Current Molecular Medicine 2022, *22*, 449-457

RESEARCH ARTICLE

Ubiquitin Mediated Degradation of EGFR by 17 β-estradiol in Triple Negative MDA-MB-231 (TNBC) Breast Cancer Cells Line

Vitthal Khode[1,2,3,*], Sumangala Patil[1], Vishwas Kaveeshwar[2], Komal Ruikar[3] Anil Bargale[2], Sarathkumar E.[4] and Satish Patil[3]

[1]*Department of Physiology, Shri B M Patil Medical College, Hospital & Research Centre, BLDE (Deemed to be University), Vijaypur, India;* [2]*Central Research Laboratory, SDM College of Medical Sciences & Hospital, Shri Dharmasthala Manjunatheshwara University, Dharwad, India;* [3]*Department of Physiology, SDM College of Medical Sciences & Hospital, Shri Dharmasthala Manjunatheshwara University, Dharwad, India;* [4]*Nitte University Centre for Science Education and Research, Mangalore, India*

ARTICLE HISTORY

Received: November 03, 2020
Revised: February 08, 2021
Accepted: February 16, 2021

DOI:
10.2174/1566524021666210729144713

Abstract: ***Background*:** Triple Negative Breast Cancer (TNBC) commonly displays Epidermal growth factor receptor (EGFR). Effective EGFR degradation results in the suppression of tumor in various models. Studies have addressed the relevance of this strategy in the treatment of TNBC. In the present study, we examined the effect of 17 β-estradiol on EGFR expression in MDA-MB-231 (TNBC) cell line and assessed whether 17 β-estradiol degrades EGFR by ubiquitination pathway.

***Objectives*:** The objective of this study is to treat MDA-MB-231 cell lines with Cycloheximide with or without 17β-estrdiol to observe whether 17β-estradiol leads to EGFR degradation and to treat with MG-132 to assess whether degradation occurs through ubiquitination pathway.

***Methods*:** MDA-MB-231 cells were treated with 17β-estradiol (E2) and EGFR expression was studied by western blotting at different intervals by using Cycloheximide chase. To assess ubiquitination pathway of degradation of EGFR in MDA-MB-231 cell line, MG-132 was used.

***Results*:** EGFR expression was reduced with β-estradiol treatment in MDA-MB-231 cell line with Cycloheximide chase. Upon Treatment with MG-132 and E2, EGFR expression did not reduce, suggesting that Estrogen degrades EGFR by ubiquitination pathway.

***Conclusion*:** Estrogen degrades EGFR in MDA-MB-231 cells and this degradation occurs by ubiquitination.

Keywords: Triple negative breast cancer, MDA-MB-231, estrogen, epidermal growth factor receptor, MG-132, ubiquitination.

1. INTRODUCTION

Breast cancer (BC) is the most common malignancy in women worldwide [1, 2]. Almost 70% of patients with breast cancer express estrogen receptor-α (ERα). Due to effective endocrine therapies, the mortality of patients with ERαtumors has reduced significantly in the past decade. Similarly, about 15% of patients have tumors that over express HER2 receptor and thus are candidates for HER2 targeted treatments. In contrast, Triple negative breast cancer (TNBC) occurs in 10–15% of patients, yet this subtype accounts for about 50% of all breast cancer deaths. TNBCs lack clinical expression of ERα, progesterone receptor, and HER2 over expression (ERα−/PR−/HER2−). Although heterogeneous, TNBCs typically occur in younger women and African American and Asian women as well as among some patients with BRCA1 gene defects [1, 2].

TNBC commonly displays epithelial growth receptor (EGFR). EGFR and human epidermal growth factor receptor 2 (HER2) are members of the ErbB family of RTKs that are of particular importance in breast cancer. Although estimates vary, EGFR over-expression is thought to present in approximately 30% of breast cancers, while HER2 positivity is detected in 25-30% of cases [3, 4]. High levels of EGFR and HER2 are associated with more aggressive cancer phenotypes and poorer prognosis [5]. When activated by ligand binding and dimerization, RTKs are internalized and

*Address correspondence to this author at the Depatment of Physiology, SDM College of Medical sciences & Hospital, Shri Dharmasthala Manjunatheshwara University, Dharwad, India;
Tel: 0836-2477280; E-mail: drkhoday@yahoo.co.n

1875-5666/22 $65.00+.00

pro-oncogenic intracellular signalling is initiated, primarily via the MAPK and PI3K/Akt pathways. The aberrant gene expression induced by enhanced expression/activation of EGFR or HER2 promotes cancer cell proliferation, survival, migration, and angiogenesis [6]. TNBC is more aggressive than other disease subtypes, and no molecular targeted agents are currently available for the treatment. Effective EGFR degradation results in the suppression of tumor in various models. Studies have addressed the relevance of this strategy in the treatment of TNBC. The ability of certain monoclonal antibody mixtures to enhance EGFR degradation raised the possibility that such a strategy would inhibit EGFR-driven tumors, including the most aggressive fraction of breast cancer [1]. Therefore, degradation of EGFR can be one of the potential targets for the treatment of TNBC.

In western countries, clinical data suggests that where OCP usage is high, prevalence of ER + breast cancer is high. In African and Asian countries where OCP usage is low, prevalence of TNBC is high [7, 8]. This clinical and molecular data had led to our hypothesis that estrogen may interact with EGFR in TNBCs and degrade it through one of the degradation pathways. The underlying mechanism is probably related to the sorting of internalized EGFRs to either recycling or degradation [9]. Sorting requires the conjugation of multiple ubiquitins, which mark the receptor for degradation [10]. We hypothesized that estrogen is involved in this mechanism, which facilitates the ubiquitination process. Therefore, we chose MDA-MB-231 cell lines treated Cycloheximide with or without β-estrdiol to observe whether β-estradiol leads to EGFR degradation. Subsequently, cell lines were treated with MG-132 to assess whether degradation occurs through ubiquitination pathway.

