Analyzing the role of non-coding RNA in conferring Tamoxifen and Trastuzumab-resistant breast cancer

A Dissertation Thesis submitted

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Institute of Science, Nirma University

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MASTER OF SCIENCE

In

BIOTECHNOLOGY

SUBMITTED BY

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NAAC ACCREDITED 'A' GRADE

CERTIFICATE

This is to certify that the thesis entitled " **Analysing the role of non-coding RNA in conferring Tamoxifen and Trastuzumab Resistant Breast Cancer**" submitted to the Institute of Science, Nirma University in partial fulfillment of the requirement for the award of the degree of M.Sc. in Biotechnology, is a record research work carried out by **Rutusha Gupta (21MBT023)** and **Muskaan Kabra (21MBT024)** under the guidance of Dr. Heena Dave. No part of the thesis has been submitted for any other degree or diploma.

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DECLARATION BY THE CANDIDATE

We hereby declare that the dissertation work entitled **Analysing the role of non-coding RNA in conferring Tamoxifen and Trastuzumab resistant breast cancer**" submitted to the Institute of Science, Nirma University is a record of the original work done by us under the guidance of **Dr. Heena Dave** and with the support of **Ms. Deepshikha Rathore, Ms. Nirali Shukla** and our lab members. The work incorporated in the thesis has not been submitted for the award of any other degree, diploma, associate, fellowship, or title in there or any other university or other institution of higher learning.

We further declare that the material obtained from other sources has been duly acknowledged in the thesis.

Date: 28/4/2023

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ABBREVIATIONS

ER- Estrogen Receptor

PR- Progesterone Receptor

HER2- Human Epidermal Growth Factor Receptor 2

GEO- Gene Expression Omnibus

BC- Breast Cancer

has- Homo Sapiens

PARP- Poly-ADP Ribose Polymerase

MCC- Maximal Clique Centrality

GEPIA- Gene Expression Profiling Interactive Analysis

TAM- Tamoxifen

cDNA- Complementary DNA

DMEM- Dulbecco's modified Eagles' Medium

PBS- Phosphate buffer saline

FBS- Fetal bovine serum

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ABSTRACT

Reports from WHO (2020) indicate that breast cancer is the prevalent cause of mortality amongst females across the globe. Breast cancer (BC) is a heterogeneous disease and is histologically classified into Luminal A, Luminal B, HER2+, and Triple Negative Breast Cancer (TNBC). Currently, the treatment regime of breast cancer is dependent on the presence of hormone receptors – Estrogen (ER), Progesterone (PR), and Human epidermal growth factor (Her2/neu). 60-70% of BC patients are diagnosed as ER-positive and are treated with selective estrogen receptor modulators (SERMs), chiefly Tamoxifen whereas, 15-20% of the patients show HER2 positive and are managed mainly by Trastuzumab. Tamoxifen and Trastuzumab is a universally accepted drug that is cost-effective and with minimum side effects. Resistance to treatment modalities is a major obstacle in the therapeutic regime from which 20-30% of tumors develop resistance due to tamoxifen while 2-3% of the tumors acquire resistance to Herceptin. Hence, it has become the need of the hour to identify the underlying mechanisms that cause tamoxifen and Herceptin resistance in ER+ tumors and HER2 + tumors respectively. Studies now focus on deciphering the role of non-coding RNAs in causing resistance in breast cancer. Non-coding RNAs are classified into small non-coding RNAs such as microRNAs (miRNAs) and long non-coding RNAs (lncRNAs). We are focusing on microRNAs that are playing role in the resistance and by using bioinformatics tools we have identified miRNA as a therapeutic target which was later validated by in-vitro analysis. Our study identified that the upregulation of miR-190b and miR-375 and downregulation of miR-135b are responsible for causing tamoxifen resistance in breast cancer patients. While miR-1276 was found to be downregulated in trastuzumab resistance breast cancer cell lines. Further, we validated miR-375 in an acquired tamoxifen-resistant MCF-7 breast cancer cell line. By in vitro analysis, it was observed that miR-375 was upregulated in the tamoxifen-resistant breast cancer cell line as compared to untreated cells.

INTRODUCTION

BREAST CANCER

Breast cancer is the most common type of cancer among women worldwide, with an estimated 2.3 million new cases and 685,000 deaths reported in 2020 according to GLOBOCAN (DeSantis et al.).

Breast cancer is a heterogeneous disease that forms in the cells of the breast tissue. It occurs when abnormal cells in the breast begin to grow uncontrollably, forming a tumor. It can also occur in men, although it is rare. Breast cancer can be classified into several subtypes based on the way the cancer cells look under a microscope and the presence or absence of certain proteins. Breast cancer can be categorized as either invasive or non-invasive based on how the cancer cells have grown and spread. Non-invasive breast cancer is further classified into ductal carcinoma in situ and lobular carcinoma in-situ, where the cancerous cells are confined to milk ducts and lobules. Invasive breast cancer is further classified into Invasive ductal carcinoma and Invasive lobular carcinoma, where the cancer cells have grown beyond the milk ducts or lobules and have invaded surrounding tissues, such as the fatty tissue or muscles (Yersal and Barutca).

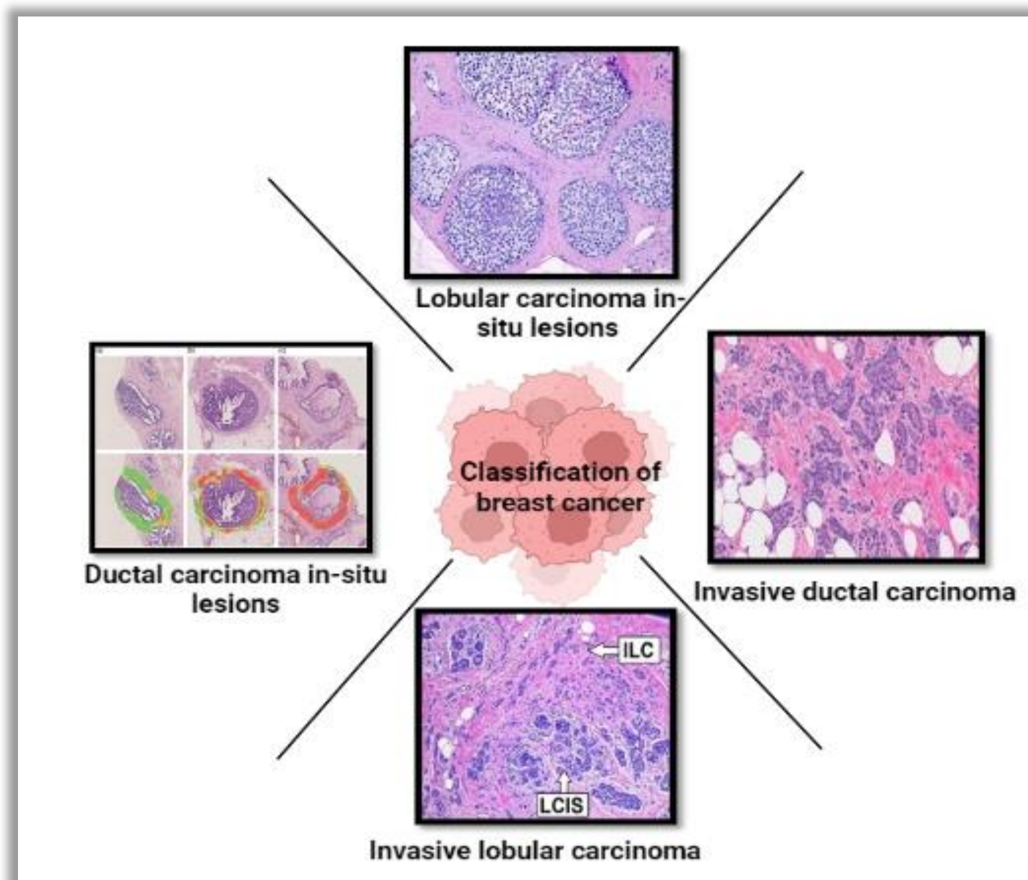
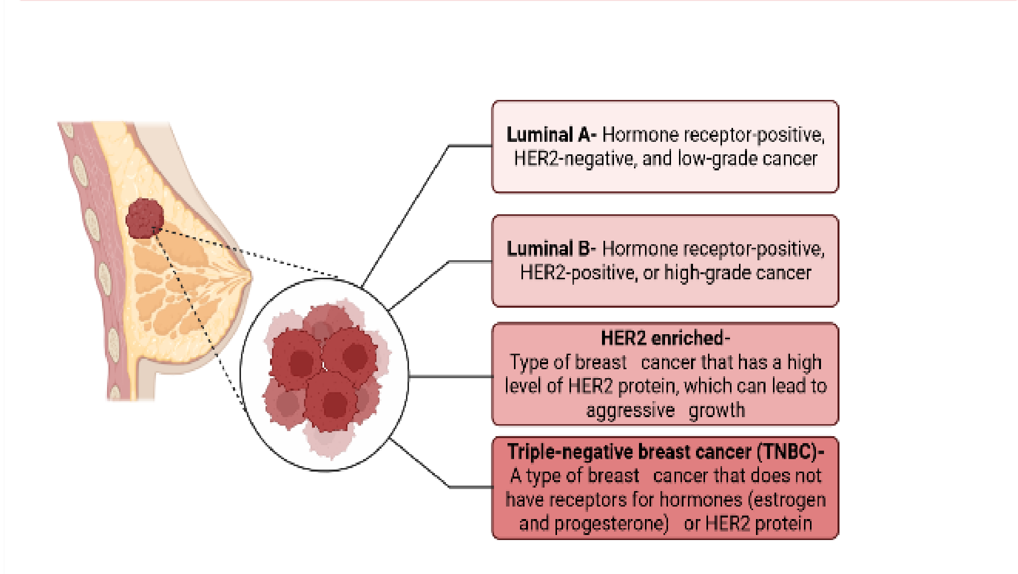


Figure 1- Classification of breast cancer- Invasive carcinoma & Non-Invasive carcinoma. Adapted from (Ehteshami Bejnordi et al.)

In addition, the expression of certain genes, such as estrogen receptor (ER), progesterone receptor (PR), and human epidermal growth factor receptor 2 (HER2), play critical roles in breast cancer pathogenesis and are used as biomarkers for diagnosis and treatment.

Breast cancer can be intrinsically divided into 4 molecular subtypes – **Luminal A, Luminal B, Human epidermal growth factor receptor 2, and Triple-negative breast cancer (TNBC).**

Table1- Types of breast cancer based on molecular subtypes. Adapted from (Tsang and Tse)



Staging

The purpose of staging is to classify patients into risk categories based on their prognosis and guide treatment recommendations for those with a similar prognosis. TNM staging is a common system used to stage breast cancer. TNM stands for Tumor, Node, Metastasis, and the system takes into account the size of the tumor, whether cancer cells have spread to nearby lymph nodes, and whether cancer has metastasized (spread) to other parts of the body.

Table 2- TNM staging. Adapted from (Cserni et al.)

Tumor (T)	Nodes (N)	Metastasis (M)
Tis: Carcinoma in situ (non-invasive cancer)	**N0**: No cancer cells in nearby lymph nodes	**M0**: No evidence of distant metastasis
T0: No evidence of primary tumor	**N1**: Cancer cells present in 1-3 nearby lymph nodes	**M1:** Cancer has spread to other parts of the body, such as the lungs, liver, or bones
T1: Tumor is less than or equal to 2 cm in size	**N2:** Cancer cells present in 4-9 nearby lymph nodes	
T2: Tumor is between 2-5 cm in size	**N3:** Cancer cells present in 10 or more nearby lymph nodes	
T3: Tumor is larger than 5 cm in size		
T4: Tumor has invaded nearby tissue, such as the chest wall or skin		

Based on the TNM staging system, breast cancer stages can be categorized into four: Stage 0, Stage I and II, Stage III, and Stage IV.

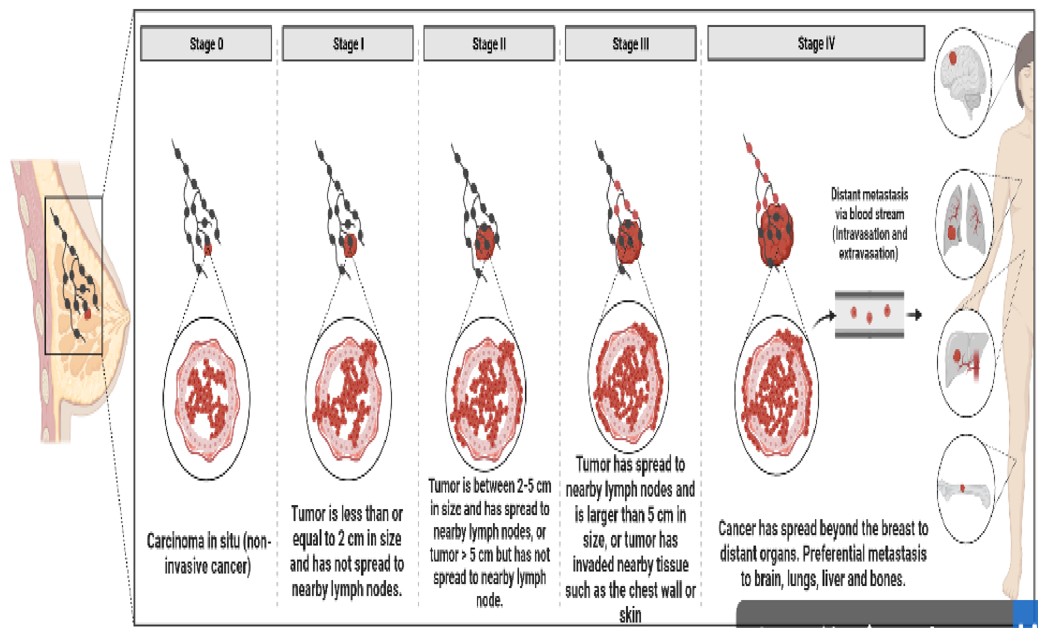


Figure 2- Stages of breast cancer. (Created in BioRender)

MANAGEMENT OF BREAST CANCER TREATMENT

The management of breast cancer typically involves a multidisciplinary approach that includes various treatment modalities such as surgery, radiation therapy, chemotherapy, targeted therapy, and hormone therapy.

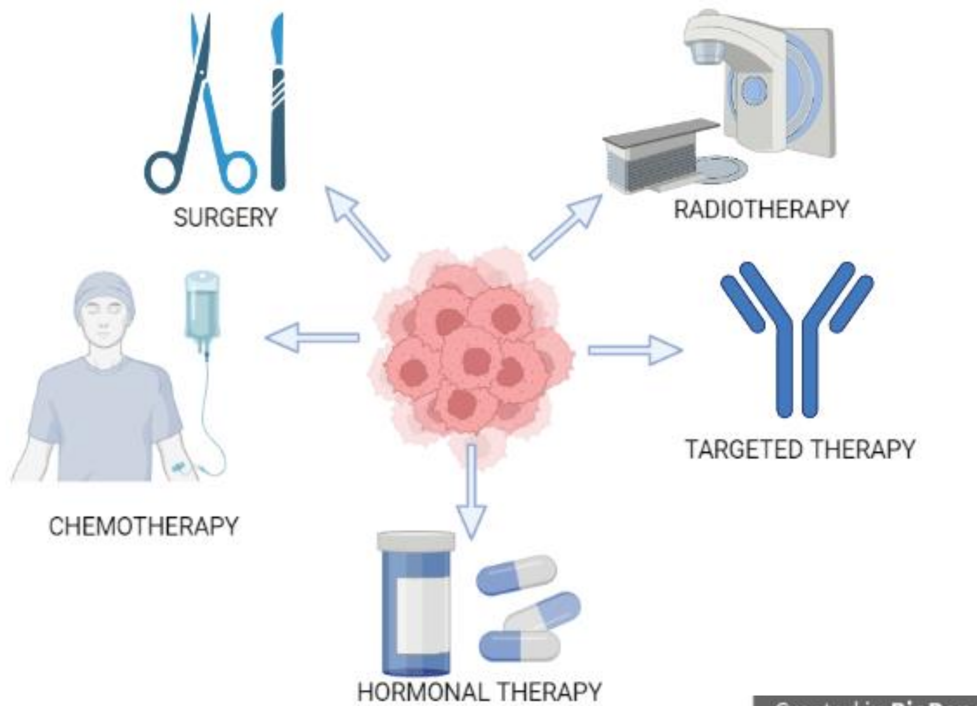


Figure 3- Treatment modalities of breast cancer. (Created in BioRender)

1. SURGERY

1A. Breast-conserving surgery- excision of the tumor and some surrounding healthy tissue but not the breast itself.

