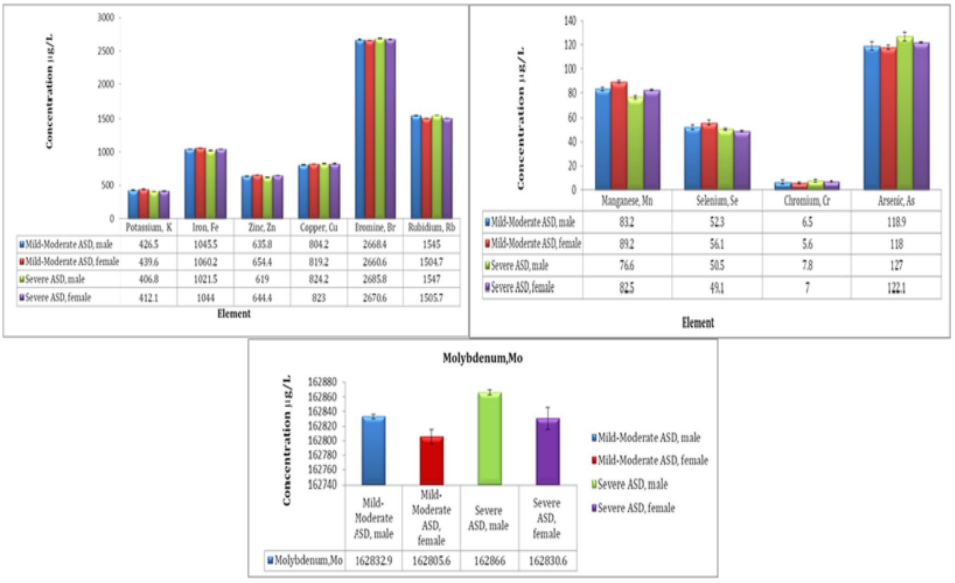

Fig. 7 Plasma element levels (µg/L) in male children with mild-moderate and severe ASD and female children with mild-moderate and severe ASD

in the blood disrupts the signals that help to maintain the proper functioning of the brain [8]. About 20% of the individuals with mental disorders have potassium deficiency [9]. The present study showed a significant decrease in the plasma concentration in autistic children (440.1 ± 25 vs 525.0 ± 20, *p*-value 0.005). Male autistic children showed significantly low concentration of potassium in comparison to control male children but female autistic children showed non-significant low concentration (Table 3). Plasma concentration of potassium was found to be significantly decreasing in both male and female children as severity increased from mild-moderate to severe (Table 4). A similar result was recorded in a previous study done in 2016 [10] but Wecker L et al. [11] recorded a high concentration of potassium in hairs of autistic children.

The human body requires *iron* as a trace metal to regulate a variety of metabolic activities, including the electron transport chain and oxygen metabolism [12, 13]. Our cells absorb iron from our food and transfer it in the form of ferritin. Maintaining homeostasis necessitates proper iron metabolism. Iron insufficiency can lead to a variety of problems, the most common of which is iron deficiency anaemia. Iron deficiency has been linked to ASD symptoms, and is notably connected with the severity of emotional and behavioural issues, as well as developmental delay in autism [2, 14]. The current study supports that idea by finding that autistic children have considerably lower iron levels than the control group (1039.6 28 vs 2372.2 35, *p*-value 0.001). In comparison to the respective control groups, severe autistic children from both the male and female groups had considerably lower iron concentrations (Table 4). In comparison to ASD females, ASD males had a lower concentration (Table 2).

Zinc is a very essential element for spermatogenesis and maturation, genomic integrity of sperm and normal functioning of neurotransmitters. However, data on serum Zn levels in ASD are more contradictory. In particular, certain studies have demonstrated the absence of significant changes or a significant increase in serum Zn levels in ASD patients as compared to the controls [15, 16]. Several studies have showed lower concentration of zinc from plasma samples of children with ASD [17, 18]. Our study supports these findings by recording a significantly lower concentration of zinc in plasma of autistic children (635.7 ± 21 vs 1024.1 ± 15, *p*-value 0.001) compared to healthy control group. Both the sexes showed significantly very low concentration with respect to their control group (Table 2). Zinc is involved in the gut-brain interaction, and many ASD patients also have gastrointestinal symptoms [19, 20]. There are considerable evidences for an association between zinc deficiency and ASD [17, 21]. Zinc levels may also be correlated to the severity of ASD [22]. Our study records a significantly low concentration of plasma zinc in mild-moderate autism to severe autism and this zinc concentration decreases gradually with increase of ASD severity.