2. MATERIALS AND METHODS

2.1. Cell Culture

Human breast cancer cell lines MDA-MB-231, MCF-7 were obtained from the National Centre for Cell Sciences, Pune, India. Cells were grown in the respective medium as prescribed by the supplier. MCF-7, MDA-MB-231 were cultured in Roswell Park Memorial Institute media (RPMI) containing phenol red and supplemented with 10% Fetal Bovine Serum (FBS). Cell lines were cultured in a 5% CO_2 humidified atmosphere at 37°C. Cells were used up to a maximum of 28 passages and were subject to regular mycoplasma testing. β-estadiol, Cyclohexamide and MG-132 were obtained from the Aldrisch sigma.

2.2. Effect of 17 β-estrdiol on MDA-MB-231 and MCF-7 Cell Lines on EGFR Expression

EGFR is a transmembrane receptor and it is over expression in breast cancer predicts for poor prognosis. This study was designed to investigate whether estrogen plays an active role in the expression or suppression of EGFR in MCF-7 and MDA-MB-231 cell lines. We hypothesized that 100 nM of 17β-estradiol will lead to the over expression of EGFR in MCF-7 and suppresses in MDA-MB-231 cell lines. We standardised the prior concentration of estrogen to be used *i.e*. 100nM in prior.

Technique: MDA-MB-231 and MCF-7 cell lines were cultured in a large flask. At 70-80% confluence cell were trypsinized twice, centrifuged at 1200rcf for 4 min, supernatant was removed. FBS was added to approximately 4×10^6 cells/well were placed in 6 well plates and starved for 12 hours. Both cell lines were treated with 100 nM β-estradiol. Expression of EGFR at 0 and 3 hours time intervals was assessed by western blot.

2.3. Cycloheximide Chase to Assess the Effect of 17 β-estradiol on MDA-MB-231 Cells on EGFR Expression

A difference in a steady state protein levels after inhibiting degradative pathways provides strong proof for the contribution of proteolysis to the control of protein abundance [11]. However, such an analysis still does not furnish information regarding the kinetics of protein turnover. Cycloheximide chase followed by western blotting overcomes this deficiency by allowing researchers to visualize protein degradation over time [12-14]. Further, because protein detection following Cycloheximide chase is typically carried out by western blotting, radioactive isotopes and lengthy immune-precipitation steps are not needed for Cycloheximide chase, unlike many commonly used pulse chase techniques, which are also performed to visualize protein degradation [15]. Cycloheximide chase is suitable for analyzing protein stability over a short time course (*i.e.,* up to two hours). Over longer time courses (*i.e.*, two hours to days), Cycloheximide, a global inhibitor of translation, is toxic to cells, likely due to depletion of ubiquitin [16]. Further, analyses of protein stability over longer time courses are more likely to be compromised by indirect effects of globally reduced protein synthesis on the degradation of the protein of interest (*e.g.,* degradation of a short-lived protein taking part in the degradation of the protein of interest). Other techniques, such as pulse chase metabolic labeling experiments, are therefore better suited for studying the degradation of long-lived proteins and may be carried out to corroborate results obtained in Cycloheximide chase experiments.

Technique: MDA-MB-231 cell lines were cultured in a large flask. At 70-80%, confluence cells were trypsinized twice, centrifuged at 1200 rcf for 4 min, supernatant was removed. FBS added approximately 4×10^6 cells/well were placed in 6 well plates and starved for 12 hours. Cells were treated with 100 nM 17β-estradiol. Expression of EGFR at 0, 0.5, 1, 2, 3 and 4 hours' time intervals with or without 50μg cycloheximide was assessed by western blot.

2.4. EGFR Degradation is Due to Ubiquitination

The ubiquitin-proteasome system (UPS) is a major protein degradative pathway involved in the preservation of cellular structure and function [17, 18].

While the 20S proteasome is involved in direct protein hydrolysis, degradation of ubiquitinated proteins by the 26S proteasome is a relatively more important process in protein turnover [19-21]. Ubiquitination of proteins designed for degradation is an ATP-dependent process and involves cooperation of three ubiquitin ligase enzymes. In particular, the ubiquitin moiety is transferred by Ubiquitin-activating enzyme E1 to the Ubiquitin-conjugating enzyme E2 followed by formation of ubiquitin chain ligation on target proteins by a substrate specific E3 ubiquitin ligase [22]. Selected components of the 26S cap proteins are involved in the recognition and transport of ubiquitinated proteins for degradation by the 26S proteasome [23, 24]. MG-132 is a potent, reversible, and cell-permeable proteasome inhibitor. It reduces the degradation of ubiquitin-conjugated proteins in mammalian cells and permeable strains of yeast by the 26S complex without affecting its ATPase or iso-peptidase activities.

Technique: MDA-MB-231 cell lines were cultured in a large flask. At 70-80%, confluence cells were trypsinized twice, centrifuged at 1200rcf for 4 min, supernatant was removed. FBS was added to approximately 4 × 10^6 cells/well were placed in 6 well plates and starved for 12 hours. Cells were treated with 100 nM 17β-estradiol and 50 µg Cycloheximide. Expression of EGFR at 0, 0.5, 1, 2, 3 and 4 hours time intervals with or without MG-132 was assessed by western blot.

2.5. Total Cell Extraction and Western Blotting

Cells were obtained from 6-well plates after the treatment after adding lysate. Cell lysates were collected on ice by washing x1 in ice-cold PBS then scraping in 100 µL of lysis buffer (150 mM NaCl, 50 mM Tris base pH 8, 1 % NP-40 containing protease and phosphatase inhibitors. The lysates were centrifuged at 14,000 rcf for 10 min at 4 °C and protein concentration of the supernatants was determined by bicinchoninic acid assay (BCA) assay. For the expression analysis in different breast cancer cell lines, total protein was extracted and quantitated as described previously [25]. Total protein was separated on 10 % Bis–Tris PAGE gel using Tris–Hcl buffer and the proteins were transferred to nitrocellulose membranes (Himedia) using a transfer apparatus at 65 V for 90 min. The antibodies were used against EGFR (rabbit monoclonal, BD Biosciences, CA-9061) and GAPDH (Santa Cruz Biotechnology, CA-166574). Appropriate secondary antibodies conjugated to horseradish peroxidase (BioRad) were incubated with respective membranes for 2 h at room temperature. The membranes were developed using ECL plus (BioRad), and the image was captured using enhanced Chemi-luminescence system, G: BOXChemi XX6/XX9. Immunoblot for GAPDH was considered as an internal control for loading. The protein bands were quantified and normalized relatively as the control band with Image J, version 1.35d (National Institutes of Health Image software).

