# *In silico* Analysis Revealed hsa-miR-19a-3p and hsa-miR-19b-3p As Two Potential Inhibitors of EGF mRNA in Breast Cancer

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A thesis submitted to the Department of Mathematics and Natural Sciences in partial fulfillment of the requirements for the degree of
Bachelor of Science in Biotechnology

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# Declaration

It is hereby declared that,

**1.** The submitted thesis is my original work while studying at Brac University.

**2.** The thesis does not contain previously published material, nor was it written by a third party, unless appropriately cited through full and accurate reference.

**3.** No content in the thesis has been approved or submitted for any other degree or certificate at a university or other institution.

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# Ethics statement

While conducting the thesis, I was committed to uphold the highest ethical standards in all aspects of my actions and decisions. My commitment to ethical practices underscores my dedication to trust, responsibility, and the pursuit of moral excellence.

# Approval

The thesis titled "*In silico* Analysis Revealed hsa-miR-19a-3p and hsa-miR-19b-3p As Two Potential Inhibitors of EGF mRNA in Breast Cancer" submitted by Nabiha Tahsin Nuha (20136013) of Spring, 2024 has been accepted as satisfactory in partial fulfillment of the requirement for the degree of Bachelor of Science in Biotechnology on 15th February, 2024.

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# ABSTRACT

Breast cancer (BrC) is a highly prevalent and often fatal disease affecting both women and, to a lesser extent, men worldwide. There were about 2.3 million new breast cancer cases worldwide in 2020, resulting in approximately 685,000 deaths. According to the American Cancer Society data, 42,250 women are estimated to die due to breast cancer in the year 2024. A major focus of current research in breast cancer prognosis revolves around chemotherapy, radiotherapy, hormonal therapy, and so on. However, microRNAs (miRNAs) have recently emerged as therapeutic agents, inhibiting pathological pathways by targeting specific cancer-related genes. In this study, we analyzed differentially expressed genes (DEGs) from four datasets of BrC patients to identify potential miRNA to block pathways associated with highly expressed DEGs. The Epidermal Growth Factor (EGF) gene was found to be a common factor in most of these datasets, indicating its potential importance in breast cancer. The overexpression of Epidermal Growth Factor Receptor (EGFR) has been observed in numerous breast cancer patients. The overexpression of EGFR occurs when EGF binds with the receptor and activates it. The activation and overexpression of EGFR eventually leads to the initiation of two common pathways named: PI3K/AKT/mTOR (PAM) pathway and Ras/Raf/MEK/ERK signaling pathway which promotes tumor growth, invasion, and metastasis in breast cancer. In order to inhibit BrC cell growth, we explored potential miRNAs targeting the EGF gene mRNA. Two miRNAs identified by our analysis bind to the conserved region at the 3' UTR of the EGF gene mRNA, namely hsa-miR-19a-3p and hsa-miR-19b-3p. Perhaps inhibiting the EGF activity might effectively inhibit EGFR activation and consequently impede the initiation of pathological pathways in breast cancer using these miRNAs. Overall, our study highlights the importance of the EGF gene in breast cancer and suggests using miRNAs hsa-miR-19a-3p and hsa-miR-19b-3p to inhibit its activity. However, further research is required to fully comprehend the mechanisms underlying the EGF gene's role in cancer development and progression.



**Keywords:** Breast cancer; differentially expressed gene; epidermal growth factor; microRNA; mRNA

# Dedication

I dedicate this piece of work to my late maternal grandmother who is still and will remain alive in my memory till my last breath.

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# List of Acronyms

BrC Breast cancer

miRNA microRNA

mRNA messenger RNA

EGF Epidermal Growth Factor

EGFR Epidermal Growth Factor Receptor

3' UTR 3' untranslated region

DCIS Ductal carcinoma in situ

LCIS Lobular carcinoma in situ

TNBC Triple-negative breast cancer

HER2 human epidermal growth factor receptor

HER2+ human epidermal growth factor receptor positive

WHO World Health Organization

FDA Food and Drug Administration

ErbB Erythroblastic leukemia viral oncogene homologue

Ras Rat sarcoma

Raf Rapidly accelerated fibrosarcoma

MEK Mitogen-activated protein kinase kinase

ERK Extracellular signal‐regulated kinase

PI3K Phosphatidylinositol-3 kinase

AKT/PKB Protein kinase B

mTOR mammalian target of rapamycin

PI3K/AKT/mTOR pathway PAM pathway

PIP2 phosphatidylinositol 4,5 bisphosphate

PIP3 phosphatidylinositol 3,4,4-triphosphate

PDK1 phosphoinositide-dependent protein kinase 1

PTEN Phosphatase and tensin homolog

RTKs Receptor tyrosine kinases

Grb2 Growth Factor Receptor-bound protein 2

SOS Son of Sevenless

GTP Guanosine triphosphate

GDP Guanosine diphosphate

MEK Mitogen-activated protein kinase

Ago Argonaute

GW182 Glycine-Tryptophan Protein of 182 kDa

RISC RNA-induced silencing complex

OncomiRs Oncogenic miRNAs

DEGs Differentially expressed genes

GEO Gene Expression Omnibus database

KEGG Kyoto Encyclopedia of Genes and Genomes

GED Gene Expression Display Server

BRCA1/2 Breast Cancer Gene ½

MAPK Mitogen-activated protein kinase

# 1. INTRODUCTION

Cancer is an indicator term used for diseases in which abnormal cells develop and grow in uncontrollable fashion in a person. These abnormal cells can form tumors and interfere with the normal functioning of organs and tissues in the body. The disease can easily spread throughout the body and decreases the chance of survival for a person. There are multiple types of cancer that have been observed in humans, among which - breast cancer, is one of the most common ones. Breast cancer is one of the most prevalent cancers diagnosed in women (Alkabban & Ferguson, 2022). Breast cancer develops in any of the breast tissues where uncontrollable cell growth can result in the development of this disease. Many factors such as- age, gender, family history, exposure to exogenous hormones such as- estrogen, lack of physical activities, alcoholism etc., can be a cause of this disease (Alkabban & Ferguson, 2022). According to the American Cancer Society (n.d.) data, lump or mass in the breast is one of the common signs of having breast cancer. Other than this, the change in the breast shape, nipple discharge and dimpling, red swollen breast are among some other symptoms of this disease. There is an approximately 2.5% chance of a woman dying due to breast cancer (Ma & Jemal, 2013). Apart from women, men also can fall victim to this disease in their lifetime. As per the data of the Centers for Disease Control and Prevention (2023), approximately 1 in 100 cases of diagnosed breast cancer in the United States is identified in males. Hence, to combat this disease, many therapeutic approaches have been taken so far, which include- radiation therapy, chemotherapy, targeted therapy, surgery, hormonal therapy, and more (Breast cancer - types of treatment, 2012). For example, in hormone therapy, production of hormones that participate in tumor growth and development are inhibited or hormonal behavior is interrupted. One such example is- Tamoxifen, a drug that blocks the binding of estrogen (a catalyst which stimulates breast tissue proliferation and division) to breast cancer cells (Breast cancer - types of treatment, 2012). However, these therapies do also have certain side effects and affect the whole body as well (Team, 2024). Hence the targeted therapeutic approaches are now getting much more attention since these therapies are site specific and designed to target specific molecules or pathways involved in the growth and survival of cancer cells (Zipes, 1990). Hence, the other cells of the body do not get affected in the process, unlike the common therapeutic approaches. The aim of this study was to find a targeted technique to combat breast cancer cell proliferation using microRNA. Recently many

studies suggested microRNA(miRNA) as a potential solution to impede cancer in which they bind to the messenger RNA (mRNA) of the target gene (Macfarlane & Murphy, 2010). This binding with mRNA via a specific region does not allow further transitional activities, inhibiting the protein synthesis as well. Our study suggests that two miRNAs: hsa-miR-19a and hsa-miR-19b-3p can potentially inhibit epidermal growth factor (EGF) mRNA by binding to its 3' untranslated region (3'UTR). However, to confirm this finding, further research in both in vivo and in vitro needs to be conducted.