Copper is an essential element that plays a significant role in cellular functioning and required for the normal development and functioning of the brain. As a cofactor of several enzymes and/or as a structural component, copper is involved in many physiological pathways in the brain. Our findings showed a significantly increased concentration of copper in autistic children compare to healthy age-matched controls (1227.8 ± 17.8 vs 811.9 ± 14.5, *p*-value 0.001). Both male and female groups also recorded increased concentration of copper. A previous study by Qin Y yan et al. (2018) results supports our study results and a study of 79 autistic individuals found a similar pattern, in which autistic and pervasive developmental disorder-not otherwise specified (PDD-NOS) patients had significantly higher plasma levels of copper [16]. But a study by A.V. Skalny et al. [10] showed no significant difference between the autistic and control group. It is supposed that altered copper metabolism in autism may be related to impaired metallothionein system functioning and activation of free radical oxidation [23]

Copper and zinc play competing roles physiologically, such that an increase in copper leads to zinc deficiency [20]. Our findings support this by recording significantly higher copper concentration in autistic children and a significantly low concentration of zinc (Table 2).

Manganese (Mn) is an essential element that plays a fundamental role in brain development and its functioning. It acts as an activator of enzyme and as a component of metalloenzymes. They have a role to play in oxidative phosphorylation, fatty acids and cholesterol metabolism, mucopolysaccharide metabolism and the urea cycle [24]. Abnormal concentrations of manganese in the brain, especially in the basal ganglia, are associated with neurological disorders similar to Parkinson's disease. The present finding of decrease manganese concentration in plasma of autistic children (95.5 ± 14 vs 117.6 ± 12) was supported with previous data, in particular, a significant association between blood Mn and ASD [25, 26]. The observed decrease of Mn levels may be hypothetically associated with oxidative stress and an accompanying decrease in Mn-SOD activity, an Mn-dependent antioxidant enzyme widely distributed in brain structures [10]. The male ASD group shows low concentration compared to the female ASD group (Table 3).

Selenium is a vital metalloid for a variety of biological functions [10]. Selenium is required for neurobehavioural development in the foetus as well as cognitive performance in later life. It also plays a role in the central nervous system's various tasks, including motor performance, coordination, memory and cognition [27]. Selenium and selenium-dependent proteins are important for brain development and oxidative damage management, and dyshomeostasis in selenium has been linked to an increased risk of ASD [28]. There was a considerable drop in plasma selenium concentration in children with autism, according to existing studies [29, 30]. In comparison to a healthy age-matched control group, autistic children had significantly lower selenium concentrations (52.38.5 vs. 69.610, *p*-value 0.001). The concentration of selenium in males decreased significantly (*p*-value 0.001), but the difference was not significant in females (*p*-value 0.83). In the mild-moderate autism group and the severe autism group, there was a substantial steady drop in both male and female (Table 4).

Zn, Se and Mn shortage can affect metabolic processes and exacerbate heavy metal toxicity, impairing brain function and neural plasticity [31].

The production of glucose tolerance factor requires chromium. Chromium shortage results in impaired glucose tolerance, while intoxication causes renal failure, dermatitis and lung cancer [32]. In this study, we found that autistic children had a greater chromium concentration (7.1 ± 2.5 vs. 2.8 ± 1.1), and that male autistic children have a higher concentration than female autistic children (7.4 ± 2.1 vs. 6.2 ± 1.4). In both males and females with severe autism, the concentration of chromium was high. Previous research (https://www.Who.int/classifications/icd/icd/onlineversions/en/) [33] backed up this conclusion. Other investigations found reduced chromium levels in autistic children's hair and serum [10, 34, 35].