2.6. Wound Healing Assay

The wound healing assay is a standard *in vitro* technique for probing collective cell migration in two dimensions. In this assay, a cell-free area is created in a confluent monolayer by physical exclusion or by removing the cells from the area through mechanical, thermal, or chemical damage. The exposure to the cell-free area induces the cells to migrate into the gap.

Technique: Briefly, the MDA-MB-231 cells (4 × 10^6 cells/well) were plated in 6-well plates for 48 h to a confluence of about 80%, then wounded by scratching with a p200 pipette tip. Thereafter, the debris was removed and we washed the cells once with 1 mL of the growth medium to assure the edges of the scratch were smoothed by washing. We took utmost care to make the wounds of the same dimensions, both for the experimental and control cells, to minimize any possible variety resulting from a difference in scratch width. The cells were then incubated with DMEM medium containing 0.5% FBS and treated with 100 nM of 17β-estradiol. The control sample harboured the cells and a standard medium without any active agents. The MDA-MB-231 cell migration was assessed by gap closure migration assay, embedded by free ImageJ software (version 1.50i, National Institute of Health, Bethesda, MD, USA). The area of the initial wound was measured, followed by gap area measurements after 24 h. The migration factor was represented as the gap area value over the initial scratch area.

2.7. Statistical Analysis

Statistical analysis was carried out by using Graph Pad Prism version 7.04. Statistical analysis of expression of EGFR. $P<0.05$ was considered to be statistically significant. Results for normally distributed data were analysed using student t test and ANOVA.

3. RESULTS

3.1. Effect of 17β-estrdiol on MCF-7 and MDA-MB-231 Cell Lines on EGFR Expression

There was increased expression of EGFR in 17β-estradiol treated MCF-7 cell lines at 0, 6 and 12 hours of interval ($P=0.0008$). A statistical significant 1.12 and 1.4 fold increased expression of EGFR at 6 hours and 12 hours was observed, respectively. There was reduced expression of EGFR in 17β-estradiol treated MDA-MB-231 cell lines at 0, 6 and 12 hours of interval. ($P=0.0001$) A statistical significant 0.41and 0.38 fold decreased expression of EGFR at 6 hours and 12 hours was observed, respectively (Fig. **1**).

3.2. Cycloheximide Chase to Assess the Effect of β-estradiol on MDA-MB-231 Cells on EGFR Expression

There was reduced expression of EGFR at 3 hours in cells treated with Cycloheximide and 17β-estradiol compared to cells treated with Cycloheximide alone. With Cycloheximide alone expression of EGFR reduced significantly to 1.29 fold. With Cycloheximide

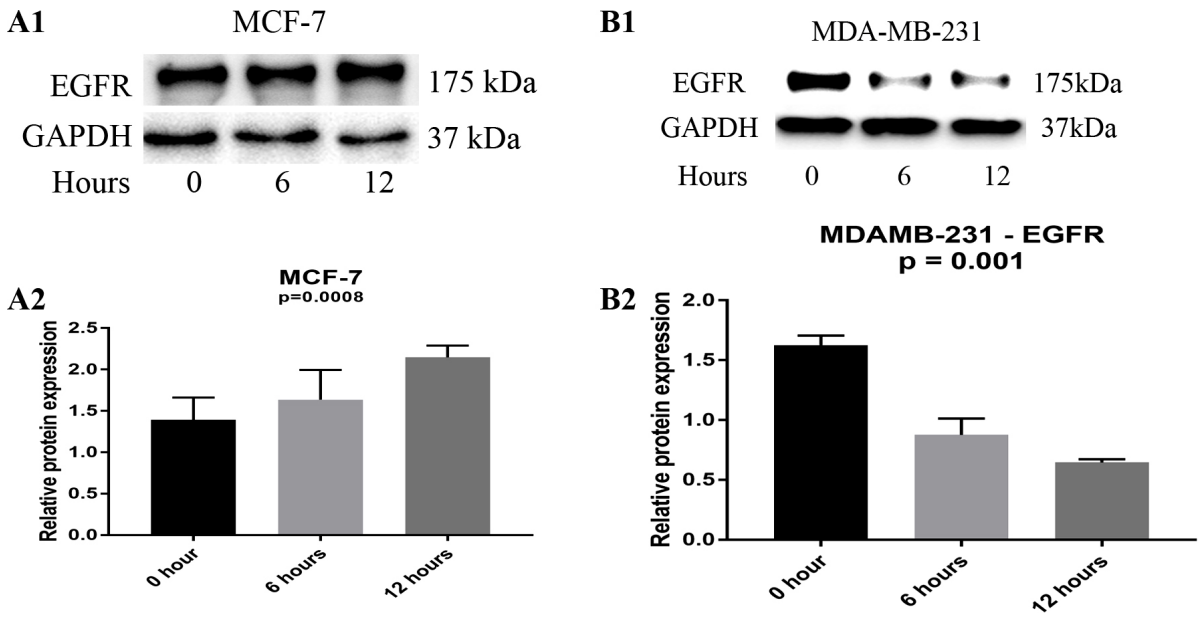

Fig. (1). A1. Effect of 100 nM 17β-estradiol on MCF-7 cell line at 0, 6 and 12hours. (Representative blot) **A2**. Effect of 100 nM 17β-estradiol on MCF-7 cell line at 0, 6 and 12hours. Statistical analysis performed was ANOVA (*p*=0.0008). **B1**. Effect of 100 nM 17β-estradiol on MDA-MB-231 cell line at 0, 6 and 12hours. (Representative blot) **B2**. Effect of 100 nM 17β-estradiol on MDA-MB-231 cell line at 0, 6 and 12hours. Experiment was done thrice in triplicates. Statistical analysis performed was ANOVA (*p*=0.001).

