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Involvement of EZH2-MYC loop and SALL4 in Epithelial-Mesenchymal Transition (EMT) and trastuzumab resistance process in HER2+ breast cancer

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That the present doctoral thesis, entitled "Involvement of EZH2-MYC loop and SALL4 in Epithelial-Mesenchymal Transition (EMT) and trastuzumab resistance process in HER2 + breast cancer", presented by Ms Chaudhuri Birlipta Pattanayak to apply for the degree of Doctor from the University of Valencia, has been carried out under the direction in the Department of Medicine of the Faculty of Medicine within the doctoral program 3139 as well as in the INCLIVA Biomedical Research Institute, and that it is completed and ready for presentation so that the corresponding court can judge it.

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La Comisión Académica del Programa de Doctorado 3139 en Medicina, en su reunión de 25 de febrero de 2020, a la vista del certificado de realización de la estancia emitido por el responsable en el centro de la misma y de la memoria de la actividad e informe de los Directores/as, y en su caso del tutor/a, presentada por D/Dª CHAUDHURI BIRLIPTA PATTANAYAK, emite informe favorable sobre la estancia realizada para la obtención de la Mención Internacional al título de doctor.

Valencia, 25 de febrero de 2020

Pilar Codoñer Franch
Coordinadora Programa Doctorado en Medicina

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” If the facts don’t fit the theory, change the facts.”