# 1.1 BACKGROUND

### 1.1.1 Breast cancer and available therapies:

Breast cancer is an intense medical condition which is prevalently diagnosed in women and less observed in men. The breast is composed of glands that produce milk (lobules), ducts that transport milk to the nipple, and supportive tissue, including fatty and connective tissue (Dense breast tissue, n.d.). Breast cancer can develop in any of these tissues. According to Clark and Fallowfield (2002), breast cancer can be divided into two categories based on its relation with the basement membrane: noninvasive and invasive. The noninvasive neoplasms in the breast can be subdivided into: ductal carcinoma in situ (DCIS) and lobular carcinoma in situ (LCIS) among which DCIS and invasive ductal carcinoma (IDC) are the common ones. The less common breast cancer types include: Triple-negative breast cancer (TNBC), Inflammatory breast cancer (IBC) and Paget's disease of the breast (Clark & Fallowfield, 2002). The cause of BrC is most commonly associated with the dysregulation of estrogen receptor and progesterone receptor (Masoud & Pagès, 2017). Generally, to categorize BrC, the expression/ status of both these receptors and human epidermal growth factor receptor 2 (HER2) is checked (Miricescu et al.,2020). The mutation in oncogenes (accelerates cell division) and tumor suppressor genes (inhibits tumor cells) also plays a key role in the development of BrC in humans (Osborne et al., 2004).

Despite being one of the most common and most treated cancer types, BrC is yet a life-threatening disease in women. Unfortunately, approximately 270,000 women are diagnosed with BrC yearly in the USA (Miricescu et al., 2020). According to the World Health Organization (WHO) data (2023), in the year of 2020, 2.3 million women were diagnosed with BrC and approximately 685,000 deaths occurred alongside. The prediction by the American Cancer Society data (n.d.) estimates the death of 42,250 women in the year of 2024. BrC even though is prevalent in women, this can also be observed in men. According to WHO, 0.5–1% of breast cancers occur in men. Apart from the molecular reasons, BrC can occur due to family history, postmenopausal women, alcohol and tobacco use, diabetes mellitus (type II), physical activities etc. are the primary causes of BrC in humans (Miricescu et al.,2020). Currently there are multiple treatment options that are available to treat BrC patients. These include- local

treatment (surgery, radiation therapy) in which the cancer cells are treated without affecting the body and systematic treatments (hormone therapy, targeted drug therapy, immunotherapy, chemotherapy) in which cancer cells can be treated in every part of the body (Breast cancer treatment, n.d.). However, these treatments can equally cause potential side effects and can have limited effectiveness over time. Moreover, these therapies can also have an immense impact on healthy cells as well. Hence, the targeted therapy-based approach has been the center of attention for the longest time.

### 1.1.2 Targeted therapy for breast cancer

Targeted treatment for breast cancer is the utilization of medications that selectively target substances or processes implicated in the proliferation and viability of cancerous cells. These medicines are specifically intended to disrupt the distinctive traits of cancer cells while minimizing harm to normal, healthy cells. According to Masoud & Pagès (2017), the FDA (Food and Drug Administration) authorized the use of the recombinant antibody trastuzumab (Herceptin) in 1998 as the first medicine specifically designed to target HER2 in therapeutic treatment HER2+ (HER 2 positive) breast tumors. Also, promising outcomes were shown in a Phase II study of TNBC research when the monoclonal antibody cetuximab was used in combination with cisplatin treatment. These are few of many examples of targeted therapy that have been used and approved for the therapy of BrC. Targeted treatments represent a notable breakthrough in the treatment of breast cancer, offering more accurate and efficient therapy alternatives. Using microRNA as a site-specific therapy is hypothesized to also inhibit the BrC cell proliferation and, consequently, cancer progression via turning off prominent pathways. Generally, in BrC, two pathways, PI3K/Akt/mTOR pathway and Ras/Raf/MEK/ERK pathway, show more prevalence among others.

### 1.1.3 Prevalent pathways of breast cancer

#### 1.1.3.1 PI3K/AKT/mTOR pathway:

Phosphatidylinositol 3-kinase (PI3K), protein kinase B (PKB/AKT) and mammalian target of rapamycin (mTOR)- PAM pathway or PI3K/AKT/mTOR pathway plays a key role cell proliferation, apoptosis resistance, progression and development of tumor cells. Generally, PAM pathway plays an essential role in cellular activities, which includes cell growth, metabolism,

proliferation etc (Miricescu et al., 2020). The abnormal prevalence of this pathway is seen in almost 70% of breast cancer (BrC) patients (Zhu et al., 2022). The PI3K is a plasma membrane bound lipid kinase which falls into three classes, - class I, class II and class III, in mammals (Miricescu et al., 2020). Moreover, PI3K class I is subdivided into three classes- PI3Ks class IA, PI3Ks class IB and PI3Ks class IC. According to Paplomata & O'Regan (2014), the class IA of PI3Ks contains a heterodimer which consists of one regulatory subunit (p85α, p85β, p85γ), encoded respectively by PIK3R1, PIK3R2 and PIK3R3, and one catalytic subunit p110 (p110α, p110β, p110γ or p110δ) encoded by PIK3CA, PIK3CB and PIK3CD respectively. The study also suggests that, regulatory subunit controls the activation of the catalytic subunit based on the availability or the absence of upstream stimulation by RTKs. Generally, the class I PI3Ks, PKB/AKT and mTOR in the PAM pathway, shows abnormal activation in human cancers and influences cell proliferation and survival (Zhu et al., 2022). According to Miricescu et al. (2020), PIK3CA is the commonly mutated gene in human neoplasms and 30-40% of BrC patients have been seen to have PIK3CA mutation in them. The study reveals that PI3K gets activated upon the binding of ligands such as - growth factors (EGF), insulin, hormones to receptor tyrosine kinase (RTKs) and also to G-protein-coupled-receptors (GPCR). The activated receptor then activates the PI3K which in return phosphorylates phosphatidylinositol 4,5 bisphosphate (PIP2) at the 3' position of the inositol head group. Consequently, this activity generates phosphatidylinositol 3,4,4-triphosphate (PIP3) which recruits, AKT (a serine-threonine kinase) and PDK1(phosphoinositide-dependent protein kinase 1) to the plasma membrane using respective pleckstrin homology interaction domains (PH domains). Following the recruitment to plasma membrane, the AKT is activated by phosphorylation on the hydrophobic motif, Ser473 by mTOR complex 2 (mTORC2). This allows further phosphorylation of AKT by PDK1 at Thr308 position, resulting in fully activated AKT. The activated AKT inhibits tuberous sclerosis 1/2 (TSC1/2) by phosphorylating TSC2 at the serine 939 and threonine 1462 sites (Paplomata & O'Regan, 2014). The study of Miricescu et al. 2020, observed that, Rheb (a Ras-related GTPase) cannot remain in its inactive state due to consequent events within TSC2. As a result, Rheb-GTP is converted from Rheb-GDP, which was previously inactive, thereby aiding in the activation of mTORC1, which influences cellular metabolism and promotes anabolic growth. Thus, phosphorylation of target proteins by AKT leads to the activation of cell survival, growth, and proliferation. According to the authors, the activation of AKT facilitates the control of various