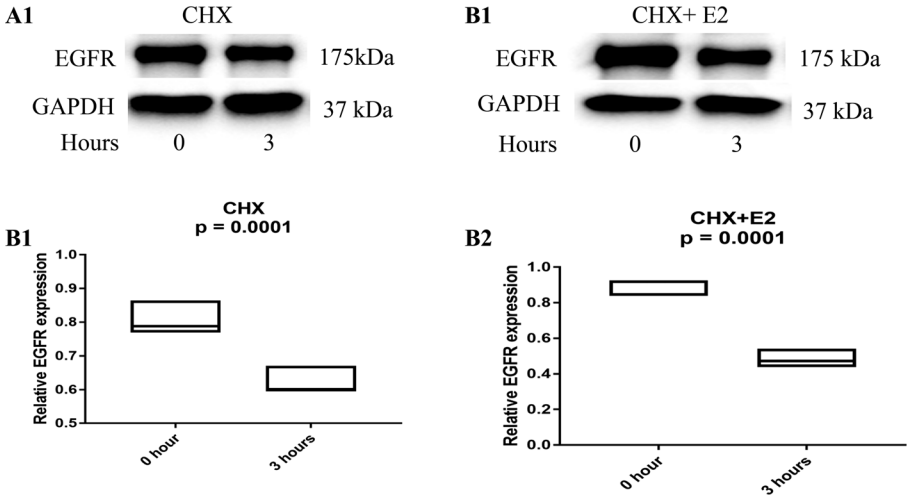

Fig. (2). A1. Expression of EGFR in MDA-MB-231 cell line with 50 µg Cycloheximide at 0 and 3 hours. (Representative blot) **A2**. Expression of EGFR in MDA-MB-231 cell line with 50 µg Cycloheximide at 0 and 3 hours. Statistical analysis was performed by independent t test (*p*=0.0001). **B1**. Expression of EGFR in MDA-MB-231 cell line with 100 nM E2+ 50 µg Cycloheximide at 0 and 3 hours. (Representative blot) **B2**. Expression of EGFR in MDA-MB-231 cell line with 100 nM E2+ 50 µg Cycloheximide at 0 and 3 hours. Experiment was done thrice in triplicates. Statistical analysis was performed by independent t test (*p*=0.0001).

and 17β-estrdiol expression was further reduced significantly 1.73 fold (Fig. **2**).

3.3. Cycloheximide Chase to Assess Ubiquitin Mediated EGFR Degradation

There was a significant reduced expression of EGFR at 1, 2, 3 and 4 hours in cells treated with 17β-estradiol, Cycloheximide (*P*= 0.001). There was a 1.52 fold decrease in the expression of EGFR from 0 hour to 4 hours. There was no significant reduced expression of EGFR at 1, 2, 3, and 4 hours in cells treated with 17β-estradiol, Cycloheximide and MG-132. (P=0.05) There was a meagre 0.7 fold decrease in the expression of EGFR from 0 hour to 4 hours (Fig. **3**).

3.4. Wound Healing Assay

The results of the wound healing assay are presented in Fig. **4**. In the control group, cell migration was very dynamic and the ratio of 0 hour to 24 hour gap was 1.09 after 24 hrs. Using a 100nM estrogen, the motility of the MDA-MB-231 cells was inhibited and the ratio of 0 hour to 24 hour gap was 0.88. Therefore,

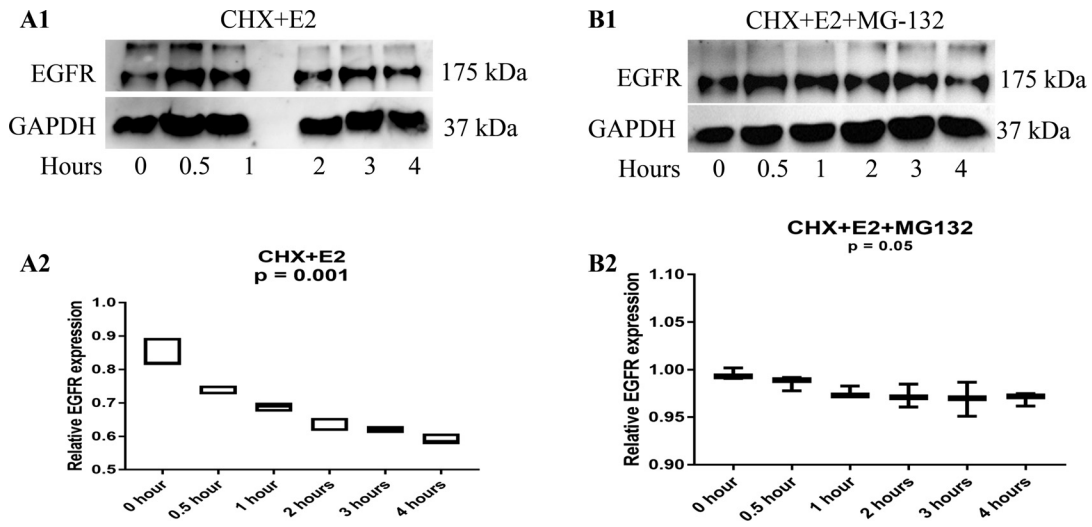

Fig. (3). A1. Expression of EGFR in MDA-MB-231 cell line with 100 nM E2+ 50µg Cycloheximide at 0, 0.5, 1,2, 3 and 4 hours. (Representative blot) **A2**. Expression of EGFR in MDA-MB-231 cell line with 100 nM E2+ 50µg Cycloheximide at 0, 0.5, 1, 2, 3 and 4 hours. Statistical analysis was performed by one way ANOVA (p=0.001). **B1**. Expression of EGFR in MDA-MB-231 cell line with 100 nm E2+ 50 µg Cycloheximide +MG-132 at 0, 0.5, 1,2, 3 and 4 hours. (Representative blot) **B2**. Expression of EGFR in MDA-MB-231 cell line with 100 nm E2+ 50 µg Cycloheximide + MG-132at 0, 0.5, 1,2, 3 and 4 hours. Experiment was done thrice in triplicates. Statistical analysis was performed by one way ANOVA (p=0.05).

it can be interpreted that 17β-estradiol promoted migration and inhibition of the MDA-MB-231 cells (Fig. **4**).

