Influence of Antiestrogen on Transforming Growth Factor-β signaling mediators in Triple Negative Breast Cancer

A dissertation thesis submitted to Nirma University for

partial fulfilment for the degree of

MASTER OF SCIENCE IN BIOTECHNOLOGY, BIOCHEMISTRY, MICROBIOLOGY

Submitted by

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NIRMA UNIVERSITY
INSTITUTE OF SCIENCE
NAAC ACCREDITED 'A+' GRADE

CERTIFICATE

This is to certify that the thesis entitled " **Influence of Antiestrogen on TGF- β Signalling mediators in Triple Negative Breast Cancer** " submitted to the Institute of Science, Nirma University in partial fulfilment of the requirement for the award of the degree of M.Sc., in Biochemistry, Biotechnology , Microbiology. The dissertation project is jointly carried out by **Shloka Kedar Majmudar** (20MBT026) **Nishtha Nischal Thakar** (20MMB033) **Devanshi Rajeshkumar Thakker** (19MBC022). Under the guidance of Dr. Heena V. Dave. No part of the thesis has been submitted for any other degree or diploma.

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DECLARATION

The above dissertation project was jointly completed by **Shloka Kedar Majmudar** (20MBT026) **Nishtha Nischal Thakar** (20MMB033) and **Devanshi Rajeshkumar Thakker** (19MBC022), under the guidance of Dr. Heena Dave.

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ABBREVIATIONS

AF-1: Activation Function-1

AF-2: Activation Function-2

AI: Aromatase Inhibitor

BSA: Bovine Serum Albumin

CDK : Cyclin Dependent Kinases

DBD: DNA-binding domain

DCIS: Ductal carcinoma in situ

DMEM: Dulbecco's Modified Eagle Medium

DMSO: Dimethyl Sulfoxide

E2: 17-estradiol

ER : Estrogen Receptor

ERE : Estrogen Response Elements

EGFR: Epidermal Growth Factor Receptor

EMT: Epithelial to Mesenchymal transition

ER: Estrogen Receptor

ERE: Estrogen Response elements

FBS: Fatal Bovine Serum

HER-2: Human Epidermal Growth Factor Receptor-2

Hsp90/70: Heat Shock protein

HMG1/2 : High Mobility Group Box 1 Protein

MAPK: Mitogen-Activated Protein Kinases

MRI: Magnetic Resonance Imaging

NTD : N-Terminal Domain

PBS: Phosphate Buffer Saline

PI3K: Phosphatidylinositol-3-Kinases

PR: Progesteron Receptor

Ras: Rat Sarcoma

SERM: Selective Estrogen Receptor Modulators

TAE Buffer: Tris Acetic acid EDTA Buffer

TAM: Tamoxifen

4-OH TAM: 4-Hydroxy Tamoxifen

TGF-β1/2/3: Transforming Growth Factor-β1/2/3

TβRI/II : Transforming Growth Factor Receptor I/II

TNBC: Triple negative breast cancer

Trypsin-EDTA: Trypsin Ethylene Diamine Tetra Acetic Acid

SMAD : MAD genes from *Drosophila* and Sma gene from *Caenorabditis elegans*

SMURF-1/2 : SMAD specific E3 Ubiqutin Ligase ½

Abstract

Breast cancer which is a form of malignancy and has been observed to be one of the most common form of malignancies in the world. It has been affecting around 2.3 million women every year. Among these 1 million cases are of Triple-Negative Breast Cancer (TNBC) worldwide. In India, around 27% to 35% of women among breast cancer patients are diagnosed with TNBC. TNBC is an aggressive type of cancer and lacks three kinds of receptors which are Estrogen receptor (ER), Progesterone Receptor (PR), and Human Epidermal Growth Factor Receptor 2 (HER-2). It is one of the most challenging malignancies that is difficult to treat. There are various hormone therapies available for breast cancer but as TNBC lacks those receptors very fewer treatments or targeted therapies are available. Antioestrogens like Tamoxifen act on the ER-beta receptors which are present. Tamoxifen is a Selective Estrogen Receptor Modulator (SERM) and hence is used as one of the treatments for breast cancer. This study will focus on the effect of tamoxifen on ER signalling which follows or is activated by two pathways viz. Genomic and Non-genomic. In the genomic pathway ER-E2-ligand complex directly moves to the nucleus and binds to DNA for transcription transactivation. It also interacts with Activating Protein AP-1 through the indirect genomic pathway. AP-1 is involved in the auto-induction of Transforming Growth Factor-β (TGF-beta) which has a dual role as a tumor suppressor during the initial stages of cancer progression and as a tumor promoter during later stages. There might be a cross-talk between TGF-β and ER signalling which helps in tumor progression. Therefore, in the presence of antioestrogens like Tamoxifen, this study aimsto study the exact down-stream mechanism and the levels of TGF-β. This may provide us withTGF-β as a novel biomarker for TNBC.

Introduction

1.1 Cancer

A cancer is a condition in which cells develop abnormally. The cells have lost their capacity to inhibit cell proliferation, allowing them to acquire immortality, invade neighbouring tissues, acquire malignancy and induce angiogenesis through which they obtain nourishment. Malignant cells can arise from any tissue in the body.

As cancerous cells grow and proliferate, tumor is formed. This tumor then spreads to other parts of the organ/body. Tumor is defined as "An abnormal mass of tissue that forms when cells grow and divide more than they should or do not die when they should." (www.cancer.gov/publicatiopns/dictionaries/cancer-terms/def/tumor). Cancer and tumor are two different terms. When cells divide uncontrollably in solid tissues or organs it is known as tumor and when cells almost everywhere in the body divide uncontrollably is known as cancer which is a disease. Primarily cancers are divided into two types viz. Benign, which are cancer cells that do not spread to other parts of the body or to any other tissue and the other is Malignant, in which cancer EMT (Epithelial to Mesenchymal Transition
) is seen and cancer cells affect the other healthy parts or organs of the body. (https://jamanetwork.com/journals/jamaoncology/fullarticle/2768634)

Cancer is divided into majorly four categories:

(i) Carcinoma: This type of cancer usually begins on the tissue covering the internal organs or the skin. Examples are lung cancer, colorectal cancer etc.

(ii) Leukaemia: This type of cancer in blood. Healthy blood cells are affected by this type of cancer. Examples include lymphocytic leukaemia and myeloid leukaemia.

(iii) Sarcomas: This type of cancer starts in the connective tissue of the body. Examples include cancer in nerves etc.

(iv) Lymphomas: This type of cancer begins in the lymphatic system. Examples include Hog din's lymphoma and non-Hog din's lymphoma. (www.cancer.net/navigating-cancer-care/cancer-basics/what-c%C3%A1ncer)

There were 10 hallmarks of cancer given by Hanahan and Weinberg in 2000 and 2011. Recently 7 hallmarks of cancer are stated which are (1) Selective growth and proliferation (ii) Changed stress response (iii) increased vascularisation (iv) Invasion and metastasis (v) Metabolic changes (vi) immune modulation (vii) Abetting microenvironment. (Found Y. et. al., 2017)

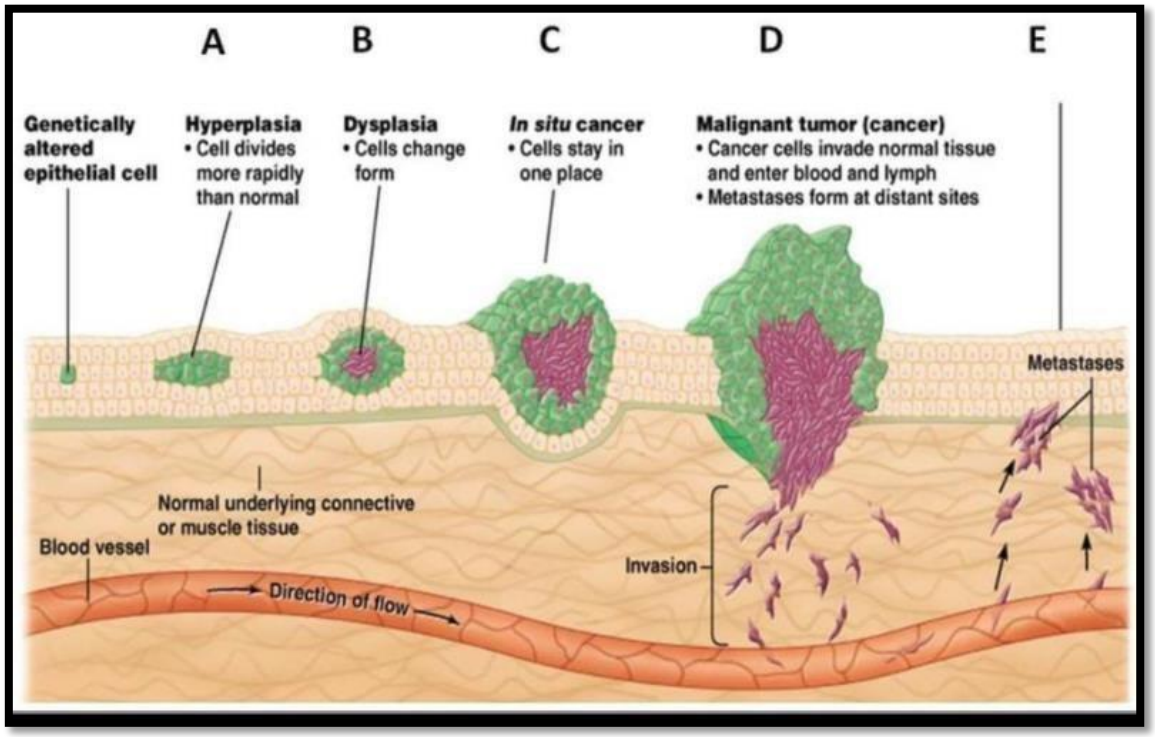

Figure 1– Stages of Cancer Development

1.2 Incidence of Breast Cancer

1.2.1 Global -

Breast cancer will overtake lung cancer as the most common form of the worldwide. A total of 2.3 million new cases were reported, accounting for 11.7 percent of all cancer cases. It is sixth highest causes of cancer death worldwide, accounting for 68500 deaths. Breast cancer affects one out of every six women and one out of every six women dies from it. In the vast majority of countries (159 of 185) and mortality in 110 countries, it has rated first. In Australia, New Zealand, Western Europe, North America and North Europe, the rate of occurrence is 88 percent higher than in central nations such as South America, Middle Africa and South-Central Asia. In countries like South America, Africa, as well as in high-income Asian countries, have shown a lower trend. (https://acsjournals.onlinelibrary.wiley.com/)

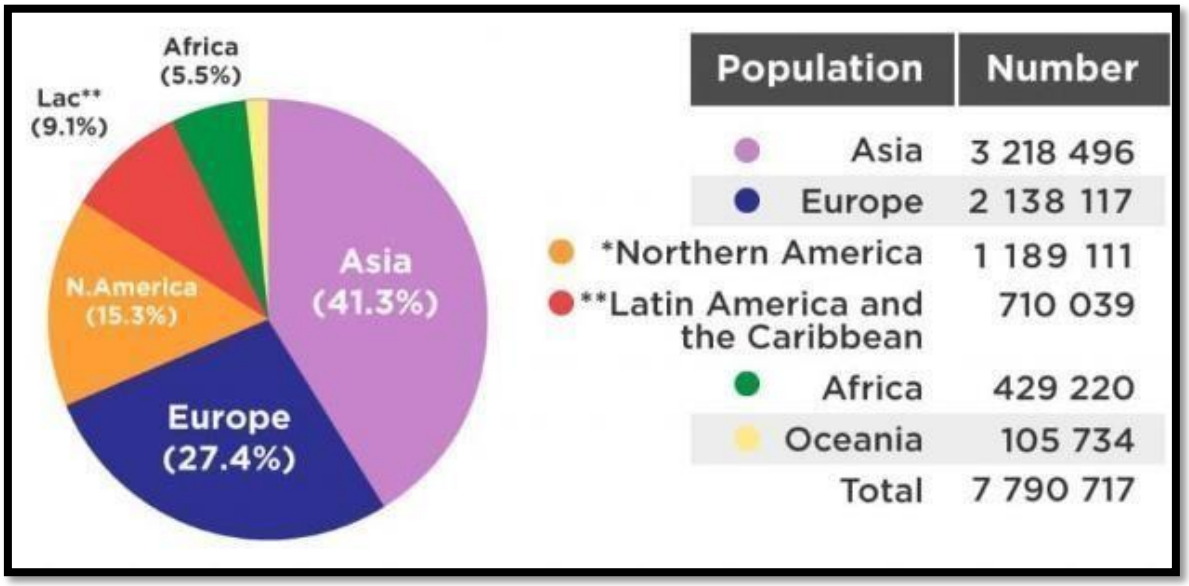

Figure 2 – Incidence of Breast cancer five-year Prevalence of both sexes

The incidence rates have skyrocketed in the higher human development index carrying countries that reflect a prevalence of the following factors.