- Albert Einstein

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List of Abbreviations

AD: Anno Domini

AREG: Amphiregulin

ALDH1: Aldehyde dehydrogenase 1

ATP: Adenosine triphosphate

ALK: Anaplastic lymphoma kinase

ADCC: Antibody-dependent cellular cytotoxicity

AML: Acute myeloid leukaemia

ADR: Adriamycin

ADSC: Adipose-derived stem cell

BChr: Before Christ

BC: Breast cancer

BTC: Betacellulin

BCA: Bicinchoninic acid assay

BSA: Bovine Serum Albumin

BCL2: B-cell lymphoma 2

BCSC: Breast cancer stem cell

C: Carboxyl

CHIP: Chromatin immunoprecipitation

CSC: Cancer stem cell

CTr: Carboxyl-terminal region

CT: Cycle threshold

CML: Chronic myeloid leukaemia

CAFs: Cancer-associated fibroblasts

CO_2: Carbon dioxide

CM: Condition medium

DAPI: 4′,6-diamidino-2-phenylindole

DCIS: Ductal carcinoma in situ

DNA: Deoxyribonucleic acid

DRC: DNA repair capacity

DMEM: Dulbecco's modified Eagles medium

DMFS: Distant metastasis-free survival

DNMT1: DNA (cytosine-5)-methyltransferase 1

DMSO: Dimethyl sulfoxide

DT: Dissecting tumour

ECM: Extracellular matrix

ECL: Enhanced chemiluminescence

EMT: Epithelial to Mesenchymal transition

ES: Embryonic stem

EGFR: Epidermal growth factor receptor

EREG: Epiregulin

EZH2: Enhancer of Zeste Homolog 2

FGFR: Fibroblast growth factor receptor

FDA: Food and Drug Administration

FITC: Fluorescein isothiocyanate

FACS: Fluorescence-activated cell sorting

FBS: Fetal bovine serum

GPCRs: G-protein-coupled receptors

GAP: GTPase-activating protein

GDP: Guanosine diphosphate

GTP: Guanosine triphosphate

GAPDH: Glyceraldehyde 3-phosphate dehydrogenase

HBV: Hepatitis B virus

HB-EGF: Heparin-binding epidermal growth factor

HCC: Hepatocellular cancer

HER2: Human epidermal growth factor receptor 2

HGFR: Hepatocyte growth factor receptor

HRP: Horseradish peroxidase

HR: Hazard ratio

HSCs: Hematopoietic stem cells

IARC: International agency of research on cancer

ICC: Immunocytochemistry

IDC: Infiltrating ductal carcinoma

IGFR: Insulin and Insulin-like receptor

IHC: Immunohistochemical

JM: Juxta membrane region

kDa: Kilodalton

KM plotter: Kaplan-Meier plotter

LCIS: Lobular carcinoma in situ

MDS: Myelodysplastic Syndromes

MiRNAs: MicroRNAs

mRNA: Messenger RNA

mTOR: Mammalian target of rapamycin

NC: Negative control

NGS: Next-generation sequencing

NK cells: Natural killer cells

NPM: Nucleophosmin

NRG: Neuregulin

OS: Overall survival

PAM 50: Prediction Analysis of Microarray 50

PBS: Phosphate-buffered saline

PDGFR: Platelet-derived growth factor receptor

PDK1: 3-phosphoinositide-dependent protein kinase 1

PDX: Patient-derived xenograft

PH: Plexin homology

PI3K: Phosphoinositide 3-kinase

PI: Propidium iodide

PIP2: Phosphatidylinositol 4, 5 – biphosphate

PIP3: Phosphatidylinositol 3,4,5 – triphosphate

PKB: Protein kinase B

PK: Protein kinase

PTEN: Phosphatase and tensin homolog

P/S: Penicillin-Streptomycin

Q-PCR: Quantitative polymerase chain reaction

RBBP4: Retinoblastoma binding protein 4

RFS: Relapse free survival

RIPA: Radioimmunoprecipitation assay

RNA: Ribonucleic acid

RTK: Receptor tyrosine kinase

RT: Reverse transcription

RT: Room temperature

SDS-PAGE: Sodium Dodecyl Sulfate–Polyacrylamide Gel Electrophoresis

siRNA: Small interfering RNA

T: Trastuzumab

TDM1: Trastuzumab emtansine1

TGF-α/β: Transforming growth factor α/β

TKD: Tyrosine kinase domain

TKI: Tyrosine kinase inhibitor

TKs: Tyrosine kinases

TMA: A tissue microarray

TME: Tumour-microenvironment

TNBC: Triple-negative BC

TNFα: Tumour necrosis factor α

TNM: Tumour (T), Nodes (N), and Metastases (M)

TSC1/2: Tuberous sclerosis complex 1/2

UTR: Untranslated region

VEGFR: Vascular endothelial growth factor receptor

WST assay: Water Soluble Tetrazolium Salt assay

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ABSTRACT

Breast cancer (BC) is the most common type of cancer in females worldwide. It is also the second leading cause of death in women. BC is covered with heterogeneity properties, that leads to poor prognosis and therapeutic resistance. It has always been essential to unveil the different molecular mechanisms involved in BC cancer progression, finding a suitable treatment for the patients. This thesis focuses on unwrapping the various molecular mechanisms involved in HER2+ BC subtypes, as this denotes an aggressive phenotype among other subtypes of BC. Downregulation of miR-33b has been documented in many types of cancers and involves proliferation, migration, and epithelial-mesenchymal transition (EMT). Furthermore, *enhancer zeste homolog 2-gene (EZH2)* is a master regulator of controlling the stem cell differentiation and cell proliferation processes. The implication of miR-33b in the EMT pathway and analyze the role of EZH2 in this process and interaction between them is one of the main spotlights of the thesis. miR-33b is downregulated in HER2+ BC cells vs healthy controls, where EZH2 has an opposite expression in vitro and patients' samples. The upregulation of miR-33b suppressed proliferation, induced apoptosis, reduced invasion, migration and regulated EMT by an increase of E-cadherin and a decrease of ß-catenin and vimentin. The silencing of EZH2 mimicked the impact of miR-33b overexpression. Furthermore, the inhibition of miR-33b induces cell proliferation, invasion, migration, EMT, and EZH2 expression in non-tumorigenic cells. Notably, the Kaplan–Meier analysis showed a significant association between high miR-33b expression and better overall survival. These results suggest miR-33b as a suppressive miRNA that could inhibit tumour metastasis and invasion in HER2+ BC partly by impeding EMT through the MYC–EZH2 loop's repression. On the other hand, treatment for the HER2+ BC subtype is minimal. Trastuzumab is a monoclonal antibody, regularly used for the treatment of this specific subtype of BC. Although trastuzumab is currently considered one of the most effective oncology treatments, a significant number of patients with HER2-overexpressing breast cancer do not benefit from it. The other part of the thesis focuses on finding a novel molecular mechanism of one transcription factor (TF), Sal-like protein 4 (SALL4), a critical regulator of cancer aggressiveness and resistance treatment. HER2+ BC cells with acquired resistance to trastuzumab express a higher level of SALL4 as compared to the wild type cells. Gain and loss function experiments showed that less SALL4 expression conducted the restoration of the trastuzumab

sensitivity significantly; however, the transient overexpression of SALL4 in parental cell lines induced high proliferation of the cells, resulting of the reduction of trastuzumab efficacy. Furthermore, SALL4 expression regulates the PI3K/AKT pathway, through controlling of PTEN expression. Moreover, AKT phosphorylation activated many downstream targets, such as BCL2, resulting in increased cell survival and proliferation. It has been observed that SALL4 expression regulates EMT pathway via controlling the MYC expression. SALL4 showed a physical interaction with RBBP4, a NuRD complex member, and regulates the downstream proteins such as PTEN and BCL2. This interaction also helps cells to be escaped from the trastuzumab treatment and therefore, targeting the SALL4–NuRD pathway in HER2+ BC, mostly in acquired resistance cell lines would be a promising therapeutic approach and better treatment for this specific type of cancer in future. SALL4 also predicted as a prognostic factor in all subtypes of BC through KM plotter. This study provides a viable molecular mechanism-drive therapeutic strategy for the significant subset of patients with HER2+ BC whose malignancies are driven by SALL4 expression.

