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EGF and IGF-1 Signaling Cross-Talk Enhances Expression of EMT Promoting Genes: Implications on Morphology and Proliferation in Breast Cancer Cells

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1. Abstract

EMT is involved in malignancies like breast cancer and aids in invasion and metastasis. Epidermal growth factor (EGF) and insulin-like growth factor (IGF-1) enhance EMT in breast cells (MDA-MB-468, MDA-MB-231 and MCF10A). IGF-1 and EGF increase expression of EMT inducing transcription factors and cell proliferation in metastatic breast cancer cells. This study indicates that targeting the EGFR/ IGF-R signaling in addition to conventional therapies can reduce tumor growth and progression and prolong survival of cancer patients.

1.1. Background

Breast cancer (BC) is one of the most prevalent cancer-related deaths, and it continues to pose a severe threat to women's health and well-being around the world. Despite significant advancements in breast cancer treatment, metastatic breast cancer remains a fatal disease. To produce next-generation therapies, a thorough understanding of the mechanism of systemic cancer cell dissemination is necessary. A slew of experimental evidence suggests that an epithelial to mesenchymal transition (EMT) plays a crucial role in the multistep process of metastasis development. Epithelial-mesenchymal transition (EMT) is a prerequisite step in various aggressive malignancies, including breast cancer that aids in invasion and metastasis.

1.2. Methods

In the present study, we evaluated the effect of epidermal growth factor (EGF) and insulin-like growth factor (IGF-1) on breast cells (MDA-MB-468, MDA-MB-231 and MCF10A). We used qPCR, western blotting followed by gene-gene interaction analysis. We also performed cell migration assay and cell proliferation assay to elucidate the downstream effects of EGF and IGF-1 on breast cells.

1.3. Results

It was found that IGF-1 enhances EMT stimulated by EGF. Also, expression of EMT inducing transcription factors viz, snail, slug, zeb1, and zeb2 were found enhanced in breast cells exposed to EGF or IGF-1. In addition, we also found that cell proliferation in metastatic breast cancer cell lines increases substantially in response to EGF and IGF-1 treatment. Interestingly, this increase in cell proliferation was not observed in normal breast cells. We also observed that the morphology of breast cancer cell lines changes when exposed to EGF and IGF-1, whereas in normal breast cells morphology remains unchanged, indicating that growth factors viz EGF and IGF-1 stimulate EMT in breast tumor cells.

1.4. Conclusions

Together these studies indicate that targeting the EGFR/ IGF-R signaling in addition to conventional therapies will substantially reduce tumor growth and progression and prolong survival of cancer patients.

2. Background

Breast cancer (BC) is now one of the most common cancers in women worldwide, as well as the main cause of tumor-related death in women. Due to significant changes in lifestyle, social, and built environments brought about by emerging economies, BC incidence rates are increasing in transitional countries such as South America, Africa, and high-income Asian nations (Japan and the Republic of Korea)[1].Furthermore, BC mortality has increased rapidly in Sub-Saharan African countries, mainly owing to under developed health infrastructure circumstances that result in poor survival [1]. BC is an incredibly heterogeneous solid cancer with over twenty different subgroups that vary in appearance, genetics, and clinical behavior. Based on gene expression, BC is categorized into HER2-enriched, luminal A, luminal B, and triple-negative breast cancer (TNBC) subtypes[2]. Despite significant advances in diagnostics, surgery, and medication research, BC continues to be an issue due to poor clinical outcomes and the occurrence of frequent and metastatic malignancies[3].The epithelial to mesenchymal transition (EMT) is a fundamental biological mechanism with well-established roles in embryonic morphogenesis, wound healing, and organ fibrosis[4]. Tumor cells have long been known to exploit the EMT process to gain the ability to spread and colonize distant organs[5]. EMT has been widely associated with metastatic cancer progression and the acquisition of stem-cell properties, bolstering the theory that reprogramming from epithelial to mesenchymal phenotype results in acquired migration and self-renewal abilities, which promote the formation of secondary tumors at distant sites [6].Numerous factors have been implicated in triggering the EMT phenomenon, including epidermal growth factor (EGF),fibroblast growth factor (FGF), and Insulin growth factor 1 (IGF-1). These factors activate downstream pathways that control epithelial to mesenchymal plasticity, including the PI3K/AKT pathway, MAPK pathway, and src pathway, by stimulating their respective receptors[6-8].In the present study, we evaluated the effect of epidermal growth factor (EGF) and insulin-like growth factor-1 (IGF-1) on the EMT process in breast cancer cells and breast normal cells. We analyzed the combinational impact of the two growth factors on morphology and proliferation in breast carcinoma and normal breast cell lines and also on the expression of genes regulating the EMT phenomenon in the same cell lines. We found that the morphology of breast carcinoma cells changes when treated with EGF and IGF-1. We also observed that the rate of proliferation increased in breast carcinoma cells as compared to normal breast cells, when treated with EGF and IGF-1. In addition to this, the expression of genes such as MAPK, TGF-β, and Notch,

which are central to tumor-promoting pathways, was also analyzed in cells exposed to the two growth factors. The expression of EMT inducing transcription factors viz, snail, slug, zeb1, and zeb2 was found to be enhanced in all the cell lines exposed to EGF or IGF-1, indicating that growth factors viz EGF and IGF-1 stimulate EMT in breast tumor cells. It was found that IGF-1 enhances EGF stimulated EMT in breast cancer cells.