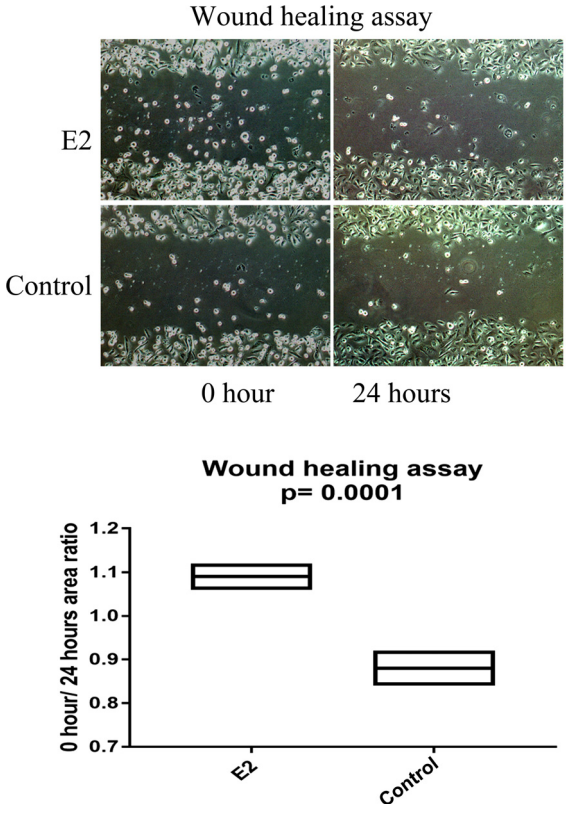

Fig. (4). Effect of 100 nM 17β-estradiol on MDA-MB-231cellline with 100 nM of E2 on wound healing. Experiment was done thrice in triplicates. Statistical analysis was performed using unpaired t test (p=0.0001).

4. DISCUSSION

In this study, we observed that there was a reduced expression of EGFR in MDA-MB-231 cell lines compared to MCF-7 cell lines upon treatment with 17β-estradiol. This indicates that estrogen behaves differently with ER+ and TNBC cell lines. There was a reduced expression of EGFR in MDA-MB-231 cell lines upon treatment with β-estradiol and Cycloheximide when compared to cell lines treated with Cycloheximide alone. This further confirms that estrogen certainly degrades EGFR. We also observed that there was no reduced expression of EGFR in MDA-MB-231 cells treated with 17β-estradiol, Cycloheximide and MG-132 compared to cells treated with 17β-estradiol and Cycloheximide. These observations suggest that β-estradiol degrades EGFR in MDA-MB-231Cells and degradation is mediated by ubiquitination.

There were few limitations of the study. We did not use other types of TNBC cell lines. We did not perform RT-PCR to estimate mRNA levels. We did not change the levels of 17β-estradiol to the exact concentration EGFR inhibited. We did no elucidate the effect of estrogen antagonist. We did not study downstream molecules of EGFR.

Although breast cancer subtypes are genetically linked, environmental factors play a key role. Oral estrogen (OCP) consumption in western countries has been high and in developing countries is low. Accordingly, incidence of ER+ breast cancer is high and low, respectively. But, TNBC incidences are low and high respectively. This has led us to think that more usage of oral estrogens may lead to less incidences of TNBC. There are evidences that ER+ tumours and TNBC behaves indifferently in presence of estrogen. In our study we too tried to demonstrate that

17β-estradiol can act indifferently in different phenotypic breast cancers *in vitro*. We demonstrated that 17β-estradiol augment the proliferation of MCF-7 cells by increasing the expression of EGFR whereas β-estradiol decreases the proliferation of MDA-MB-231 cells by decreasing the production of EGFR.

TNBCs typically occur in younger women And African American women as well as among some patients with BRCA1 gene defects [1, 2]. Population-based data show that African American women have a higher incidence of TNBC and present with more advanced stages than Caucasian women [26]. This cancer subtype also associates with adverse biological features, including high mitotic count and very aggressive behavior. Based on current data, estradiol regulates gene expression of EGFR and other several proteins by genomic and non-genomic inputs [27, 28]. Genomic signals involve the direct action of nuclear-localized ERα as an estradiol regulated transcription factor or co-regulator. By contrast, non-genomic signaling involves extra nuclear events mediated by extra nuclear ERs often in cooperation with co activator or adaptor proteins [29]. Thus, estrogens promote the progression of ER+ breast cancers through predominant ERα. In TNBC, second type of estrogen receptor, termed estrogen receptor-beta (ERβ). ERα and ERβ have reciprocal actions. Studies have demonstrated that ERβ1 inhibits epithelial mesenchymal transition (EMT) and invasion in basal-like breast cancer cells when they grow esther *in vitro* or *in vivo* in zebrafish. EMT is also because of hypoxia known in cancer development [30]. Thus, activation of ERβ in TNBC probably reduces the expression of EGFR [31]. EGFR expression can also be degraded by the activation of non-genomic pathways. In our study, we demonstrated that β-estradiol indeed causes degradation, also we tried to analyse how degradation occurs. There are various steps involved in degradation of EGFR. Upon activation, EGFR is tyrosine-phosphorylated, and subsequently recruits Cbl, an E3 ubiquitin ligase, and Grb2, an adaptor protein, for assembly of the ubiquitination complex, and interacts with Eps15 and AP-2, two endocytic adaptor proteins, to form clathrin-coated endocytic vesicles [32- 35]. The endocytic vesicles or endosomes containing ubiquitinated EGFR are recognized by the ubiquitin-binding protein Hrs and transported to multi-vesicular bodies (MVBs) [36, 37]. Finally, the MVBs fuse with lysosomes to complete the degradation of EGFR. We hypothesized that estradiol inhibits ubiquitination. To test our hypothesis, we treated MDA-MB-231 cells with cycloheximide and estradiol and observed estrogen degrades EGFR. There was a 1.52 fold significant decrease in the expression of EGFR. Subsequently, to test another hypothesis that degradation of EGFR occurs due to facilitation of ubiquitination, we treated MDA-MB-231 cells with cycloheximide, estradiol and MG-132 where MG-132 effectively blocks the proteolytic activity of the 26S proteasome complex reduces the degradation of ubiquitin-conjugated proteins. We observed that there was a statistically insignificant 0.7 fold decrease in EGFR expression. This indicates that degradation of EGFR occurs upon treatment with 17β-estradiol, where degradation is mainly mediated by ubiquitination. We also tested our hypothesis on wound healing test. We treated MBA-MB-231 cells with or without estradiol; we observed that without estradiol, cell migration was very dynamic, a ratio of 0 hour to 24 hour gap was 1.09 after 24 hrs and motility of the MDA-MB-231 cells was inhibited and ratio of 0 hour to 24 hour gap was 0.88 with estradiol. This observation also proves that esrogen delays the proliferation of MDA-MB-231 cells.