cellular processes, including the cell cycle, growth, proliferation, and energy metabolism. After the phosphorylation in plasma membrane, AKT loses its connection with it and continues phosphorylation of other target proteins in cytosol and nucleus. The PTEN (phosphatase and tensin homolog) is a phosphatase which specifically dephosphorylates PIP3 to PIP2, resulting in the inactivation of AKT and down regulation of the PAM pathway. According to Carbognin et al. 2019, the PTEN thus works as a negative regulator which has been mutated in many breast cancer cases. Their study also discovered that mutation of PTEN gene results in abnormal transduction of PI3K signaling which ultimately leads to uncontrollable cell growth and tumor progression.

#### 1.1.3.2 Ras/Raf/MEK/ERK pathway:

The Ras/Raf/MEK/ERK pathway has been shown to get activated in tumors via BCR-ABL chromosomal translocation, cytokine receptor mutation, i.e- Flt-3, Kit or receptor overexpression, such as- EGFR (McCubrey et al., 2007). Dysfunction of this pathway is the key reason behind many types of cancer, which includes breast cancer. This pathway also has cell proliferation and anti-apoptotic effect, which causes cancer cells to survive. The various elements of the cascade exhibit significant variability in human malignancies (McCubrey et al., 2007).

When any ligand such as- viruses, cytokines, growth factors or G-protein-coupled receptors bind to the RTKs (receptor tyrosine kinases), it activates the Ras/Raf/MEK/ERK pathway (Guo et al., 2020). Binding of the ligand to its receptor initiates RTK activation, dimerization and autophosphorylation of tyrosine residues (Ullah et al., 2022). The binding of ligand-receptor also initiates the Shc protein to bind with the receptor consequently which allows the Grb2(Growth Factor Receptor-bound protein 2) and SOS (Son of Sevenless) to form a complex (McCubrey et al., 2007). Subsequently the phosphorylation of tyrosine residue is recognized by Grb2 (Ullah et al., 2022; Guo et al., 2020). According to Guo et al. 2020, The receptor-Grb2-SOS complex is formed when Grb2 binds to the activated receptor and associates with the proline-rich sequence located at the C-terminus of SOS. According to their study, Ras, is an upstream GTP-binding protein involved in this cascade, which is encoded by Hi-ras, Ha-ras, and N-ras oncogenes (ras gene family). Also, Ras has an inactive GDP binding conformation and an active GTP binding

conformation. Ras protein can alternatively change its conformation to regulate the signal transduction. The study revealed that a high concentration of SOS is found in close proximity to Ras when it is translocated from the cytoplasm to the membrane via its binding to the tyrosine phosphorylation site on the receptor or receptor substrate protein. Thus, the Ras pathway is initiated when Ras is stimulated to replace GDP with GTP, which is facilitated by SOS and Ras-GDP. This activity consequently makes Ras active and allows the GTP bound Ras to recruit Raf protein into the cell membrane (McCubrey et al., 2007). According to Guo et al. 2020, the Raf protein is serine/threonine kinase which is composed of 648 amino acids. In this cascade, the Ras protein translocates Raf protein from cytoplasm to cell membrane, using two domains: cysteine-rich domain at the N-terminus of Raf-1 and the Ras-binding domain. According to the study of McCubrey et al. 2007, the activated Raf then activates dual specificity protein kinases: MEK1 (Mitogen-activated protein kinase/ERK kinase) and MEK2. Upon activation of Raf, its C-terminal catalytic domain can bind to MEK, leading to phosphorylation of the serine residue in its catalytic VIII subregion, therefore activating MEK. According to the study of Guo et al. 2020, ERK (Extracellular-signal-regulated kinases 1,2) are serine/threonine kinases that are positively controlled by phosphorylation, which is facilitated by MEK1 and MEK2. In addition, when several kinases phosphorylate MEK, the active MEK directly interacts with ERKs through its N-terminal region. The authors also discovered that this interaction catalyzes the phosphorylation of Tyr and Thr residues in a bispecific manner. Specifically, ERK1 is phosphorylated on T202/Y204 and ERK2 is phosphorylated on T183/Y185. This action triggers the activation of ERKs, which are then transported to the nucleus (Ullah et al., 2022; Guo et al., 2020). The study also reveals that the activated ERKs facilitate the phosphorylation of target proteins in the cytoplasm or modulate the function of other protein kinases. In addition to activating ERKs, transcription factors are also activated, which in turn control several physiological processes by modifying the profile of gene expression (Ullah et al., 2022). In addition, ERK has the ability to activate p90Rsk, a 90 kDa ribosomal S6 kinase, which in turn activates CREB, a transcription factor (McCubrey et al., 2007). Thus, ERK/MAPK signaling cascade is responsible for the cell proliferation and differentiation signals which ultimately contribute to cancer cell development.

### 1.1.4 Function of EGF and EGFR in breast cancer

These two pathways are initiated by ligand-receptor binding in which the epidermal growth factor (EGF) binds to the epidermal growth factor receptor (EGFR) which leads to its overexpression. The EGFR belongs to the ErbB receptor (erythroblastic leukemia viral oncogene homologue) family, which is a subset of the receptor tyrosine kinase superfamily (Purba et al., 2017). EGFR is a transmembrane glycoprotein of 170,000 D which contains a transmembrane domain, an extracellular domain, a kinase domain, a juxtamembrane (JM) segment & a C-terminal regulatory tail (Boonstra et al., 1995; Purba et al., 2017). This receptor tyrosine kinase (RTK) works as a receptor for EGF and becomes activated subsequently by ligand-induced dimerization. Generally, the epidermal growth factor, which is a single polypeptide consisting of 53 amino acid residues, binds to EGFR (Boonstra et al., 1995). The EGF binding domain has been found in between two cysteine-rich domains of the external domain (Boonstra et al., 1995). Numerous site-specific mutagenesis investigations of the ligand have demonstrated that a number of EGF amino acid residues—including Tyr13, Ile23, Arg41, and Leu47—are essential for EGFR binding (Ogiso et al., 2002). According to Boonstra et al. (1995), the Arg41 has been seen to play an important role in ligand-receptor interactions. The binding of EGF to EGFR leads to a conformational shift of the receptor molecule. Consequently, this activity results in an enhanced affinity of the receptor towards adjacent receptors (Boonstra et al., 1995). According to Magkou et al. (2008), this activity promotes the hetero and homodimer formation between receptors. This receptor dimerization induces the autophosphorylation of the tyrosine kinase domain. The phosphotyrosine residues allow EGFR to activate downstream signaling pathways, including Ras/Raf/MEK/ERK and PI3K/AKT/mTOR signaling pathways (Magkou et al., 2008).