- Menarche at a very early age
- A usually large number of children.
- Decreased breastfeeding.
- Contraceptive pills
- The postponement of childbearing.
- Hormone treatment for menopause.
- Alcohol consumption, Obesityand physical inactivity are lifestyle risk factor.

Growing economics have influenced the prevalence of breast cancer risk factor by causing dramatic changes in life style, social culture and the built environment, as well as an increase in the number of women in the industrial labour force. (https://acsjournals.onlinelibrary.wiley.com/)

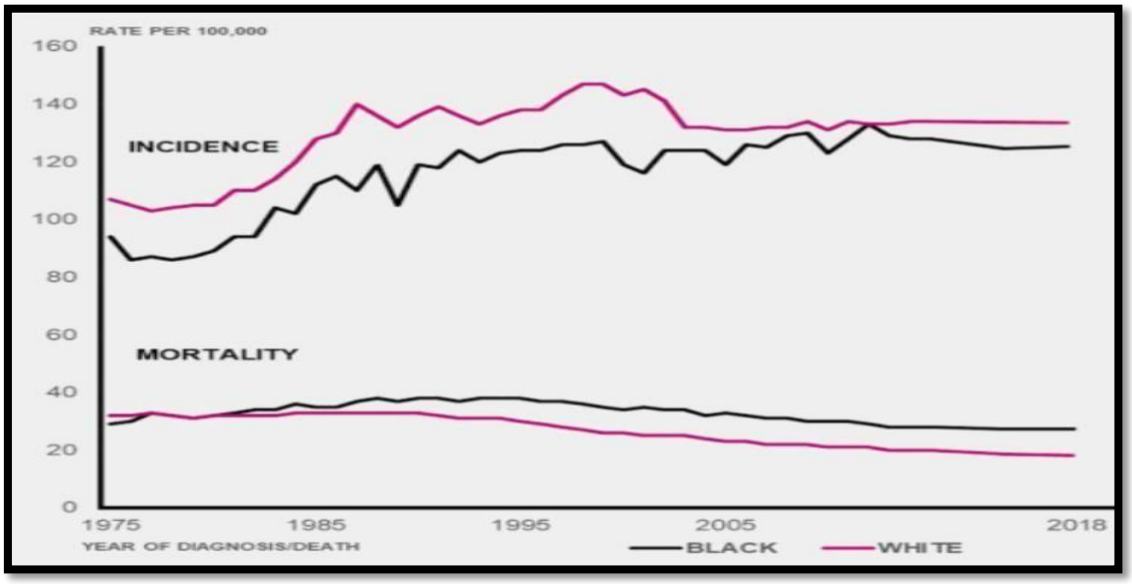

Figure 3 – Incidence of Breast cancer

1.2.2 Local -

- Mumbai, Delhi, Bengaluru, Bhopal, Kolkata, Chennai and Ahmedabad account for more than a quarter of all the female cancer cases. In 2012, 1.67 million new cancer cases were anticipated to have been diagnosed. It is becoming the most frequent cancer in women all over the world. The number of cases among women in developing countries (883000) is slightly greater than in industrial countries (794000).
- While India's age-adjusted breast cancer incidence rate is lower (25,8 per 100,000) than United kingdom's (95 per 100,000), there is a significant increase in the cancer related morbidity and mortality across the Indian subcontinent, according to goal and Indian studies.
- According to Globo can 2012, India is responsible for roughly a third of all the breast cancer cases worldwide. Breast cancer incidence and death in India increased by 11.54 percent and 13.82 percent respectively between 2008 and 2012.
- Decrease in breast cancer screening, illnessdiagnosis at an advanced stage, and a lack of sufficient medical facilities are the main causes of the reported increase in death. Breast cancer was leading causes of death among women in five major Indian cities between 2012 and 2014 (Mumbai, Bangalore, Chennai, New Delhi and Dibrugarh)
- Breast cancer is one of the major reason of deaths in all urban registries, owing to the nation of urbanization and westernization as well as altering lifestyles and eating habits. among the different registrations. Thiruvananthapuram had the highest crude rate (43,9 per 100,000), followed by New Delhi (34.8), Mumbai (33.6) and Chennai (406).

Population based cancer registration's top four places are occupied by Delhi with 41.0 age -adjusted rates (AAR) (per 100,000). Chennai with 37.9, Bangalore with 34.4 and Thiruvananthapuram with 37.7 AAR. (https://acsjournals.onlinelibrary.wiley.com/)

1.3 Breast Cancer:

Breast cancer is a kind of carcinoma which starts in one of the either breast. There are more than 1 million cases per year in India. Breast cancer lump may be benign or malignant depending on the stage. There are certain cases in which breast cancer exist but still the patient is unable to feel the lump and hence other signs and symptoms are important to take under consideration. Some common symptoms include: (https://www.cancercenter.com/cancer-types/breast-cancer/symptoms)

(1) Turning of nipple inwards
(2) Some rashes or abnormal skin around the cancer lump
(3) Dimpled skin
(4) Increased nipple discharge
(5) Swollen breast
(6) Swelling in armpit
(7) Swollen collarbone

1.3.1 Anatomy of breast:

(1) Nipple: The central dark part of the areola. This is where the milk is secreted out of the body.
(2) Areola: this is the dark central part of the whole breast which encircles the nipple. It also contains sweat glands.
(3) Lobules: This part of the breast is responsible for the production of milk. Cancers that originate from this part of breast are called lobular cancers.
(4) Ducts: They are responsible for carrying the milk that is produced in the lobules to the nipple.
(5) Fatty and connective tissue: it protects the ducts and lobules. It actually supports the whole breast. A cancer found in this tissue is called Phyllodes tumor which is a very rare kind of cancer.

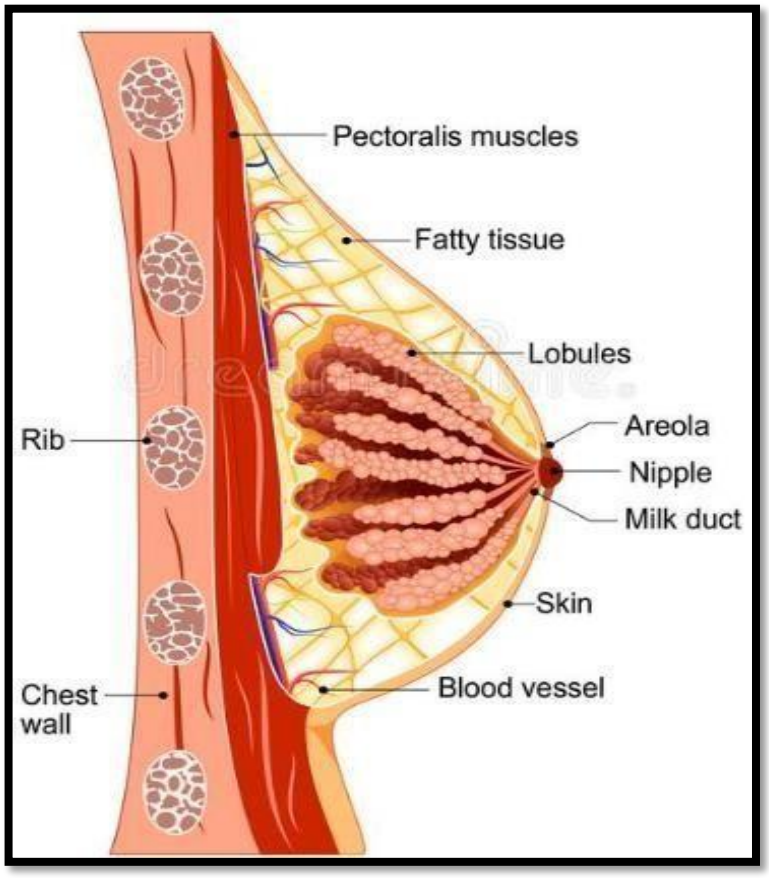

Figure 4: Anatomy of breast

1.3.2. The Lymphatic System of Breast:

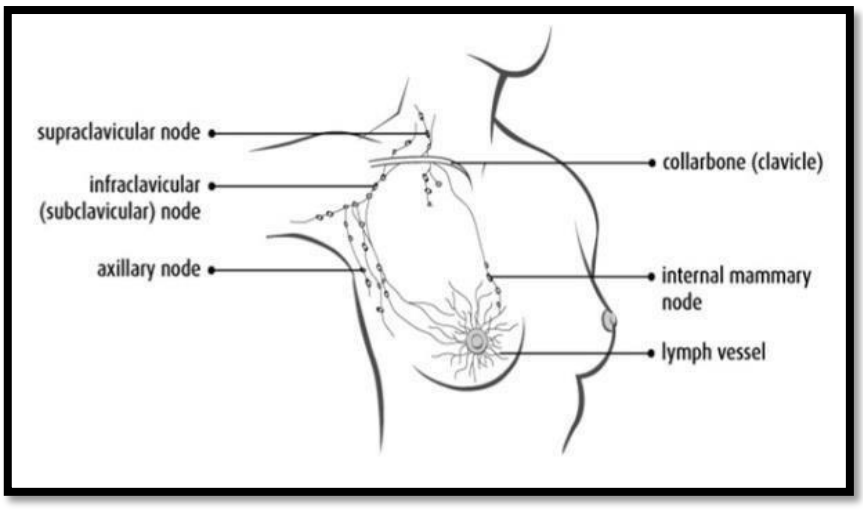

Figure 5: The lymphatic System of Breast

The main function of lymph vessels is to carry the lymph from the breast to lymph nodes present around the breast. There are various kinds of lymph nodes present in the breast.

(1) Supraclavicular nodes: it is located on superior position of the collarbone.

(2) Sub-clavicular nodes: it is located below the clavicle, besides the cephalic vein.

(3) Internal mammary nodes: they are located on the left side, in the intercoastal space.

(4) Axillary lymph nodes: these lymph nodes run from breast into the armpits.

1.3.3 Types of breast cancer

The first point is the epithelial cells that organs and tissues. Sometimes they are adenocarcinomas, which usually occur in glandular tissue. Adenocarcinomas of the breast originate in milk-producing glands called lobules or milk ducts. Among the other types are sarcomas, which begin in connective tissues, adipose tissues, and muscle cells. Sometimes it may be a mixture of in-situ cancer and invasive in a single breast tumor. If gone to get treated, a doctor first goes through the specifics which includes whether the cancer cells have moved beyond the breasts and the tissue from where it actually began. (https://www.cancer.net/cancer-types/breast-cancer/stages)

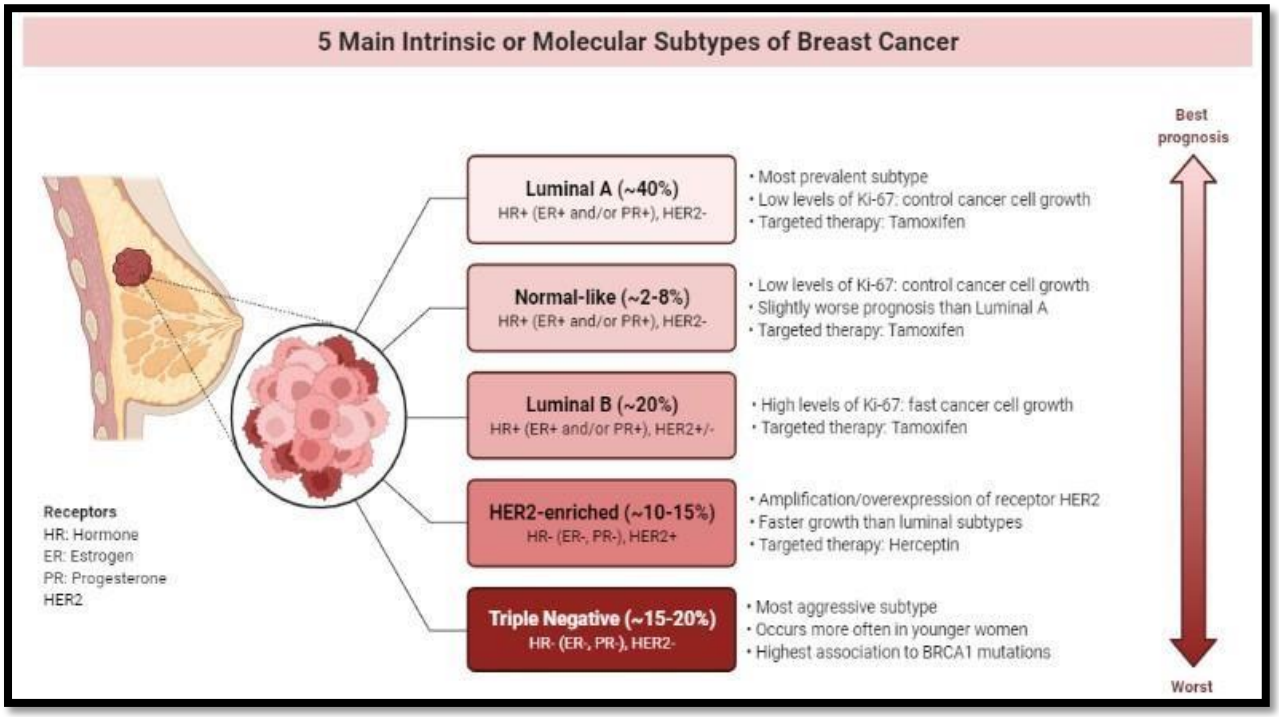

Figure 6 – Subtypes of Breast cancer

1) **Ductal carcinoma in situ:** Several types of the breast cancer are pre-invasive. The cancer cells develop in the milk ducts and do not spread to other parts. Also, it can be called stage 0 cancer.