EGFR is one of the receptors most commonly associated with human tumors and has been shown to correlate with the progression of many tumor types, including breast tumors [38-40]. Most often associated with aspects of tumor growth (*i.e*., proliferation, apoptosis, and cell survival), little emphasis has been placed on the effects of EGF on breast cancer cell migration. The complex process of cell migration is a critical component of many normal and patho-physiological processes, and its central role in the progression of tumors from a noninvasive to an invasive and metastatic phenotype is well known [41]. Epidermal growth factor receptor (EGFR) levels predict a poor outcome in human breast cancer and are most commonly associated with proliferative effects of epidermal growth factor. In this study, we tried to demonstrate the effect of estrogen with EGFR expression in different subtypes of breast cancers, mainly ER+ and TNBC tumours, whether they respond differently. We found that estradiol degrades EGFR in MDA-MB-231 and we could also demonstrate that degradation occurring through ubiquitination. The underlying mechanism of degradation appears related to the sorting of internalized EGFRs to either recycling or degradation [9]. Sorting EGFR requires conjugation of multiple ubiquitins, which mark the receptor for degradation [10]. Therefore, our study demonstrated that EGFR is degraded by ubiquitination.

Several lines of evidence support the possibility that EGFR plays a the role of a driver in a large fraction of TNBC. For example, EGFR gene amplification is commonly identified in metaplastic breast carcinoma, a basal-like fraction of tumors [42]. Likewise, gene expression signatures correlated TNBC with modules comprising EGF-like ligands, EGFR, and several downstream effectors [43]. Although TNBC clinical trials using EGFR inhibitors, including cetuximab, reported a lack of clinical benefit [44], our study offers an alternative strategy by degrading EGFR by estrogen. Experiments that used TNBC line indicated that down regulation of EGFR through ubiquitination can retard motility and proliferation of TNBC cell line.

By adding estogen, we detected EGFR degradation and degradation occurs through ubiquitination in MDA-MB-231 cellline. Our *in vitro* study require confirmation in animal models. Assuming confirmation *in vivo*, this study may help in understanding alternative pathway where degradation of EGFR by estrogen or other specific molecule receptors can be a targeted in the treatment of TNBC. In the future, estrogen like

molecules may be used as adjunct in the treatment of TNBC.

CONCLUSION

We concluded that β-estradiol degrades EGFR in MDA-MB-231 cells and this degradation occurs by ubiquitination. This study may help in understanding alternative pathway where degradation of EGFR by estrogen or other specific molecule receptors can be targeted in the treatment of TNBC.

LIST OF ABBREVIATIONS

AKT	=	Ak strain transforming murine thymoma viral oncogene
ANOVA	=	Analysis of variance
AP-2	=	Adaptor protein 2
Cbl-gene	=	Casitas B-lineage lymphoma
DMEM	=	Dulbecco's modified Eagle's medium
E2	=	17β-estradiol
EGFR	=	Epidermal growth factor receptor
Erb2	=	Erythroblastic oncogene B, also Her2
Erα	=	Estrogen receptorα
ERβ	=	Estrogen receptor β
Esp15	=	Epidermal growth factor receptor substrate 15
GAPDH	=	Glyceraldehyde 3-phosphate dehydrogenase
Grb2	=	Growth factor receptor-bound protein 2
HER2/neu	=	Human epidermal growth factor receptor 2
MAPK	=	Mitogen-activated protein kinase
MCF-7	=	Michigan Cancer Foundation-7,
MD-AMB-231	=	MD Anderson-Metastatic Breast-231
MG-132	=	Proteosome inhibitor
OCP	=	Oral contraceptive pill
PI3K	=	Phosphoinositide 3-kinases
PKA	=	protein kinase A
PR	=	Progesterone receptor
RTK	=	Receptor tyrosine kinases
TNBC	=	Triple negative breast cancer

ETHICS APPROVAL AND CONSENT TO PARTICIPATE

Ethical committee approval was obtained from the institution (Ref:SDMIEC:0741:2016).

HUMAN AND ANIMAL RIGHTS

Not applicable.

CONSENT FOR PUBLICATION

We declare that we have consent to publish this article.

AVAILABILITY OF DATA AND MATERIALS

Not applicable.

FUNDING

This study was not funded by funding agencies.

CONFLICT OF INTEREST

The author declares no conflict of interest, financial or otherwise.

ACKNOWLEDGEMENTS

We Acknowledge Central Research Laboratory, SDM College of Medical sciences & Hospital, Dharwad, India for providing infrastructure.