3. Material and Methods

3.1. Cell Culture

Human breast cancer cell lines, MDA-MB-231, and MDA-MB-468 were procured from National Centre for Cell Science (NCCS) Pune.MDA-MB-468 and MDA-MB-231 cells were cultured in DMEM (Sigma-Aldrich, St Louis, MO, USA) supplemented with 4mM L-glutamine (Invitrogen, Waltham, MA, USA) and 10% fetal bovine serum (FBS) (Sigma-Aldrich) and kept in a humidified incubator at 37°C with 5% CO2.The identity of these cells was regularly assessed based on cell morphology. MCF10A was procured from NCCS Pune and cultured in MEBM Basal Medium supplemented with BPE, EGF, Insulin, Hydrocortisone, GA-1000 (CC-3150, Lonza Walkersville, Inc. USA), and cholera toxin (Sigma Chemicals, St. Louis, MO, United States).

3.2. Induction of EMT

Cells were seeded in a six-well plate, allowed to adhere for 24 hours, and serum-deprived (0.5% FBS) for a period of 24 h. After 24 hrs, media was replaced with fresh media supplemented with EGF (0.1 µg/mL) and/or IGF-1 (0.3 µg/mL). The doses of EGF and IGF11 that we have used have already been standardized for the induction of EMT [9,10]. The morphological changes in the cells were observed after treatment under an inverted light microscope.

3.3. RNA Isolation and cDNA Synthesis

Total RNA was extracted using TriZol (Invitrogen), and the purity of obtained RNA was verified using a nano-spectrophotometer (Thermo Fisher Scientific USA) and electrophoresis. Complementary DNA (cDNA) was reverse-transcribed from 1 microgram of total RNA in a 20µl reaction mixture containing 1µl random hexamer primer, 4µl of 5x reaction buffer, 1µl RNase inhibitor, 2 µl dNTP 10mM, 200 unit RevertAid M-MuLV reverse transcriptase enzyme, and appropriate RNase/DNase free water. The mixture was then incubated at 25°C for 5 min, 42°C for 1 hour, and 70°C for 5 min using the Thermo Scientific RevertAid first-strand cDNA synthesis kit (Thermo Scientific, Massachusetts, USA). The sequences of the PCR primers used are listed in Table 1. PCR (Applied Biosystems) condition was 95 °C for 10 min, followed by 40 rounds of 95 °C for 15 s and 60 °C for 1 min.

3.4. Quantitative Real-Time PCR

The mRNA expression levels of EMT-related genes in breast cell lines were determined by Real-Time PCR (Roche, Germany), using SYBR green as a fluorescent dye. GAPDH and β-actin were used as an internal control. When we are dealing with cell culture, the expression of housekeeping genes may change due to their immortal nature and potentially high number of generations undergone by them. Thus, it is best to use multiple housekeeping genes (in our case we used 2) and to check how they behave relative to one another. The relative quantification of selected mRNAs was determined by the $2^{-\Delta\Delta CT}$ method [11], where ΔΔCT corresponded to the difference between the CT measured for the mRNA level of each tissue and the CT measured for the mRNA level of the reference gene, ΔCT = CT(target gene)—mean of CT(β-Actin) and CT(GAPDH).

3.5. Western Blotting

Cells were lysed in NP-40 lysis buffer (1% NP-40, 150 mM NaCl, 2 mM EDTA, 20 mM Tris-Cl, 10% glycerol) complimented with protease and phosphatase inhibitors (50 mM NaF, 1 mM Na3VO4, 1 mM PMSF, 10 ml of 1000X PIC stock/ml of lysis buffer). The cell lysates were centrifuged at 10,000 rpm for 20 min at 4°C. The protein concentration of the supernatants was determined by using the Bradford assay. The protein samples were denatured by adding 1X Laemmli sample buffer (50 mM Tris-Cl [pH 6.8], 10% glycerol, 2% SDS, 5% b-mercaptoethanol, 0.01% bromophenol blue) followed by boiling at 100°C for about 5 min. Equal amounts of proteins were loaded onto SDS-PAGE gels and the resolved proteins were electroblotted on PVDF membranes. The blots were then blocked with 5% skim milk or BSA in 1X PBS containing 0.1% Tween-20 (PBS-T) for about 1 hour at room temperature, followed by overnight incubation with specific primary antibodies at 4°C. Antibodies against E-Cadherin, Vimentin, Snail1 protein, NF-κB protein, Notch1 protein and β-actin were used at a dilution of 1:1000. Afterward, blots were washed three times with chilled 1X TBS containing 0.05-0.1% Tween-20 (TBS-T) and incubated with secondary antibody (DyLight 800) for 1-2 hours. The blots were again washed three times with 1X TBS-T, followed by infrared detection using the Li-Cor Odyssey imaging system.