2) **Invasive Ductal carcinoma:** In these types of the breast cancer that develops in fat tissue generally breaks through the duct wall. It usually starts in the milk ducts. It can spread to other parts of the body through bloodstream. It is easy to detect by a mammogram than the invasive lobular carcinoma.

3) **Invasive lobular carcinoma:** Invasive lobular carcinoma begins in the lobules. It can easily spread to different parts of the body.

4) **Inflammatory cancer:** In this type breast cancer, cells can block the lymph vessels of breast and are usually seen rarely but aggressive. The name inflammatory is given because inflammation is seen on the breast.

5) **Special types of invasive breast carcinoma:** There are several subtypes of invasive carcinoma. When viewed under the microscope, they are named according to the arrangement of cells witnessed.

1.3.4 TNM staging system

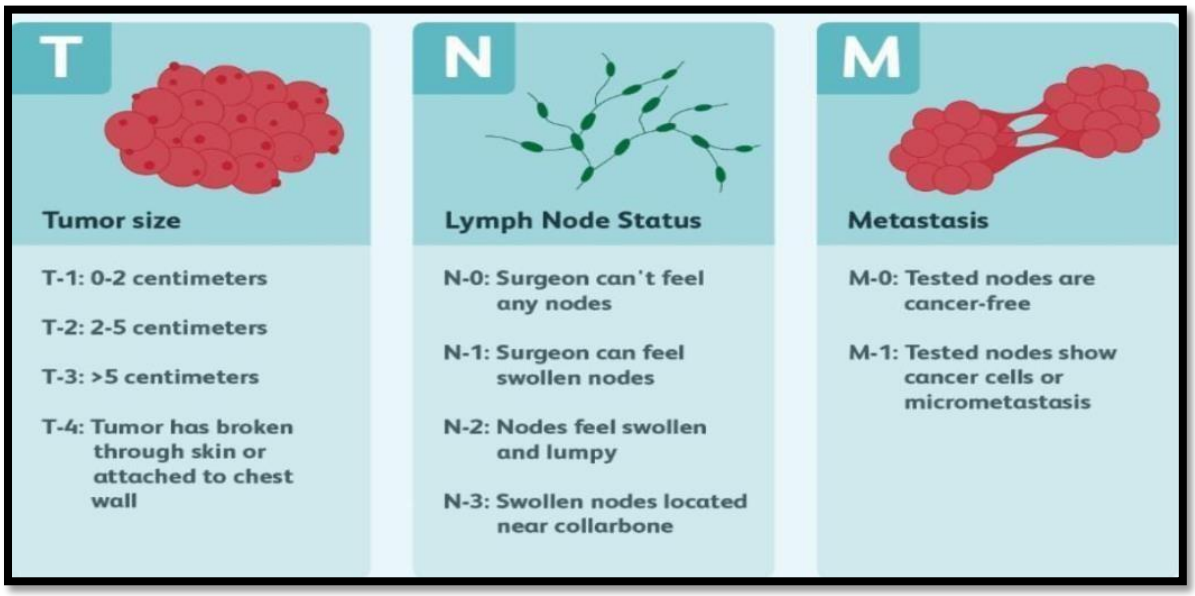

Figure 7: TNM Staging of Breast Cancer

The above abbreviation TNM stands for the following:

T: Tumor: the size of detected primary tumor.

- When the tumor size cannot be detected the stage is denoted by Tx. A term 'Tis (DCIS)' is used when breast ducts are affected by cancer but still it has not affected any other organ. A term 'Tis (Paget)' is used when cancer has affected only the nipple of the entire breast.

- When the stage is given as T1, it means that the cancer is around or less than 2cm in dimension which is further divided into 4 categories (T1mi, T1a, T1b and T1c). When the stage is given as T2, it means that the cancer is more than 2 cm in dimensions and T3 means the cancer is larger than 5 cm. T4 stage is divided into four other categories.

N: Nodes: cells spread to the lymph nodes.

- There are various lymph nodes affected by cancer. When 1-3 lymph nodes are affected by cancer it is staged as pN1. This stage is further divided into four categories named pN1mi, pN1a, pN1b and pN1c. When cancer cells are present in 4-9 lymph nodes the cancer is staged as pN2 which is further divided into two groups namely, pN2a and pN2b.
- When 10 or more than that lymph nodes are affected, the cancer is staged as pN3. This group is further divided into three categories namely, pN3a, pN3b and pN3c.

M: Metastasis: the cells spread to other parts of the body.

- M0 shows that the cancer has not affected any other part of the body.
- come(i+) stage indicates that the cancer is physically absent but is present far from the site of origin.
- cM1 means that the cancer has spreads to other parts of the body.
- cM2 stage is given when the cancer has spread and secondary tumor is found to be around 0.2mm. (https://www.cancerresearchuk.org/about-cancer/breast-cancer/stages-types-grades/tnm-staging)

1.4 Diagnosis of Breast cancer-

1. **Mammography-** Mammography is defined as an X ray image of breast. Nipple discharge or a breast lump are some common symptoms experienced by women during diagnosed initial stage and hence mammogram is used as screening method. These X ray images are used by doctors in diagnosing abnormalities in breast. Women may feel a sense of uncomfortableness during a mammogram , although the procedure takes less time and feeling of discomfort is over soon. An abnormality seen in the mammogram may not always mean that the patient is suffering from cancer. There is technique called screening mammography which uses low X ray dose for detection of cancer. https://www.radiologyinfo.org/en/info/mammohttps://www.cdc.gov/cancer/breast/basic_info/mammograms.htm

2. **Magnetic Resonance Imaging (MRI) –**MRI is also used as diagnosis method for breast cancer. Radio waves and strong magnetic force is used in this technique. This technique creates a detailed image of breast cancer. Soft image of breast sometimes be hard and this can be detected by MRI. This technique can be detected cancer which are not detected by Mammography. Sometimes other techniques detect something use as cancer which is called False positive cases and this can be diagnosed by MRI.https://www.cancer.org/cancer/breast-cancer/screening-tests-and-early-detection/breast-mri-scans.html

3. **Molecular Breast Imaging (MBI) –**

Molecular breast imaging (MBI) is the current state of nuclear breast cancer imaging allows superior imaging at low radiation dose than breast specific gamma imaging. The MBI system has two heads and apply to only milder compression to immobilize the breast. Similar to mammography, it is Similar to mammography, it provides craniocaudal and mediolateral oblique view of breast, which facilitates comparison between MBI and mammographic photos.https://www.snmmi.org/AboutSNMMI/Content.aspx?ItemNumber=36606

4. **Breast Biopsy –**

A simple medical procedure named Biopsy means a sample of breast tissue is removed and has been sent to the laboratory to be testing procedure. The best way to evaluate is Breast as if a portion of breast is cancerous or not. It is not necessary that breast lumps are always Cancerous sometimes they are not. There are many factors and several conditions involved that can cause growth and lumps in the breast. If a lump in your breast is cancerous or benign which means noncancerous that can be determined by breast biopsy.https://www.healthline.com/health/breast-biopsy

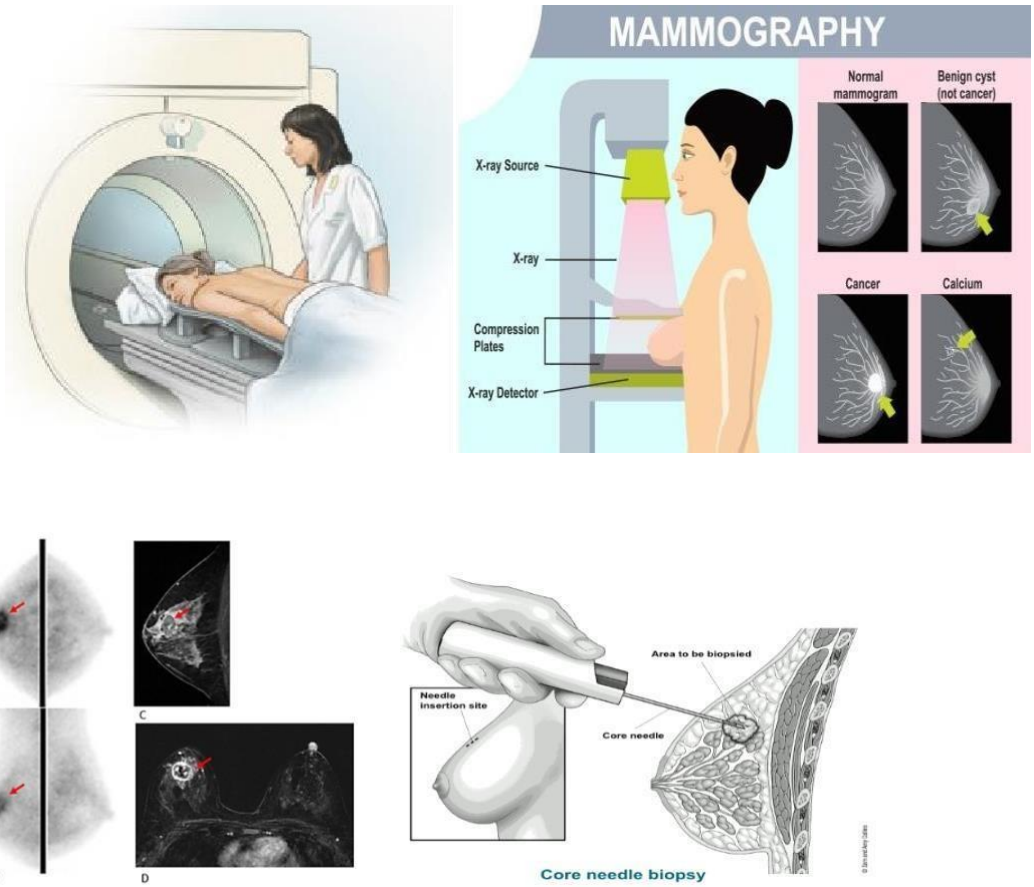

Figure 8 : Diagnosis of Breast Cancer

1.5 Treatments of Breast Cancer

1) Surgery : The most common approach is the surgical removal of tumor it is used when medications are unsuccessful to treat cancer. There have been reported many cases of recurrence after many years of surgery even after removing the entire tumor. Surgery is the most common treatment of breast cancer in that the tumor is removed with the help of surgery . Even though there have been many cases which are reported of recurrence of cancer.

2) Mastectomy: Mastectomy is the procedure to remove all of your breast. Mastectomy is an operation in which the removal of your Breast Tissue is done. In order to improve the appearance of the breast newer surgical techniques may be a selected option in selected cases. The two most common operations for breast cancer are skin sparing and

nipple sparing mastectomy . Breast tissue – The lobules, ducts, fatty tissue, skin, nipple and areola are removed by Most Mastectomy procedures.

3) Immunotherapy –To fight against cancer immunotherapy uses your Immune system. Immunotherapy is a type of cancer treatment which helps your immune system to fight cancer. Immune system helps your body fight infections. Your body's disease fights with your immune system may or may not attack cancer as the cancer cells produce proteins that blind the immune system cells. And the same way Immunotherapy works by interfering in that process.

4) Radiation Therapy – A large machine that aims the energy at your body is called Radiation therapy. By Other ways radiation can also be done by placing radioactive material inside the body. After lumpectomy, External beam radiation of the whole breast is commonly used. After lumpectomy , Breast Brachy therapy may be an option if you have a low risk of cancer Recurrence.

5) Chemotherapy – In some women Chemotherapy is also used in which cancer has spread to other parts of the body . It is tried to control the cancer and it decreases the symptoms of cancer caused. Sometimes chemotherapy is given before the surgery to the women with large breast tumors. To shrink the size of the tumor is the major goal that makes it easier to overcome the surgery. After surgery chemotherapy is given so that the chance of cancer will reoccur. It uses drug so that it destroys fast growing cancer cells. Chemotherapy should be given If your cancer has high risk of returning or spreading the disease.