1B. Total mastectomy- whole breast is removed along with lymph nodes present under the armpit

1C. Modified radical mastectomy- excision of the majority of the lymph nodes beneath the arms as well as the whole cancerous breast.

2. RADIATION THERAPY- High levels of radiation doses are used to destroy cancer cells and reduce tumor size. This therapy damages the DNA but the cells are not killed immediately. There are two types of radiation therapy:

External beam- Machine that directs a beam of radiation at the tumor.

Internal beam- A catheter or other injectable tool is filled with radioactive particles, which delivers radiation straight to or close to a tumor.

3. CHEMOTHERAPY- a medication that kills rapidly proliferating cells within the human body using strong chemicals. Before surgery, neoadjuvant chemotherapy can reduce the size of the tumor. Adjuvant chemotherapy can eliminate cancer cells that are still present post-surgery. Drug names include vinorelbine, paclitaxel, doxorubicin, and methotrexate.

4. TARGETED THERAPIES- Targeted therapy refers to a type of medical treatment that focuses on specific molecules or genetic changes that are involved in the development and progression of certain diseases, such as cancer, autoimmune disorders, and other chronic diseases. Unlike traditional chemotherapy, which can affect both healthy and cancerous cells, targeted therapy is designed to selectively target the cancer cells or disease-causing cells while minimizing damage to normal cells.

Some examples of targeted therapies include: -

1. **Small molecule inhibitors** – These are drugs that can target specific enzymes or signaling pathways inside cancer cells, thereby blocking their growth or ability to spread.
2. **Immunotherapies**: These are treatments that stimulate the body's immune system to attack cancer cells. Examples include immune checkpoint inhibitors, CAR T-cell therapy, and cancer vaccines.
3. **Gene therapy**: This involves altering the genes inside cancer cells to either kill them or make them more susceptible to other types of treatment.

4. **Hormone therapies**: These are drugs that can block the effects of certain hormones that promote the growth of certain cancers, such as breast or prostate cancer (Lee et al.).

4A: HORMONAL THERAPY FOR ER+ BREAST CANCER

Hormonal therapy is a widely used treatment for estrogen receptor-positive (ER+) breast cancer, as it aims to block the effects of estrogen in the body and prevent the cancer cells from growing and spreading. Selective Estrogen Receptor Modulator (SERMs) such as Tamoxifen, Raloxifene, etc. blocks the estrogen receptor in breast cancer cells and has been shown to reduce the risk of developing contralateral breast cancer in women with ER+ tumors. Tamoxifen as an adjuvant endocrine therapy administered beyond a period of 5–10 years is regarded as conventional treatment for all luminal early breast cancers, i.e., those that are hormone-receptor positive (ER or PR positive, or both) . The conventional endocrine treatment for people who already are premenopausal is 20 mg of tamoxifen each day (Colleoni et al.).

Tamoxifen and aromatase inhibitors are both acceptable treatment alternatives for postmenopausal individuals, whether used consecutively or as monotherapy for a period of five years. When compared to tamoxifen, aromatase inhibitors dramatically lower relapses by roughly 30%, but not death. Although contrast to 5 years of tamoxifen treatment, 5 years of treatment with aromatase inhibitors greatly decreases breast cancer mortality by 15% (Bradley et al.).

5. **Monoclonal antibodies:** These are lab-made antibodies that target specific proteins on the surface of cancer cells or immune cells. They can block the growth of cancer cells or enhance the body's immune response against cancer.

5A. TARGETED THERAPY (Monoclonal Antibodies) FOR HER2+ BREAST CANCER

Trastuzumab, a monoclonal antibody, is the standard drug given to treat patients with HER2+ breast cancer. It binds to the extracellular domain of HER2 receptors preventing its dimerization and inhibits intracellular signalling pathway. They are injected in conjugation of other drugs like taxane for better efficiency. Trastuzumab along with Docetaxel and carboplatin (TCH) show

reduced cardiac toxicity (Harbeck and Gnant). Hence, TCH represents a viable treatment option for HER2-positive cancer, especially for those with cardiac complications.

ESTROGEN RECEPTOR

Estrogen controls the expression of numerous genes, promoting the survival and proliferation of breast cancer cells. Estrogen effect is regulated by its two receptors, ERα and ERβ, which can change gene transcription by interacting with other transcription factors or binding to DNA at specific sequences known as estrogen response elements (ERE). Based on gene expression profiles tumours that are both ER+ and ER- are classified based on luminal A and luminal B (Baumgarten and Frasor).

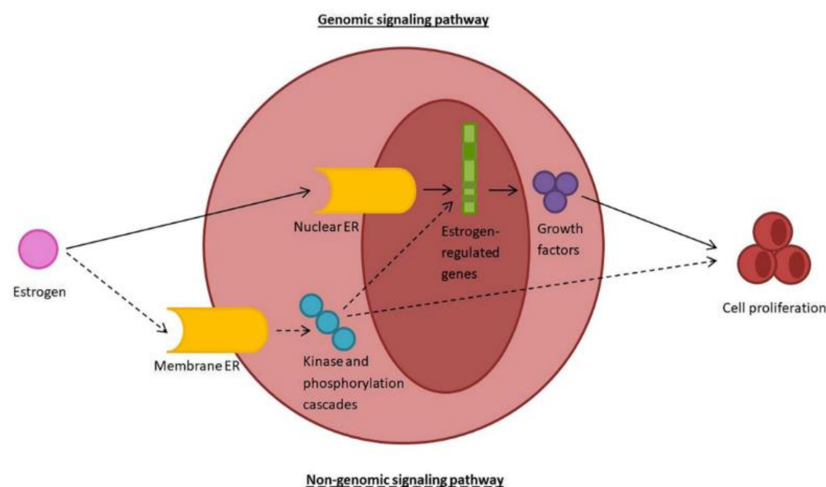


Figure 4 – Estrogen signalling pathway (Schuurman et al.)

The estrogen receptor was identified by Elwood Jensen in 1958. RNA from the human breast cancer cell line MCF-7 was used to clone the first estrogen receptor in humans, also known as ERα. Similarly, 10 years later, the second estrogen receptor, also known as ERβ, was discovered. The ESR1 gene, found on chromosome 6, encodes the ERα in humans. On the contrary end, the ESR2 gene on chromosome 14 encodes the protein ERβ. As a steroid hormone, estrogen can penetrate through the plasma membrane, engage with intracellular ERα and ERβ to bind to DNA sequences.(Barazetti et al.).

CHALLENGES

Tamoxifen's estrogenic characteristics cause both favourable and unfavourable effects. They include detrimental endometrial and thromboembolic repercussions, but also desirable reductions in bone turnover and stability of lipid metabolism. Hot flushes, vaginal dryness, and urogenital and vasomotor symptomatology leading to urinary symptoms are other undesirable effects of tamoxifen's antagonistic qualities that are frequently linked to menopause (Mouridsen et al.). The chance of getting endometrial cancer rises as a result of prolonged treatment and may also result in resistance(Fisher, Dignam, et al.).

TAMOXIFEN

Depending on the presence of biomarkers the treatment can be planned accordingly. Biomarkers expression level like ER, PR, HER2 and also Ki-67 enables correct treatment modalities. (Goldhirsch et al.)

Almost 66% of the patients who are diagnosed with breast cancer are ER+ and they are given hormone therapy(Abe et al.). Out of all the endocrine therapy, tamoxifen is highly recommended for premenopausal women while for postmenopausal women aromatase inhibitor is the most preferred form of treatment. Resistance for hormonal therapy may be de novo or can be acquired (Musgrove and Sutherland).

For ER+ pre-menopausal women, tamoxifen is the most popular anti-estrogen adjuvant therapy. Tamoxifen can either serve as an agonist or an antagonist, luring coactivators or corepressors to the ER transcription complex, depending on the tissue(Hayes and Lewis-Wambi). Tamoxifen exerts antagonistic actions in breast tissue, which have both preventative and cytotoxic effects on breast cancer cell growth (Fisher, Costantino, et al.). Moreover, tamoxifen has agonistic effects in the uterus that raise the danger of endometrial hyperplasia and cancer (Deligdisch et al.). Overall 30% of the ER+ breast cancer patients acquire tamoxifen resistance thus this endocrine treatment can’t treat them (Egeland et al.).

MODE OF ACTION OF TAMOXIFEN-

Tamoxifen is most frequently used selective estrogen receptor modulator which is used to treat ER positive breast cancer patients. In breast cancer cells, estrogen binds to estrogen receptor in breast cancer thereby cell proliferation takes place. Tamoxifen is antiestrogen and thus blocks the binding of estrogen to the receptors. If the estrogen is not attached to a breast cell, the cell will not receive signal to grow and multiply. Thus, the cell proliferation is stopped in G1 phase. They serve as antagonist for breast cancer but agonist for bone, lung, uterus and liver.

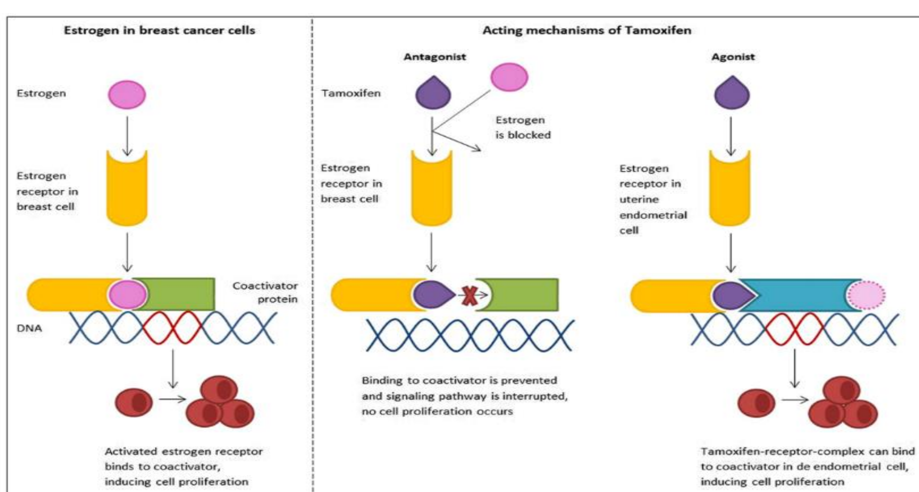


Figure 5- Tamoxifen- Mode of action (Schuurman et al.)

HUMAN EPIDERMAL GROWTH FACTOR RECEPTOR 2

HER2+ breast cancer is a type of breast cancer in which the cancer cells overexpress the HER2 protein due to the amplification of the HER2 gene. This leads to uncontrolled cell growth and division, making cancer more aggressive and faster-growing than other types of breast cancer. HER2-positive breast cancer accounts for approximately 20% of all breast cancer cases and is associated with a more aggressive tumor behavior, higher risk of recurrence, and poorer overall prognosis compared to HER2-negative breast cancer (Yersal and Barutca). HER2 is a transmembrane receptor tyrosine kinase that is a member

of the HER/EGFR family and is encoded by the ERBB2 gene. Unlike other HER receptors, HER2 does not require a ligand to activate and is always in an open conformation that enables it to dimerize with other HER receptors, such as EGFR, Her3, and Her4 (Graus-porta et al.). This dimerization leads to the activation of downstream signaling pathways, including the PI3K/Akt pathway involved in cell survival and MAPK-mediated pathways that promote cellular proliferation.

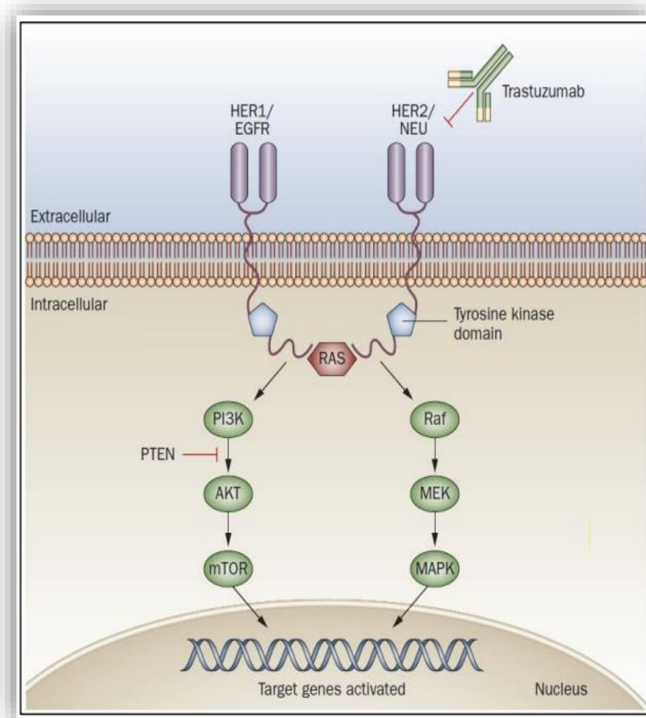


Figure 6- Human epidermal growth factor signaling cascade (La Thangue and Kerr).

HER2 amplification causes the receptor to be constantly active, which triggers certain signaling pathways. These pathways lead to uncontrolled transcription of genes and increase cell survival and growth. Additionally, HER2 amplification induces an increase in VEGF expression, which is responsible for the creation of new blood vessels. This provides the rapidly growing cancer cells with the necessary O_2 supply for tumor growth and invasion. Breast cancers that are HER2-positive are linked with a poorer prognosis and earlier metastasis. This information was reported in studies conducted in 1987 by (Slamon et al.) and (Yen et al.) in 2000. However, the introduction of HER2-targeted therapies,

such as trastuzumab, pertuzumab, and ado-trastuzumab emtansine (T-DM1), has dramatically improved the treatment outcomes for patients with HER2-positive breast cancer (Pernas and Tolaney).

TRASTUZUMAB

Trastuzumab is a humanized monoclonal antibody of the IgG1 kappa light chain class that binds to the HER2 receptor, causing programmed cell death of the HER2 overexpressed cancer cells. A modified version of Trastuzumab was created for the first time in 1992 (Carter et al.) and was initially approved by the US FDA in 1998 (Kreutzfeldt et al.) for use in the metastatic setting. It is created through genetic engineering, where the CDR regions of a mouse monoclonal antibody specific to HER2 were fused with the framework regions of a human antibody. Trastuzumab is administered through intravenous infusion either weekly or every three weeks. For the weekly schedule, the initial loading dose is 4 mg/kg, followed by a weekly dose of 2 mg/kg starting one week later. On the other hand, for the three-weekly schedule, the loading dose is higher at 8 mg/kg, followed by a dose of 6 mg/kg administered every 21 days. It is usually given together with chemotherapy in all registered indications. However, for postmenopausal women with breast cancer that is positive for hormonal receptors, trastuzumab can be combined with an aromatase inhibitor (Meijerman et al.).

MODE OF ACTION OF TRASTUZUMAB

Trastuzumab's exact mechanism of action is not fully understood, but some possible ways it works have been identified. These include inhibiting downstream signalling pathways like MAPK and PI3K/AKT by blocking HER2 cleavage and dimerization, activating ADCC, and promoting HER2 internalization and degradation. Other proposed mechanisms include inhibiting DNA repair and reducing angiogenesis by inhibiting VEGF. Overall, trastuzumab's mechanism of action is multifaceted and involves various pathways to target HER2+ breast cancer cells (Hudis).