3.6. Gene-Gene Interaction Analysis

Gene MANIA, an Online web resource was accessed to study the Gene-gene interaction (GGI) network of EMT-related genes and IGF-1 and EGF. The GGI network of IGF-1 and EGF was established with a confidence score ≥ of 0.70, and the network was constructed using Cytoscape software (version 3.8.2). Also, the plug-in of Molecular Complex Detection (MCODE) in Cytoscape software was used to explore the remarkable modules in the GGI network.

3.7. Cell Proliferation Assay

The cell proliferation rate was measured using MTT cell proliferation assay. MCF10A, MDA-MB-468 and MDA-MB-231 cells cultured in 24-well plates (~1×105 cells per well) were treated with EGF, IGF-1 and EGF in combination with IGF-1, followed by incubation with 10 µl of MTT (50mg/ml) in a humidified atmosphere (37°C, 5% CO2) for 3-4 hours. Afterward MTT was removed and 200 µl of an acidic DMSO solution was added to each well. Plates were incubated for 30 min in dark on a shaker. The absorbance of the resulting-colored solution was measured at 570 nm using a microplate spectrophotometer (Bio-Rad Laboratories, Hercules, CA, USA).

3.8. In Vitro Wound Healing Assay

The standard protocol for an in vitro scratch assay was followed. The cells were treated with EGF, IGF-1 and EGF and IGF-1 together and incubated at 37ºC until the cells reached 100% confluence and formed a monolayer. A P-10 pipette tip was used to create a scratch in the monolayer and cells were again incubated for 24 hours. Pictures were taken using a camera fitted to a microscope. The gaps were measured, and the % wound closure was calculated by using the formula, percent Closure (%) = Migrated Cell Surface Area / Total Surface Area x 100.

3.9. Statistics

The statistical significance in the treated vs control groups was analyzed using two-way ANOVA followed by Dunnett's multiple comparisons test or Sidak's multiple comparisons test was performed using GraphPad Prism, 9.3. $P < 0.05$ was considered significant.

4. Results

Morphological changes in Breast Cells upon EGF/IGF-1 treatment: EGF and IGF-1 are the most prevalent growth factors identified in the tumor microenvironment and cause EMT in a variety of cancer cells. To mimic the in vivo condition, cells are primarily exposed to EGF and IGF-1 in the tumor microenvironment, we treated breast cells with EGF and IGF-1 for 24 hours. After 24 hours, the morphologies of MDA-MB-468 and MDA-MB-231 cells were changed entirely from spindle to a circular form (Figure 1). The morphological change was high in the cells treated with the combination of EGF and IGF-1, indicating that the combinational

treatment with both EGF and IGF-1 stimulated phenotypic change in breast tumor cells more as compared to the treatment with EGF and IGF-1 alone. Also, post treatment with EGF and IGF-1 breast cancer cells showed a reduction in cell-cell contacts as compared to the normal breast cell line MCF10-A. In the breast control cell line (MCF10A), EGF & IGF-1 did not induce change in cell morphology as can be seen in Figure 1.

4.1. Characterization Of Epithelial and Mesenchymal Cells

Breast cells (MDA-MB-231 and MDA-MB-468) were characterized by analyzing the expression of cell surface markers expression, Vimentin (VIM), and E-Cadherin (E-CAD) using RT-PCR analysis. Results demonstrated that EGF and IGF-1 treatment enhances the expression of mesenchymal markers and reduces the expression of epithelial marker E-CAD. Moreover, the combinational treatment of EGF and IGF-1 synergistically augmented VIM expression and downregulated E-CAD, further validating their cross-talk in EMT. We also examined the change in endogenous protein expression of some E-cadherin and Vimentin via western blotting in MDA-MB-231 cells. We saw that the expression of E-Cadherin decreased in response to treatment with EGF and IGF-1, with the least expression observed in cells treated with EGF and IGF-1 together. The expression of Vimentin increased in response to the same treatments, with the highest expression observed in cells treated with EGF and IGF-1 together. β-actin was used as loading control (Figure 2).

4.2. IGF-1 Enhances EGF Stimulated Expression of EMT Transcription Factors

MDA-MB-231 and MDA-MB-468 cells were treated with EGF (100ng/ml) alone in combination with IGF-1 (300ng/ml) for 24 hours. After treatment, relative gene expression of snail, slug, zeb1, and zeb2 was analyzed using RT-PCR analysis (Figure3). Gene expression analysis revealed that exposure of MDA-MB-231 and MDA-MB-468 cells to EGF or IGF-1 increased the expression of snail, slug, zeb1, and zeb2. Additionally, the expression of EMT-related Transcription factors (TFs) enhanced significantly upon treatment with both EGF and IGF-1. These results indicate that EGF or IGF-1 upregulates the expression of transcription factors, which in turn can promote the epithelial to mesenchymal transition by promoting the mesenchymal phenotype and suppressing the epithelial characteristics of tumor cells. The increase in the expression of EMT-related TFs upon exposure to EGF and/or IGF-1 was witnessed in MCF10A cells as well(Figure 3).