6) Hormone Therapy – Before or after surgery hormone therapy is used or other treatment is used so that the chance of returning of cancer does not exist. Even though cancer has spread to other parts of the body only hormone therapy can control it. The other name of hormone therapy is blocking therapy which is used to treat breast cancer and that are very sensitive to hormones. This type of cancer is referred to as ER Positive and PR positive cancers. https://www.cancer.net/cancer-types/breast-cancer/types-treatment

https://www.mayoclinic.org/diseases-conditions/breast-cancer/diagnosis-treatment/drc-20352475

7) Targeted Therapy Drugs –

Targeted therapy drug is an active area of cancer Research. Targeted therapy drugs that focus on other abnormalities within cancer cells are available. Targeted drug treatment

that Attacks specific abnormalities within cancer cells. For an example, several targeted therapy drugs target on some proteins that some breast cancer cells overproduce called Human epidermal growth factor receptor 2 (HER 2) Gene. The protein helps breast cancer cells grow and survive. The drugs that can damage cancer cells spare healthy cells , by targeting cells that can make too much HER 2. https://jnm.snmjournals.org/content/jnumed/57/Supplement_1/9S.full.pdf

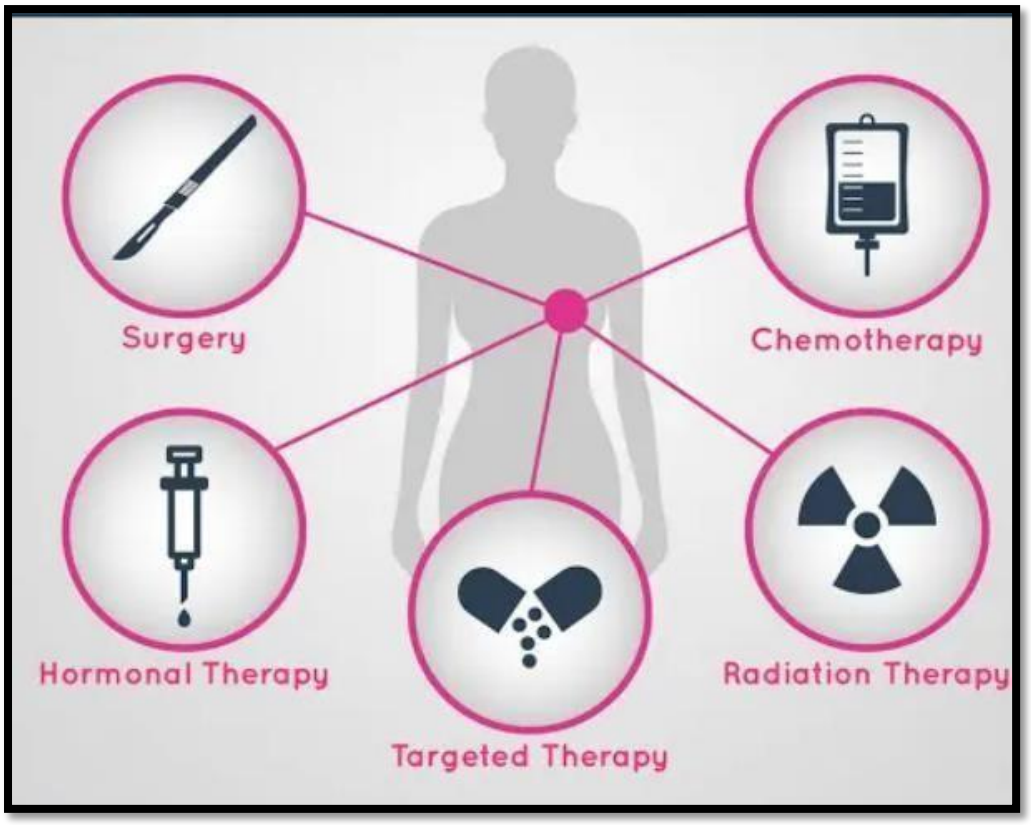

Figure 9 : Treatments of Breast Cancer

1.6 Biomarkers:

The biomarkers belong to the family of transcription factors of the nuclear receptors and human epidermal growth factors.

It involved in the metabolic homeostasis, reproduction, differentiation and eukaryotic development.

Single polypeptide chains of the nuclear receptor superfamily have many domains:

1. Less conserved carboxy – terminal ligand binding domain.
2. A variable amino – terminal domain
3. A DNA binding domain which is highly conserved

4. The use of clinical biomarkers is critical in the diagnosis and treatment of breast cancer. Three molecular biomarkers for breast cancer provide prognostic information and predict the response to specific therapies, which are of great importance for required clinical decision making. These biomarkers comprise of Estrogen receptor (ER), progesterone receptor (PR), and human epidermal growth factor receptor 2 (HER2).(https://www.cancer.org/cancer/breast-cancer/understanding-a-breast-cancer-diagnosis/breast-cancer-hormone-receptor-status.html)
5. **Receptors** are proteins inside or found on the cells which can bind to certain blood substances. Normal cells and few cancer cells in the breasts consist of receptors that attach to the hormones Estrogen and progesterone, and are require these hormones for the cells to achieve their utmost desire. (https://www.cancer.org/cancer/breast-cancer/understanding-a-breast-cancer-diagnosis/breast-cancer-hormone-receptor-status.html)

The cells involved in the development of breast cancer may show one of the following receptors, both or none of the receptors mentioned below:

- **ER-positive:** Breast cancers cells which possess Estrogen receptors are termed as ER-positive (or **ER+**) cancers.
- **PR-positive**: Breast cancers cells which possess progesterone receptors are termed as PR-positive (or **PR+**) cancers.
- **Hormone receptor-positive**: the cells are termed as Hormone-Receptor Positive when both the above-mentioned receptors are present (HR-positive or HR+).
- **Hormone receptor-negative**: the cancer is termed as Hormone-receptor negativewhen the above-mentioned receptors are absent. (HR- or HR-negative).
- Preventing the hormones Estrogen and progesterone from binding to their respective receptors can help limit the cancer from enhancing. (https://www.cancer.org/cancer/breast-cancer/understanding-a-breast-cancer-diagnosis/breast-cancer-hormone-receptor-status.html)

    1) **Estrogen Receptor –**

        It is important for sexual and reproductive function. In the body 17 Beta-Estradiol (E2) Is the most patent. They are having effect on Musculoskeletal, immune, cardiovascular and central nervous in women as well as men. In various organs like mammary gland, uterus, ovary, lung, brain and prostate they play major role in morphogenesis so they are known as morphogen.

Figure 10 -Schematic representation of Estrogen receptors isoforms:

There are two subtypes of estrogen receptor: ER-α and ER-β receptors. ER-α(NR3A1) and ER-β(NR3A2) are subtypes of cellular signalling. They are structurally and functionally distinct. DNA recognition and binding is done by the central DNA binding domain which is the most conserved domain. Ligand–binding domain is carboxy-terminal where multi-functional ligand binding is done. Most variable domain is NH-2 Terminal domain. Constitutively AF-1 is located at amino terminus and AF-2 in the carboxy terminus. Except amino terminal domain ER-α and ER-β share sequences of the homology. Affinities for Estradiol are same for both the receptors. ER binds to the ERE present on the DNA in nucleus. DBD, LBD are the domains of the receptors. AF-1 AND AF-2 are the two main transcriptional activation functions.

Both isoforms α (ERα) and β (ERβ) have AF-1 and AF-2: distinct transcription activation sites; DBD—DNA-binding domain; LBD—ligand-binding domain; NH 2 – and –COOH are terminal regions of protein; ERα is 595 amino acids long, ERβ is 530 amino acids long.

2) **Progesterone Receptor –**

It is belonging to the steroid family receptorand also, it is very helpful in pregnancy and helps in reproductive events. The reproductive behaviour is controlled by progesterone receptor. They are generally prescribed for postmenopausal or hormone replacement and contraception. Estrogen receptor is present for activation of progesterone receptor. Aggressive tumours and up regulated growth factor are associated by low progesterone receptor. Breast cancer patients have been observed to be dependent on hormone receptor associated biological growth which can be defined by progesterone receptor. ER activation is considered as a marker because PR can activate by it and ER majorly controls the actions of PR.

3) **Human epidermal growth factor receptor (HER-2):**

HER-2 belongs to the Epidermal growth factor family of tyrosine kinase receptors. HER-2 is encoded by ERBB2 gene which is also known as CD340 or ERBB2. It is Essential in regulating survival, and differentiation which is done by receptor tyrosine kinases (ErbBs).

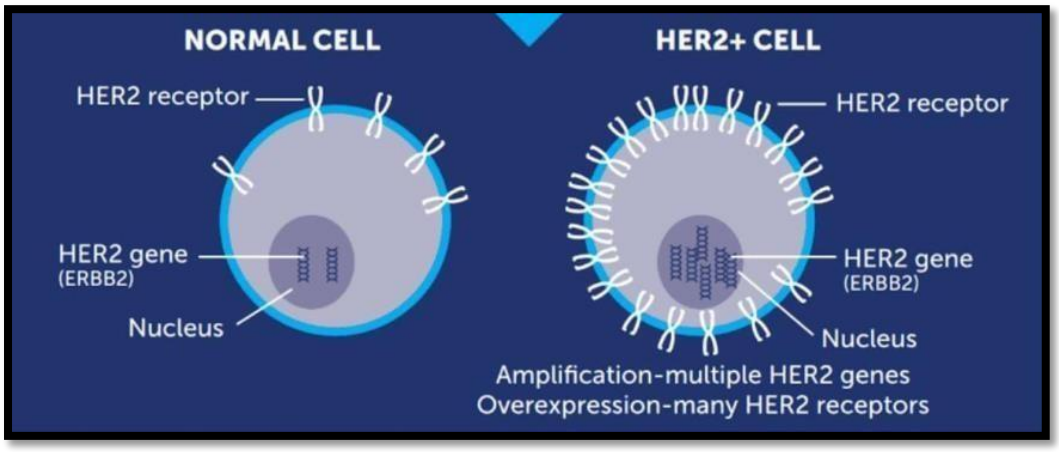

Figure 11: HER-2 receptor

They are made up of glycoproteins which usually contains transmembrane domain, extracellular ligand – binding ectodomain, a tyrosine kinase, tyrosine containing C - Terminal tail and a short juxta-membrane section. Receptor form heterodimer once bound to the ligand. Activation and phosphorylation of C-Terminal tail and intracellular receptor dimerization is important. Activation is done directly or through adaptor proteins due to phosphotyrosine residues. Ras/ STAT / PLC, PI (3) Kinase /Akt pathways these are signalling cascades involved.

1.4 Triple Negative Breast Cancer:

- An example of aggressive breast cancer subtype is TNBC (Triple Negative breast cancer) and HER 2 (Human epidermal growth receptor 2) , PR (Progesterone receptor) , ER (Estrogen receptor) are absent.
- Those women who are having TNBC With very bad prognosis it happened due to Aggressive nature as well as not having proper targeted therapy . TNBC has 15 % of breast cancer cases all over the world and it is seen more in young and African – American women. TNBC is cancer that affects 13 in 10,0000 women each year.
- TNBC is hard to cure and it is challengeable one. But researches are working very hard to make steady progress to make more effective treatment. Mostly 77% women who are having TNBC are alive only five years after being diagnosed.

- It is known as Triple negative breast cancer (TNBC) as TNBC doesn't have three markers which are associated with other types of breast cancer and it is important for treatment and prognosis.
- TNBC is seen more frequently in women aged 40 and in younger women than in old women. TNBC is more likely to develop in black and Latina women rather than white women.
- Those women who are having genetic Alteration in BRAC 1 gene are more too vulnerable to develop TNBC than any other women. When BRAC 1 gene mutation occurs, it promotes cancer and appear to make your body's own cell more vulnerable to cancer.
- The breast cancer cells may have hormone receptors, this hormone receptors may help us determine the prognosis of the disease and which treatment can be used. TNBC has recently become more prevalent in premenopausal women among all breast cancer cases.
  (https://journals.lww.com/md-journal/fulltext/2016/11150/trends_of_triple_negative_breast_cancer_research.55.aspx)
  https://my.clevelandclinic.org/health/diseases/21756-triple-negative-breast-cancer-tnbc

1.5 Estrogen receptor signalling:

- Estrogens are female hormones including estrone, Estradiol, estriol, Estretol. They belong to steroids family.
  Ovaries primarily produce estrogens but adrenal gland and adipose tissue also produce estrogens.
- The main function of estrogen is the maintenance of menstrual cycle and reproduction. Besides these it regulates bone density, brain function, cholesterol mobilization, development of breast tissue and sexual organs and control of inflammation. (Liang et. al., 2013).
- Estrogen receptor was discovered by Elwood Jensen in 1958 and more than 20 years later the first estrogen receptor (currently known ER-alpha) was cloned from RNA which was derived from MCF-7 cell line which is a human breast cancer cell line. 10 years later second estrogen receptor was discovered which is currently known as ER-beta.