### 1.1.5 Overexpression of EGFR in breast cancer

EGFR has a crucial role in several cellular processes such as cell proliferation, differentiation, motility and survival. Multiple studies have revealed the significant contribution of overexpressed EGFR in the development and proliferation of breast cancer. EGFR gene amplification has been reported to cause excessive production of EGFR in several prevalent malignancies, such as breast carcinomas (Purba et al., 2017). This phenomenon is particularly prominent in aggressive inflammatory breast cancer (IBC) and triple negative breast cancer

(TNBC) (Masuda et al., 2012). Also, TNBC exhibits EGFR overexpression in a minimum of 50% of cases, surpassing the rate in that of other subtypes of breast cancer. In normal cells, EGFR expression is 40,000–100,000 receptors per cell whereas cancer cells express more than $10^6$ receptors per cell (Wee & Wang, 2017). EGFR overexpression can occur due to EGFR gene amplification and also due to mutation in EGFR (Purba et al., 2017). However, mutations in the EGFR gene are rare, therefore the receptor's broad overexpression in such malignancies is unknown.

### 1.1.6. Role of microRNA against messenger RNA (mRNA)

Since the binding of a ligand (EGF) to its receptor (EGFR) causes the initiation of cancerous pathways, targeting EGF could be a potential solution to inhibit breast cancer cell proliferation. To conduct the solution, microRNAs are hypothesized to be used. MicroRNA or miRNAs are short (21-24 nucleotides) non coding RNAs which are synthesized by endonucleolytic cleavages by two RNase III enzymes named, Dicer and Drosha (Davis-Dusenbery & Hata, 2010). According to this study, the cleavage results in the formation of double stranded RNA products (approximately 22 nucleotides) consisting of miRNA passenger strand and mature miRNA guide strand. The single stranded mature miRNA forms an RNA-induced silencing complex (RISC) with Argonaute (Ago) proteins (Gu & Kay, 2010). The miRNAs bind to the 3’ untranslated region (3’UTR) of the target gene mRNA by imperfect base pairing to regulate post-transcriptional expression (Singh & Mo, 2013). The binding of miRNA to mRNA is not perfect across the mature miRNA sequence and is dominated by the seed region of miRNAs in mammals (Kehl et al., 2017). The seed region is a heptametrical sequence that is typically located at nucleotides 2-7 from the 5' end of the miRNA (Chipman & Pasquinelli, 2019; Kehl et al., 2017). It is highly conserved among different miRNAs and is essential for the specific recognition and binding to complementary sequences in the target mRNA by Watson-Crick pairing (Peterson et al.,2014). Following this binding, the miRNA-RISC complex can inhibit transcriptional expression mainly by: enhanced mRNA degradation and translational inhibition (Davis-Dusenbery & Hata, 2010). The mismatch between miRNA and target sequence is commonly seen in two of these processes in mammals (Gu & Kay, 2010). The mRNA degradation can occur by deadenylation from 3′ end and/or decapping from 5′ end by cellular exonucleases, such as- DCP1/2 (Valinezhad Orang et al., 2014). For transnational inhibition,

both AGO-2 and GW182 are required at the initiation (Cannell et al., 2008). However, the exact mechanism of miRNAs blocking a specific mRNA is still unknown.

### 1.1.7 Role of microRNA in Breast cancer

The significant contribution of miRNAs to the development of cancer, such as cell proliferation, growth, apoptosis, invasion, metastasis etc. has been well demonstrated. The miRNAs can further regulate medication sensitivity/resistance by controlling genes associated with biological activities linked to the response to therapies.

There are three types of microRNA: tumor suppressor miRNAs, metastatic miRNAs and oncogenic miRNAs (oncomiRs) (Szczepanek et al., 2022). The oncomiRs and tumor suppressor miRNAs function as oncogenes and tumor suppressor based on the inhibition of tumor-suppressive and oncogenic target mRNAs (Davis-Dusenbery & Hata, 2010). For example: let-7 expression in lung cancer has been shown to reduce tumor growth in vivo whereas mir-155 was seen to be highly expressed in many cancers which promoted oncogenesis. However, the categorization of miRNAs remains ambiguous due to the fact that certain miRNAs, such as miR-30b/30d and mir-125b , exhibit dual functionality (both as oncogene and tumor suppressor gene) (Szczepanek et al., 2022). In this study, it was also observed that miRNAs can act in sensitizing cancer cells to treatment. One such example would be mir-155 which increases sensitivity of BrC patients in ionizing radiation therapy. miRNAs are highly stable, making them potentially effective non-invasive biomarkers for treatment strategies and early diagnosis in cancer (Garrido-Cano et al., 2022). The main aim for miRNA-based therapies includes two approaches- restoration of the function of tumor suppressor miRNAs or inhibition of oncomiRs by small molecule inhibitors, miRNA sponges, miRNA masking, anti-miRNA oligonucleotides and so on (Menon et al., 2022; Szczepanek et al., 2022) . An approach called “replacement therapy” is used to reintroduce tumor suppressing miRNAs into the tumor microenvironment to control cell proliferation and reduction of oncogenesis (Davey et al., 2021).

Apart from using microRNA as a biomarker, this technique can be employed to inhibit the EGF mRNA in BrC patients. This technique is hypothesized to inhibit the expression of target genes since it is associated with post transcriptional regulation of gene expression. The aim of this

study is to determine the suitable miRNAs that can potentially inhibit the expression of EGF gene and consequently prevent it from binding to its receptor -EGFR and initiating pathways.

# 2. METHODOLOGY

## 2. 1 Identification of specific cancer datasets and gene expression profiles

The Gene Expression Omnibus database (GEO) was used to accumulate the data of BrC patients (Clough & Barrett, 2016) against "breast cancer" as the query word. Selecting the entry type as "datasets "provided 184 data among which only 155 were of humans (*Homo sapiens*). The microarray datasets of four BrC based projects with gene expression profiles were downloaded which excluded cell line experiments and contained a maximum number of samples.