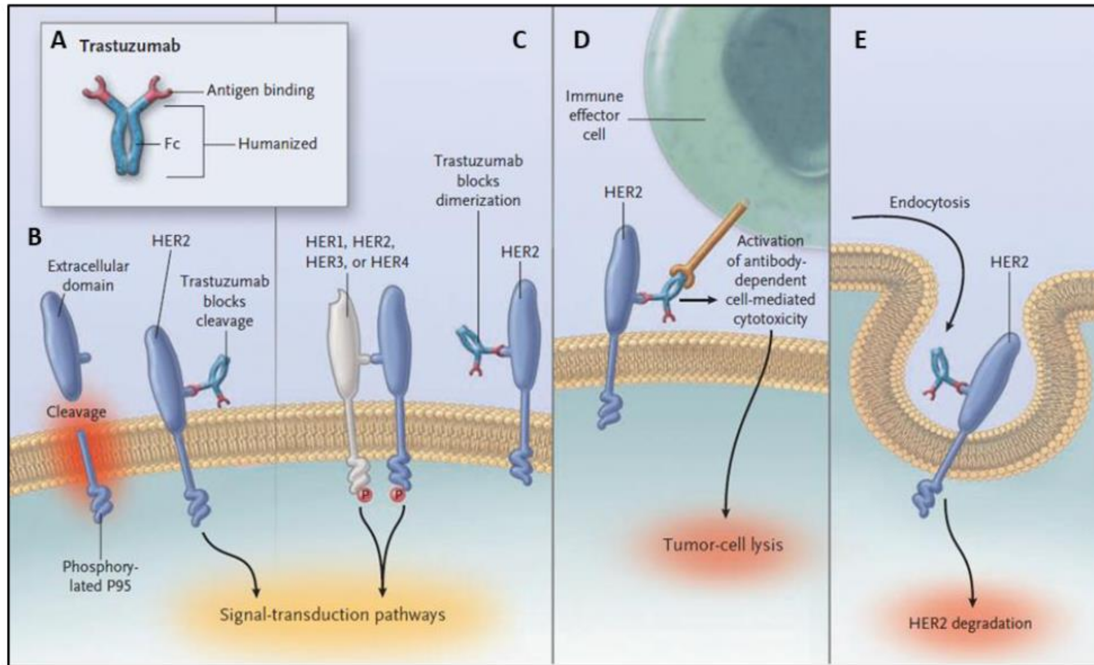


Figure7 - Trastuzumab can reduce signaling in various ways. The figure illustrates the various ways in which Trastuzumab functions. Firstly, it binds to the HER2 extracellular domain. Secondly (B), it blocks the parting of the extracellular region, thereby preventing the production of membrane-bound phosphorylated p95, which can activate signal-transduction pathways. Thirdly (C), it physically blocks homo- or heterodimerization. Fourthly (D), it triggers antibody-dependent cell-mediated cytotoxicity (ADCC) by recruiting Fc-competent immune effector cells. Lastly (E), it facilitates receptor internalization by endocytosis, which leads to its degradation.. This information is adapted from (Hudis).

RESISTANCE

Approximately 75% of breast cancer cases are ER-positive, and approximately 20% of breast cancer cases are HER2-positive (Yersal and Barutca). Treatment options for ER-positive breast cancer include endocrine therapies such as tamoxifen and aromatase inhibitors, while HER2-positive breast cancer is treated with targeted therapies such as trastuzumab in addition with chemotherapy. However, despite the initial response to treatment, resistance to therapy may develop, leading to disease progression.

There are two types of resistance in breast cancer: de novo resistance and acquired resistance.

1) De novo resistance - It refers to the presence of drug resistance before any exposure to the drug. It can occur due to various mechanisms such as genetic mutations, epigenetic changes, or alterations in signaling pathways. De novo resistance can participate to the inability to eradicate minimal residual disease, which is the small number of cancer cells that remain after treatment, and also facilitate acquired drug resistance emergence.

2) Acquired resistance - Occurs when cancer cells initially respond to treatment but eventually become resistant to it.

TAMOXIFEN- MECHANISM OF RESISTANCE

Pharmacological research, structural changes, abnormal microRNA expression, ER function in the tumour microenvironment, and genetic modifications linked to tamoxifen resistance can all be used to better understand this phenomenon. Tamoxifen metabolism is influenced by changes in the expression of ERα or ERβ, modifications to co-regulatory molecules, aberrant production of microRNA, and genetic polymorphisms(Ali et al.). In-depth analysis into the causes of tamoxifen resistance has shown a number of intricate mechanisms, including ER signalling modification, growth factor receptor overexpression (HER2, EGFR, FGFR, IGFIR), changes to the PI3K-PTEN/AKT/mTOR pathway, and NFkB signalling (Wong et al.). Various miRNA has also shown to cause tamoxifen resistance in ER positive MCF-7 cells (Rao X, Di Leva G, Li M, Fang F, Devlin C, Hartman-Frey C, Burow ME, Ivan M, Croce CM). When ERα is absent or negative in tumors then in these patients' tamoxifen and many other anti-estrogens doesn't work(Jaiyesimi et al.). It is also observed that changes or absence in expression of ERα might lead to tamoxifen resistance (Gutierrez et al.). Reduction in the expression of ERα protein is due to miR-221 and miR-222 thus they are sought to have effect in cells getting resistant to tamoxifen (Zhao et al.). It was also demonstrated that miRNA regulates tamoxifen resistance for instance family of miR-200 was sought to be reduced in breast cancer cells which are tamoxifen- resistant. Cells sensitivity for

tamoxifen was increased when miR-200b or miR-200c was upregulated (Manavalan et al.). And when miR-375 was again expressed, tamoxifen could affect tamoxifen- resistant cells making it sensitive to tamoxifen (A. Ward et al.). Researchers hypothesised that in breast cancer cells, miR-301 activity could increase the tamoxifen sensitivity when it is inhibited and this is possible by regulating PTEN/Akt pathway(Shi et al.). There are various factors that affects the regulation of tamoxifen resistance. For instance, it was stated that miR-519a is increased in cells which are resistant to tamoxifen and also it is highly expressed in wild type cells of MCF-7 contributing resistance towards tamoxifen. Blocking this miRNA action can overrule the resistance in tamoxifen- resistant cells(Aoife Ward et al.). In MCF-7 cells, inhibition of miR-101 by the use of an anti-miR-101 blocks nucleic acid oligo that increases the sensitivity to tamoxifen. Researchers also found that miR-221 present in breast cancer patients is a versatile non- coding RNA which is linked to various resistance like tamoxifen, fulvestrant and transtuzumab (W. Zhang et al.).

TRASTUZUMAB- MECHANISM OF RESISTANCE

Despite trastuzumab effectiveness, some patients can develop resistance, leading to disease progression and poorer outcomes. Trastuzumab resistance is a complex and multifactorial process, involving various mechanisms that can lead to the decreased efficacy of the drug. These mechanisms fall into three major categories: steric effects caused by structural mutations in HER2 protein, alternative elevations of other tyrosine kinase receptors like IGFR, and intracellular changes in HER2 downstream signaling, particularly PTEN deficiency and/or PI3K/Akt constitutive activation.

Structural mutation in HER2 receptor does not allow the binding of trastuzumab on the receptor thus inhibiting the effect of drug on HER2+ cancerous cells. This forms the truncated p95HER2 having kinase activity which leads to continuous oncogenic signalling, bypassing the effect of trastuzumab and leads to resistance. This process is observed by (Scaltriti et al.) in 2007.

Trastuzumab, is ineffective in preventing the pairing of HER3 and HER2 proteins. When HER3 is overexpressed, it becomes more phosphorylated

leading to activation of downstream signaling pathways and thus overcome the effects of trastuzumab on HER2 signaling (Aubele et al.). Notably, another study suggested the role of overexpressed insulin like growth factor 1 receptor(IGF-1R)(Köstler et al.) and the tyrosine kinase c-Met (Shattuck et al.) as a potential target that could lead to resistance against trastuzumab due to its abnormal expression.

Trastuzumab-resistant cells undergo intracellular changes where they lose the function of PTEN, leading to the constant activation of Akt(Nagata et al.). These cells may also contain mutations that activate PI3K or Akt, causing the activation of other signaling pathways even when trastuzumab blocks HER2 or PTEN is active. Another such study claims the role of Src in trastuzumab resistance breast cancer cell line (S. Zhang et al.). The researchers discovered that individuals who had active Src responded less effectively to trastuzumab treatment compared to those without Src activation Src was identified as the central mediator of all resistant pathways, making it a promising therapeutic target for trastuzumab-resistant patients. In both laboratory experiments and animal studies, it was demonstrated that inhibiting Src can overcome resistance to trastuzumab by preventing Akt phosphorylation and the downstream signaling of EGFR. This effect was observed even in cells lacking the PTEN gene. Combining trastuzumab and Src inhibition could be a promising approach to improving the clinical benefit for a large population of patients with poor response to trastuzumab.

NON-CODING RNA (ncRNA)

The RNA which does not code for a protein are generally denoted as non-coding RNA (ncRNA), but these do contain some sort of information and they do perform function too (Mattick and Makunin). ncRNA can act as regulatory signal hence they have ability to work as receivers and transmitters and binds to UTR of mRNA (Frith et al.). Compared to mRNA, ncRNAs are expressed at less amount and this type of RNA perform more regulatory functions. ncRNA is classified mainly into two groups, first group is small ncRNA and another one is long ncRNA. This category is on the basis of size of transcript (Sana et al.).

This small ncRNA is grouped into Dicer-dependent and Dicer- independent

small ncRNAs. Dicer- dependent ncRNA includes miRNA (microRNAs), siRNAs (small interfering RNAs) and snoRNAs (small nucleolar RNAs) while Dicer- independent small ncRNAs include piRNAs (PIWI- interacting RNAs) (Houwing et al.).

IMPORTANCE OF NON-CODING RNAs IN BREAST CANCER DIAGNOSIS AND MANAGEMENT

Any particular component that identifies the existence or advancement of human tumours is referred to be a tumour marker. The US Food and Drug Administration (FDA) has accepted carcinoembryonic antigen (CEA) and cancer antigen 15-3 (CA15-3) in serum as diagnostics markers for breast cancer. However, they have several drawbacks, mostly in the form of poor sensitivity and specificity. Hence, finding new molecular markers with enhanced diagnostic utility is therefore crucial (Lu et al.). Dysregulated lncRNA can beneficial as an effective diagnostic tool. For instance, serum MALAT1 has demonstrated its diagnostic accuracy with respect to sensitivity of 83.7% and specificity of 81.2% for breast cancer (Zidan et al.).

MicroRNAs

In eukaryotes, miRNA length usually is of 18-25 nucleotide long, whose sequence is conserved evolutionarily which is single stranded having specialized roles in gene expression regulation(Feinbaum et al.). Biogenesis of miRNA takes place via two locations, first in nucleus where pre-miRNA (precursor miRNA) is formed from pri-miRNA (primary miRNA) using Drosha and other RNA binding proteins. This is then transported to cytoplasm by exportin 5 and now here it is processed into mature miRNA duplex. One of its strand act as guide strand while the other strand act as passenger miRNA which is degraded by RISC complex(Krol et al.).

On the other hand, long non-coding RNA (lncRNAs) are of length 200 nucleotides and are upto 100 kb and are like mRNA transcripts that doesn’t have ORF (open reading frame). lncRNA includes long intergenic non-coding RNAs (lincRNAs), telomere-associated ncRNAs (TERRAs), transcribed-ultra

conserved regions (T-UCRs).

Some microRNAs playing role in tamoxifen resistance- When ERα is absent or negative in tumors then in these patients' tamoxifen and many other anti-estrogens doesn't work(Jaiyesimi et al.). It is also observed that changes or absence in expression of ERα might lead to tamoxifen resistance (Gutierrez et al.). Reduction in the expression of ERα protein is due to miR-221 and miR-222 thus they are sought to have effect in cells getting resistant to tamoxifen (Zhao et al.). It was also demonstrated that miRNA regulates tamoxifen resistance for instance family of miR-200 was sought to be reduced in breast cancer cells which are tamoxifen- resistant. Cells sensitivity for tamoxifen was increased when miR-200b or miR-200c was upregulated (Manavalan et al.). And when miR-375 was again expressed, tamoxifen could affect tamoxifen- resistant cells making it sensitive to tamoxifen (A. Ward et al.). Researchers hypothesised that in breast cancer cells, miR-301 activity could increase the sensitivity to tamoxifen when it is inhibited and this is possible by regulating PTEN/Akt pathway(Shi et al.). There are various factors that affects the regulation of tamoxifen resistance. For instance, it was stated that miR-519a is increased in tamoxifen resistant cells and also it is highly expressed in wild type cells of MCF-7 contributing resistance towards tamoxifen. Blocking this miRNA action can overrule the resistance in tamoxifen- resistant cells(Aoife Ward et al.). In MCF-7 cells, inhibition of miR-101 by the use of an anti-miR-101 blocks nucleic acid oligo that increases the sensitivity to tamoxifen. Trastuzumab is an effective targeted therapy for breast cancer patients with HER2-positive tumors. However, a significant proportion of patients eventually develop resistance to trastuzumab, leading to disease progression. Emerging evidence suggests that miRNAs may play a critical role in the development of trastuzumab resistance in breast cancer (Si et al.). Specifically, aberrant expression of certain miRNAs has been shown to impact the signaling pathways involved in trastuzumab resistance, contributing to the development of treatment resistance. Researchers also found that miR-221 present in breast cancer patients is a versatile non-coding RNA which is linked to various resistance like tamoxifen, fulvestrant and transtuzumab (W. Zhang et al.).

RATIONALE

Tamoxifen and **Trastuzumab** has significantly improved the overall survival of Luminal-A and HER2 expressing breast cancer patients. However, in past years the percentage of patients developing resistance to the therapy are increasing due to varying molecular mechanisms. Thus identifying the main cause of this resistance and eliminating it can make tumors sensitive to tamoxifen and trastuzumab.

AIM AND OBJECTIVE

The study aims to analyze the role of small non-coding RNAs in tamoxifen and trastuzumab resistance breast cancer patients. To uncover the miRNA implicated in resistance, we utilized two methods:

1. Bioinformatics approach – In the process of identifying miRNAs that are implicated in drug resistance, we used a range of bioinformatics tools such as gene expression omnibus (GEO database), target prediction, functional enrichment analysis, and network analysis. These tools aided in the analysis and interpretation of large-scale biological data and helped us to narrow down the list of miRNA candidates involved in drug resistance.

2. In-vitro analysis - After identifying the potential miRNA molecules involved in drug resistance through in-silico analyses, we conducted in-vitro experiments to confirm their functions. These experiments were carried out using various cell lines and involved the manipulation of miRNA expression levels to observe the effect on drug resistance. By combining both in-silico and in-vitro approaches, we were able to gain a better understanding of the role of miRNA in drug resistance and identify potential targets for further research.

Materials & Methods

BIOINFORMATICS TOOLS

Tool	Description
NCBI	Stands for National Center for Biotechnology Information. NCBI provides free access to PubMed, GenBank, BLAST, Gene, and RefSeq, as well as tools for analyzing and visualizing genomic data.
GEO	The NCBI GEO dataset is a collection of gene expression data, including microarray and next-generation sequencing data, that is publicly available through the NCBI's Gene Expression Omnibus (GEO) database.
TargetScan	TargetScan is a bioinformatics database that provides information on predicted microRNA targets in various species.
miRDB	miRDB is an online bioinformatics database that provides information on microRNA-target interactions with high accuracy.
STRING	The STRING (Search Tool for the Retrieval of Interacting Genes/Proteins) database is a bioinformatics resource that provides information on protein-protein interactions in various organisms.
Cytoscape	Cytoscape is a bioinformatics tool for visualizing and analyzing complex networks, including biological networks such as protein-protein interaction networks and gene regulatory networks.
GEPIA	Gene Expression Profiling Interactive Analysis) is a web-based tool. It compares the expression of a gene of interest across multiple cancer types or between tumor and normal tissues and generates Kaplan-Meier survival plots based on gene expression.