- A new type of Estrogen Receptor was found by molecular cloning methods which is GPER1 (G Protein-Coupled Estrogen Receptor), a membrane bound receptor. (Filardo et. al., 2012).
- There are two major kinds of receptors viz. ER-alpha and ER-Beta. ER-alpha is of 595 amino acids long. ER beta is having shorter amino acids terminal hence it is 530 amino acids long. Both the receptors belong to the superfamily of Nuclear Hormone Receptor superfamily of transcription factors.
- There are various domains in the ER receptor each having its own function in ER signalling. The functional domain is named as A/B, C, D, and E/F domains. The A/B domain is amino terminal domain (NTD) which is responsible for the gene transcription transactivation. The C domains is the DNA binding domain (DBD). This domain is responsible for the dimerization of estrogen receptor and binding to specific sequences. The D domain is a hinge region that connects C domain to E/F domain. The E/F domain is the Carboxy terminal which is a Ligand binding domain. It contains estrogen binding sites for Co-activators and repressors. Two regulators are present which are AF1 and AF2 (Activation function domains) in which AF1 is present in the NTD region and AF2 is present in DBD region. (Kumar et. Al 2011). The DBD region binds to the ERE (Estrogen Response Elements) which is present in DNA. (Scheidereit et. Al 1986, Truss et. al., 1993).
- In humans ESR1 gene located on chromosome 6 encodes for ER-alpha. There are various shorter isoforms of ER-alpha which lack AF1 and AF-2 but they cannot activate Transcription rather they bind to the ER-αfull-length isoform and inhibit its activity.
- ESR2 gene located on chromosome 14 encodes for ER-beta. It has 5 known isoforms. The shorter isoform which has no or less transcriptional activity can interfere with ER-alpha signalling and block ER alpha signalling by dimerizing with it. (Vrtacnik et. al., 2014).
- The gene encoding for GPER1 is present on chromosome 7 and it does not share similarities with ER-alpha and ER-beta.
- Estrogen is a Hormone and hence it can cross the cell membrane and enters into the cytoplasm. ER-alpha and ER beta are predominantly present in the nucleus but also present in the cytoplasm. Estrogen will cross the membrane and bind to its estrogen receptor. Estrogen receptor is a ligand activated receptor.
- There are two types of pathways followed by ER viz. Genomic pathway and non-Genomic pathways.

- In genomic pathway the ER moves to the nucleus and ER pathway involves indirect regulation of gene expression.

1.5.1 GENOMIC PATHWAY:

1) **Direct Genomic pathway :** In genomic signalling estrogen crosses the membrane and binds to the ER in cytoplasm or nucleus. This causes conformational change. It also causes Hsp90 and hsp70 dissociation and dimerization. The ER-alpha gets phosphorylated when they are bound to its Ligand. They can also be phosphorylated in absence of the Ligand. (Klinge, 2001). The E2-ER-ligand complex binds to the DNA sequence at ERE site. There are two ways in which ER complex binds to the ERE: (1) E2-ER-ligand complex directly binds to the ERE (2) ER associates with the regulatory sequences due to interaction with other DNA binding proteins. (O'lane et. al., 2004). The Ligand used is tissue specific. There are various kinds of ligands for ER. They are basically divided into two groups viz. Steroidal and Non-Steroidal. A series of compound called 11beta-ether-17alpha-ethinyl-3, 17beta Estradiol have been observed to show Strong ER antagonist activity. (Zhang et. al., 2014). Combretastatin A4 analogies have also been reported to show anti-breast cancer properties. Two new series of compounds C6-piparazine-substituted purine steroid-nucleosidesand C6-cyclosecondary amine-substituted purine steroid-nucleoside analogues have been reported in development of anticancer drugs. (Farzaneh. al., 2016).

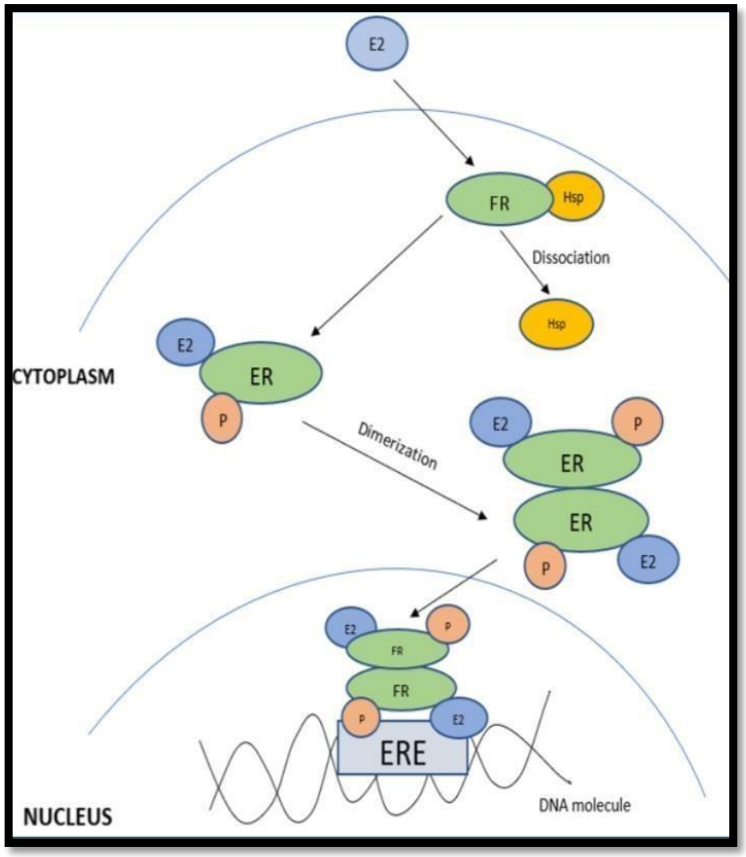

Figure 12 – Direct ER Signalling (Klinge, Carolyn M. "Estrogen receptor interaction with estrogen response elements." *Nucleic acids research* 29.14 (2001): 2905-2919.)

There is an interaction between the sugar phosphate backbone of ERE and ER dimer. This interaction was found to be important for recognition of the ERE sequence and binding of the receptor with high affinity to the sequence. Majorgroove of the DNA is the place where ER dimer binds. There is zinc finger in the 'P box' of the receptor. This zinc finger has three specific amino acids which interact with the major groove in sequence specific manner. A positive contact for the 'P box' is provided by fourth base pair of the ERE half-site (AGGTCA) and binding energy is provided by third base pair (AGGTCA). CII zinc finger is involved in ER dimerization. (Klinge C. et. al., 2001).

HMG-1 and HMG-2 have been shown to decrease the rate of dissociation of ER-alpha- ERE and hence facilitates the ER-ERE interaction.

1.5. Antiestrogen Treatment and Resistance –

Antiestrogens includes agent such as Tamoxifen , Toremifen, Fulvestrant, Raloxifen . Drug named Tamoxifen is used in chemoprevention of breast cancer and mostly women who are having hormone receptor positive , invasive breast cancer they preferred to go with the treatment of Tamoxifen. Antiestrogen act with estrogen and binds with Estrogen Receptor (ER), is widely used administrated endocrine agents for expressing ER Breast cancer. Most frequently prescribed Antiestrogen is Tamoxifen (TAM) . There are Two Types of Antiestrogen Resistance they are De novo and acquired . The common mechanism of De novo Resistance is the absence of both ER and PR Expressions. loss of Antiestrogen unresponsiveness is done by initially responsiveness tumors and it is the most common acquired phenotype. Antiestrogen resistance tumors retains levels of ER expression on antiestrogen therapy would define still them as ER positive. HTTPs

In adjuvant, metastatic, chemo preventive settings and induce increase in survival of breast cancer patients in these areas Antiestrogen are Effective.

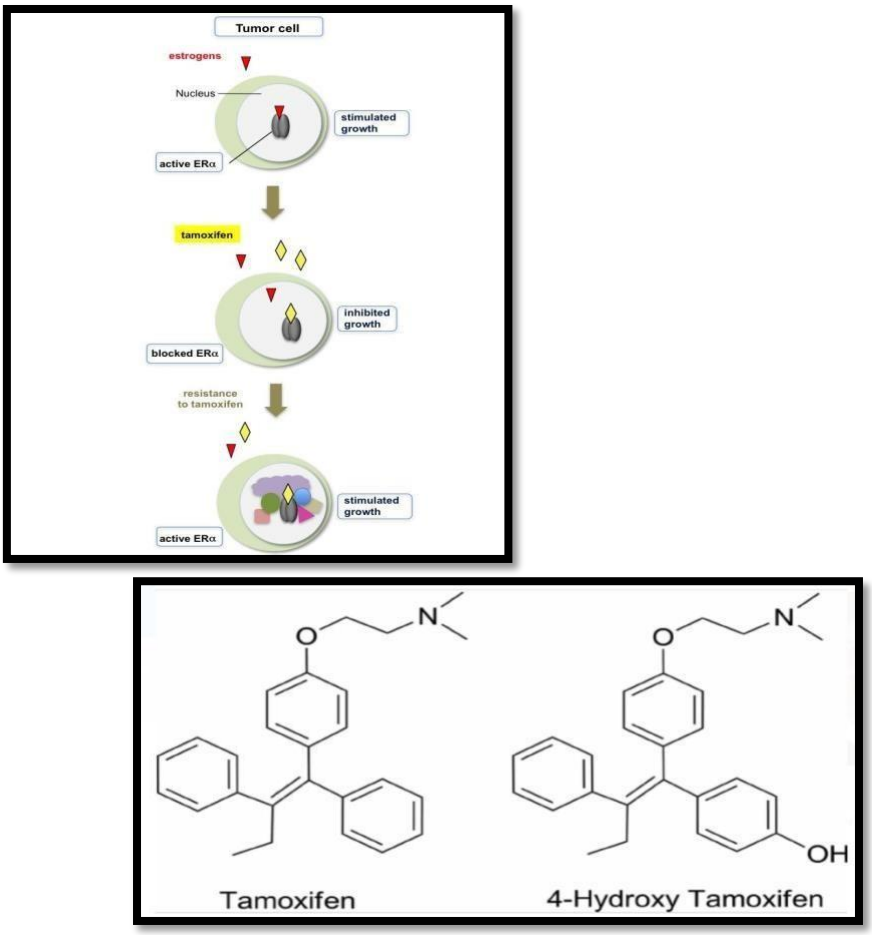

Figure 13: Adopted from: https://www.eurekalert.org/multimedia/711217

Figure 14: Antiestrogen role

1.6. Transforming Growth Factor – beta:

Transforming growth factor- beta (TGF-β) regulates cell proliferation, cell differentiation and apoptosis. It is synthesized as Pro-proteins which consist of large amino terminal and this is called Latency Associated Proteins (LAPs). The whole complex is called 'Small Latent Complex' (SLC). Furin type enzymes are present in the Golgi apparatus which cleaves the complex to give a TGF-beta dimer but still it is attached to its pro-peptide by non-covalent interaction creating 'Large Latent Complex' (LLC).(Miyazono. et. al., 1988). Cells release TGF-β into extracellular matrix as Latent TGF-β Binding Proteins (LTBP). It is further activated by various molecules such as retinoic acid, fibroblast growth factor 2 (FGF-2), plasmin, MMP-2, MMP-9, thrombospondin 1, various integrins and reactive oxygen species. TGF-β are of three types of receptors viz. TGF-β receptor-1 (TβRI), receptor-2 (TβRII) and receptor-3 (TβRIII). Among all three the most abundant form which is TβRIII is called beta glycan. Although being the most abundantly found receptor it does not mediate signal transduction. The other two forms I.e., TβRI and TβRII are responsible for signal transduction intracellularly. It consists of extracellular Ligand binding domain which is-terminal domain and an intracellular N-terminal domain which is serine/threonine kinase domain. TGF-β receptor 1 is of 85 to 110kDa which is larger than TGF-β receptor II which is of 65 to 70kDa. TβR1 has a characteristic highly conserved GS domain which is 30 amino acids long in the cytoplasmic part which is required to be phosphorylated to fully activate TβR1. TβRII has a polyadenine repeating unit which is 10 base pairs long present in the extracellular domain. Frameshift missense mutation or early protein termination frequently occur in this region which has led to the production of defected or faulty products. (Kubiczkova L. et. al., 2012).The receptors have a cytoplasmic domain which is called Dual Specificity kinases. They are so called because they contain serine/threonine kinase activity as well as tyrosine kinase activity. (Hata A. et. al., 2016).

The Ligand initially binds to TBRII and gets phosphorylated which in turn phosphorylates the Gly-Deregulatory region (G-S domain) of TβR1. This leads to the formation of large ligand-receptor complex which consist of dimeric TGF-β and two pairs of TβR1 and TβRII and this complex is extremely stable upon solubilizing. TGF-β2 binds to TβRII only when it binds to TβRI, on contrary, TGF-β1 and TGF-β3 binding to the receptor does need the dimerization of

both the receptors. Affinity of TGF-β2 is low with TβRII, TβRIII (beta glycan) increases the affinity and aids binding to the receptor.(Kubiczkova L. et. al., 2012)

There are two pathways followed by TGF-beta viz. Canonical pathway which includes SMAD proteins and a non-canonical pathway which does not include the SMAD proteins.

1.7.1. CANONICAL PATHWAY:

SMAD PROTEINS

The Smad proteins are directly activated by serine phosphorylation at their cognate receptors. They are latent cytoplasmic complexes because they are present in the cytoplasm and function when they are phosphorylated. There are two prototypic members of SMAD family which are called Mad and Sma. Mad was derived from *Drosophila* and Sma was derived from Caenorabditis elegans. All SMADs range from 400 to 500 amino acids long. *SMADs* are divided into three subfamilies viz. (i) the receptor regulated *SMADs* (R-*SMADs*) (ii) common *SMADs* (Co-*SMADs*) (iii) Inhibitory *SMADs* (I-*SMADs*). There are eight *SMAD* proteins found in humans. *SMAD2*, *SMAD4* and *SMAD7* are found on chromosome 18. These are frequently deleted in cancers. *SMAD3*, *SMAD5* and *SMAD6* are found on chromosome 15. *SMAD1* is found on chromosome 4. *SMAD8* is found on chromosome 13. (Attisano. et. al., 2001). R-SMADs include SMAD1, SMAD2,SMAD3, SMAD5 and SMAD8/9. From this SMAD2 and SMAD3 are Activin regulated R-SMADs and SMAD1, SMAD5 and SMAD8 are BMP-regulated(Bone morphogenetic protein) R-Smashed only known Co-SMAD in humans is SMAD4. SMAD6 and SMAD7 are I-SMADs.