**Table 01: The attributes of the selected datasets**

| Dataset | Total Samples | Selected Samples | Platform | Reference |
|---|---|---|---|---|
| GSE27567 | 162 | Normal = 31<br>Carcinoma = 131 | GPL570<br>[HG-U133_Plus_2] | (LaBreche, H. G. et al., 2011) |
| GSE10797 | 66 | Normal = 10<br>Carcinoma = 56 | GPL571:<br>[HG-U133A_2] | (Casey, T. et al., 2009) |
| GSE9574 | 29 | Normal = 15<br>Carcinoma = 14 | GPL96<br>[HG-U133A] | (Tripathi, A. et al., 2008) |
| GSE26910 | 24 | Normal = 12<br>Carcinoma= 12 | GPL570<br>[HG-U133_Plus_2] | (Planche A et al., 2012) |

### 2.2 Detection of differentially expressed genes (DEGs) in selected datasets

To identify and normalize differentially expressed genes (DEGs) in the selected datasets, GEO2R (Hunt et al., 2022) tool was used. This tool uses both the limma R package and GEOquery tool to perform differential expression analysis (Ritchie et al., 2015). To perform this analysis, the cut off conditions were set as log fold change (log FC) > 1 or (log FC) < -1 and the default p value <0.05 was selected to attain differentially expressed genes in the selected 4 datasets. The volcano plots of DEGs were generated using GEO2R with the negative logarithm (base 10) of the p-values on the y-axis (representing statistical significance) and the log2 fold changes on the x-axis (representing magnitude of change).

### 2.3 Functional enrichment analysis of the differentially expressed genes

The selected genes were arranged in descending order according to their log fold change to analyze their expression levels. The top 20 DEGs were selected for enrichment analysis to gain insights into the biological significance of them. The Enrichr, a web based tool was used to annotate top 20 or in total 80 DEGs from the selected datasets (Kuleshov et al., 2016).

### 2.4 Exploration of the associated pathways in breast cancer

In order to identify and analyze the enriched pathways in breast cancer, the Kyoto Encyclopedia of Genes and Genomes (KEGG), an online database was used. This database facilitates the scientific assessment of gene activities by connecting genomic information with more complex functional information (Kanehisa, 2000). This helped to identify a unique DEG responsible for the progression of breast cancer.

### 2.5 Gene expression analysis between normal and tumor tissues

To validate the hypothesis, the unique DEG- epidermal growth factor (EGF) and its receptor epidermal growth factor receptor (EGFR) gene was selected for gene expression analysis. Gene Expression Display Server (GEDs) (Xia et al., 2019) was used to estimate the gene expression

levels of both EGF and EGFR in both normal and tumor tissues. The log2 FC against The Cancer Genome Atlas (TCGA) (Hutter & Zenklusen, 2018) of all cancer types allowed to distinguish the expression level of both EGF and EGFR gene, particularly in breast cancer .

### 2.6 Prediction and enrichment of the target microRNAs

The potential microRNAs against the EGF gene were predicted using the TargetScan (Agarwal et al., 2015). This online web tool utilizes an algorithm to identify potential biological targets of miRNAs by detecting the existence of conserved 8mer, 7mer, and 6mer regions corresponding to the seed region for every miRNA (Lewis et al., 2005).

# 3. RESULT

### 3.1 Selection and analysis of microarray datasets

The selected 4 microarray datasets were analyzed separately which showed the prevalence of significantly different gene expression of both upregulated and downregulated genes. From the selected data sets, only 20 DEGs from each dataset were selected for further analysis (Table 02). From these selected genes, 04 volcano plots were generated which showed both upregulated and downregulated genes in the selected data sets (Figure 01).

**Table 02: Top 20 DEGs from selected 4 microarray datasets**

| Gene symbol | | | |
|---|---|---|---|
| **GSE27567** | **GSE10797** | **GSE9574** | **GSE26910** |
| ZNF160, PIGL, LOC101930071///LIPE-AS1, DNAJC30, KLHDC10, EVPLL, FOS, USMG5, MEGF6, CCNY, LINC00685, YPEL5, IMMP1L, PTGER1, ALDH1L1, PRKD2 | KIT, CX3CL1, ROPN1B, NTRK2, NDRG2, MFGE8, CX3CL1, SIK1, JUN, KRT15, **EGF** IGHA2///IGHA1///IGH, SLC6A14, DST, NCALD BBOX1, ALDH1A3, JUN MYH11 | PTP4A1, IER2, FOSB NR4A3, ATF3, BTG2 NOL12, FOS, TACSTD2 MIR4738///H3F3B///H3F3A, JUN, EIF1, NR4A2, JUN DUSP1, EIF5, TGFB2, APOH, NR4A2, PTP4A1 | C16orf89, GRK5, PDLIM5, CFD, PENK, MAL2, BMPR1B, BCO2, GFOD1 PDLIM5, KLHL14 GARNL3, SMCO4, BMPR1B, TFPI, RHOJ PPL, GOLM1, CCDC178, FAM189A2 |

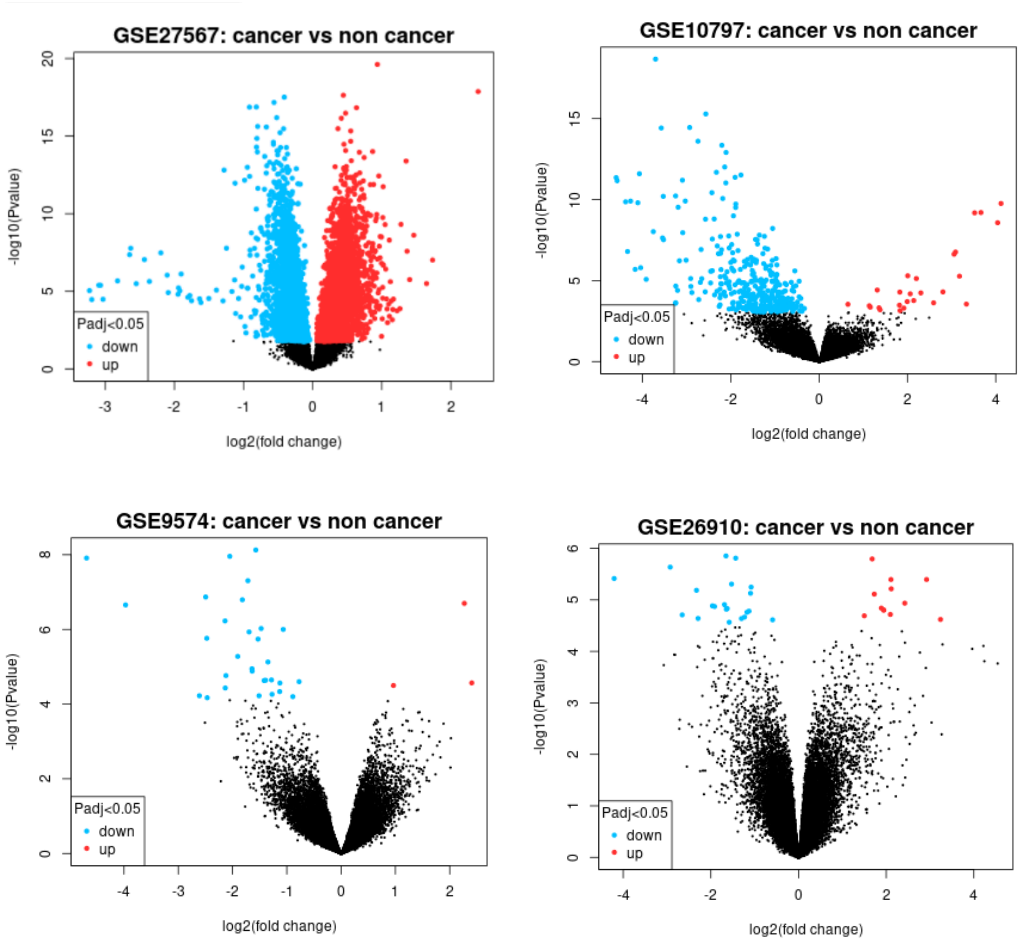


**Figure 01:** Volcano plots of differentially expressed genes (DEGs) from selected 4 datasets wherein blue dots represent downregulated genes and red dots represent upregulated genes.