In-Vitro Methods & Materials

1) Establishment and Maintenance of Human Breast Adenocarcinoma cell line (MCF-7)
2) Induction of acquired resistance in MCF-7 cells through prolonged tamoxifen exposure
3) RNA isolation and quantitative Real Time Polymerase Chain Reaction for evaluating the quantification of microRNA playing role in tamoxifen resistance breast cancer cell line.

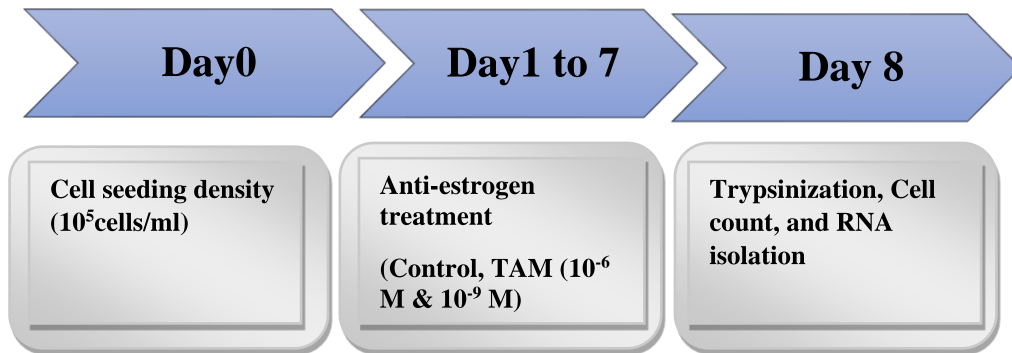
Establishment and maintenance of MCF-7 breast cancer cell

In our study, we used MCF-7 cell line to determine the action on miRNA in the tamoxifen resistant breast cancer. These cells were cultured in duplicates in 6 well plates. They were given complete media (DMEM media, FBS & anti-mycotic and antibiotic) along with tamoxifen with varied concentration. Cell line was maintained for 7 days in 5% CO2 incubator at 37°C in a humidified environment.

Induction of acquired resistance in MCF-7 cells through prolonged tamoxifen exposure

In a 6-well plate, MCF-7 cells were seeded at a cell density of 10^5 cells with complete media in duplicates. On the second day, two concentrations of tamoxifen (10^{-6}M & 10^{-9} M) were added to each group of cells in duplicates to initiate the treatment. This process was repeated until day 7, with 1 mL of media being withdrawn from each well every day and replaced with 1 mL of tamoxifen of each concentration.

Long term treatment of anti-estrogen to MCF-7 cells



MCF-7 cells were seeded in 6 well plates in duplicate with a cell density of 10^5 cells in complete growth medium and the cells were allowed to grow in CO2 incubator. After 24 hours, Anti-estrogens (Doses for TMX (10^{-6}M & 10^{-9}M) were added daily from day 1 to day 7. After day 7, cells were taken out of the CO2 incubator and were trypsinized and counted using hemocytometer.

RNA isolation using RNeasy kit

Requirements-

1. RNeasy Kit
2. QIAzol lysis Reagent
3. Microcentrifuge tubes
4. 70% chilled ethanol
5. RPE Buffer
6. RW1 buffer
7. RNase- free water

Procedure-

1. Prepare cell lysate using 500ul QIAzol lysis reagent and store it in -80°C.
2. During RNA isolation, incubate the sample at room temperature (15-25°C) for 5 to 10 min.
3. Add 500ul chilled 70% ethanol in RNA lysate and mix thoroughly with pipette.
4. Transfer 800ul of solution in 2ml column and centrifuge it for 20 seconds.
5. Discard flowthrough along with the collection tube.
6. Transfer the column in a new collection tube.
7. Add 700ul RW1 and centrifuge it for 20 seconds.
8. Add 500ul RPE buffer and centrifuge it for 20 seconds.
9. Discard the flowthrough.
10. Again, add 500ul RPE buffer and centrifuge for 3 min.
11. Discard the collection tube.
12. Transfer the column in new collection tube.
13. Give dry spin for 2 min to elute the excess contents.
14. Discard the collection tube and transfer the column in new collection tube.
15. Add 30ul of RNase free water in column.
16. Short spin it for 2 min.
17. Now the RNA will be present in collection tube after the spin.
18. Transfer the RNA content into a new 0.5ml mini centrifuge tube and short spin it.
19. Check the concentration and purity in nanodrop.

cDNA synthesis using miRscript kit.

Requirements-

Components	Control	For 10^{-6} tamoxifen	For 10^{-9} tamoxifen
1. 5x miR script Hispec Buffer	4µl	4 µl	4 µl
2. 10x miR Script nucleus mix	2µl	2 µl	2 µl
3. miScript RT mix	2µl	2 µl	2 µl
4. Template RNA	8µl	1 µl	1 µl
5. RNase- free water	4µl	11 µl	11 µl

Procedure-

1. Place the template on ice for thawing. Hispec buffer and nucleus mix should be defrosted at room temperature (around 25°C).
2. Mix each of the solution by flicking the tube.
3. Briefly centrifuge the tubes.
4. On ice assemble RT master mix depending on the volume set above. (Note: RT mix must be taken out from -20 freezer at the time of preparation only and must be placed on ice.)
5. To the RT master mix tube, add template RNA.
6. Mix them thoroughly and feebly. Centrifuge them and place on ice.
7. Set the lid of thermocycler more than 95°C.
8. For next 60 min incubate the tubes at 37°C.
9. To inactivate the RT mix again incubate for 5 mins at 97°C.
10. Hold at 4°C.
11. Later, place them on ice and store in -20°C.

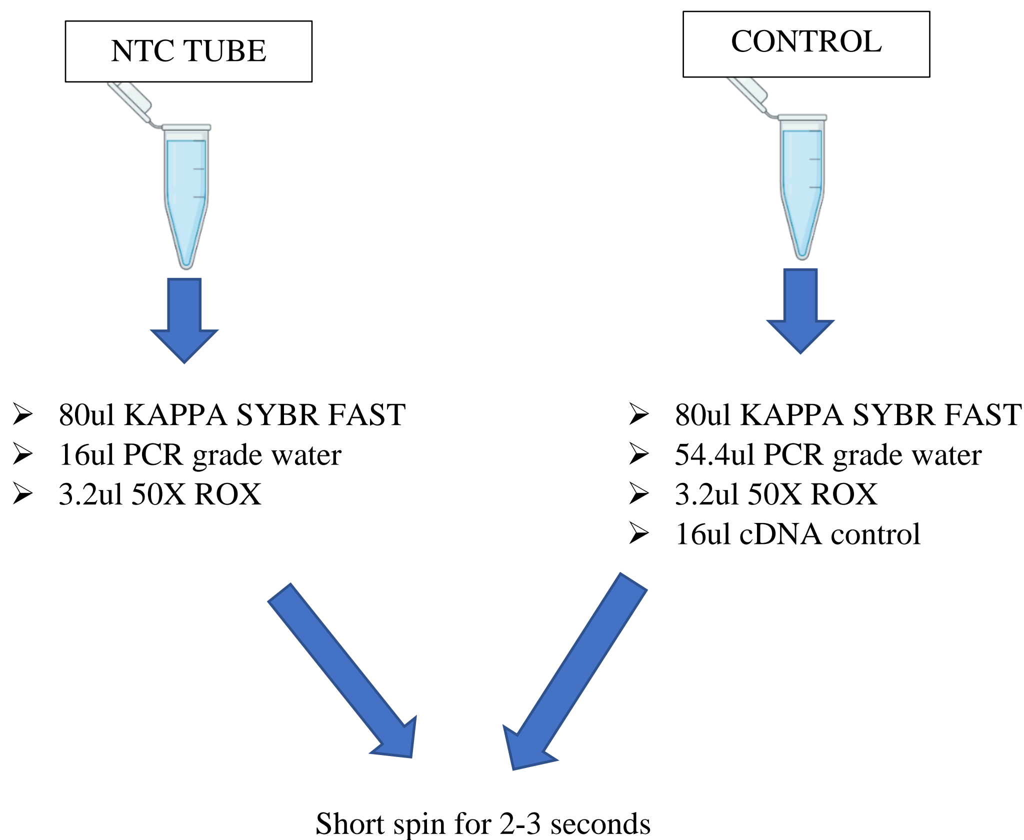
(Steps 7–10: Cycling condition)

Real time PCR (q-PCR)

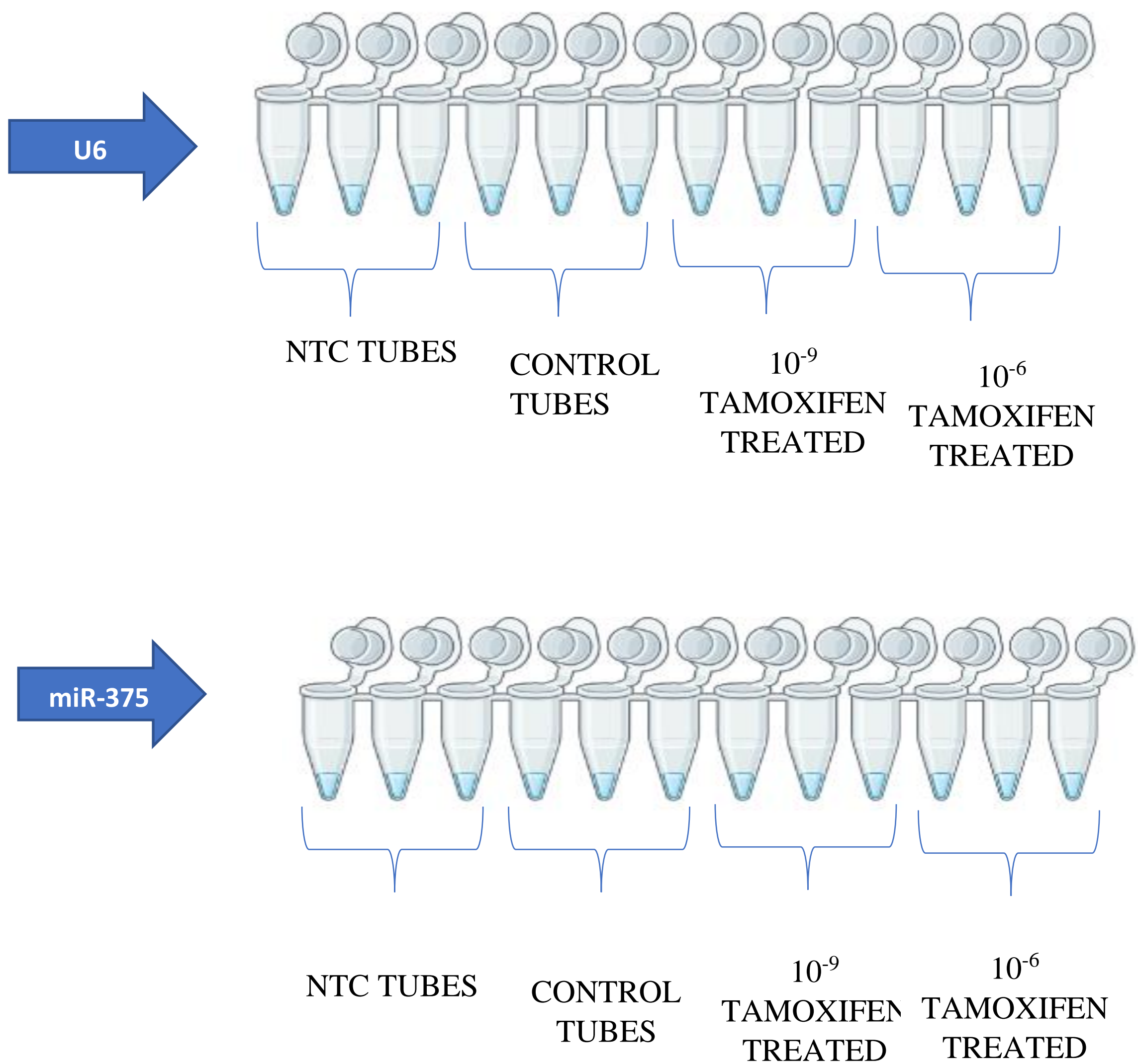
- Thaw forward and reverse primers of U6 and miR-375 at room temperature.
- Short spin the materials present in the kit.
- Dilute U6 forward primer with 681ul of NFW (nuclease free water) and reverse primer with 657ul of NFW.
- Dilute miR-375 forward primer with 780ul NFW and reverse primer with 520ul NFW.
- Short spin all the primer tubes.
- For 10uM working solution the contents are as follows-

	U6(forward primer)	U6(reverse primer)	miR-375 (forward primer)	miR-375 (reverse primer)
NFW	9ul	9ul	9ul	9ul
Primers	1ul	1ul	1ul	1ul
Sequence	GCTTCGGCAGCACAT ATACTAAAAT	CGCTTCACGAATTTG CGTGTCAT	TGCGGTTTGTTCGTTC GGCT	CAGTGCAGGGTCC GAGGT

- Prepare a tube of NTC (negative control) and 2nd tube for control.



❖ Preparation of master mix in triplets.



- Add 10ul KAPPA SYBR fast in all U6 tubes and miR-375 tubes.
- Add 6.8ul NFW in all U6 tubes and miR-375 tubes.
- Add 0.4ul 50X ROX all U6 tubes and miR-375 tubes.
- Add 6ul of 10^{-9} cDNA sample in 10^{-9} tamoxifen treated tubes.
- Add 1ul of 10^{-6} cDNA sample in 10^{-6} tamoxifen treated tubes.
- Add 19.2ul of NTC solution in NTC tubes.
- Add 19.2ul content of control tubes in control strip PCR tubes.
- Add 0.4ul U6 forward and reverse primer in all U6 tubes.
- Add 0.4ul miR-375 forward and reverse primer in all miR-375 tubes.
- Now, short spin all the tubes.

CYCLYING CONDITION

- Volume: 20ul
- Lid(cover) temperature: 105°C

	HOLD STAGE	PCR STAGE	MELTING STAGE
	Enzyme activation	Denaturation	Annealing and extension
Temperature	95°C	95°C	60°C
Duration	3 min	1-3 seconds	20 seconds

RESULTS
&
DISCUSSION

RESULTS OF TAMOXIFEN RESISTANCE BREAST CANCER- IN SILICO ANALYSIS

Breast cancer is a complex disease, despite the successful used of tamoxifen in the treatment of ER +ve breast cancer, resistance to this drug remains a major clinical challenge. Thus, in this study, we have directed our attention towards identifying the specific miRNA and genes involved in the tamoxifen resistance breast cancer patients.

Data collection from GEO database

The analysis of miRNA has been facilitated through the use of GEO database. The data was found using the search term- "miRNA AND tamoxifen resistance breast cancer patients". In this study we solely focused on patient data

Total number of obtained datasets were bifurcated on the basis of two criteria: -

1. The datasets should be "*Homo sapiens"*
2. The datasets must have GEO2R analysis option

- Out of all the datasets found, we selected one dataset. And two different type of grouping was done of the same dataset.