4.3. Combinational Treatment with EGF and IGF-1 Promotes EMT Via Enhanced Activation of Notch and TGF- B Signaling

We further analyzed the expression of TGF-β and Notch genes in breast cells, and it was revealed that treatment with EGF or IGF-1 stimulates Notch and TGF-β. Also, the mRNA levels of MAPK1 and NFκB were assessed in the treated cells, and significant upregulation was observed. Additionally, combinational treatment with EGF and IGF-1 promotes EMT via enhanced activation of Notch and TGF-βsignaling in TNBC cells (Figure 3). Cells treated with both EGF and IGF-1 had significantly higher mRNA levels of both NFκB and MAPK1. Additionally, the breast control cell line (MCF10A) also showed enhanced activation of Notch and TGF-β on exposure to EGF and IGF-1 (Figure 3).

4.4. Increase in Expression of EMT Promoting Proteins in Response to EGF and IGF-1 Treatment

In order to further investigate the role of EGF and IGF-1 in regulating the EMT promoting genes, we examined the change in endogenous protein expression of some EMT promoting genes in response to treatment with EGF and IGF-1 via western blotting in MDA-MB-231, MDA-MB-468 and MCF10A cells. The expression of all the three proteins studied varied in response to the treatment with EGF and IGF-1. All the cells were treated with EGF alone, IGF-1 alone and EGF in combination with IGF-1. The expression of Snail1, NF-κB, Notch1 proteins was examined in the cell lines post treatment. β-actin was used as loading control. We saw that the expression of all the three proteins increased in response to treatment with EGF and IGF-1, with the expression being the highest when cells were exposed to EGF and IGF-1 together (Figure 4).

4.5. Gene-Gene Interactions (GGI) Analysis of EMT - Related Genes

We performed GGI analysis to study the interactions of EMT-related genes viz snail1, snail2, zeb1, and zeb2 with EGF and IGF-1. GGI analysis revealed key associations between these genes with TGF-β central to IGF-1 mediated and EGF-mediated signaling cascades. Furthermore, pathway enrichment analysis via Gena Mania revealed the involvement of IGF-1 in the positive regulation of cell migration. Also, IGF-1 activated SNAIL1 was found to regulate the canonical Wnt signaling pathway. TGF-β, which is started by both EGF and IGF-1, was found associated with epithelial to mesenchymal transition, canonical Wnt signaling pathway, positive regulation of migration, by pathway enrichment analysis (Figure 5). Furthermore, IGF-1 showed a high score with KPNA3 and IGFBP5 genes.

4.6. EGF and IGF-1 Treatment Increases Cell Proliferation in Breast Carcinoma Cells

We next analyzed the consequences of EGF and IGF-1 treatment on cell proliferation in MDA-MB-231, MDA-MB-468 and MCF10A cell lines. The proliferation levels were determined in these cells after they were exposed to EGF, IGF-1 and EGF in combination with IGF-1 using the MTT assay. Results showed that treatment of cells with EGF, IGF-1 and EGF/IGF-1 together markedly increased cell proliferation in MDA-MB-231 and MDA-MB-468 cell lines, with the highest proliferation seen in cells exposed to EGF and IGF-1 simultaneously. The proliferation levels post-treatment was considerably lower in breast control cell line MCF10A. These results demonstrate that exposure to EGF and IGF-1 leads to higher cell proliferation in metastatic cells as compared to non-cancerous cells (Figure 6).

4.7. EGF and IGF-1 Treatment Increases Cell Migration in Breast Carcinoma Cells

We analyzed the migratory potential of MDA-MB-231 and MCF10A cells treated with EGF, IGF-1 and EGF and IGF-1 together using wound healing assays Results of the wound healing assay revealed that cell migration into the wound gap was less in MCF10A cells as compared to the breast cancer cell line MDA-MB-231 cell line under the same treatments. The maximum migration was seen when MDA-MB-231 cells were treated with EGF in combination with IGF-1, when compared to their control counterparts. The wound healing assay suggests that treatment with EGF and IGF-1 increased the migration potential of MDA-MB-231 breast cancer cells as compared to the normal MCF10A cell line (Figure 7).