REFERENCES

[1] Foulkes WD, Smith IE, Reis-Filho JS. Triple-negative breast cancer. N Engl J Med 2010; 363(20): 1938-48. http://dx.doi.org/10.1056/NEJMra1001389 PMID: 21067385

[2] Morris GJ, Naidu S, Topham AK, *et al.* Differences in breast carcinoma characteristics in newly diagnosed African-American and Caucasian patients: a single-institution compilation compared with the National Cancer Institute's Surveillance, Epidemiology, and End Results database. Cancer 2007; 110(4): 876-84. http://dx.doi.org/10.1002/cncr.22836 PMID: 17620276

[3] Bolla M, Chedin M, Souvignet C, Marron J, Arnould C, Chambaz E. Estimation of epidermal growth factor receptor in 177 breast cancers: correlation with prognostic factors. Breast Cancer Res Treat 1990; 16(2): 97-102. http://dx.doi.org/10.1007/BF01809293 PMID: 2176111

[4] Slamon DJ, Godolphin W, Jones LA, *et al.* Studies of the HER-2/neu proto-oncogene in human breast and ovarian cancer. Science 1989; 244(4905): 707-12. http://dx.doi.org/10.1126/science.2470152 PMID: 2470152

[5] Rimawi MF, Shetty PB, Weiss HL, *et al.* Epidermal growth factor receptor expression in breast cancer association with biologic phenotype and clinical outcomes. Cancer 2010; 116(5): 1234-42. http://dx.doi.org/10.1002/cncr.24816 PMID: 20082448

[6] Nicholson RI, Hutcheson IR, Jones HE, *et al.* Growth factor signalling in endocrine and anti-growth factor resistant breast cancer. Rev Endocr Metab Disord 2007; 8(3): 241-53. http://dx.doi.org/10.1007/s11154-007-9033-5 PMID: 17486454

[7] DeSantis CE, Fedewa SA, Goding Sauer A, Kramer JL, Smith RA, Jemal A. Breast cancer statistics, 2015: Convergence of incidence rates between black and white women. CA Cancer J Clin 2016; 66(1): 31-42. http://dx.doi.org/10.3322/caac.21320 PMID: 26513636

[8] Brewster AM, Chavez-MacGregor M, Brown P. Epidemiology, biology, and treatment of triple-negative breast cancer in women of African ancestry. Lancet Oncol 2014; 15(13): e625-34. http://dx.doi.org/10.1016/S1470-2045(14)70364-X PMID: 25456381

[9] Spangler JB, Neil JR, Abramovitch S, *et al.* Combination antibody treatment down-regulates epidermal growth factor

receptor by inhibiting endosomal recycling. Proc Natl Acad Sci USA 2010; 107(30): 13252-7.
http://dx.doi.org/10.1073/pnas.0913476107 PMID: 20616078

[10] Goh LK, Huang F, Kim W, Gygi S, Sorkin A. Multiple mechanisms collectively regulate clathrin-mediated endocytosis of the epidermal growth factor receptor. J Cell Biol 2010; 189(5): 871-83.
http://dx.doi.org/10.1083/jcb.201001008 PMID: 20513767

[11] Crowder JJ, Geigges M, Gibson RT, *et al.* Rkr1/Ltn1 Ubiquitin Ligase-mediated Degradation of Translationally Stalled Endoplasmic Reticulum Proteins. J Biol Chem 2015; 290(30): 18454-66.
http://dx.doi.org/10.1074/jbc.M115.663559 PMID: 26055716

[12] Tran JR, Brodsky JL. Assays to measure ER-associated degradation in yeast. Methods Mol Biol 2012; 832: 505-18.
http://dx.doi.org/10.1007/978-1-61779-474-2_36 PMID: 22350909

[13] Kao SH, Wang WL, Chen CY, *et al.* GSK3 β controls epithelial-mesenchymal transition and tumor metastasis by CHIP-mediated degradation of Slug. Oncogene 2014; 33(24): 3172-82.
http://dx.doi.org/10.1038/onc.2013.279 PMID: 23851495

[14] Hampton RY, Rine J. Regulated degradation of HMG-CoA reductase, an integral membrane protein of the endoplasmic reticulum, in yeast. J Cell Biol 1994; 125(2): 299-312.
http://dx.doi.org/10.1083/jcb.125.2.299 PMID: 8163547

[15] Hochstrasser M, Varshavsky A. In vivo degradation of a transcriptional regulator: the yeast alpha 2 repressor. Cell 1990; 61(4): 697-708.
http://dx.doi.org/10.1016/0092-8674(90)90481-S PMID: 2111732

[16] Hanna J, Leggett DS, Finley D. Ubiquitin depletion as a key mediator of toxicity by translational inhibitors. Mol Cell Biol 2003; 23(24): 9251-61.
http://dx.doi.org/10.1128/MCB.23.24.9251-9261.2003 PMID: 14645527

[17] Glickman MH, Ciechanover A. The ubiquitin-proteasome proteolytic pathway: destruction for the sake of construction. Physiol Rev 2002; 82(2): 373-428.
http://dx.doi.org/10.1152/physrev.00027.2001 PMID: 11917093

[18] Hershko A. The ubiquitin system for protein degradation and some of its roles in the control of the cell division cycle. Cell Death Differ 2005; 12(9): 1191-7.
http://dx.doi.org/10.1038/sj.cdd.4401702 PMID: 16094395

[19] Ravid T, Hochstrasser M. Diversity of degradation signals in the ubiquitin-proteasome system. Nat Rev Mol Cell Biol 2008; 9(9): 679-90.
http://dx.doi.org/10.1038/nrm2468 PMID: 18698327

[20] Gallastegui N, Groll M. The 26S proteasome: assembly and function of a destructive machine. Trends Biochem Sci 2010; 35(11): 634-42.
http://dx.doi.org/10.1016/j.tibs.2010.05.005 PMID: 20541423

[21] Hershko A, Heller H, Elias S, Ciechanover A. Components of ubiquitin-protein ligase system. Resolution, affinity purification, and role in protein breakdown. J Biol Chem 1983; 258(13): 8206-14.
http://dx.doi.org/10.1016/S0021-9258(20)82050-X PMID: 6305978

[22] Metzger MB, Pruneda JN, Klevit RE, Weissman AM. RING-type E3 ligases: master manipulators of E2 ubiquitin-conjugating enzymes and ubiquitination. Biochim Biophys Acta 2014; 1843(1): 47-60.
http://dx.doi.org/10.1016/j.bbamcr.2013.05.026 PMID: 23747565

[23] Finley D. Recognition and processing of ubiquitin-protein conjugates by the proteasome. Annu Rev Biochem 2009; 78: 477-513.
http://dx.doi.org/10.1146/annurev.biochem.78.081507.101607 PMID: 19489727