Bromine is a naturally occurring element that can be found in a variety of chemicals such as insecticides, flame retardants and water treatment chemicals. Bromine toxicity is caused by a greater quantity of bromine in the body, which damages the membranes of neurons, affecting neuronal transmission over time. Bromism is the term for bromine toxicity. Bromism can also produce neurological, mental and psychotic symptoms, as well as convulsions, dermatological and gastrointestinal issues [36]. In this study, autistic children had a substantially higher concentration of bromine (2695.124 vs 2157.818.4, *p*-value 0.018), and there was no significant difference in concentration between the sexes (Table 3).

Rubidium is present in large concentrations in muscle tissue, red blood cells and viscera. Our study recorded lower concentration in autistic children compared to age-matched healthy children (1528.9 ± 28 vs 1725.9 ± 34.6). Interestingly female ASD children showed low concentration compare to male ASD children (Table 3).

Molybdenum is a trace element that is essential for life and it functions as a cofactor for sulphite oxidase, xanthine oxidase, aldehyde oxidase and mitochondrial amidoxime reducing component. Molybdenum deficiency and toxicity are rare but a varying concentration of molybdenum in the body is associated with reduced growth, histological changes in kidney and renal failure, reproductive abnormalities, bone deformities and anaemia [37]. Studies have shown that molybdenum toxicity appears to be a cause of some cases of autism. This is consistent with

toxic leukoencephalopathy caused by heavy metal toxicity. Our findings are contradictory to this because significantly low concentration of molybdenum is observed in autistic children compared to age-matched healthy children (162,800.8 ± 14 vs 187,684.3 ± 17.2 *p*-value 0.001). Male autistic children showed a significant difference with healthy male children (162,841.7 ± 30 vs 187,650 ± 16, *p*-value 0.001). Female autistic children showed a non-significant decrease compare to healthy female children (162,798.6 ± 45 vs 187,750.7 ± 15.6, *p*-value 0.34).

Arsenic is a human developmental neurotoxicant. Arsenic appears to have toxic effects on neurotransmitters involved in cell-to-cell signalling within the brain. A study of rats demonstrated that arsenic induces regional increases in levels of dopamine, serotonin and their metabolites and also induces a decrease in norepinephrine levels in discrete brain regions [38]. Autistic children from our study group showed a significantly very high concentration of arsenic in the plasma compared to age-matched healthy children (126.3 ± 10 vs 28.6 ± 5.9 *p*-value 0.001). Male autistic children showed high concentration compare to female autistic children (124.9 ± 8.9 vs 120.9 ± 3.5). Mild to moderate autistic children from both male and female groups showed similar arsenic concentration (118.9 ± 3.5 vs 118.0 ± 1.5). Similar results were recorded in a previous study carried in 28 autistic children from Italy [39].

Conclusion

For the first time present study investigated the concentrations of trace elements in blood plasma of children with ASD and unaffected children from the North Karnataka population from India. These altered concentrations in trace elements in autistic children compared to normal healthy children affect the physiological processes and metabolism. Further studies are needed to clarify the association between the altered trace elemental concentration and physiology of autism in the North Karnataka population in India.

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Declarations

Statement of Ethics Ethical approval for the study was taken from the Institutional Ethical Committee of Shri B.M Patil Medical College, Hospital and Research Centre, BLDE (Deemed to be University), Vijayapura (Ref No: BLDE (DU) IEC/337–2018-19). Informed consent was obtained from parents/guardians before the collection of blood samples.

Conflict of Interest The authors declare no competing interests.

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Cytogenetic analysis of Autistic children with Down syndrome features

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Abstract:

INTRODUCTION: Autism is a heterogeneous neurodevelopmental disorder that influences a child's ability to think, learn, communicate, and interact socially and possess behavioral problems. It has also been observed to be associated with several medical conditions, including certain chromosomal disorders like Down syndrome (DS). Several studies have shown autistic individuals with DS. The main objective of the present study is to screen for chromosomal abnormalities in autistic children with DS characteristics in the North Karnataka population, India.