Creation of Venn Diagram of this dataset

- Two different groups were formed in the Define Groups section. Their grouping was done on the basis of:

Group 1	Group 2
Luminal A, Normal like	Luminal A, ERBB2/basal

- They were further shortlisted on the basis of p value and log FC. **(p-value ≤ 0.05 or Fold change ± 1)**

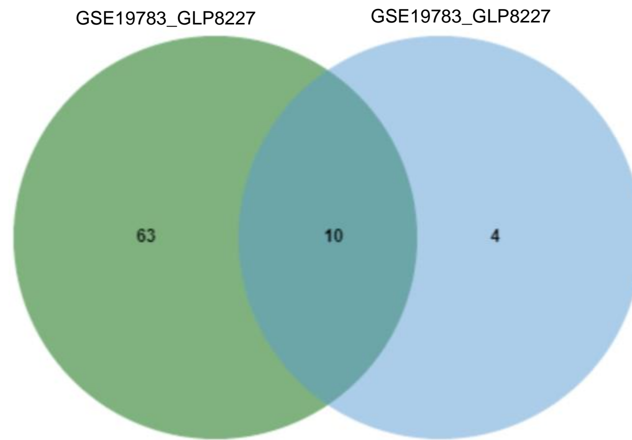


Figure 8: - Venn Diagram of miRNA in GSE 19783 GLP 8227

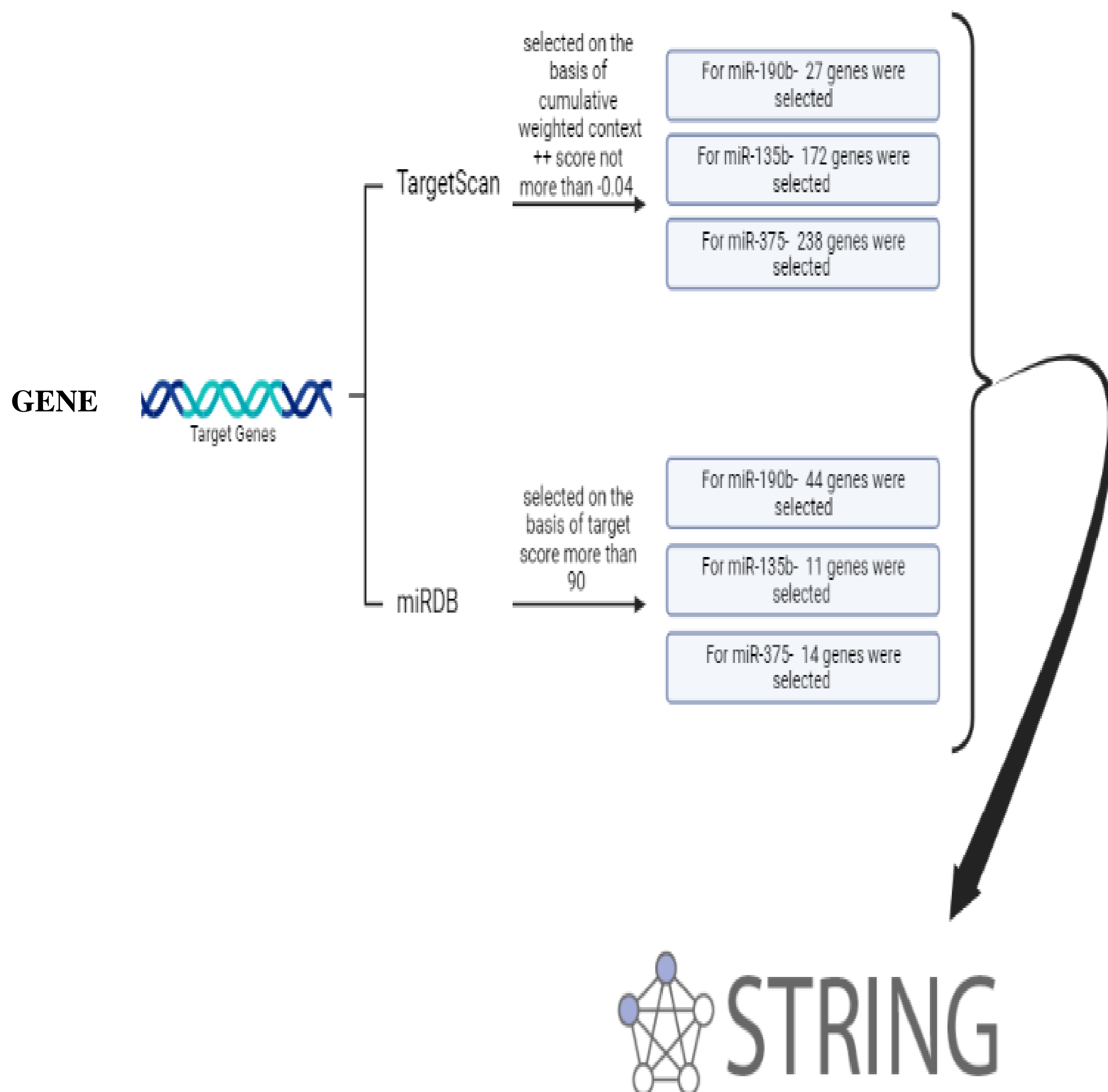
- Out of these 10 miRNAs, only 5 miRNAs were considered based on higher fold change difference.
- Their literature search was conducted where we found that out these 5, only 3 miRNAs showed resistance towards tamoxifen in breast cancer.

1. **miRNA-190b (upregulated)**
2. **miRNA-135b (downregulated)**
3. **miRNA-375 (upregulated)**

Table: - Significant miRNA based on p-value ≤ 0.05 or Fold change ± 1

	Luminal A+ Normal like		Luminal A+ Basal	
miRNA	p value	Log FC	p value	Log FC
miR-190b	0.0	1.1414027	1.79E-13	2.59794
miR-135b	0.0	-1.6482121	1.86E-13	-4.40228
miR-375	0.01	1.4980797	2.85E-09	3.32364

Target search of miRNA using TargetScan and miRDB databases



Interaction network using Cytoscape.

1. Each miRNAs shortlisted genes were put in the STRING (Search Tool for Retrieval of Interacting Genes/Proteins) tool.
2. From the export option data was downloaded in TSV form that can be opened in excel and cytoscape.
3. Top 20-100 genes were selected for different miRNAs in Gene Interaction Network which was ranked according to the MCC (Maximal Clique Centrality) method.

- Expression analysis of genes regulated by miRNA-190b

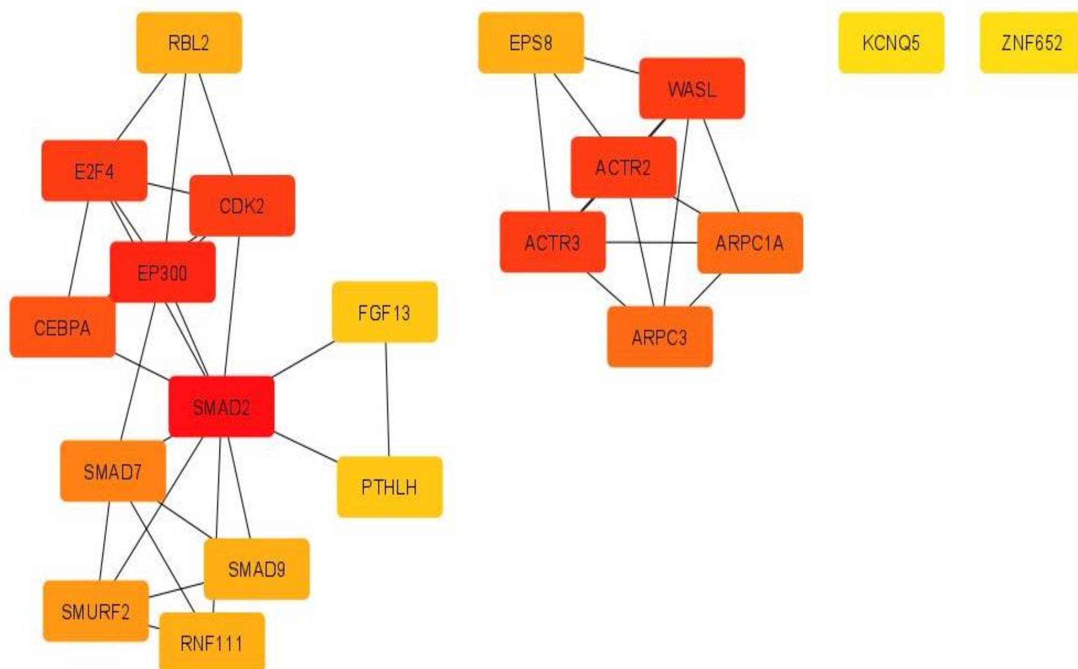


Figure 9: The Gene Interaction Network showing the interconnection between top 20 genes acquired from TargetScan and miRDB, using Cytoscape.

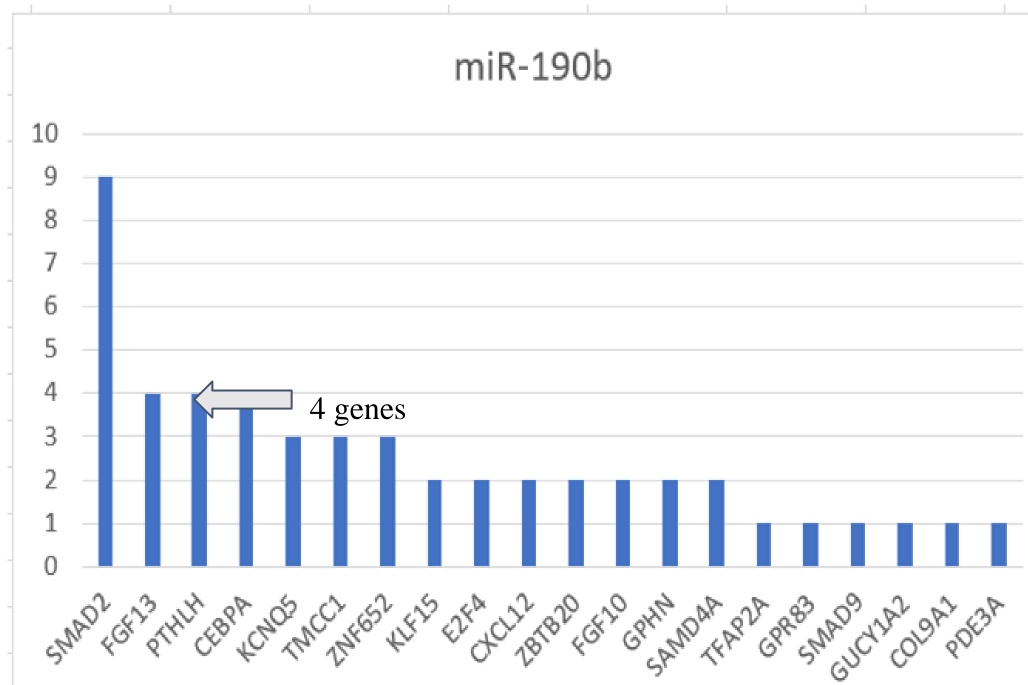


Figure 10: Line chart of top 20 genes in network string interactions ranked by MCC method (x-axis- Scores, y-axis- Genes)

- <u>Expression analysis of genes regulated by miRNA-135b</u>

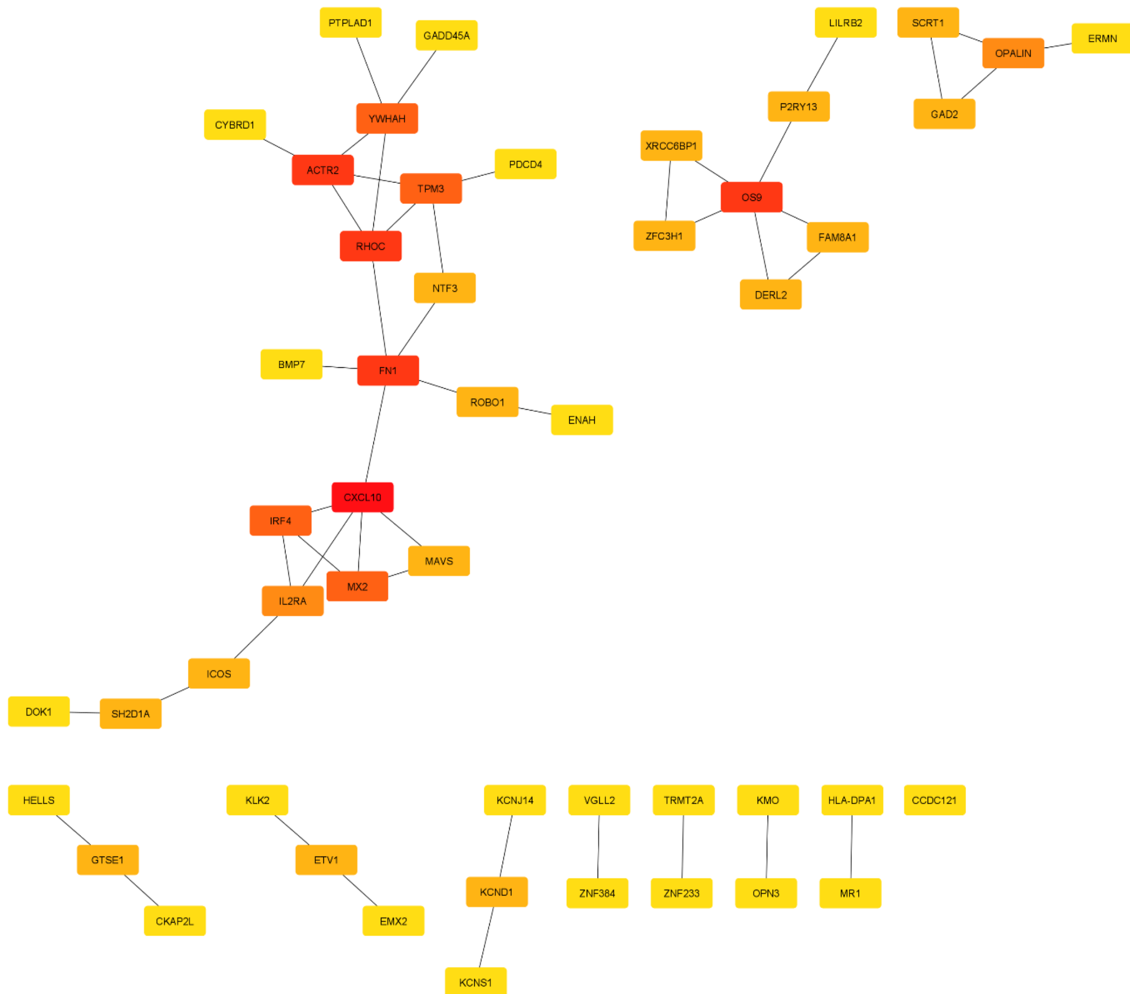


Figure 11: The Gene Interaction Network showing the interconnection between top 50 genes acquired from TargetScan and miRDB, using Cytoscape.