CHAPTER 1

1.1 INTRODUCTION

1.1.1 Origin of Cancer and BC

Cancer occurrence has been noted throughout history as far back as 3000 BChr (before Christ). It is not a modern malady. Fossilized bone and Egyptian mummies have shown the first evidence of tumour; even though the word cancer was not derived during those periods[1]. The origin of word cancer is designated by Greek physician Hippocrates (460-370 BChr) who was considered the father of Medicine. He used the term karkinos and karcinoma to describe non-ulcer and ulcer forming tumours[2]. A crab chased those words because of its finger-like spreading anatomy, which can be related to the complex form of tumour[3]. Afterwards, the Roman physician Cornelius Celsus translated the Greek term Karkinos to the Latin word for crab, called Cancer[4], during (25 BChr-Anno Domini (AD) 50). Eventually, the term cancer became well recognizable in the field of Medicine. Around 200AD one of the Greek physicians named Galen had applied this term to medicine field using a word called "Onkos" which later on was adopted as the root of word "Oncology" the most popular used phrase in the field of disease and Medicine in today´s world[5].

The International Agency for Research on Cancer (IARC) predicted in 2020, that one in five men and one in six women worldwide have cancer throughout their lives and that one in eight males and one in eleven females die from the disease[6]. In developing countries, cancer is the second cause of death, and in developed countries, it is the first cause of death. The constant increase in cancer burden is due to smoking, pollution, sedentary lifestyle, and western food and population aging[7]. Lung and BC are the world's leading types in terms of the number of new cases; in 2018, approximately 2.1 million diagnoses are estimated for each of these types, contributing about 11.6% of the total incidence burden of cancer. Out of the 26 different types of cancers, BC is one of the most commonly diagnosed cancers in women worldwide. There were 268,600 new cases in 2019, according to the World Cancer Research Fund International[8]. Current treatment therapy and different molecular approaches towards developing new strategies for treating this disease have become the most challenging subject.

Breast carcinoma is the most advanced disease among women in world wide. It is not a single disease, which comprises only a single treatment. Instead, it is a heterogeneous disease that implies many biological entities with distinct pathological features and

diverse clinical implications. **Figure 1** shows the evolution of BC elaborately from 3000 Before Common Era (BCE) to 2016.