### 3.2 Functional enrichment analysis of DEGs in breast cancer:

After conducting the functional enrichment analysis of the top 20 DEGs from each of the 4 datasets, the EGF gene was seen to be more enriched than other DEGs. Moreover, it was seen to be associated with the cancer pathways: PI3K/AKT/mTOR pathway and MAPK signaling cascade.

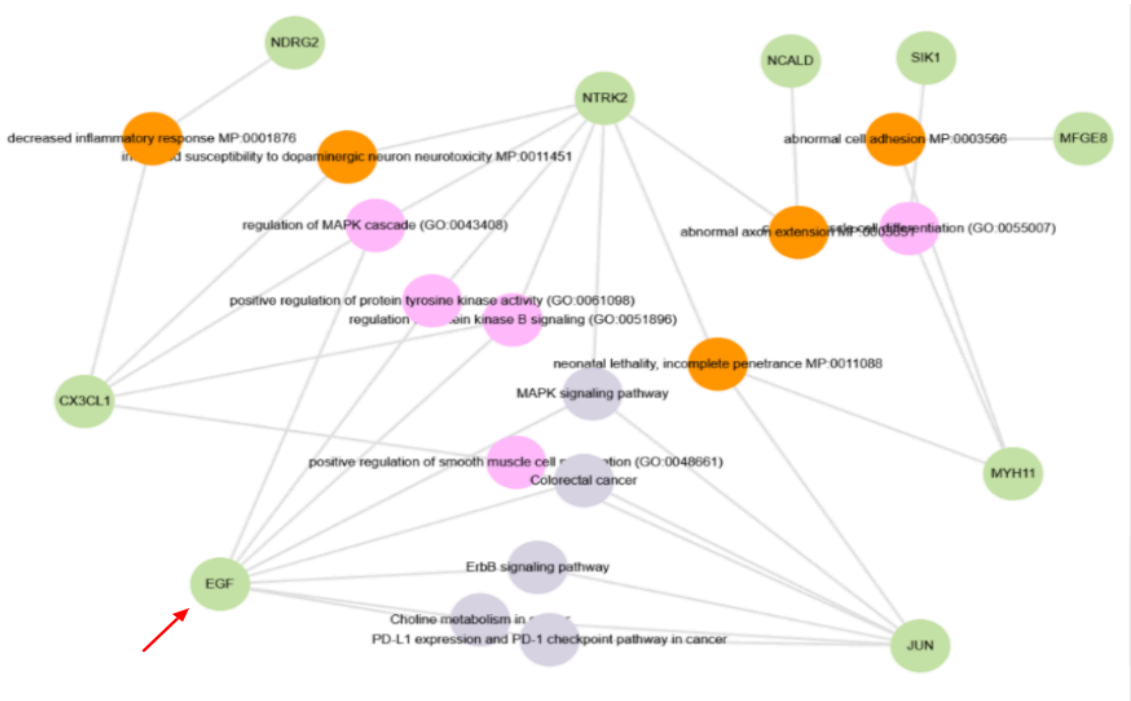


**Figure 02:** The functional enrichment analysis shows EGF to be enriched in PAM and MAPK signaling cascade

### 3.3 The presence of PI3K/AKT/mTORC and Ras-Raf-Mek-ERK pathway in breast cancer:

The enrichment analysis using ENRICHR showed the EGF gene to be significantly associated with breast cancer and its associated - PI3K/AKT/mTORC and Ras/Raf/MEK/ERK pathways. The enrichment analysis showed that the MAPK cascade and PI3K/AKT signaling pathways were enriched which coaligned with the previous findings. The KEGG pathway enrichment analysis showed the involvement of the EGF gene in BrC via two of the above-mentioned pathways. The EGF gene binds as a ligand to the EGFR and HER2, which results in the overexpression of HER2 and EGFR. This initiates the PI3K/AKT/mTOR (PAM) pathway and Ras/Raf/MEK/ERK pathway, which results in cell proliferation, survival, and translation of the oncogenic protein in HER2+ and basal-like/triple-negative breast cancer. Two of these outcomes provided a basis for further hypothesis testing in which the EGF gene would be the prime target for the inhibition of breast cancer.

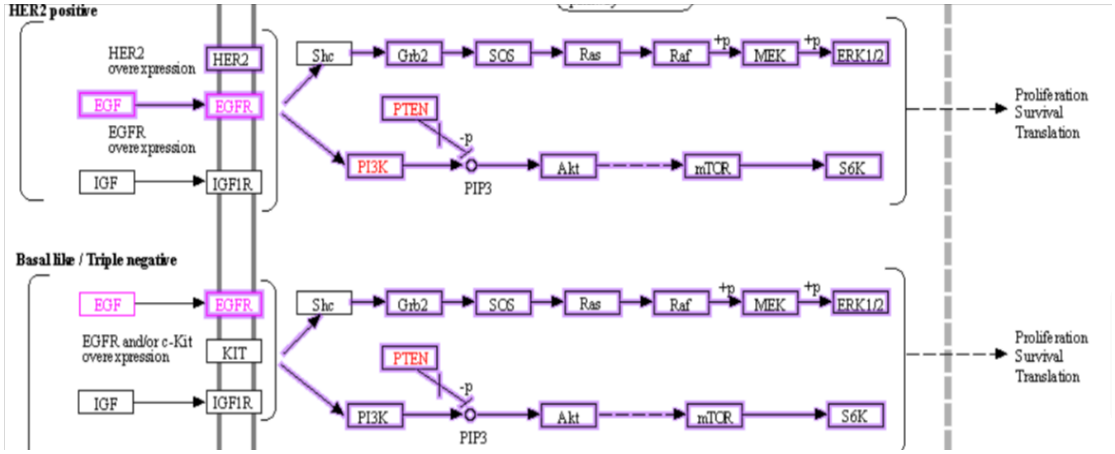


**Figure 03:** The binding of EGF to EGFR initiates the PAM pathway and MAPKcascade.

### 3.4 Analysis of the of the relationship between EGFR and EGF in BrC:

The GEDs output regarding the expression of EGF in both normal and tumor tissues was comparable. The EGF gene expression was significantly higher in tumor tissues comparatively.