[24] Lander GC, Estrin E, Matyskiela ME, Bashore C, Nogales E, Martin A. Complete subunit architecture of the proteasome regulatory particle. Nature 2012; 482(7384): 186-91.
http://dx.doi.org/10.1038/nature10774 PMID: 22237024

[25] Das S, Roth CP, Wasson LM, Vishwanatha JK. Signal transducer and activator of transcription-6 (STAT6) is a constitutively expressed survival factor in human prostate cancer. Prostate 2007; 67(14): 1550-64.
http://dx.doi.org/10.1002/pros.20640 PMID: 17705178

[26] Amirikia KC, Mills P, Bush J, Newman LA. Higher population-based incidence rates of triple-negative breast cancer among young African-American women : Implications for breast cancer screening recommendations. Cancer 2011; 117(12): 2747-53.
http://dx.doi.org/10.1002/cncr.25862 PMID: 21656753

[27] Pietras RJ, Márquez-Garbán DC. Membrane-associated estrogen receptor signaling pathways in human cancers. Clin Cancer Res 2007; 13(16): 4672-6.
http://dx.doi.org/10.1158/1078-0432.CCR-07-1373 PMID: 17699844

[28] Hammes SR, Levin ER. Extranuclear steroid receptors: nature and actions. Endocr Rev 2007; 28(7): 726-41.
http://dx.doi.org/10.1210/er.2007-0022 PMID: 17916740

[29] Yan Y, Li X, Blanchard A, *et al.* Expression of both estrogen receptor-beta 1 (ER- β 1) and its co-regulator steroid receptor RNA activator protein (SRAP) are predictive for benefit from tamoxifen therapy in patients with estrogen receptor-alpha (ER- α)-negative early breast cancer (EBC). Ann Oncol 2013; 24(8): 1986-93.
http://dx.doi.org/10.1093/annonc/mdt132 PMID: 23579816

[30] Pezzuto A, Carico E. Role of HIF-1 in Cancer Progression: Novel Insights. A Review. Curr Mol Med 2018; 18(6): 343-51.
http://dx.doi.org/10.2174/1566524018666181109121849 PMID: 30411685

[31] Thomas C, Rajapaksa G, Nikolos F, *et al.* ERbeta1 represses basal breast cancer epithelial to mesenchymal transition by destabilizing EGFR. Breast Cancer Res 2012; 14(6): R148.
http://dx.doi.org/10.1186/bcr3358 PMID: 23158001

[32] Waterman H, Levkowitz G, Alroy I, Yarden Y. The RING finger of c-Cbl mediates desensitization of the epidermal growth factor receptor. J Biol Chem 1999; 274(32): 22151-4.
http://dx.doi.org/10.1074/jbc.274.32.22151 PMID: 10428778

[33] Jiang X, Huang F, Marusyk A, Sorkin A. Grb2 regulates internalization of EGF receptors through clathrin-coated pits. Mol Biol Cell 2003; 14(3): 858-70.
http://dx.doi.org/10.1091/mbc.e02-08-0532 PMID: 12631709

[34] Confalonieri S, Salcini AE, Puri C, Tacchetti C, Di Fiore PP. Tyrosine phosphorylation of Eps15 is required for ligand-regulated, but not constitutive, endocytosis. J Cell Biol 2000; 150(4): 905-12.
http://dx.doi.org/10.1083/jcb.150.4.905 PMID: 10953014

[35] Ramaekers CH, Wouters BG. Regulatory functions of ubiquitin in diverse DNA damage responses. Curr Mol Med 2011; 11(2): 152-69.
http://dx.doi.org/10.2174/156652411794859269 PMID: 21342128

[36] Lu Q, Hope LW, Brasch M, Reinhard C, Cohen SN. TSG101 interaction with HRS mediates endosomal trafficking and receptor down-regulation. Proc Natl Acad Sci USA 2003; 100(13): 7626-31.
http://dx.doi.org/10.1073/pnas.0932599100 PMID: 12802020

[37] Bache KG, Brech A, Mehlum A, Stenmark H. Hrs regulates multivesicular body formation via ESCRT recruitment to endosomes. J Cell Biol 2003; 162(3): 435-42.
http://dx.doi.org/10.1083/jcb.200302131 PMID: 12900395

[38] Radinsky R, Risin S, Fan D, *et al.* Level and function of epidermal growth factor receptor predict the metastatic potential of human colon carcinoma cells. Clin Cancer Res 1995; 1(1): 19-31.
PMID: 9815883

[39] Harris AL, Nicholson S, Sainsbury R, Wright C, Farndon J. Epidermal growth factor receptor and other oncogenes as prognostic markers. J Natl Cancer Inst Monogr 1992; (11): 181-7.
PMID: 1627426

[40] Jain A, Jain SK. Advances in Tumor Targeted Liposomes. Curr Mol Med 2018; 18(1): 44-57.

http://dx.doi.org/10.2174/1566524018666180416101522 PMID: 29663884

[41] Price JT, Bonovich MT, Kohn EC. The biochemistry of cancer dissemination. Crit Rev Biochem Mol Biol 1997; 32(3): 175-253. http://dx.doi.org/10.3109/10409239709082573 PMID: 9239493

[42] Reis-Filho JS, Pinheiro C, Lambros MB, *et al.* EGFR amplification and lack of activating mutations in metaplastic breast carcinomas. J Pathol 2006; 209(4): 445-53. http://dx.doi.org/10.1002/path.2004 PMID: 16739104

[43] Hoadley KA, Weigman VJ, Fan C, *et al.* EGFR associated expression profiles vary with breast tumor subtype. BMC Genomics 2007; 8: 258. http://dx.doi.org/10.1186/1471-2164-8-258 PMID: 17663798

[44] Burness ML, Grushko TA, Olopade OI. Epidermal growth factor receptor in triple-negative and basal-like breast cancer: promising clinical target or only a marker? Cancer J 2010; 16(1): 23-32. http://dx.doi.org/10.1097/PPO.0b013e3181d24fc1 PMID: 20164687