5. Discussion

Metastasis is the chief cause of mortality among cancer patients. As a result, determining the origins of metastasis is an essential topic in cancer research. Cancer cells have hijacked the epithelial-to-mesenchymal transition (EMT), which was first observed during embryogenesis as a process of cellular phenotypic trans differentiation from immobile epithelial cells to motile mesenchymal cells[6,23]. Epithelial tumor cells lose their polarity and close connections with adjacent cells due to EMT but gain the capacity to move and invade, avoid apoptosis, and reclaim stemness characteristics. EMT has been eagerly advocated as a necessary stage in the creation of metastasis because EMT-associated characteristics fit the parameters for metastasis formation. WNT, TGF- β, NOTCH, and growth factor receptor tyrosine kinases are only a few embryonic signal transduction pathways involved in initiating EMT under specific

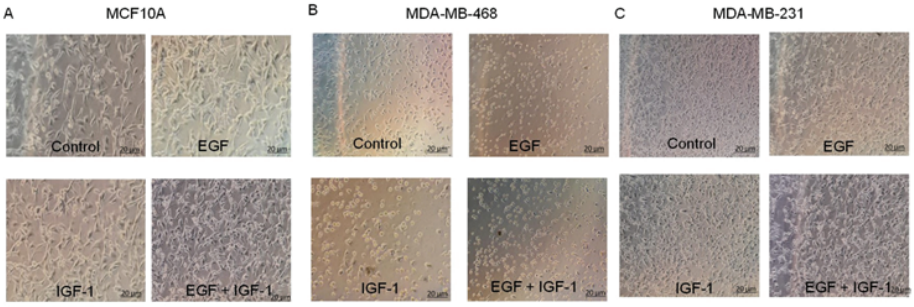

Figure 1: EGF and IGF-1 induced morphological change of MDA-MB-468 and MDA-MB-231. The morphology of MCF10A remains unchanged post treatment. Cells were treated with different doses of EGF and IGF-1 for 24 h, and the change in morphology was imaged by inverted microscopy.

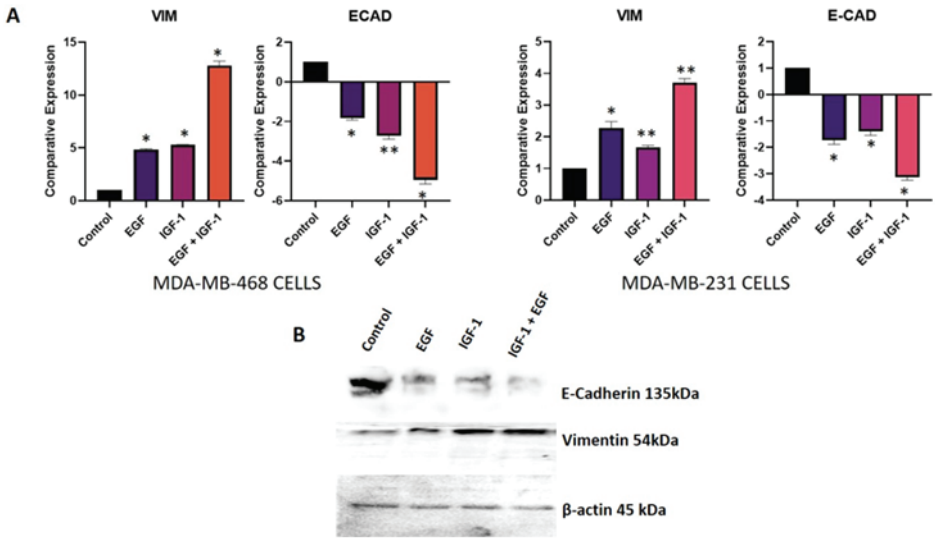

Figure 2: Exposure of breast cancer cells to EGF and IGF-1 decreased the expression of epithelial markers. (A)The expression of E-cadherin significantly reduced in MDA-M-231 and MDA-MB-468 cells upon exposure to EGF or IGF-1, while the expression of Vimentin increased under the similar conditions. (B) The protein expression of E-Cadherin and Vimentin was examined in MBA-MD-231 cells before and after treatment with EGF and IGF-1. Protein expression of all Vimentin protein increased in response to the treatment and the highest expression was observed in the cells treated with EGF and IGF-1 together, while as the protein expression of E-Cadherin decreased under the similar conditions in MDA-MB-231 cells. B-actin was used as a loading control. Data are mean ± SEM. p values determined by two-way ANNOVA followed by multiple comparisons test (* = p value 0.001, ** = p value 0.0001).