SMAD proteins have an amino terminal which is Mad Homology-1 domain (MH1) and a carboxy terminal which is Mad Homology-2 domain (MH2) which is separated by a proline rich linker region which is of varying length. (Attisano L. et. al., 2001). The domain responsible for DNA binding is MH-1 domain and MH-2 domain binds to the nucleoproteins for further signaling. R-SMADs and Co-SMADs contain MH-1 as well as MH-2 domains whereas I-SMADs consist of only MH-2 domain. The I-SMADs bind to the MH2 domain of the TβRI and hence compete with R-SMADs for binding with the receptor and once they are bound, they prevent the phosphorylation of the R-SMADs. (Hayasnhi H. et. al., 1997).

PATHWAY:

Binding of the TGF-B to the receptor causes the heterodimerization of TβR-1 and TβR-II. (Kubiczkova L. et. al., 2012). TβR-II are active kinases which phosphorylate the juxtamembraneregion of the cytoplasmic domain of TβR-I. TBR-I are also serine/threonine kinases and hence they phosphorylate the serine residue in the Ser-Ser-X-Ser sequence which is called SSXS motif of theR-SMAD2 and SMAD3. SMAD4 does not have the motif and hence cannot be phosphorylated. TβR-I phosphorylates the carboxy terminal of SMAD2 and SMAD3. (Hata A. et. al., 2016). TβR-I also contains a zinc double finger which consist of FYVE domain. This domain has a protein named SARA (The SMAD Anchor for Receptor Activation). This protein is responsible for recruiting the non-phosphorylated SMADs to the receptor for phosphorylation. (Tsukazaki T. et. al., 1998).Once the R-SMADs (SMAD2 and SMAD3) are phosphorylated the binding affinity towards SMAD4 increase and they bind to the MH2 terminal of the SMAD4 forming a complex of two R-SMADs and one Co-SMAD. This complex now further moves to the nucleus. The complex binds to the DNA through its MH1 domain.(Moustakas et. al.,2009). There are importins present on the nuclear membrane which recognize nuclear localization like sequence present on the SMADs. These are importantfor the proper translocation of SMADs on the DNA. There is various interaction with protein binding partners and DNA to hold the SMAD complex in the nucleus. (Kubiczkova L. et. al., 2012)

1.7.2. NON-CANONICAL PATHWAY

There are various pathways which are activated by TGF-β other than the classic canonical pathway including SMAD proteins. It interacts with MAPK pathway (Mitogen Activated Protein Kinases).(Kubiczkova L. et. al., 2012).There are two points of interaction between the TGF-β signaling and MAPK signaling (i) phosphorylation of co-activators of R-SMADs by ERKs, (ii) phosphorylation of SMAD2/3 by ERKs in the linker region. The interaction between the p53 and TGF-B was observed to the regulated by Ras-MAPK pathway. (Chapnick D. et. al., 2011) . Various branches of MAPK pathways are activated which includes ERK1/ERK2, Jun-N Terminal Kinase (JNK) and p38 and PI3K kinases. (Bakin et. al., 2002)

TGF-B also interacts with Protein kinase B(AKT/PKB), the small GTP-binding proteins, Rat Sarcoma Virus (RAS), RAS homology family member A(RhoA), Ras related C3 bolulinum toxin substrate1 (RAC1) as well as Cell Division Control Protein 42 homolog(CDC42)and Mammalian target of rapamycin (mTOR). (Kubiczkova L. et. al., 2012)

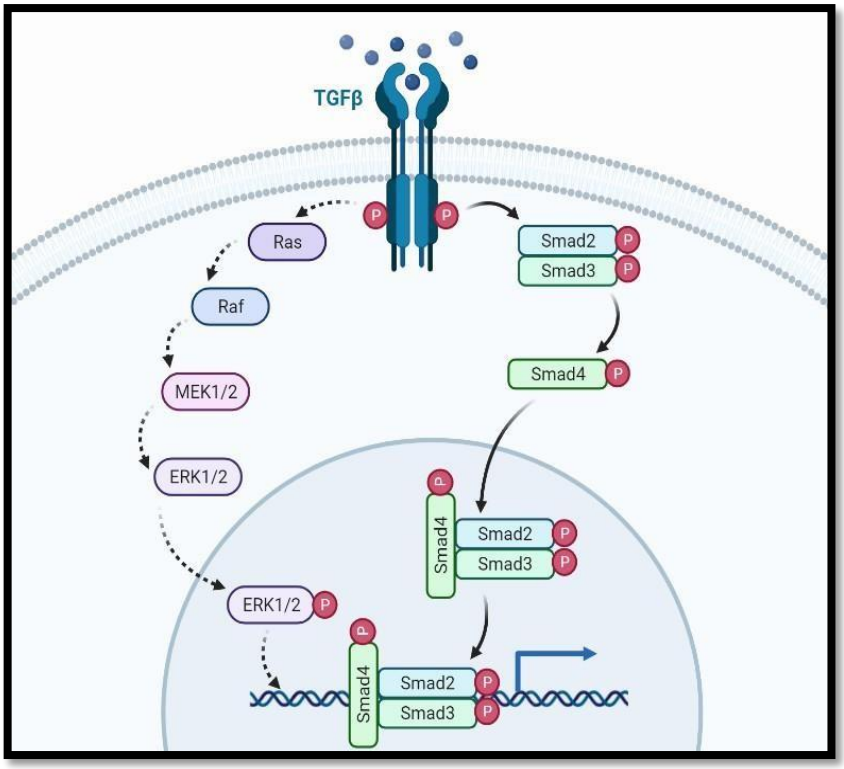

Figure 15 – TGF β signaling Pathway (Kubiczkova, Lenca, et al. "TGF-β–an excellent servant but a bad master." *Journal of translational medicine* 10.1 (2012): 1-24.)

Dual role of TGF-β during cancer:

TGF-β plays a primary role of inhibitor of cell proliferation and induces apoptosis. TGF-β plays a dual role in cancer. It acts as tumor promoter during early stages of cancer progression and as tumor promoter in later stages of cancer. The primary role of TGF-β is arrest of cell cycle progression and hence functions primarily as a tumor suppressor. TGF β promotes the function of CDK (cyclin dependent kinase) inhibitors and suppresses the function of c-Myc (Cellular myelocytomatosis oncogene) and CDKs. Also, the function of the TGF-β is tissue specific i.e., it suppresses the growth of epithelial, endothelial, hematopoietic cell and neural cells but it promotes proliferation in mesenchymal cell types like fibroblasts. TGF-β inhibits cell cycle progression in the G1 phase. TGF-β induces the expression of CDK inhibitor p15 ^{INK4B} and p21 ^{CIP1} , inhibits CDK4 expression and down-regulates CDC25A. (Kubiczkova L. et. al., 2012). Inactivation of suppressor genes and mutations of the receptors or signaling molecules

leads to the divergence of the role of TGF-β from tumor suppressor to tumor promoter.(Lebrun J. et. al., 2012).

During later stages of cancer various activities of TGF-β have shown to be tumor promoting. (Kubiczkova L. et. al., 2012)Some studies indicate that increases with the levels of TGF-β has been related to poor prognosis, on contrary some studies have shown increase in TGF-β levels are related to worst outcome of the disease. (Chang H. et. al., 1999). Loss mutation or attenuation of TBRI, TBRII, SMAD2 and SMAD4 was seen. Downregulation of TBRII is seen majorly which affects the actual role of TGF-β . (Kubiczkova L. et. al., 2012) . TGF- β is known to interact with SRC which plays a crucial role in cancer metastasis. (Zhange H. et. al.,2015).

1.8. Crosstalk between Estrogen Receptor and Transforming Growth Beta –

Estrogen receptor signalling and Transforming Growth factor-β play crucial roles in mammary gland development. TGF-β plays various roles in early embryonic development, cell growth, differentiation, motility and apoptosis. It inhibits cell proliferation by inducing apoptosis in cancer cells. Major role of ER includes promoting cell proliferation in cells. Both TGF-β and ER have contradictory roles in tumor progression. There is a crosstalk between ER signalling and TGF-β. Also, TGF-β is known to play a dual role in tumorigenesis. These pathways interact at SMADs and ER-α is known to inhibit the TGF-β signalling. When cancer cell lines are administered with antioestrogens there has been observed a marked increase in TGF-β1, TGF-β2 and also TGβ-RII. p38MAPK is responsible for the activity of antioestrogen. (Buck MB et. al., 2004). SnoN is a negative regulator of TGF-β. Smurf-2 (SMAD-SMAD-ubiquitin-related-factor 2) is E3-ubiquitin ligase which interacts with SnoN. SMURF-2 domain interacts with SMAD2 and this SMURF-2/SMAD2 complex recruits the SnoN protein. (Tecalco-Cruz et. al.,2018). SMURF-1 (SMAD specific E3 Ubiquitin ligase 1) interacts with ER-α and increase the ER signalling which leads to increased cell proliferation. This interaction causes degradation of SMAD2/3. (Yang H. et. al., 2018).ER-β may also function in a similar manner in degradation of SMAD2/3. (Ito et. al., 2010). SMURF-1 interacts with SMAD7 which is an inhibitory SMAD. This association causes degradation is TBR-I and blocks TGF-β pathway. (Ebisawa. et. al., 2001). ER inhibits TGF-β through non-genomic pathway. Also, SMAD4 interacts with ER-alpha and inhibits ER- alpha activity. This interaction is activated by antioestrogens. (Wu et. al., 2003).

Hypothesis:

The role of ER and TGF-β are contradictory. ER plays a part in cell proliferation and TGF-β plays a part in blocking the cell proliferation. But at the same time, we also know that the TGF-β plays a dual role of tumor suppressor and tumor promoter. So, the exact mechanism of their interaction may not know. Some studies have stated that they interact through Non-Genomic ER pathway through the involvement of AP-1. TGF-β auto-induction may take place through AP-1. Hence in this manner, ER and TGF-β may be interconnected. Some studies have also suggested that ER and TGF-β are connected through SMAD proteins. R-SMADs may be a target of ER. There are evidences in the literature suggesting that when antioestrogens like Tamoxifen is administered ER-alpha targets SMAD4 and decreases the efficacy of TGF-β signalling or completely aborts its function. This may be one of the reasons in shift of role TGF-β. We hypothesize that the levels of TGF-β may decrease with increase in the tamoxifen treatment.

Objectives:

(1) To Analyse the Cytotoxic effects of Tamoxifen on Human Triple Negative Breast cancer Cell line MDA-MB-231.

(2) To analyse the transcript levels of TGF-β during Tamoxifen treatment in Triple Negative Breast Cancer Cell Line MDA-MB-231.

Material and Methods:

1.1. Cell line:

In our current experiment we have used MDA-MB-231 cell line. It is a human breast cancer cell line. It is derived from pleural effusion of a female patient with Metastatic Mammary Adenocarcinoma.

MDA-MB-231 was gifted to us by IIT Gandhinagar. The cells were cultured and grown as a monolayer in complete growth medium namely, Dulbecco's Modified Eagle's Medium(DMEM).The cell line was maintained in a tissue culture flask (T-25, T-75).

1.2. Media preparation

Media used for MDA-MB-231 cell line is Dulbecco's Modified Eagle's Medium (DMEM) media. Dulbecco's Modified Eagle's Medium (44.5ml) is supplemented with 5ml 10% Fetal Bovine Serum (FBS) and 0.5ml Antibiotic and Antimycotic solution. Mix the contents well. Keep it in CO_2 incubator for some time. Store it at 4°C.

1.3. Cell revival

Requirements:

1) Water bath
2) Growth media
3) Pipette
4) 70% alcohol
5) T-25 flask

Procedure:

1) Carefully take out the cryovial from liquid nitrogen.
2) Quickly thaw the cells. Place the cryovial in 37C water bath and agitate gently while the contents of the cryogenic vial are thawed completely.
3) Dilute the contents of the cryogenic vial with growth media. wipe the vial with 70% alcohol and transfer the contents into 15mL centrifuge tube containing 4-5mL media.
4) Harvest cells by centrifugation to remove the freezing medium.
5) Discard the supernatant.
6) Add media to the pellet and resuspend the pellet using pipette.
7) Transfer resuspended cells to T-25 flask.

1.4. Sub-culturing

Materials:

1. 1x Trypsin -EDTD

2. Complete Growth Medium.
3. Water Bath.
4. CO_2 Incubator
5. Cooling Centrifuge.
6. Biosafety cabinet.
7. BSL Laminar Flow Chamber.