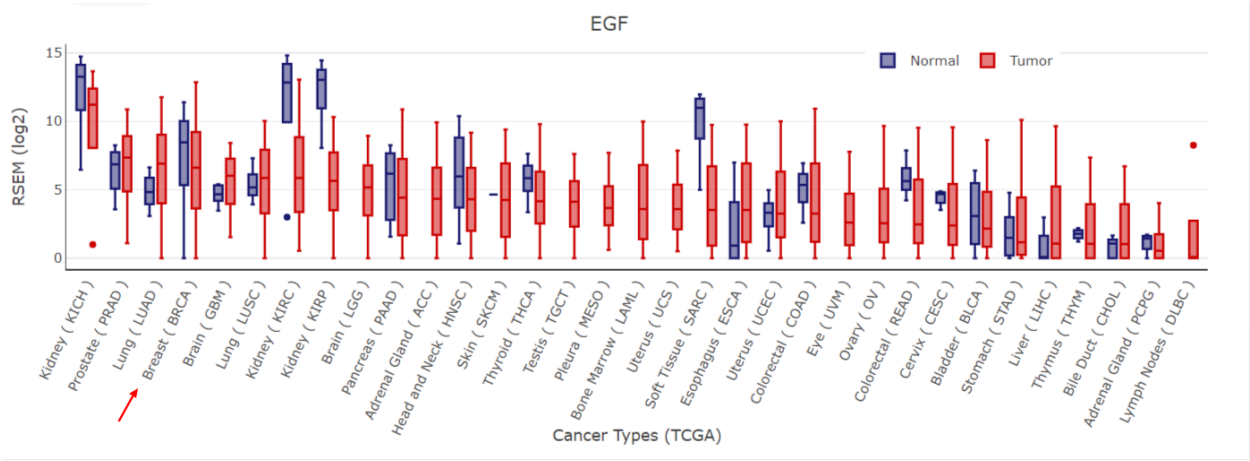


**Figure 04:** Elevated expression of EGF gene in BrC cells compared to normal cells.

However, the binding of EGF to EGFR is the core reason which activates the receptor and leads to its overexpression, consequently initiating cancerous pathways. Hence, to analyze and validate the hypothesis regarding the inhibition of the EGF gene, the expression level of the EGFR gene was compared in both normal and cancerous breast tissues using the GEDS tool. The findings indicate that EGFR genes exhibit elevated expression levels in cancerous tissues compared to normal tissues. Therefore, according to this finding, downregulation of the EGF gene will

consequently prohibit the activation and overproduction of EGFR protein expression, resulting in the inhibition of the breast cancer pathways.

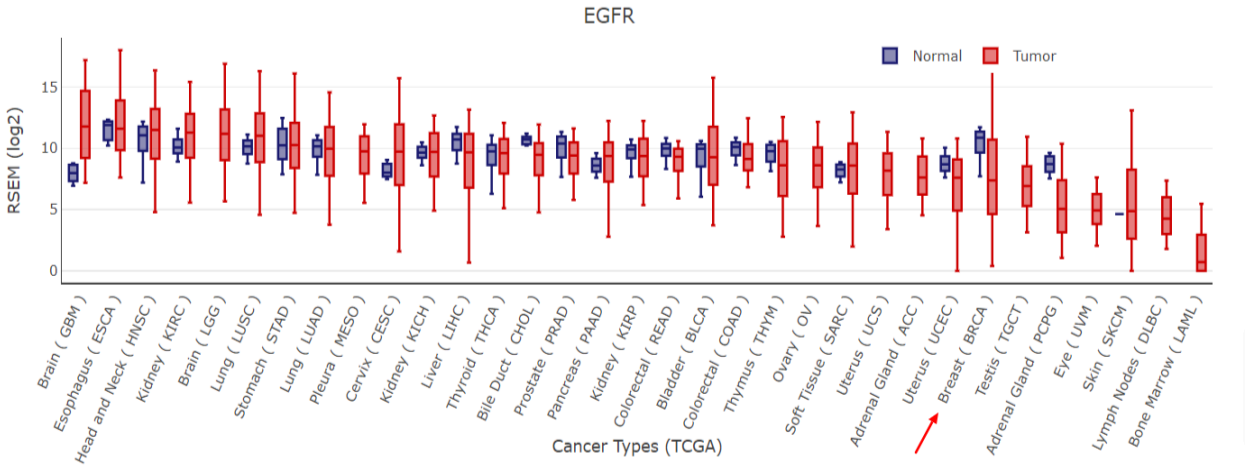


**Figure 05:** The elevated expression of EGFR in breast cancer compared to normal cells.

### 3.5 Inhibition of EGF gene using potential miRNA

Several studies have implicated the role of EGF binding with EGFR which can result in overexpression of the receptor. This overexpression, as analyzed, can initiate oncologic pathways resulting in breast cell tissue proliferation, translation and survival. Hence to combat this situation, we chose to work with microRNAs which have been proven to downregulate the activity of certain genes by binding to the specific 3' untranslated region (3'UTR) of target genes mRNA. Computational analysis from TargetScan analysis showed that two miRNAs- hsa-miR-19a-3p and hsa-miR-19b-3p, both bind to two specific conserved positions on EGF mRNA at 3'UTR. Binding of miRNA to target gene's mRNA allows the repression of target gene by inhibiting translation/ promoting mRNA degradation.

**Conserved**

| | Predicted consequential pairing of target region (top) and miRNA (bottom) | Site type | Context++ score | Context++ score percentile | Weighted context++ score | Conserved branch length | $P_{CT}$ | Predicted relative $K_D$ |
|---|---|---|---|---|---|---|---|---|
| Position 691-697 of EGF 3' UTR<br>hsa-miR-19a-3p | 5' ...UCCCUCUUGGUGUGAUUGCACAG...<br>3' AGUCAAAACGUAUCUAAACGUGU | 7mer-A1 | -0.21 | 86 | -0.21 | 3.706 | 0.58 | -4.013 |
| Position 691-697 of EGF 3' UTR<br>hsa-miR-19b-3p | 5' ...UCCCUCUUGGUGUGAUUGCACAG...<br>3' AGUCAAAACGUACCUAAACGUGU | 7mer-A1 | -0.21 | 86 | -0.21 | 3.706 | 0.58 | -4.013 |