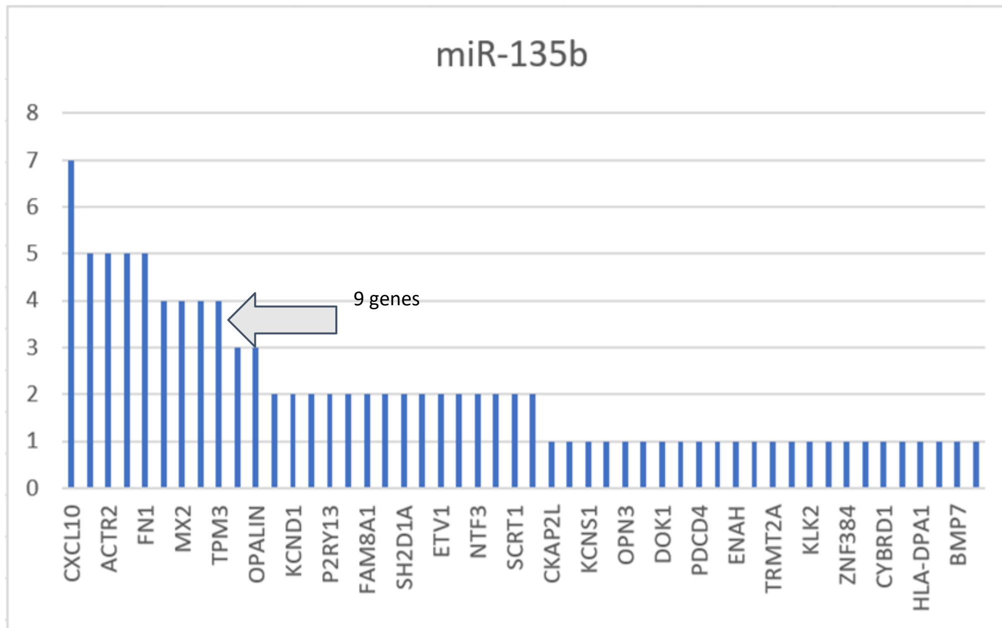


Figure 12: Line chart of top 50 genes in network string interactions ranked by Maximal Clique Centrality (MCC) method (x-axis- Scores, y-axis- Genes)

- <u>Expression analysis of genes regulated by miRNA-375</u>

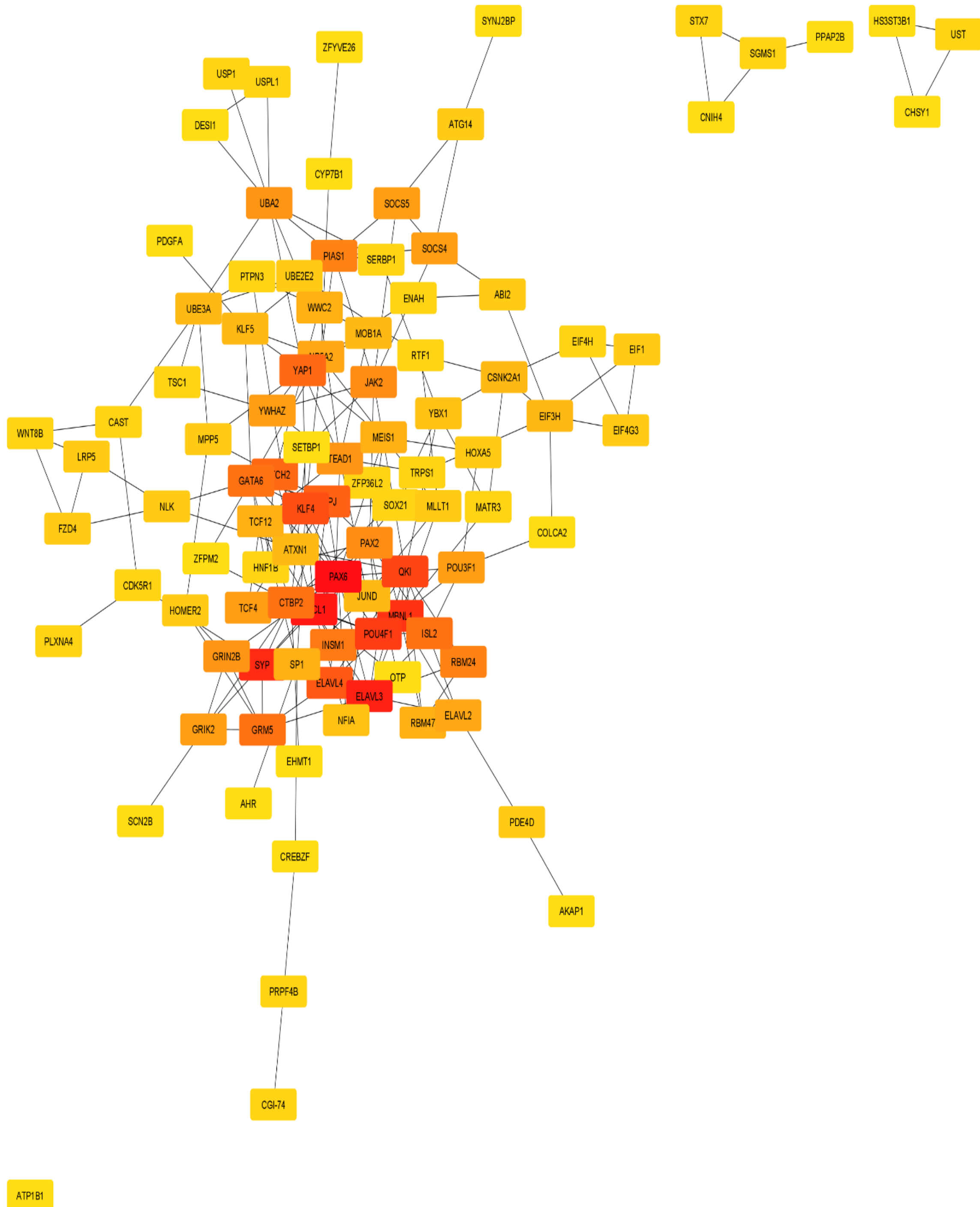


Figure 13: The Gene Interaction Network showing the interconnection between top 100 genes acquired from TargetScan and miRDB, using Cytoscape

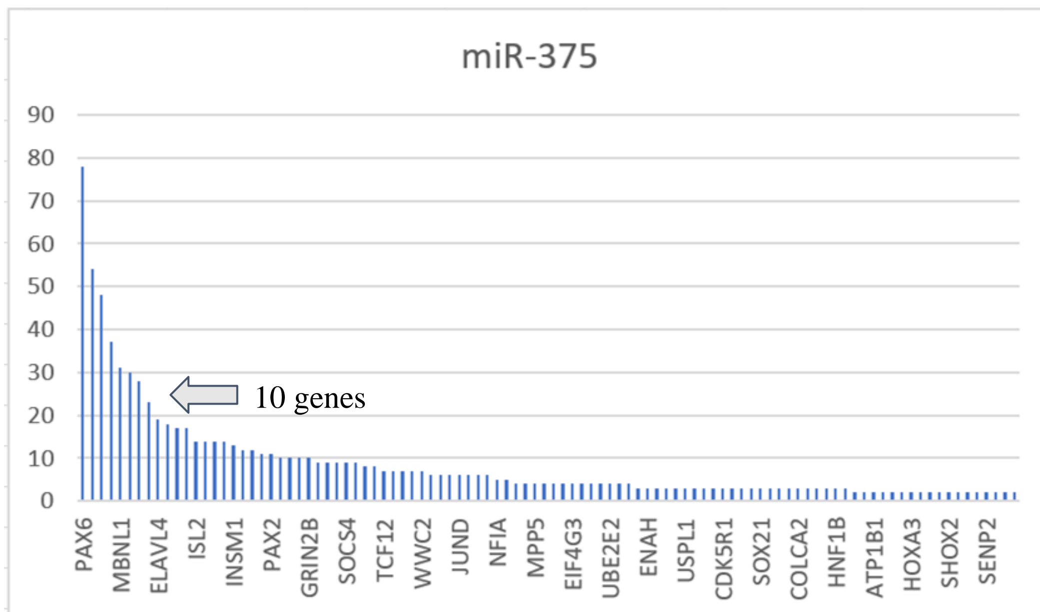


Figure 14: Line chart of top 100 genes in network string interactions ranked by MCC method (x-axis- Scores, y-axis- Genes)

Outcomes of GEPIA

- We observed the expression of all the genes using GEPIA tool with respect to BRCA gene.
- Only 8 genes showed significant level of expression change in tumor patient as compared with normal patient.

- **miR-190b regulates two genes which showed significant expression.**

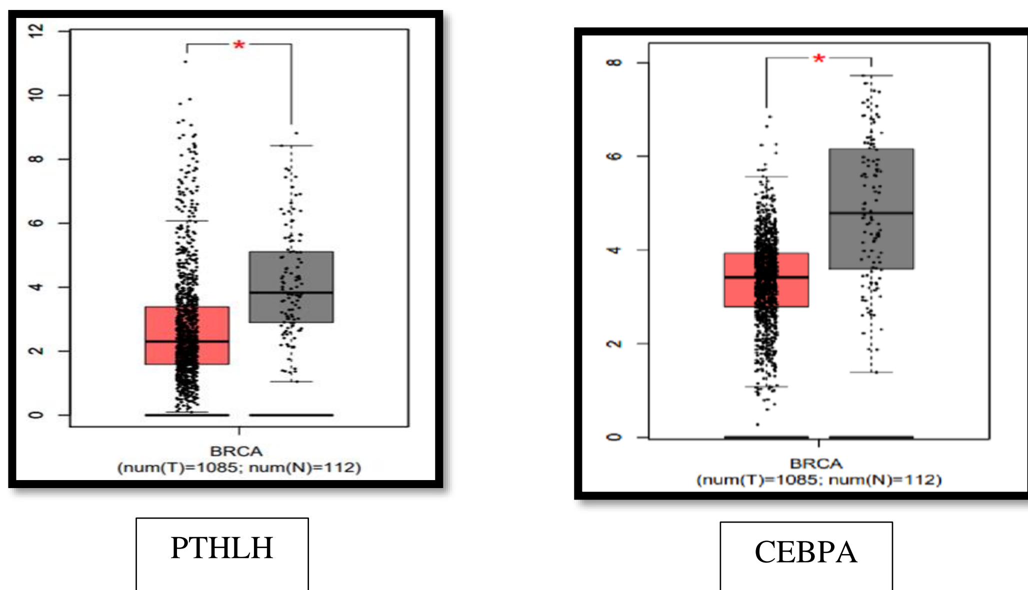


Figure 15: Gene expression of PTHLH and CEBPA

- **miR-135b regulates three genes which showed significant expression.**

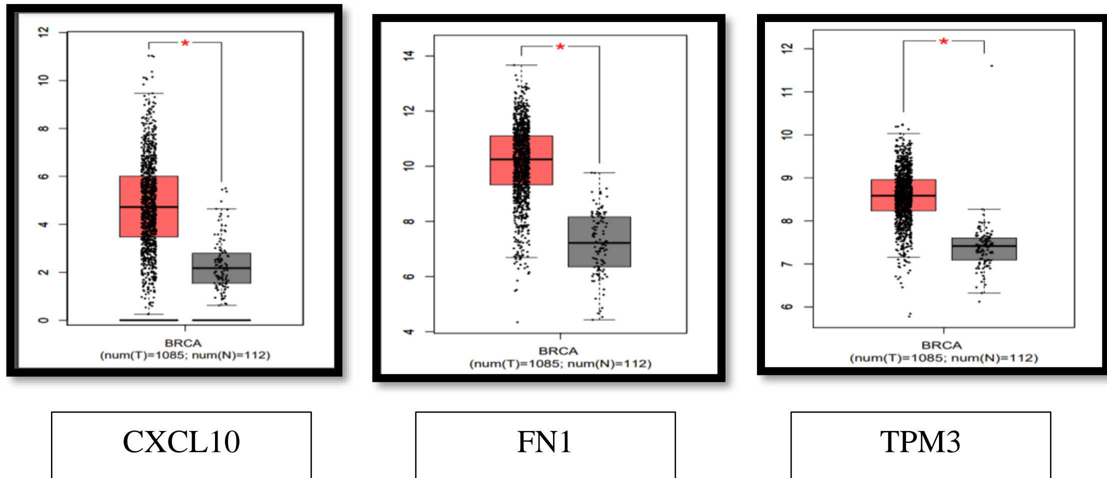


Figure 16: Gene expression of CXCL10, FN1 and TPM3

- **miR-375 regulates three genes which showed significant expression.**

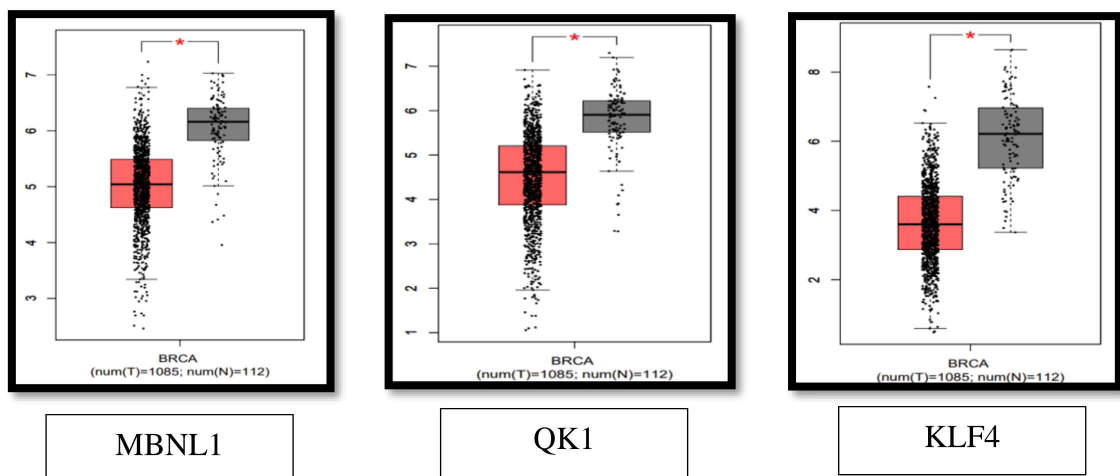


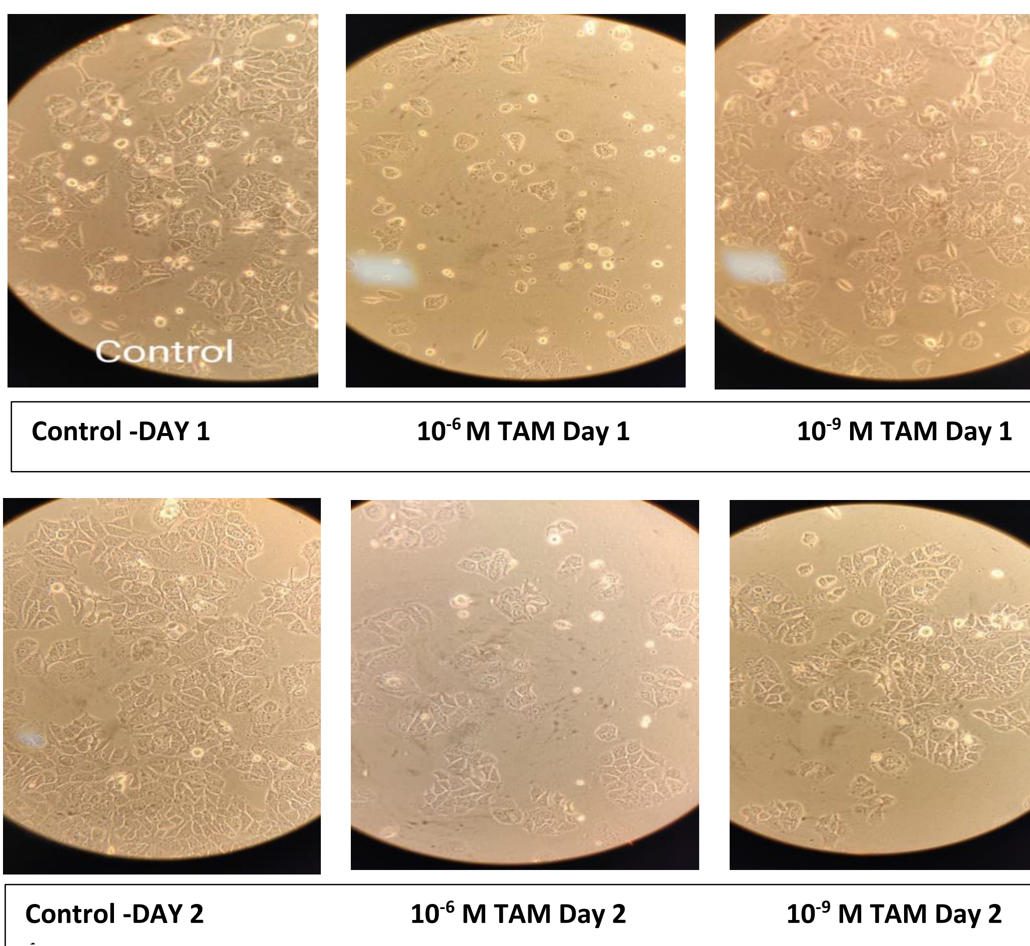
Figure 17: Gene expression of MBNL1, QK1 and KLF4

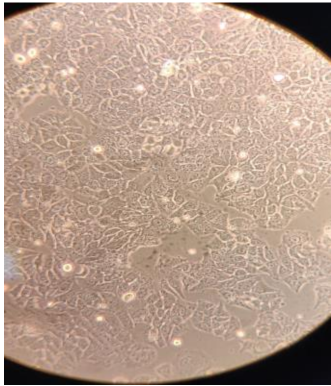
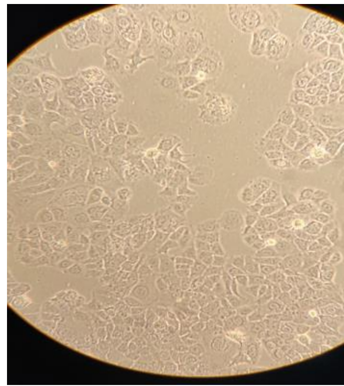
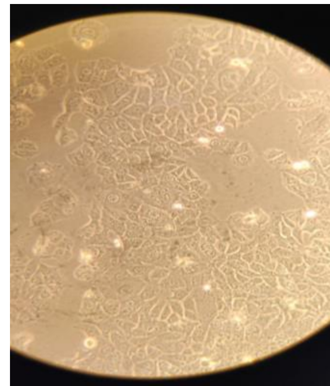
RESULTS OF IN-VITRO ANALYSIS (TAMOXIFEN RESISTANT BREAST CANCER CELL LINE)

The present study exhibits the current problem of the anti-estrogen resistance in the human breast cancer cells. In the present study we have treated MCF-7 cells with long term treatment of tamoxifen concentration of 10^{-6}M and 10^{-9}M. Expression of miR-375 was then validated in anti-estrogen treated cell lines. We tried to mimic MCF-7 resistance cell line.