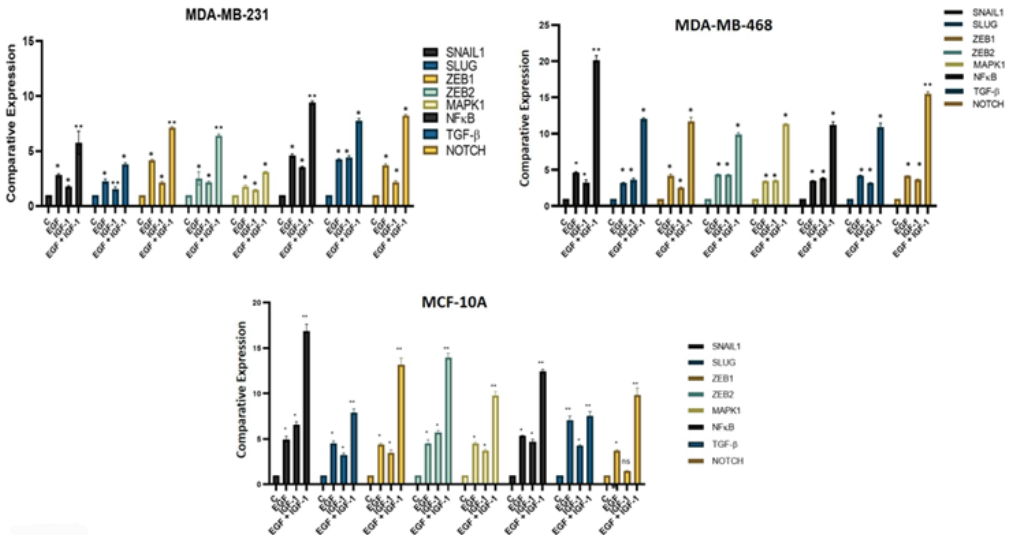

Figure 3: Exposure of breast cancer cells and normal breast cells to EGF and IGF-1 enhanced expression of EMT-related TFs. The expression of TFs in MDA-MB-231, MDA-MB-468 and MCF10A increased upon exposure to EGF or IGF-1, as compared to the control. Highest expression was observed in cells that were treated with EGF and IGF-1 together. Data are mean ± SEM. p values determined by two-way ANNOVA followed by multiple comparisons test (* = p value 0.001, ** = p value 0.0001).

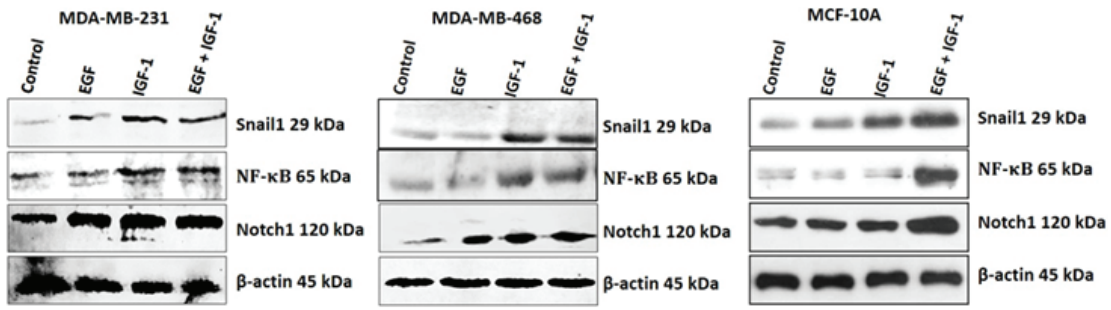

Figure 4: Treatment of MDA-MB-231, MDA-MB-468 and MCF10A cells with EGF and IGF-1 increases protein expression. The protein expression of Snail1, NF-κB and Notch1 was examined in MBA-MD-231, MDA-MB-468 and MCF10A cells before and after treatment with EGF and IGF-1. Protein expression of all three proteins increased in response to the treatment and the highest expression was observed in the cells treated with EGF and IGF-1 together. B-actin was used as a loading control.

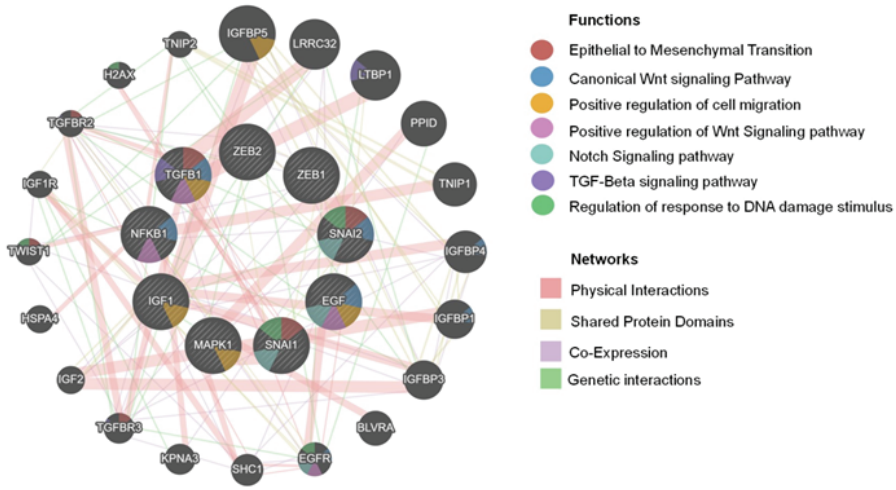

Figure 5: GGI network analysis of EMT-related genes.