MATERIALS AND METHODS: Chromosome analysis of peripheral blood of four DS children with autistic characteristics was performed to examine the chromosomal abnormalities, which were confirmed by fluorescent *in situ* hybridization.

RESULTS: Childhood Autism *Rating* Scale score was calculated initially to assess the severity of autism. All four cases were found to have autism with DS. Out of four cases, three (75%) were found to be trisomy 21 and 1 (25%) had a trisomy 21 mosaic condition.

CONCLUSION: Our study confirms the chromosomal abnormality present in autistic children with DS characteristics and these findings will contribute in several ways to the diagnosis and treatment of the genetic cause of autism with other comorbidities or vice versa.

Keywords:

Autism, chromosome analysis, Down syndrome, fluorescent *in situ* hybridization, mosaicism

In 1943, L. Kanner for the first time described the term autism.[1] This is a heterogeneous neurodevelopmental disorder that influences a child's ability to think, learn, communicate, and interact socially and possess behavioral problems.[2] The American Psychiatric Association's Diagnostic and Statistical Manual of Mental Disorders 4th Edition (DSM-Ⅳ) defines autism as a pervasive developmental disorder (PDD). PDD is a group of five disorders which includes autistic disorder, Rett disorder, PDD-not otherwise specified (PDD-NOS), Asperger's disorder, and childhood disintegrative disorder (CDD).[3] The idea of a "spectrum" autism diagnosis was introduced in the DSM-5, which combines the DSM-IV's PDD diagnoses of autistic disorder, Asperger's disorder, CDD, and PDD-NOS into a single diagnosis.[4] The prevalence of autism is estimated to be 1.5% as per a recent US-based population study.[5] The prevalence of autism in the Indian population was observed to be 0.23%.[6] However, it is gradually increasing in recent days. Autism has been associated with several medical conditions, namely, metabolic disorders, infectious diseases, and structural and numerical chromosomal disorders. Autism is more frequently observed in children with certain disorders such as congenital rubella,[7] Turner syndrome, Fragile X syndrome,

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and microcephaly.[8,9] Many case studies have also described autistic features in individuals with Down syndrome (DS).[10] or vice versa.

John Langdon Down released his classic article in 1866, in which he characterized individuals who shared a common phenotypic that would later bear his name (Down, 1866).[11] In around 95% of cases, trisomy 21 (DS) is defined by the presence of three full copies of chromosome 21, which results from nondisjunction during meiosis I.[12] DS is characterized by mental impairment, hypotonia, and facial, hand, and foot deformities. Congenital heart problems, digestive tract abnormalities, congenital cataracts, and leukemia are all variable components of the phenotypic.[11] In India, the incidence is 0.88–1.09 per 1000 people. Every year in India, an estimated 21,400 babies are born with DS.[12] Maternal age has a significant impact on the prevalence of DS. As a mother's age rises, the probability of having a miscarriage increases.[13]

For numerous reasons, the link between autism and DS is of great interest. To begin with, children with DS are frequently described as calm, pleasant, and friendly. Second, the apparent lack of such a link could point to a key topic for future research into the mechanisms behind autistic social dysfunction.[7,14] The following are some significant behavior that may indicate the presence of autistic features in a child with DS: (1) autistic aloneness to the extinction: the child does not interact properly with others and appears to prefer being left alone. Others appear to the child as objects rather than people. He refuses to play in a group with other kids. The autistic child, who is incredibly cuddly and huggable and does not want to be held, (2) has an anxiously obsessive desire to keep things the same: any changes in daily routines could cause a major upheaval, (3) lack of eye contact: autistic people rarely make eye contact with others and instead look away or "straight through" them, and (4) consistently "stereotypical" movement, such as sitting for extended periods with an object in his hand, simply waving it back and forth while staring at it.[3] The main objective of this study is to screen for chromosomal abnormalities in autistic children with DS characteristics in the North Karnataka population of India.