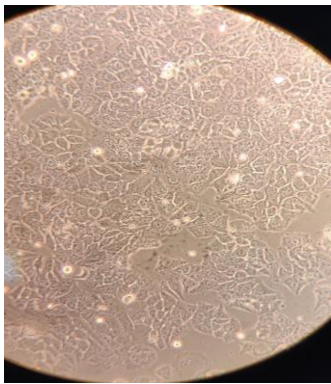
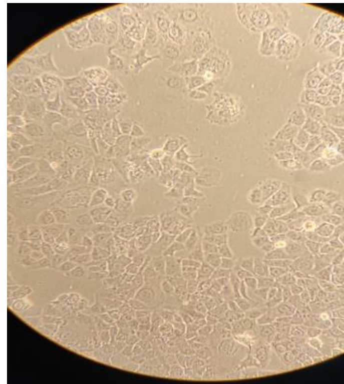
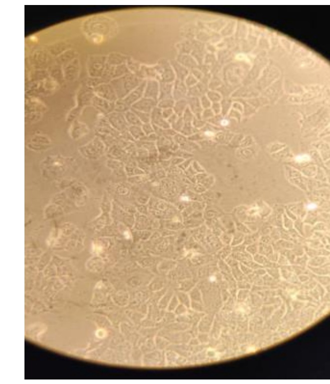
Cell characteristics of MCF-7:

MCF-7 cell line is adenocarcinoma mammalian breast cancer cell line (ER+ PR+) is believed to be a gold standard for any of the cell culture experiment on breast cancer cells. MCF-7 cells were observed after every 24 hours for 7 days. 10^5 cells were seeded with two different concentration of tamoxifen (10^{-6} and 10^{-9}). The cells were observed under inverted microscope after every 24 hours under 20X are demonstrated below in fig 18.

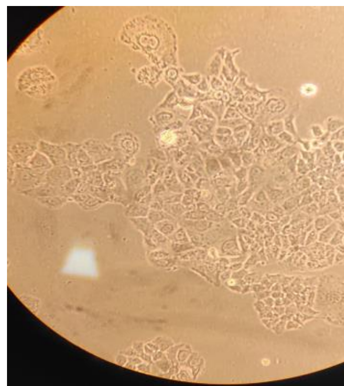
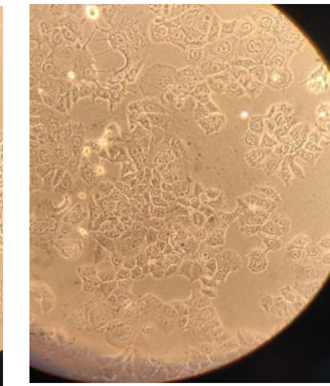


Control -DAY 3	10^{-6} M TAM Day 3	10^{-9} M TAM Day 3

Control -DAY 4	10^{-6} M TAM Day 4	10^{-9} M TAM Day 4

Control -DAY 5	10^{-6} M TAM Day 5	10^{-9} M TAM Day 5

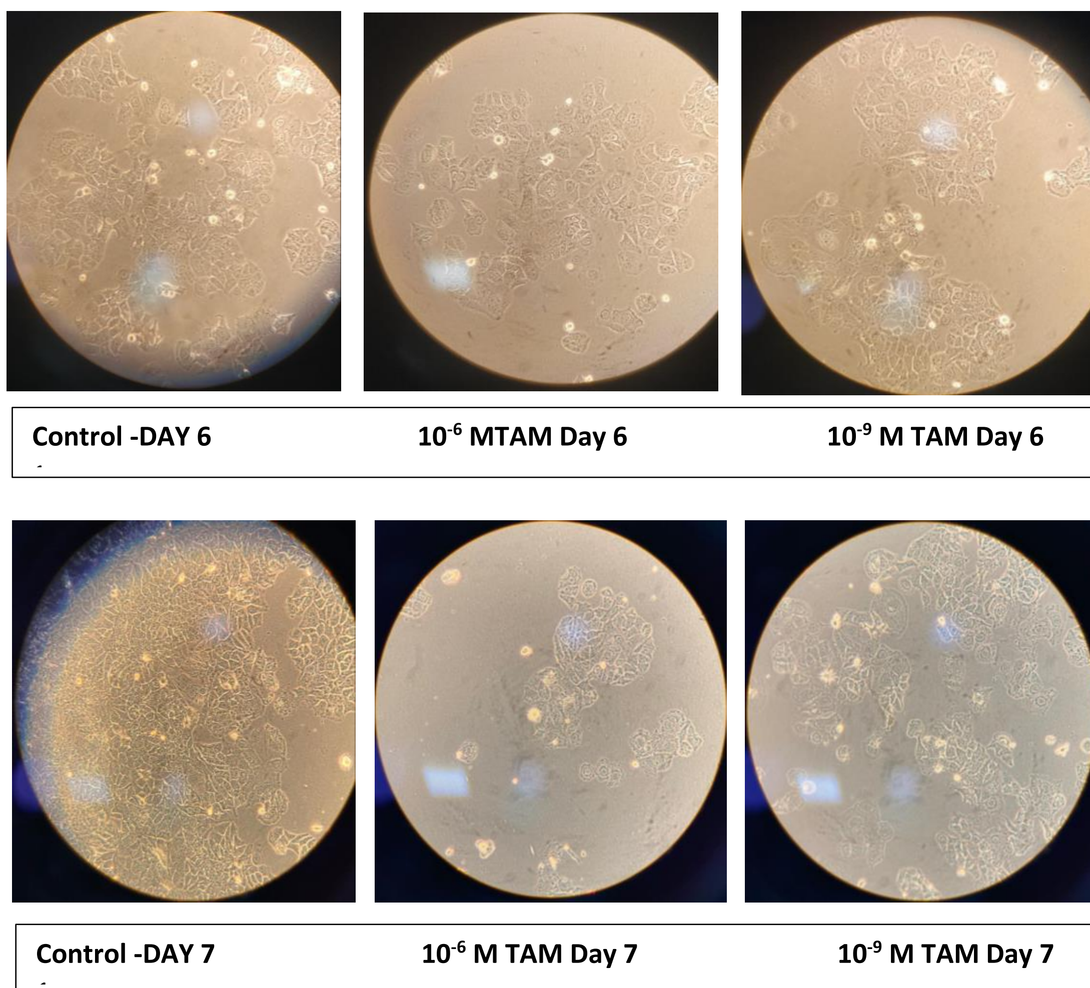


FIGURE 18: - Photomicrographs with diverse range of Confluency of MCF-7 Cells (20X)
Control: - untreated, M: - Molar (Tamoxifen concentration)

Isolation of RNA from Cells and analysis RNA concentration and its purity

Table: - Nano spectrometer Analysis

	Concentration (ng/µl)	**260/280 ratio**
Blank	0.00	0.0
Control	294.5	1.897
10^{-9} M TAM treated	184.7	1.926
10^{-6} M TAM treated	112.2	1.962

microRNA Expression using Real time-PCR

When cells were treated with a low concentration of tamoxifen (10^{-6} M), there was a significant increase in the expression of miRNA-375 compared to untreated cells, with a fold change of more than 4. However, at an even lower concentration of tamoxifen (10^{-9} M), the expression of miRNA-375 decreased compared to untreated cells. In other words, miRNA-375 was upregulated in response to low doses of tamoxifen but downregulated at even lower doses. This is illustrated in figure 19.

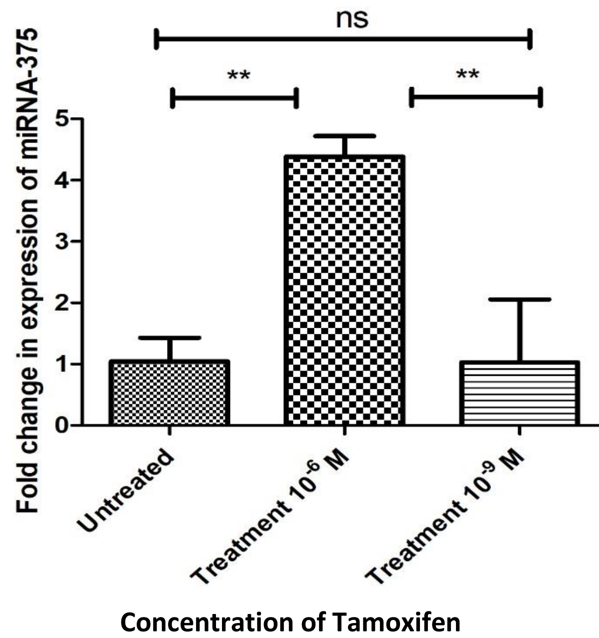


Figure 19: - Plot for the relative gene expression of miRNA-375 in MCF-7 untreated and treated with different concentrations of Tamoxifen. N=3; *p<0.05, **p<0.01, ns = p>0.05.

RESULTS OF TRASTUZUMAB RESISTANCE BREAST CANCER- IN SILICO ANALYSIS

Breast cancer is a complex disease, and despite the successful use of trastuzumab in the treatment of HER2-positive breast cancer, resistance to this drug remains a major clinical challenge. Therefore, in this study, we have directed our attention towards identifying the specific miRNAs and genes that are involved in trastuzumab resistance breast cancer cell lines.

Collection of Spatial Data from GEO database

The analysis of miRNAs has been facilitated through the use of GEO (Gene Expression Omnibus) database. The data were found using 2 search terms, the first being **'miRNA AND Herceptin resistance AND breast cancer'** and the second being **'microRNA AND trastuzumab resistance AND breast cancer'.** In this study, we solely focused on cell line data and did not obtain any patient data.

The total number of obtained datasets were bifurcated based on two criteria: -

1. The datasets should be of '*Homo sapiens'*
2. The datasets should consist GEO2R analysis

Out of all the databases disclosed we carried forward with only 2 datasets that fulfilled our selection criteria

1. **GSE38415**
2. **GSE197822**

Creation of Venn Diagram

- **'DEFINE GROUPS'** was performed of the above two datasets of trastuzumab resistance breast cancer data.
- The groups were defined as **"Trastuzumab sensitive cell line"** vs **"Trastuzumab resistance cell line"**

Group 1	Group 2
Bt474, SkBr3	**Bt474r, SkBr3r**

- After defining the groups, list of all the microRNAs of both databases were downloaded.
- The miRNA list contained data from multiple species, but the focus was on selecting only those miRNAs that are specific to humans (denoted as hsa) with a careful selection process.
- Out of all miRNAs only those miRNAs are selected whose **p-value < 0.05**.
- **Venn diagram** was constructed of those miRNAs from both datasets.
- As observed from Venn diagram, **only 1 miRNA** was found to be common from both datasets.

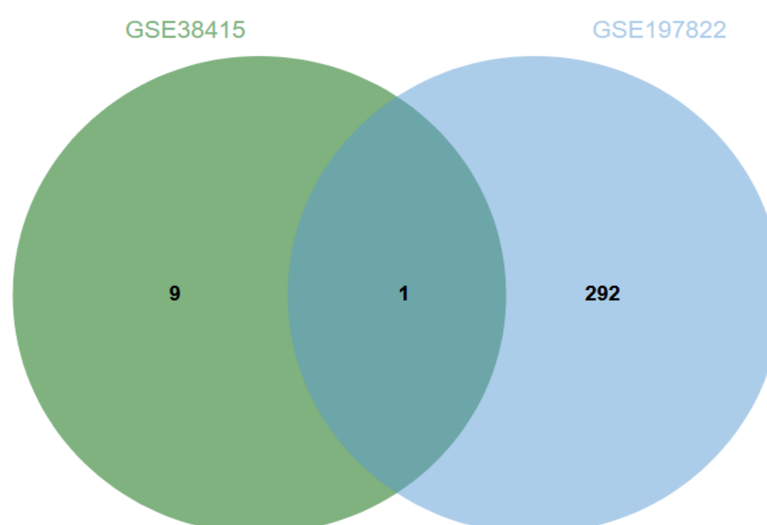


Figure 20: - Venn diagram of miRNAs in GSE38415 & GSE197822

Identification of Significance of miRNA: -

1. On comparing the common miRNA with each dataset, different p-value and log Fold Change were found.
2. A cut-off of **p-value ≤ 0.05** or **Fold change ± 1** was set.
3. **miRNA-1276** was considered to be **downregulated** based on log FC value of GSE38415.

Table 4: - Significant miRNAs based on p-value ≤ 0.05 or Fold change ± 1

	Common miRNA	**p-value**	**Log FC**
GSE38415	**miR-1276**	**0.03**	**-2.22**
GSE197822	**miR-1276**	**0.04**	**-0.01**

Target Search of miRNA using TargetScan and miRDB databases

1. Genes that are targeted by miR-1276 was analysed using TargetScan database and miRDB database and the table was downloaded.
2. In TargetScan, **Cumulative weighted context++ score** and in miRBD, **Target Score** were taken into consideration.
3. All the genes from both databases were taken into consideration and Venn diagram was constructed.
4. As shown above, **669 common genes** were found from TargetScan and miRDB database.
5. All 669 genes were further analysed using STRING and Cytoscape databases.

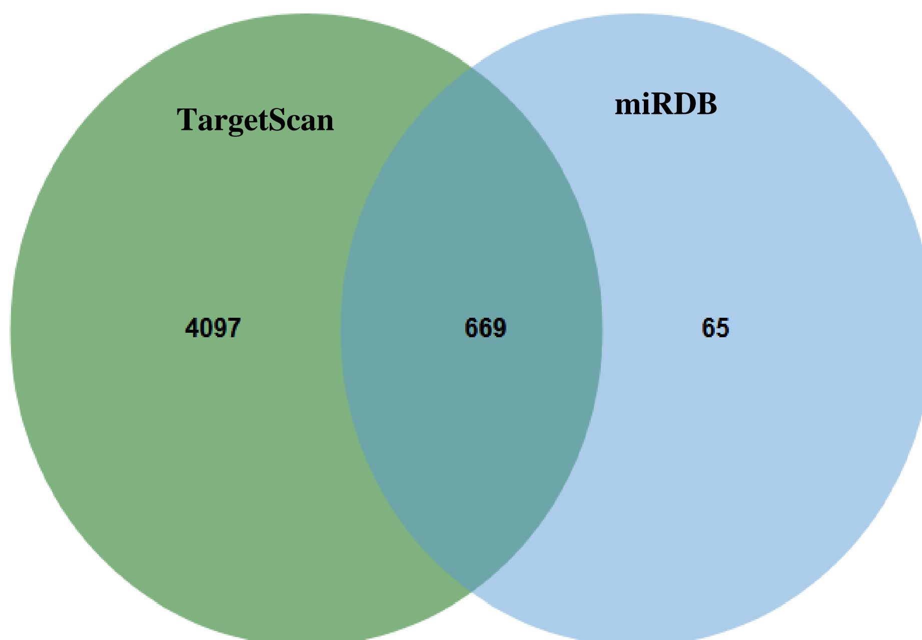


Figure 21: - Venn diagram of genes found common from TargetScan & miRDB

Gene interaction network using Cytoscape

1. All of the genes were uploaded to the STRING (Search Tool for Retrieval of Interacting Genes/Proteins) tool and the table was exported in the TSV format to make it compatible for Cytoscape analysis.