Procedure:

1. Remove old media from T-25 flask.
2. Add 5mL DMEM media into that same T-25 flask by micropipette. (From that pinkish red in colour was washing step)
3. Add trypsin to the flask 2mL and keep it in CO_2 incubator for 10 minutes.
4. Remove flask from incubator and transfer the content into falcon tube.
5. Add 4mL media into the tube.
6. Centrifuge for 5 minutes at 1000 rpm.
7. Add 5mL DMEM media into 2 respective T-25 flasks.
8. Discard the supernatant and collect pellet.
9. Add 1mL pellet into two respective T-25 flasks.

1.5. Cell Viability counting :

Requirements:

1) Trypan Blue dye
2) Hemocytometer
3) Microcentrifuge tubes
4) Micropipettes

Procedure :

1) Prepare 0.4% solution of Trypan Blue dye in buffered isotonic solution of pH 7.2 - 7.3.
2) Take 0.1mL Trypan blue stock solution in a microcentrifuge tube.
3) Add 0.1 mL of cell solution to the Trypan Blue containing microcentrifuge tube.
4) Total volume is 0.2 milk Load 0.1 mL approx. on the Hemocytometer.
5) Observe under microscope and count the total number of cells as well as viable cells for further calculation.

1.6. Tamoxifen treatment :

We had performed long term and short-term exposure assays on TNBC cell line MDA-MB-231.

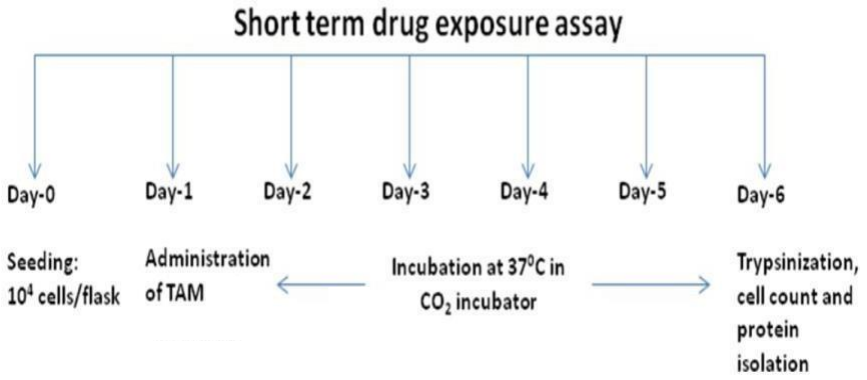

Figure 16 : Schematic representation of Short-Term Exposure Assay

Culture flask (T-25) were seeded 1X10 ^{6} cells density in DMEM media. We had administered TAM (1000nm) 100µL to the cells. The drug was administered only once for short term to check the effects of TAM on TNBC cell line MDA-MB231. The cells were incubated for 6 days in CO_2 incubator at 37ºC. Cells were later trichinized for further procedure.

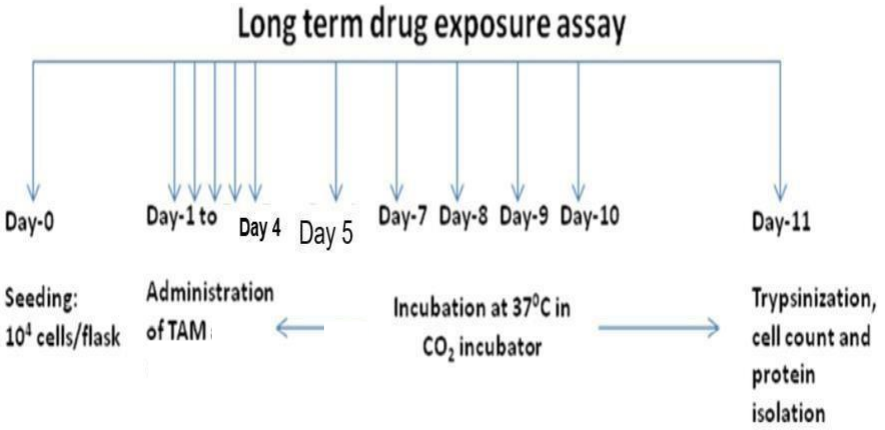

Figure 17: Schematic Representation of Long-Term Exposure Assay

Culture Flask(T-25) was seeded with 1X10 ^{6} cells density in DMEM media. We had administered TAM (1000nm) 100µL to the cells. The drug was administered for consecutive 4 days to check the effects of TAM on TNBC cell line MDA-MB231. The cells were incubated for 6 days in CO_2 incubator at 37ºC. Cells were later trypsin zed for further procedure.

1.7. RNA isolation :

Requirements:

1) Diazole reagent
2) Qiagen RNeasy Mini Kit for RNA isolation (Catalogue number : 74106)
3) 70% ethanol
4) Micropipettes
5) Microcentrifuge tubes

Procedure:

1) Harvest 1×10^6 cells in 800uL Diazole Lysis Reagent. Store this at -80°C.
2) Incubate frozen lysate at 37°C in waterbathuntil completely thawed.
3) Add 1 volume of 70% ethanol to the lysate and mic well by pipetting.
4) Divide the total volume in two columns each containing 800uL of lysate in each column. Place the column in 2mL collection tube. Close the lid and centrifuge for 15 seconds at >8000x g. Discard the flow through.
5) Add 700uL buffer RW1 to RNeasy spin column. Close the lid and centrifuge for 15s at >8000x g. Discard the flowthrough.
6) Add 500uL Buffer Repetto RNeasy spin column. Close the lid and centrifuge for 15s at >8000x g. Discard the flowthrough.
7) Add 500uL Buffer Repetto RNeasy spin column. Close the lid and centrifuge for 15s at >8000x g.
8) Place the RNeasy spin column in a new 1.5ml collection tube. Add 30-50uL RNase-free water directly to the spin column membrane. Close the lid and centrifuge for 1 min at >8000x g to elute the RNA.

1) The results of gel electrophoresis were obtained and analyzed using gel documentational system instrument under UV intensity.

1.8. cDNA synthesis:

Requirements-

1. Qiagen Quant Innov^{ate}SYBR Green PCR kit (Catalogue number : 208154)
2. Quant Studio 3 Real Time PCR System (Catalogue number : A28567)
3. Micro pipette tips
4. Microcentrifuge tubes
5. Diazole reagent
6. 70% Ethanol
7. Froward and Reverse Primers

(**TGF β1)**

(F): TCGCCAGAGTGGTTATCTT
(R): TAGTGAACCCGTTGATGTCC)

NFW (Nuclease free water)	Make up to 12µL
Primer-Oligo dt/Random Hexamer	1µL
Template RNA	50µg
5X Reaction Buffer	4µL
Rib lock RNase Inhibitor	1µL
10 mM dent mix	2µL
Revert Aid M -MULV RT(200/µL)	1µL

https://link.springer.com/article/10.1007/s10549-020-05958-y

https://www.ncbi.nlm.nih.gov/tools/primer-blast/primertool.cgi?ctg_time=1644773149&job_key=WlCFOnPxfllZZ2RiaQJAUBMZUWI-Ckp_Pw

Procedure-

1. After thawing, mix and centrifuge the kit components. Store on ice.

2. Add the components listed above into Maxi amp PCR tubes (Flat cap).
3. Incubate for 5 min at 25°C.
4. After that incubate for 60 minutes at 42°C
5. Then terminate the reaction by heating at 70°C for 5 minutes.

1.9. Gradient Gel Electrophoresis:

Requirements:

1) Agarose gel : 1.5g agarose in 100mL double distilled water
2) 1X TAE buffer

(TAE – Tris Acetate Buffer, take 16mL of 50X TAE buffer and make up volume up to 800mL by adding Distilled water to it.)

3.) Gel Electrophoresis Apparatus

4) Micropipettes

5) PCR Kit

6). Ethidium Bromide

Procedure:

2) Take PCR tubes and add 10µl PCR master mix into the tubes.
3) Add 10µl of Forward as well as reverse primers into the PCR tubes. Add 10µl each of both the primers.
4) Add 7.6 Nuclease-free water in the tubes.
5) Add 2µL of cDNA into each tube .
6) Load 6µL of ladder in the first well and then add 10µL of each sample into the wells.

Results and Discussion:

In our current study we have studied the effects of Antiestrogen named Tamoxifen on Triple Negative Breast Cancer Cell Line MDA-MB-231. RNA was isolated by Kit based method. cDNA was constructed and the transcript levels of TGF-β were analyzed using Real Time PCR technique.

1.1 Cell revival Results :

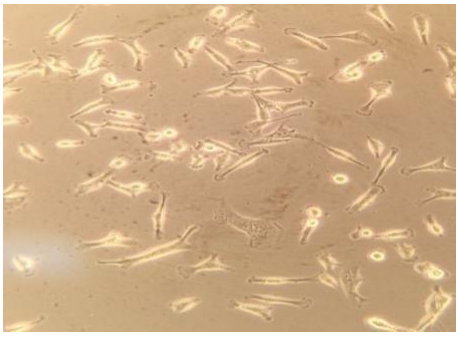

Figure 18: Cells under microscope on Next day of Revival

Cells were preserved in a vial containing DMSO and they were thawed completely and cultured in a T-25 Flask in Complete Growth medium DMEM. The figure 18 shows the cells morphology and confluency immediately on the next day of performing cell revival. The confluency was observed to be around 20%. Media was changed almost every alternate day.

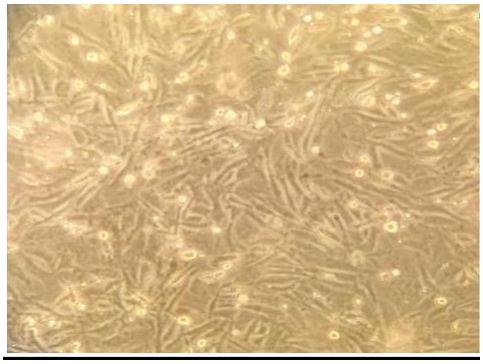

Figure 19: Cells under microscope after growth

Media was changed and cells were cultured for almost a week (7 days) and the above image shows the results of the cells morphology and confluency. The confluency was observed to be around 80-90%. The flask was confluent enough for further procedure so the cells were given further treatment.

1.2. Tamoxifen treatment results :

a) short term drug exposure results:

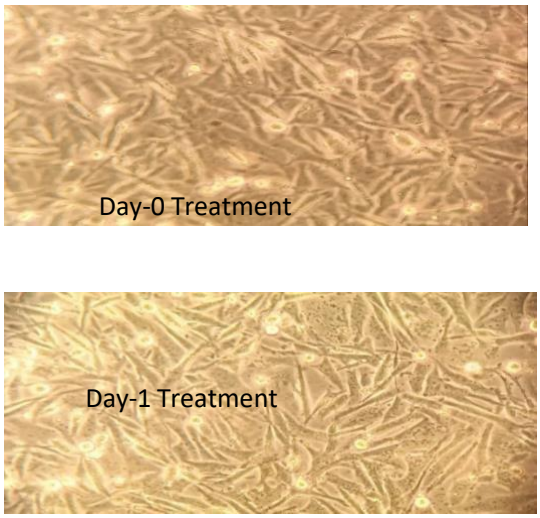

Figure 20: Short term Drug exposure result

For short term drug exposure, the drug was administered only once and the above figures shows the results. First image is of cells before TAM treatment and the second image shows the cells after 1 day of TAM treatment.

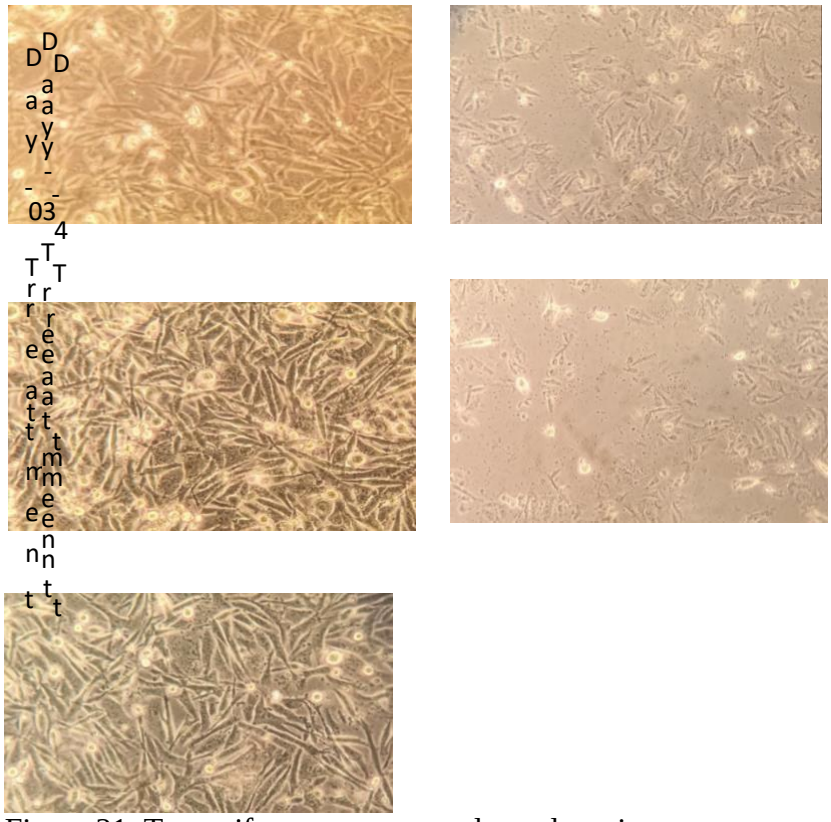

Figure 21: Tamoxifen treatment results under microscope

These are the results of long-term drug exposure of TAM. Cells were observed daily just before administering TAM again on consecutive days. The cell confluency is seen to be decreasing as the number of doses have increased.