Materials and Methods

Sample collection

The present study was ethically approved by the Karnataka Institute for DNA Research, Dharwad, and informed consent was taken from the parents/guardians of the child. As per the DSM-V and the International Classification of Diseases, 10th edition, a total of 136 autistic children were screened and included in the study. The degree of autistic psychopathology of individuals was measured using the Childhood Autism Rating Scale (CARS) scoring method.[15] A score of 30 or above shows the existence of autism, with 30–36.5 indicating mild/moderate autism and 37–60 indicating severe autism, with a score below 30 indicating the absence of autism. Individuals with other congenital defects, chromosomal disorders, and other comorbidity were excluded from the study. The present study involved four autistic children with DS characteristics under the age of 18 years. Around 2–3 ml of whole blood was collected from each child in sterile heparin vacutainers, and the samples were transported to a laboratory for further analysis.

Chromosome preparation and G-banding

With slight modifications to the previous method,[16] chromosome preparation and G-banding were performed. One milliliter of heparinized whole blood was added into a culture flask containing 4 ml of complete medium (90 ml of Roswell Park Memorial Institute 1640 [RPMI 1640], 10 ml of Fetal bovine serum [FBS]) and the lymphocytes were stimulated to proliferate with the addition of 100 µl of phytohemagglutinin. The cells were cultured for 72 h at 37°C with 5% CO_2. At 71.5 h, metaphases were arrested by adding 50 µl of colchicine, a spindle fiber inhibitor. After incubating for 0.5 h, the cultured cells were transferred to a centrifuge tube and centrifuged at 1200 rpm for 20 min. The supernatant was discarded, the pellet was disturbed, and 8 ml of 75 mM KCl was added and incubated for 35 min at 37°C. The contents were again centrifuged at 1200 rpm for 10 min. The supernatant was discarded and Carnoy's fixative was added dropwise while disturbing the pellet using a vortex to avoid clumping and incubated at room temperature for 20 min. Two to three washes were given with Carnoy's fixative to obtain a white pellet. The pellet was cast onto a clean, chilled glass slide and aged at 60°C for 72 h, and treated with Sorenson's buffer in the water bath at 37°C for 10 min. The slides were then treated with trypsin and Giemsa and analyzed under the microscope. The metaphases were analyzed, and the appropriate results were documented.

Fluorescent *in situ* hybridization

Further trisomy 21 conditions from G-banding were confirmed by fluorescent *in situ* hybridization (FISH). During prehybridization, 2 ml of blood was taken in a falcon tube and 8 ml of 75 mM prewarmed KCl was added and incubated at 37°C. Fixative was added to this solution and centrifuged to get a white pellet. The pellet was resuspended in fixative and the cells were spread on a chilled glass slide. The hybridization was performed according to the FAST FISH prenatal

enumeration probe kit (CytoCell, UK). Interphase FISH was performed in which the 21st chromosome DNA probes labeled with biotin were utilized in this preliminary study. After hybridization, the slides were washed using FISH wash buffer and dried completely. To this, 10 µl of 4',6-diamidino-2-phenylindole [DAPI] counterstain was added and incubated at 4°C for 6 h, and slide fluorescence was observed at ×40 and ×100 using a fluorescent microscope.

Results

In the present study, four autistic children with DS characteristics were taken for cytological analysis. Clinical features of autistic children with DS characteristics were summarized in Table 1. To check the chromosomal abnormalities in autistic children with DS characteristics, the peripheral blood samples from four children were screened for chromosomal abnormalities in which metaphases from each sample were examined under a microscope with ×100 and karyotyped [Figure 1]. There was trisomy 21 seen in all four autistic children with DS characteristics. This trisomy 21 was further confirmed by FISH after G-banding. Each chromosome-specific fluorescent signal obtained by FISH in a given cell nucleus indicates the existence of a 21st chromosome. When three fluorescence signals were detected, a nucleus was deemed trisomic. The FISH data from peripheral blood cells *in vivo* during interphase from individuals indicates the presence of DS. In four autistic cases, of which three cases showed trisomy 21 [Figures 2-4] condition and one case showed trisomy 21 mosaic condition [Figure 5] as detailed below:

Case 1

A 16-year-old boy with an intelligence quotient (IQ) of 20 and a CARS score of 47.5 meets the criteria of having an autistic character. This participant was born to a consanguineous couple with third-degree relatives aged 37-year-old (maternal age) and 40-year-old (paternal age), with an age gap of 3 years. The patient exhibited typical DS characteristics such as a flattened face, particularly the bridges of the nose, almond-shaped eyes that slant up, a descending angle of the mouth, brush field spots, and so on. He showed substantial feeding problems, although he did not have any

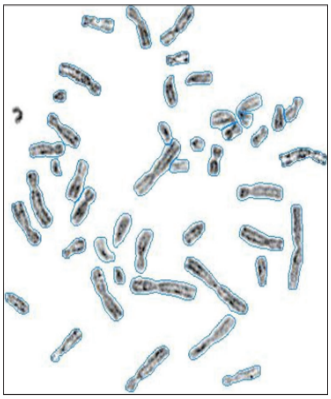

Figure 1: Chromosome analysis for autism with Down syndrome shows trisomy 21

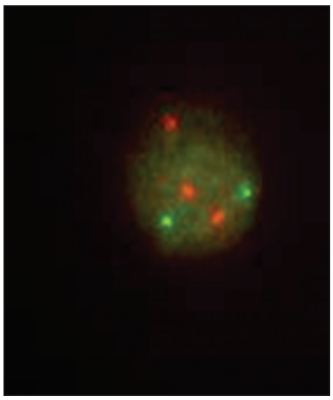

Figure 2: Interphase FISH analysis for case 1 showing trisomy 21. FISH = Fluorescent *in situ* hybridization

Table 1: Clinical features of autistic children with Down syndrome characteristics

Characteristics	Case-1	Case-2	Case-3	Case-4
Ethnic origin	Indian	Indian	Indian	Indian
Age	16	17	13	12
Sex	Male	Female	Male	Female
Paternal age at the time of childbirth	40	29	43	29
Maternal age at the time of childbirth	37	22	38	22
Consanguineous marriage	Yes	Yes	Yes	Yes
Prenatal damage	None	None	None	None
Postnatal damage	Feeding problems	Delayed crying	None	Delayed crying
IQ	20	40	<20	40
CARS score and severity	47.5, severe	35, mildly/moderate	50, severe	35, mildly/moderate
Comorbid condition	Down syndrome	Down syndrome	Down syndrome	Down syndrome

IQ=Intelligence quotient, CARS=Childhood Autism Rating Scale

Paper/Poster Presentation Certificates

1. Presented a paper in UNESCO/UNITWIN Network Web Seminar 2020 hosted by BLDE (Deemed to be University). Awarded "**Medal of Merit**" in PhD category for best presentation.

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Certificate of Merit

This is to certify that, **Mr. RAJAT HEGDE** of Department of Physiology, Shri B M Patil Medical College, Hospital & Research Centre, BLDE(DU), Vijayapura, Karnataka, India has been awarded **'CERTIFICATE OF MERIT'** in PhD category for his/her presentation entitled **"Molecular Analysis of Neuronal Cell Surface Proteingene; Neuroligin 3 in Autism Spectrum Disorder"** during UNESCO/UNITWIN Network Web Seminar during August 6 & 7, 2020.

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August 6-7, 2020

Medal of Merit

(Ph.D.)

Awarded to

Rajat Hegde

Department of Physiology,
Shri. B. M. Patil Medical College, BLDE (DU)
Vijayapura, Karnataka, India.

For his outstanding research presentation entitled *"Molecular Analysis of Neuronal Cell Surface Protein gene; Neuroligin 3 in Autism Spectrum Disorder."* during the Web Seminar

PROF. SINERIK AYRAPETYAN
UNESCO Chair-Life Sciences
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PROF. KUSAL K. DAS
UNESCO/UNITWIN Co-ordinator
Dean, Environmental Health under
UNESCO Chair Life Sciences
BLDE(DU), India

2. Oral presentation of a paper entitled “Insilco analysis of functional and structural effect of novel missense mutation of NLGN3 gene recorded in Indian autistic population” in 41 annual conference on Recent Trends in Biomedical Research organised by the Indian association of biomedical scientists from 12-14th march 2021.