2. The file was then uploaded into Cytoscape for further analaysis.

3. Top 20 genes were selected for gene interaction network which was ranked according to MCC (Maximal Clique Centrality) method.

4. All the genes were then uploaded in GEPIA for further analysis.

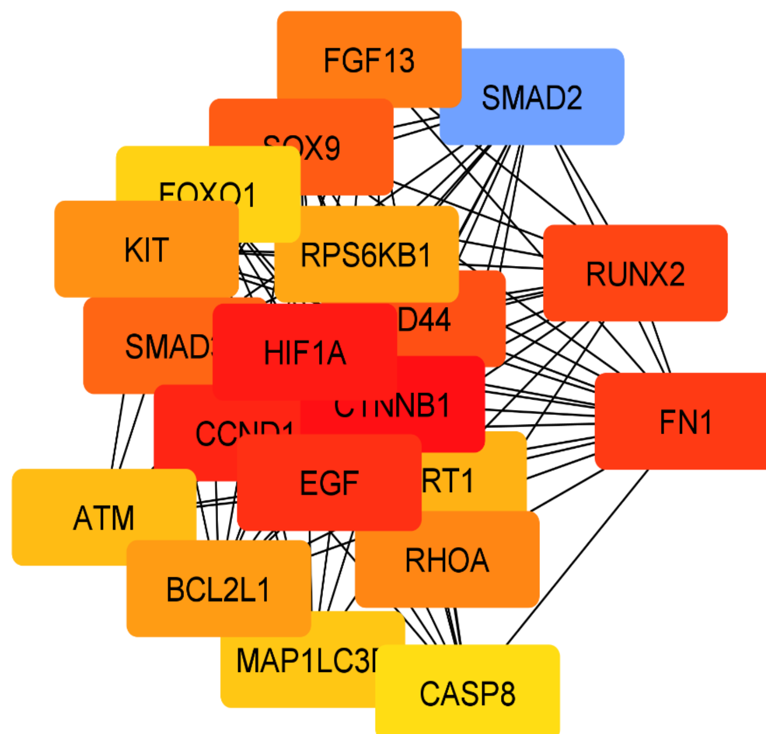


Figure 22: - Gene interaction network

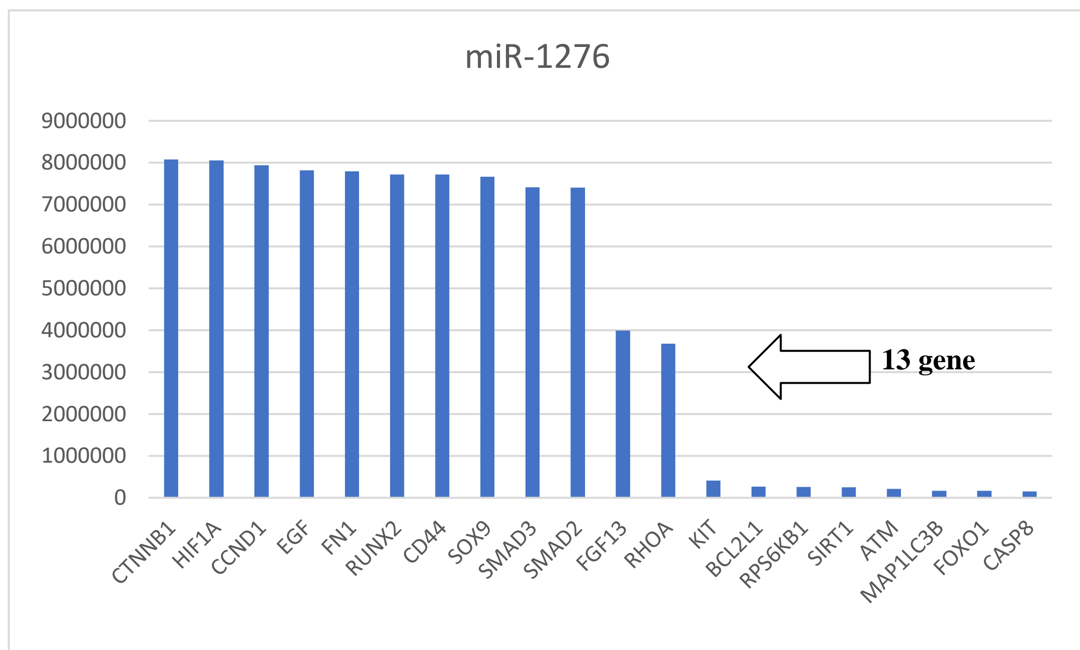


Figure 23: -Line Chart of top 20 genes based on MCC score (x-axis- Scores, y-axis- Genes)

Outcomes of GEPIA

- We observed the expression of all 13 genes in GEPIA in relation to the BRCA gene
- Only two genes were obtained using GEPIA based on the significance level of expression of genes in tumor patient as compared with normal patient.

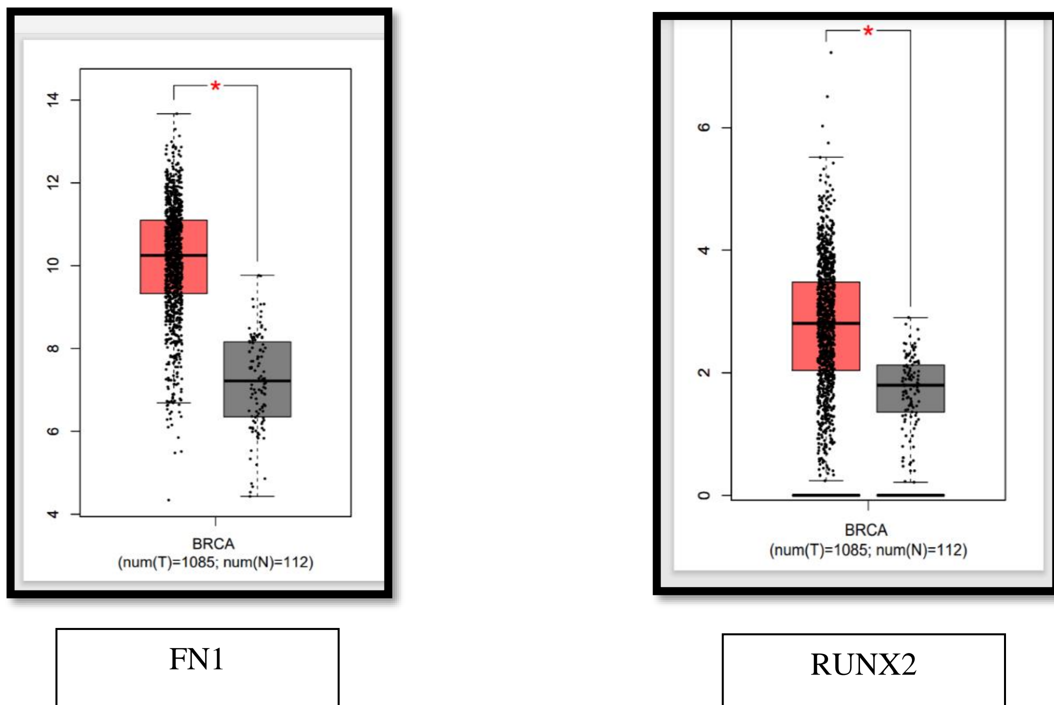


Figure 24: - Expression of FN1 & RUNX2 gene

DISCUSSION

In the current study we tried to identify the novel/unexplored miRNAs that are involved in resistance (Tamoxifen induced as well as Herceptin induced) in breast cancer. First, we have used the bioinformatics approach using the various tools, as well as review literature. Based on p-value and log FC value we have finalized miR-375 for in-vitro study. Secondly, we tried to go further in order to identify the role of miR-375 in the ER positive cell line as a mimicking model.

ER and HER2 are important targets for treating ER+ and HER2+ breast cancer patients. Tamoxifen and Herceptin are the two main targeting agent that targets estrogen receptor and human epidermal growth factor receptor 2 which are already approved by FDA and are already in market for several years. Moreover, resistance to these drugs has been a major obstacle for treatment of ER+ and HER2+ breast cancer patients. There are many reasons for development of resistance to these drugs, and one of them is due to dysregulation of microRNAs.

Here, we studied miRNAs that are playing role in tamoxifen resistance breast cancer: miR-190b, miR-135b and miR-375 act as a causative agent for tamoxifen resistance wherein, miR-190b and miR-375 are upregulated while miR-135b is downregulated in tamoxifen-resistant patients. Further, these miRNAs target certain genes that has involvement in breast cancer and mainly resistance to anti-cancer drugs are studied. By in-vitro studies, we further validate miR-375 in MCF-7 cell line.

A study involving a group of patients with ER+ breast cancer undergoing adjuvant tamoxifen treatment showed changed expression of miR-190b in TamR cell lines (Joshi et al. 2016). miR-190b was also up-regulated in ER+/Normal breast tissue. MiR-190b may potentially constitute a novel biomarker in hormone dependent breast tumours due to its selectivity and elevated expression, however its precise involvement in carcinogenesis is yet unknown (Cizeron-Clairac et al. 2015). According to *Ward et al.* miR-135b and miR-190b was downregulated in their tamoxifen resistance-models using q-PCR technique.

Bioinformatics study and laboratory validation showed HOXB3 as a direct target of miR-375, which overexpressed degraded HOXB3 mRNA in breast cancer MCF-7 cells. miR-375 reduces CSC characteristics in MCF-7 cells (Fu et al. 2017). Enhancing miRNA-375 expression again made tamoxifen-resistant cells susceptible to the drug and prevented invasion. Main reason of tamoxifen resistance is due to miR-375 downregulation. It was demonstrated that miR-375 can prevent CSC phenotype development in MCF-7 cells. Their outcomes showed that miR-375 restoration can prevent tamoxifen resistance by controlling CSCs (Ward et al. 2013). miR-375 targets MTDH (Metadherin) whose expression was increased in tamoxifen- resistant cells (Wan et al. 2014).

Our in-vitro study showed that miR-375 was upregulated when we mimic the MCF-7 resistant cell line.

ER-positive cells had higher levels of hsa-miR-375 than ER-negative cells or cells in normal condition did. This led to the hypothesis that hsa-miR-375 up-regulation is a crucial proliferation driver and an early event in cancer development in ER-positive tissues (Jonsdottir et al. 2012). According to Ward et al., miRNA-375 is directly repressed by MTDH expression in tamoxifen-sensitive cells (Wild type MCF-7), and this regulation gives the cells a tamoxifen-sensitive and epithelial phenotype. In contrast, as cells grow resistant to tamoxifen, miRNA-375 expression is reduced, increasing the expression of MTDH and other targets. As a result, the cells become more invasive, resistant, and mesenchymal-like.

In our study, using the bioinformatics tool we identify that miR-1276 is downregulated in trastuzumab resistance breast cancer cell lines. After conducting a search for potential targets of miR-1276 using target search tools, we identified two genes: Fibronectin 1 (FN1) and Runt-related protein 2 (RUNX2). We also analyzed the expression of both genes in tumor patients vs normal patients using an in-silico approach and found that upregulation of both genes is responsible for trastuzumab resistance in breast cancer cell lines.

FN1 gene is a glycoprotein and is found to be present in plasma, cell surface, and extracellular matrix. Fibronectin 1 gene plays a potential role in cell adhesion, blood coagulation, migration, and wound healing. FN1 expression

was found to be overexpressed in the acquired trastuzumab-resistant BT474 breast cancer cell line when compared with parental BT474 due to the upregulation of EGFR expression (Y.S. Jeong et.al). Another study examined the contribution of EMT in anti-HER2 drug resistance in HER2+ breast cancer cell lines. They analyzed and suggested the expression of mesenchymal marker genes such as FN1, SNAI2, and CDH2 to be highly upregulated in an acquired lapatinib-resistant BT747 HER2+ breast cancer cell line (Babak Nami et.al).

We identified RUNX2 gene to be highly upregulated and playing role in trastuzumab resistance. Runt-related protein 2 is a transcription factor that plays role in osteogenesis, but recent studies also found its role in DNA damage which is a critical factor in the development of cancer. RUNX2 has the ability to interact with important cell cycle regulators such as cyclin-dependent kinases, p21Cip1 protein, and also with p53 which is a tumor suppressor gene. A study suggested the role of the RUNX2 gene in estrogen receptor signaling, interacting with Erα and inducing estrogen levels by enhancing the level of aromatase- an estrogen-producing enzyme in ER-negative breast cancer patients (Wysokinski et al.). They also put forward the interaction of RUNX2 with HER1 & HER2 receptors and recommended the poor survival of HER2+ breast cancer patients due the high nuclear level of runt-related protein 2. Given that high HER2 and RUNX2 expression are each recognized as independent poor prognostic factors in breast cancer, evaluating both parameters in combination may offer a new and innovative approach to assessing prognosis. As such, further investigation into the interplay between these two factors is warranted.

Conclusion

The aim of this study was to identify the miRNA molecules that are involved in the mechanism of resistance induced by two drugs (1) Tamoxifen and (2) Trastuzumab. Initially,

1. In the bioinformatics study, we explored the microRNAs that are involved in breast cancer patients that are resistant to tamoxifen and trastuzumab.
2. Further, we carried forward with in-vitro validation of miRNA involved in only tamoxifen resistance breast cancer patient.

Through our analysis we discovered that in tamoxifen resistant breast cancer patients,

- The expression of miR-190b was increased, which led to the downregulation of two genes, PTHLH and CEBPA.
- The expression of miR-135b was decreased, which led to the upregulation of three genes, CXCL10, FN1 & TPM3.
- The expression of miR-375 was increased, which led to the downregulation of three genes, MBNL1, QK1 & KLF4.

Through in-vitro analysis of miRNA-375 in tamoxifen resistance MCF-7 cell line we conclude that,

miRNA-375 expression was sensitive to tamoxifen concentration, with an **upregulation** at a concentration of **10^{-6} M** of tamoxifen and no significant change in expression at a concentration of **10^{-9} M** of tamoxifen as compared to untreated cells. This suggests that miRNA-375 may play a role in the cellular response to tamoxifen treatment and could potentially serve as a biomarker for monitoring the efficacy of tamoxifen therapy.

In trastuzumab resistance breast cancer cell line we conclude that,

- The expression of miR-1276 was decreased, which led to the upregulation of two genes, FN1 & RUNX2. These findings provide valuable insight into the molecular mechanisms underlying trastuzumab resistance and could ultimately contribute to the development of more effective treatment strategies for patients with HER2-positive breast cancer.

Future Prospects

Our analysis suggests that miR-375 may play a role in the development of drug resistance in MCF-7 cells, as it is dysregulated in the resistant cell line compared to the untreated or normal breast cancer cell line. After treating with tamoxifen for 7 days, we observed an upregulation of miR-375. To establish a reliable drug-resistant cell line, we plan to administer tamoxifen treatment continuously for 3 months. Furthermore, we intend to investigate the role of miR-375 in an in-vivo model.

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