1.3. RNA isolation results :

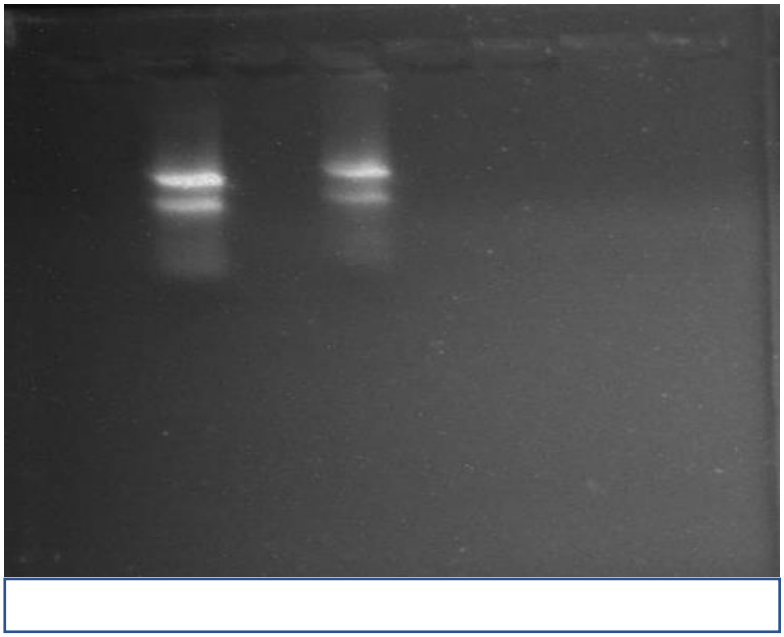

Figure 22: RNA on Agarose Gel Electrophoresis

Total RNA was isolated by Kit based technique. This figure shows the image of RNA on Agarose gel. This was done to check the integrity of RNA which we isolated. Gel electrophoresis was [performed in 1% agarose gel and TAE was used as buffer. Ethidium bromide was used as stain. 18s and 28s RNA bands are clearly visible which depicts that RNA which we isolated is intact. The gel electrophoresis was performed for 1 hour to obtain these results.

1.4. Gradient Gel results :

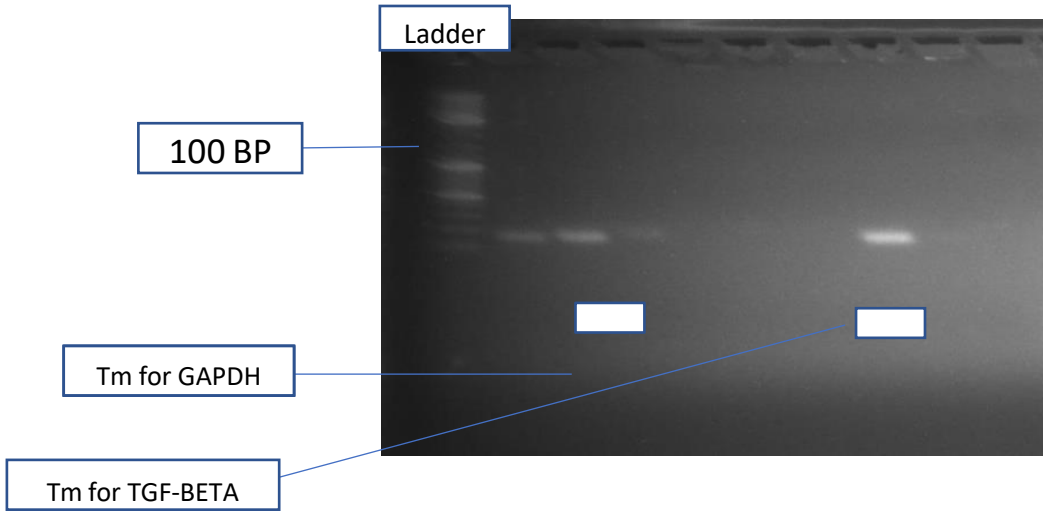

Figure 23: cDNA bands on Gradient Gel technique, Tm→ Melting Temperature

Temperature Gradient Gel Electrophoresis was performed to obtain the melting temperature. This was performed using1.5% agarose using TAE as buffer. The melting point temperature was observed to be 60ºC for TGF- beta and 64ºC for GAPDH. This temperature will be using for further procedure of PCR.

1.5. Transcript readings and calculation of cDNA:

Gene	NTC	Control CT Mean	Short Term CT Mean	Long Term CT Mean
GAPDH	34.858	15.776	22.050	21.079
TGF-β	Undetermined	28.053	27.847	27.772
ER-α	36.382	14.257	34.681	4.123

The CT values were taken for further calculation:

From CT Mean Values obtained, we calculated

1. The delta Ct for each sample

ΔCt = Ct (gene of interest) – Ct (housekeeping gene)

2. The delta detects values for each sample

ΔΔCt = ΔCt (Sample) – ΔCt (Control Mean)

3. The fold gene expression values

   Fold gene expression = 2^-(ΔΔCt)

4. **Log2 Fold Change**

Tamoxifen Treatment Change	Transcript	Log2 Fold Change
Short Term	1) ER-α 2) TGF-β	-14.28771 5.7944159
Long Term	1) ER-α 2) TGF-β	-15.02468 5.5837598

Conclusion

Antiestrogen Therapy such as Tamoxifen has been a successful therapy for hormone responsive breast cancer patients.

In our study we have evaluated that the cytotoxic effects of Antiestrogen on Triple negative breast cancer cell line MDA MB 231.We have determined the phenotypic of growth factors (TGF β1 and TGF β2) during Antiestrogen treatment (TAM)

In our current study we have studied the effects of Antioestrogen named Tamoxifen on Triple Negative Breast Cancer Cell Line MDA-MB-231. RNA was isolated by Kit based method. cDNA was constructed and the transcript levels of TGF-β were analysed using Real Time PCR technique.

There was no significant decrease in levels of TGF-β during long term exposure of TAM as compared to short term exposure of TAM.

As we have used TNBC cell line, ER -Alpha is absent but still TAM effect is seen on the cell. This shows that there may be some other pathway through which TAM is affecting the cells which is still unknown.

There is a crosstalk between ER -alpha and TGF-beta signalling. But as it a TNBC cell line ER-alpha is absent so there may be some other parts of the Chain of interaction between ER and TGF- beta which leads to the inhibition of TGF-beta during TAM treatment. Some studies suggest that SMAD proteins interact with ER and inhibit the TGF-beta signalling pathway. We would like to further study the role of SMAD proteins in this crosstalk and establish a relationship between the two-interlinking pathway.

REFERENCES:

1. Klinge, Carolyn M. "Estrogen receptor interaction with estrogen response elements." *Nucleic acids research* 29.14 (2001): 2905-2919.
2. Kubiczkova, Lenca, et al. "TGF-β–an excellent servant but a bad master." *Journal of translational medicine* 10.1 (2012): 1-24.
3. Fuentes N, Silveira P. Estrogen receptor signalling mechanisms. Adv Protein Chem Struct Biol. 2019; 116:135-170
4. Linda Bjorn storm, Maria Sjoberg, Mechanisms of Estrogen Receptor Signalling: Convergence of Genomic and Nongenomic Actions on Target Genes, *Molecular Endocrinology*, Volume 19, Issue 4, 1 April 2005, Pages 833–842
5. Elite A, D'Agostino Y, Alexandrovna E, Lamberti J, Pecoraro G, Memolo D, Rocco D, Covelo E, Gyrator G, NASA G, Tarallo R, Weisz A, Rizzo F. Insights into the Role of Estrogen Receptor β in Triple-Negative Breast Cancer. Cancers (Basel). 2020 Jun 5;12(6):1477. Doi: 10.3390/cancers12061477. PMID: 32516978; PMCID: PMC7353068.
6. Sillito, Assunta, et al. "Insights into the role of estrogen receptor β in triple-negative breast cancer." *Cancers* 12.6 (2020): 1477.
7. Neel, Jean-Charles, Laure Humbert, and Jean-Jacques Lebrun. "The dual role of TGFβ in human cancer: from tumor suppression to cancer metastasis." *International Scholarly Research Notices* 2012 (2012).
8. Farzaneh, Shabnam, and Afshin Zaria. "Estrogen receptor ligands: a review (2013–2015)." *Scientia pharmaceutical* 84.3 (2016): 409-427.
9. O'lane, Raegan, et al. "Genomic targets of nuclear estrogen receptors." *Molecular endocrinology* 18.8 (2004): 1859-1875.
10. Vrtačnik, Peter, et al. "The many faces of estrogen signalling." *Biochemical medica* 24.3 (2014): 329-342.
11. Bartella, V., et al. "Estrogen receptor beta binds Sp1 and recruits a corepressor complex to the estrogen receptor alpha gene promoter." *Breast cancer research and treatment* 134.2 (2012): 569-581.
12. Biswas, Debit K., et al. "The nuclear factor kappa B (NF-dB): a potential therapeutic target for estrogen receptor negative breast

cancers." *Proceedings of the National Academy of Sciences* 98.18 (2001): 10386-10391.

13. Kushner, Peter J., et al. "Estrogen receptor pathways to AP-1." *The Journal of steroid biochemistry and molecular biology* 74.5 (2000): 311-317.
14. Attisano, Liliana, and Si Tune Lee-Helfrich. "The shads." *Genome biology* 2.8 (2001): 1-8.
15. Hata, Akiko, and Ye-Gudang Chen. "TGF-β signalling from receptors to Smad." *Cold Spring Harbour perspectives in biology* 8.9 (2016): a022061.
16. Chapnick, Douglas A., et al. "Partners in crime: the TGFβ and MAPK pathways in cancer progression." *Cell & bioscience* 1.1 (2011): 1-8.
17. Barcelo's-Hoff, Mary Helen, and Rosemary J. Amhurst. "Transforming growth factor-β in breast cancer: too much, too late." *Breast cancer research* 11.1 (2009): 1-6.
18. Zhang, Hongqiao, Kelvin JA Davies, and Henry Jay Forman. "TGFβ1 rapidly activates Sc through a non-canonical redox signalling mechanism." *Archives of biochemistry and biophysics* 568 (2015): 1-7.
19. Patel, Aisha. "Benign vs malignant tumours." *JAMA oncology* 6.9 (2020): 1488-1488.
20. Fouad, Yousef Ahmed, and Carmen Aeneid. "Revisiting the hallmarks of cancer." *American journal of cancer research* 7.5 (2017): 1016.
21. (www.cancer.gov/publicatiopns/dictionaries/cancer-terms/def/tumor
22. (https://jamanetwork.com/journals/jamaoncology/fullarticle/2768634
23. www.cancer.net/navigating-cancer-care/cancer-basics/what-c%C3%A1ncer)
24. (https://acsjournals.onlinelibrary.wiley.com/
25. (https://www.cancercenter.com/cancer-types/breast-cancer/symptoms
26. https://www.cancerresearchuk.org/about-cancer/breast-cancer/stages-types-grades/tnm-staging)
27. https://www.cancerresearchuk.org/about-cancer/breast-cancer/stages-types-grades/tnm-staging)
28. https://www.cancer.org/cancer/breast-cancer/screening-tests-and-early-detection/breast-mri-scans.html
29. https://www.snmmi.org/AboutSNMMI/Content.aspx?ItemNumber=36606

30. .https://www.healthline.com/health/breast-biopsy
31. https://www.cancer.net/cancer-types/breast-cancer/types-treatment
32. https://www.mayoclinic.org/diseases-conditions/breast-cancer/diagnosis-treatment/drc-20352475
33. https://jnm.snmjournals.org/content/jnumed/57/Supplement_1/9S.full.pdf
34. https://www.cancer.org/cancer/breast-cancer/understanding-a-breast-cancer-diagnosis/breast-cancer-hormone-receptor-status.html)
35. (https://www.cancer.org/cancer/breast-cancer/understanding-a-breast-cancer-diagnosis/breast-cancer-hormone-receptor-status.html)
36. (https://www.cancer.org/cancer/breast-cancer/understanding-a-breast-cancer-diagnosis/breast-cancer-hormone-receptor-status.html)
37. (https://journals.lww.com/md-journal/fulltext/2016/11150/trends_of_triple_negative_breast_cancer_research.55.aspx)
38. https://my.clevelandclinic.org/health/diseases/21756-triple-negative-breast-cancer-tnbc

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