**Figure 06:** Two miRNA: hsa-miR-19a-3p and hsa-miR-19b-3p bind to the 3'UTR or the EGF mRNA at 691-697 position.

# 4. DISCUSSION

Breast cancer is one of the most common cancers in women with lower incidence in men. Annually, BrC accounts for 30% of all cancers in U.S. women and over 1.5 million of women in the world are diagnosed with BrC every year (Sun et al., 2017). Generally BrC is divided into noninvasive and invasive categories, with noninvasive neoplasms being categorized into ductal carcinoma in situ (DCIS) and lobular carcinoma in situ (LCIS) (Alkabban & Ferguson, 2022). Mutation in oncogenes and tumor suppressor genes, dysregulation of estrogen receptor and progesterone receptor etc. are few common causes of BrC in humans (Osborne et al., 2004; Masoud & Pagès, 2017). Alongside, inheritance of BRCA1/BRCA2 (Breast Cancer Gene) mutation can develop the risk of this disease. An estimate of 13-19% patients diagnosed with BrC have first degree relatives affected with BrC as well (Łukasiewicz et al., 2021). Other factors such as postmenopausal women, alcohol and tobacco use, diabetes mellitus (type II), and physical activities can also increase the risk of developing the disease (Miricescu et al.,2020). Hence, despite being a common fatal disease, the rate of deaths due to BrC is still pretty high. At present, BrC patients are offered a variety of treatment alternatives, encompassing both local procedures such as radiation therapy and surgery, as well as systemic treatments including hormone therapy, targeted medication therapy, immunotherapy, and chemotherapy. Nevertheless, these therapies may induce adverse side effects (nausea, headache, depression etc.) and exhibit reduced efficacy as time progresses. For example: Cancer chemotherapy resistance (MDR) is a term in which cancer cells acquire resistance against chemotherapeutic drugs. Thus, the efficacy of the treatment decreases over time (Alfarouk et al., 2015). Also, the interference of an antidepressant named- serotonin reuptake inhibitors (SSRI) in hormone therapy has also been observed (Hormone therapy for breast cancer, 2022) . The SSRIs have been observed to inhibit CYP2D6 enzyme activity which is utilized in effective tamoxifen (hormone) operation (Stubbs et al., 2017). Apart from such incidents, endometrial cancer has been observed in women taking tamoxifen drugs too (Tamoxifen and Uterine Cancer, 2008). Therefore, advanced therapies are required to inhibit cancer cell proliferation and progression in BrC patients. Apart from the above-mentioned therapies, many targeted therapies have been used to treat BrC patients in many cases. Targeted therapies aim at specific proteins and pathways that play a role in the cancer's development (Zipes, 1990). This precision targeting helps to minimize damage to healthy cells and reduce side effects. The U.S. Food and Drug Administration (FDA) has

approved many novel targeted therapies, which includes- cyclin-dependent kinase (CDK) 4/6 and poly (ADP-ribose) polymerase (PARP) inhibitors (Arora et al., 2022) Apart from these, aromatase inhibitors, such as-anastrozole, has been employed as an alternative targeted therapy in which the biosynthesis of androgens is impeded via inhibition of the aromatase enzyme which results in decrease in estrogen levels in estrogen dependent cancer. The initiation of BrC starts with many growth factors which can bind as ligands with their receptors (Masoud & Pages, 2017). One such ligand is the epidermal growth factor, which is a single polypeptide with 53 amino acid residues (Boonstra et al., 1995). This ligand binds with EGFR which is a receptor tyrosine kinase, and activates it by ligand binding dimerization. One of the most common receptors that has been overexpressed by 40% times in BrC is EGFR (Masoud & Pages, 2017). Therefore, impeding the ligand binding to EGFR may serve as a viable strategy to inhibit the progression of the cancerous cell and its associated pathways.

Generally, microRNA (small, non-coding RNAs) are seen to be participating in cell growth and development in BrC and so also used as potential biomarkers. However, miRNAs can also bind to the 3'UTR of the target gene mRNA by imperfect base pairing (Singh & Mo, 2013). The binding of miRNA to its target gene allows it to regulate post-transcriptional expression by enhanced mRNA degradation and translational inhibition (Singh & Mo, 2013). However, many recent studies have shown that miRNAs can also inhibit different cancers by impeding cell proliferation, metastasis, invasion and migration in different cancers. Hence miRNA was hypothesized potential solutions to target EGF gene in BrC to consequently inhibit the binding with EGFR and initiation of associated pathways.

To conduct the experiment based on this hypothesis, the expression of differentially expressed genes was analyzed by choosing 4 datasets containing maximum samples and excluding cell line experiments from GEO. Differentially expressed genes (DEGs) refer to genes whose expression levels vary significantly between different experimental conditions, such as different tissues, disease states, or experimental treatments (Anjum et al., 2016) . DEGs exhibit variability in their functions throughout distinct growth phases and time periods of cancer and their aberrant expression accelerates the progression of cancer (Li et al., 2021). Hence DEGs have been targeted as a novel treatment strategy in a number of antitumor clinical research designs as well.

The analysis of top 4 datasets showed that EGF is a highly expressed DEG in BrC patients among others. The functional enrichment analysis via Enrichr also showed EGF to be enriched and associated with multiple pathways which included both PI3K/AKT/mTOR pathway and Ras/Raf/MEK/ERK pathway. These two pathways have also been observed to be actively involved in BrC cell proliferation and progression according to many studies. These findings also coaligned with the KEGG pathway enrichment analysis in which EGF was found to activate the EGFR and lead to its overexpression in BrC. This ultimately led to the initiation of both of these pathways resulting in cell proliferation and protein translation.

Since, EGFR is overexpressed in BrC as mentioned previously, the best way to inhibit its activation would be to suppress the EGF itself. To suppress the EGF gene, two potential miRNAs were found using TargetScan web tool. It showed that two miRNAs- hsa-miR-19a-3p and hsa-miR-19b-3p (mature miRNAs of miR-17-92 cluster) (Bai et al., 2019) can bind to the 3'UTR of EGF mRNA via the 7mer-A1 binding site. This binding site indicates that nucleotides 2 to 7 of the miRNA seed region form a perfect Watson-Crick (WC) base pair with the corresponding nucleotides in the target mRNA. (Peterson et al.,2014). Recent studies have also shown that hsa-miR-19a-3p can potentially inhibit cellular proliferation and invasion of non-small cell lung cancer ((Pan et al., 2020). Moreover, this miRNA is also seen to suppress invasion and migration in multiple myeloma and prostate cancer cells (in vitro) (Wa et al., 2018; Wei et al., 2021). The hsa-miR-19b-3p was also found to suppress cell proliferation in breast cancer and gastric cancer ((Wei et al., 2020; Jin et al., 2018). These studies hence made both of these miRNAs, two potential solutions to target EGF mRNA in breast cancer to consequently inhibit the binding with EGFR and initiation of associated pathways.

Target prediction however sometimes provides false positive outcomes which is a challenging factor to determine potential solutions. Hence, the efficiency of both miRNA needs to be measured by conducting the luciferase enzyme assay. This is a biochemical assay that measures the activity of the enzyme luciferase, which catalyzes the light-emitting reaction of luciferin (Jin et al., 2013). This test can be conducted with wild type EGF mRNA and mutant EGF mRNA in which the 3'UTR would be mutated. Since the 3'UTR is mutated in the mutated EGF mRNA, both hsa-miR-19a-3p and hsa-miR-19b-3p would not be able to bind and so the luciferase

enzyme will significantly express. Whereas the binding of both these miRNAs to the predicted site (3' UTR) will significantly reduce the luciferase expression, indicating the miRNAs to be potential solution to target EGF mRNA in BrC cases. The possibility of involvement of other genes needs to be considered as well for which the involvement of maximum data would be required.

# 5. CONCLUSION

To conclude, the study aimed to prove that two miRNAs: hsa-miR-19a-3p and hsa-miR-19b-3p, both can potentially inhibit the expression of EGF mRNA. This would also not allow the EGF to bind with EGFR leading to its overproduction and initiation of both PI3K.Akt/mTOR and Ras/Raf/MEK/ERK signaling pathways. However, these predicted outcomes need to be validated by further research via in *vivo* and in *vitro* methods. Thus, these two potential solutions can be a great approach to inhibit breast cancer cell proliferation and metastasis, while also to develop advanced research in this field.

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