physiological circumstances. TGF- β, a cytokine produced in the tumor microenvironment by tumor cells and stromal fibroblasts, is considered to be a significant activator of EMT [24].The IL6/STAT signalling pathway, inflammatory cytokines such as TNFα through NFkB and hypoxia via HIF1, and extracellular matrix (ECM) stiffness are all implicated in EMT induction [25].In the present study, we explored the effect of EGF and IGF-1 on the expression of EMT-related genes in breast tumor cells and normal breast cell line MCF10A.The most abundant growth factors present in the tumor microenvironment are EGF and IGF-1, which induce EMT in a variety of cancer cells. In the present study, it was demonstrated that exposure of metastatic breast tumor cell lines to EGF and IGF-1 induced morphological changed from spindle to a circular form with cells losing cell-cell contacts and developing a mobile form. The change in morphology was not observed in case of normal breast cells and neither was there a reduction in cell-cell contact in case of MCF10A cell line. The morphological change in breast cancer cells treated with EGF plus IGF-1 was substantial, showing that IGF-1 and EGF signaling cascade cross-talks stimulated phenotypic change in breast tumor cells. In addition, in comparison to the control, EGF and IGF-1 treatment reduced cell-cell interactions indicating that growth factors in the TME prompt tumor cells to acquire a mesenchymal phenotype with enhanced migration potential. A recent study also proved that EGF promotes EMT in colorectal cancer via activation of NFkB pathway and enhanced migration and invasive potential of tumor cells [26].Gene expression studies indicated that EGF or IGF-1 treatment enhanced the expression of EMT-related TFs viz, snail, slug, zeb1, and zeb2 in breast cell lines. Furthermore, when exposed to EGF and IGF-1, the expression of EMT-related TFs increased substantially. Our findings indicate high expression of EMT TFs, viz zeb1 and zeb2, snail1and snail2, upon exposure to both EGF and IGF-1, indicating that EGF and IGF-1 signaling cascades cross-talk in tumor cells and promote a mesenchymal phenotype. EMT TFs are activated to coordinate the EMT program in response to various EMT-inducing stimuli and their downstream signaling pathways. SNAIL family zinc finger transcription factors (snail1and snail2), zinc finger Ebox binding homeobox proteins (zeb1 and zeb2), and TWIST family basic helix–loop–helix transcription factors are the most important EMT TFs (twist1 and twist2). To suppress ECAD transcription and promote EMT, the SNAIL and ZEB transcription factor superfamilies bind to Ebox domains in E-cadherin promoters. [27]. Occludin, claudins, ZO1, and connexins are members of the tight junction proteins that are suppressed by SNAIL and ZEB families [25]. Moreover, high expression of zeb1 in TNBC patients correlates with a worse prognosis, indicating that transition from epithelial to mesenchymal phenotype reduces survival probability of TNBC patients [28].Furthermore, the expression of E-cadherin, an epithelial marker, was reduced in tumor cell lines and in MCF10A exposed to EGF and IGF-1. Likewise, the combinational treatment of EGF and IGF-1 showed augmented VIM expression and downregulated E-CAD, further validating their cross-talk in EMT. A previous studies demonstrated that inhibition of IGF-1 using recombinant CCN6 protein reduced zeb1 expression in breast tumor cells which further reduced invasive potential of tumor cells, indicating the role of IGF-1 in BC [29]. Also, zeb1 has been found to interact with ELK3 and decrease expression of E-Cadherin in TNBC cells [30]. These studies indicate that TME mediated activation of signalling cascades in breast tumor cells are central to the EMT phenomenon and provide an avenue for the management of breast cancer patients.We further investigated the expression of TGF-β

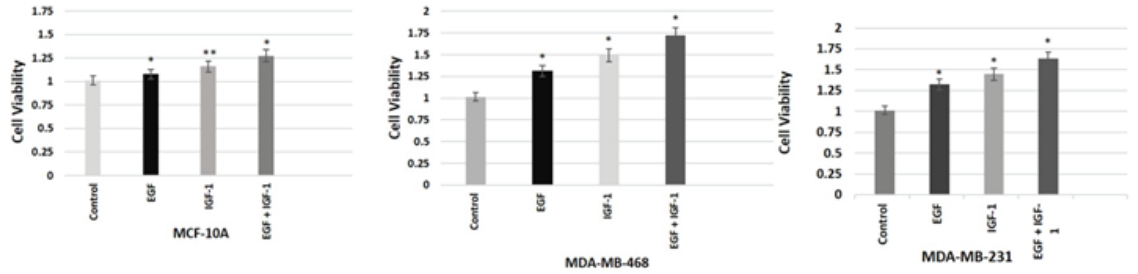

Figure 6: Cell proliferation increases in breast carcinoma cell lines in response to EGF and IGF-1. MDA_MB-231, MBA-MD-468 and MCF10A cells were treated with EGF, IGF-1 and EGF in combination with IGF-1. Cell proliferation markedly increased in metastatic breast cancer cells post treatment, as compared to normal breast cell line. Cell viability assay represented as mean ± S.E. (n=3), and $p<0.05$ was considered significant. Assay was performed in triplicate and repeated at least three times independently for each cell line. Data was analyzed using two-way analysis of variance (ANOVA) and Dunnett's multiple comparison post hoc tests. Two-way ANOVA indicated there was a significant difference overall for EGF + IGF-1 treatment ($p<0.05$) and the posttest indicated that there were differences against each control ($p<0.05$) as displayed in graph.