41 Annual Conference of Indian Association of Biomedical Scientists

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12- 14th March, 2021

CERTIFICATE

This is to certify that Rajat Veerabhadra Hegde Department of Physiology, Shri B M Patil medical college, Hospital & Research centre, BLDE (DU) Vijayapura

has participated as and presented Oral Presentation entitled

Insilco analysis of Functional and Structural effect of Novel missense mutation of NLGN3 gene recorded in Indian autistic population

in the "International Virtual Conference on "RECENT TRENDS IN BIOMEDICAL RESEARCH" Organized by the

Indian Association of Biomedical Scientists held during 12-14 March 2021.

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General Secretary, IABMS
Organizing Secretary

3. Oral presentation of a paper entitled “A novel frameshift variant in the Indian autistic community produces a binding defect of neuroligin and neurexin resulting in defective synaptogenesis” in 9th international e-conference on “Reconnecting Physiology and Nature for Healthy Life” by Federation of Indian Physiological Societies (FIPS).

Department of Pharmaceuticals Ministry of Chemicals and Fertilizers Government of India

स्वच्छ भारत

एक कदम स्वच्छता की ओर

Azadi Ka Amrit Mahotsav

HYDERABAD

9th International e-Conference of

Federation of Indian Physiological Societies (FIPS)-2022

CERTIFICATE OF APPRECIATION

This Certificate is awarded to

Rajat Veerabhadra Hegde

for the attendance and presentation entitled

“A novel frameshift variant in the Indian autistic community produces a binding defect of neuroligin and neurexin resulting in defective synaptogenesis”

under Young Scientist Oral Presentation category during the International e -Conference on

“Reconnecting Physiology and Nature for Healthy Life”

Organized by FIPS and National Institute of Pharmaceutical Education and Research (NIPER) Hyderabad

from 25th to 27th March 2022

DR. SHASHI BALA SINGH
PRESIDENT, FIPS
DIRECTOR, NIPER-HYDERABAD

DR. DHARMENDRA K. KHATRI
ORGANIZING SECRETARY, FIPS

PSI Young Scientist Awards, 2022 (Prof. Prof. Sachchidananda Banerjee Memorial Research Award)

PHYSICON -2022

XXXIII[rd] Annual Conference of The Physiological Society of India

on

RESEARCH AND TECHNOLOGICAL ADVANCEMENT IN HEALTH SCIENCES AND SUSTAINABLE DEVELOPMENT

03 - 05 March, 2023

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&
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Mr. Rajat Hegde
Department of Physiology
Shri B.M Patil Medical College Hospital and Research Centre
BLDE [DU], Vijayapura

January 20, 2023

Dear Mr. Hegde,

Greetings!

We feel happy to inform you that you have been selected for the **PSI Young Scientist Award (Prof. Sachchidananda Banerjee Memorial Research Award)** for the year 2022, and you are being invited to deliver a lecture in the XXXIII[rd] **Annual Conference of the Physiological Society of India (PSI)**, the "XXXIII[rd] PHYSICON 2022" which will be held at Vidyasagar University, Paschim Medinipur, West Bengal, India on and during 3-5 March 2023, organised by the Department of Human Physiology. We are expecting your active participation by delivering the above-mentioned lecture in this International Conference entitled: **"Research and Technological Advancement in Health Sciences and Sustainable Development"**. We will highly appreciate if you submit the **abstract of your presentation** following the **Guidelines of Paper Presentation (Oral/Poster)**.

We are hopeful that you will accept our invitation and do the needful for the good cause of the International Conference.

Enclosed please find the **brochure** and **registration form** of the conference **as attachments** for information regarding abstract(s) submission, registration and accommodation.

With warm wishes,

Prof. Chandradipa Ghosh
Convener

Dr. Sandip Kumar Sinha
Organizing Secretary

Keshab Chandra Mondal

Prof. Keshab Chandra Mondal
Organizing Secretary