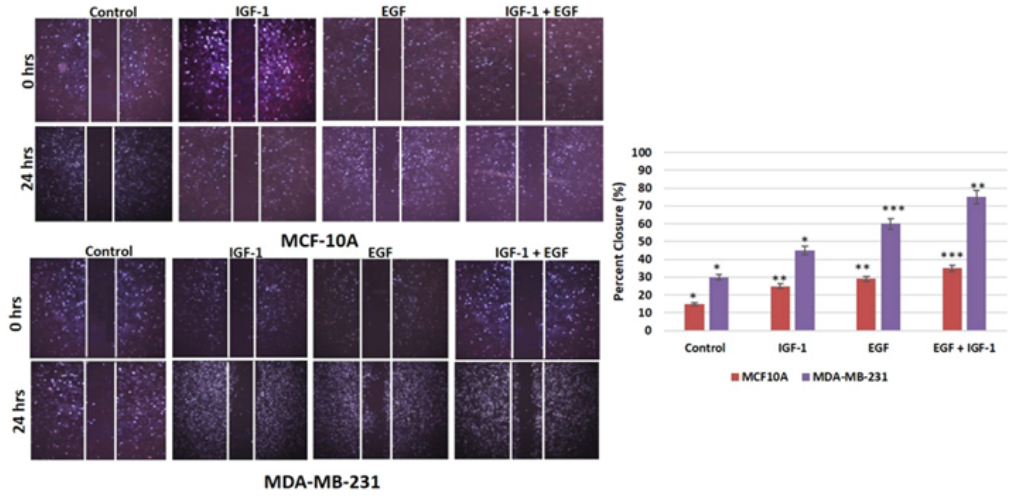

Figure 7: Cell migration increases in breast carcinoma cell lines in response to EGF and IGF1. Representative photographs depicting wound healing potential of the cells treated with EGF, IGF1 and EGF in combination with IGF-1 in comparison to control. The bar graph shows fold-change in migratory potential in treated cells in comparison to control. Cell migration markedly increased in metastatic breast cancer cells post treatment, as compared to normal breast cell line. Results are expressed as mean ± SD from three experiments. The statistical analysis was done using one-way ANOVA followed by Dunnett`s multiple comparison test to determine level of significance. (*P < 0.05, **P < 0.01, ***P < 0.001.).

and Notch signaling and found that EGF and IGF-1 combinational treatment increases the expression of Notch and TGF-β. MAPK1 and NFkB mRNA levels were also measured in the treated cells, and substantial upregulation was found. Cells treated with both EGF and IGF-1 showed markedly increased expression of NFkB, MAPK1, Notch, and TGF-β mRNA levels. Previous research has shown that co-expression of snail and slug enhances TGF-β gene expression and contributes to increased histone acetylation at the TGFBR2 locus in breast carcinomas [31]. The synthesis of TGF-β in response to the activation of the EMT programme implies a feedforward loop that helps cells sustain the EMT programme after it has been initiated. TGF-β can control the expression of EMT-TFs by inducing post-translational changes, according to many recent findings. TGF-β activates snail by promoting sumoylation at the Lys234 residue of snail, which is required for its ability to generate EMT, according to one research [32]. Also, NOTCH has been reported to cross-talk with the TGF-β pathway via SMADs stimulating EMT in tumor cells. Furthermore, TGF- β stimulates the secretion of NOTCH ligands such as Jagged1, which acts autocrine on the cells promoting EMT [33]. To conclude our study more unambiguously, we studied the protein expression of some of the most important genes of our study vis-à-vis Snail1, NF-κB and Notch1 in breast carcinoma cell lines. We found that the treatment of the cells with EGF, IGF-1 and both together lead to an increase in the protein expression of these genes with the highest expression being found in the cells treated with EGF and IGF-1 in combination. In addition to this, we also conducted cell proliferation assay, and we observed that cell proliferation increases in metastatic breast carcinoma cell lines post treatment with EGF and IGF-1, with the higher proliferation seen in cells which were exposed to EGF and IGF-1 together. Interestingly, no significant change in cell proliferation was observed in the control breast cell line, MCF10A, under the same conditions. To conclude our study, we performed wound healing assay to check the cell migration potential. We observed that cell migration increases in metastatic breast carcinoma cell lines post treatment with EGF and IGF-1, with the higher proliferation seen in cells which were exposed to EGF and IGF-1 together. No significant change in cell migration was observed in the control breast cell line, MCF10A, under the same conditions. Carcinomas with a more significant proportion of mesenchymal cells are found to be less susceptible to cytotoxic therapies. Together these studies indicate that targeting the EGFR/ IGF-R signaling in addition to conventional therapies will substantially reduce tumor growth and progression and prolong survival of cancer patients. Moreover, EMT being a requisite step in tumor development, seems a plausible target for triple-negative breast cancer treatment owing to its aggressive behavior.

6. Conclusion

The understanding of the underlying mechanistics of breast cancer will shed light on new and promising therapeutic and prognostic avenues, that will in turn alleviate the disease progression and help in better treatment. One of these strategies can be targeting the signaling cascades involved in the up-regulation of EMT related genes, viz IGF-1 or EGF mediated activation. This approach might be a potential strategy to inhibit breast cancer metastasis and prolong survival of cancer patients.

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