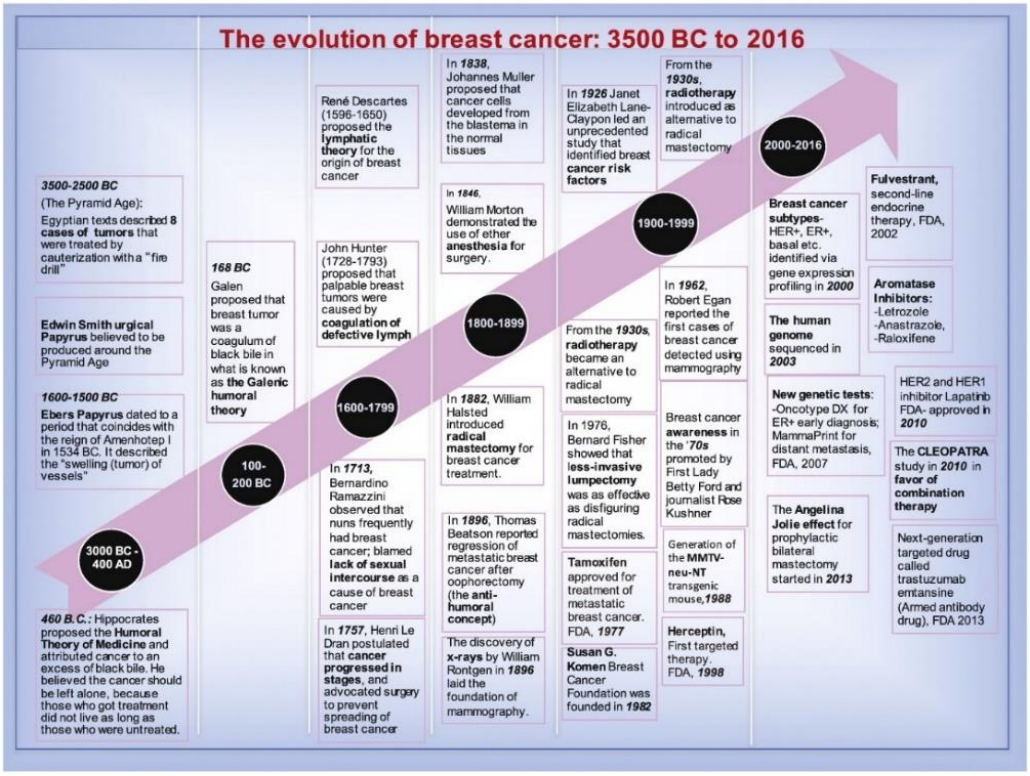


Figure 1: Timeline: The evolution of BC: 3000 BCE to 2016: The sceptical description of BC's evaluation process.

In Medicine, BC is earliest diagnosed cancer, written in Edwin Smith Papyrus during 3000 BCE[1]. Throughout the ages, nobody has been sure what causes BC. Research continues today. For quite a long time to follow, speculations by Hippocrates (460 BCE) and Galen (200 Common Era (CE)), crediting the reason for BC to an "excess of black bile" and treatment choices including the utilization of opium and castor oil, prevailed[9]. After discovering the lymphatic system by Olof Rudbeck of Sweden in 1652, René Descartes (1596–1650) proposed the lymphatic theory for BC's origin, which contradicted the prevailing humoral explanation (Galen Theory)[10]. 19th century marked the major advances in human pathology and safety during surgery and progress in oncology. In 1838, German pathologist Johannes Muller (1801–1858) suggested that cancer cells evolved from the blastema between the normal tissues and not from the lymphatic system, and later Rudolph Virchow (1821–1902) demonstrated that tumours were composed of cells[11]. In 1909 Danish botanist Wilhelm Johannsen coined the word

"gene", since then the scientists started to dig the molecular mechanisms that underlying in evolve of the BC, which still remains unclear.

1.1.2 Histological classifications of BC subtypes:

BC's distinctive histological characteristics fascinate pathologists, distinguishing particular morphological and cytological trends consistently correlated with clinical outcomes. However, BC can be broadly categorized into in situ carcinoma and invasive (infiltrating) carcinoma, as shown in **Figure 2**. In situ carcinoma is a group of abnormal cells found only in the originated place of the tumour. These abnormal cells can become cancerous and spread to normal tissue nearby. Further, it is sub-classified as either ductal or lobular depending upon their growth pattern. Ductal carcinoma in situ (DCIS) is significantly more normal than lobular carcinoma in situ (LCIS). DCIS has commonly been subclassified according to the tumour's architectural characteristics, resulting in five well-recognized subtypes: Comedo, Cribiform, Micropapillary, Papillary and Solid

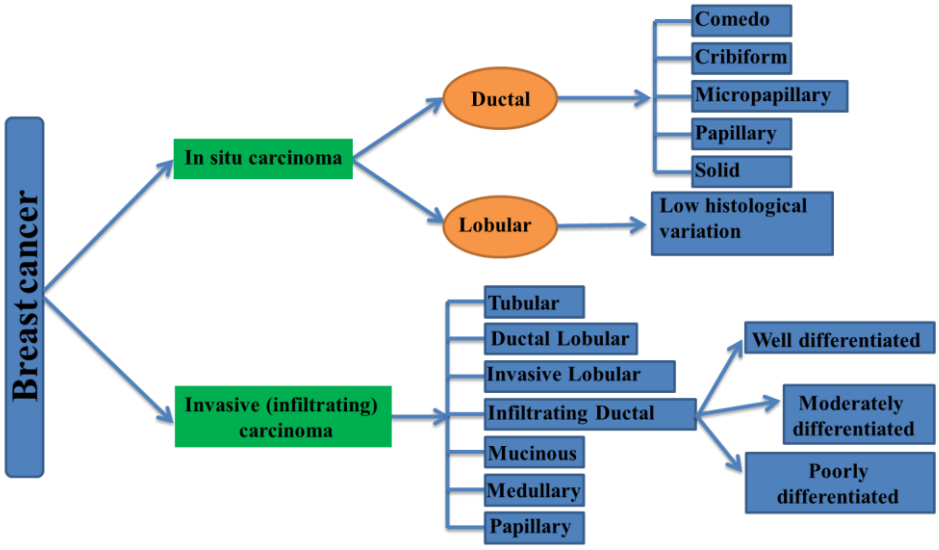


Figure 2: Histological classification of BC: The schematic representation of the various sections under the BC histological classification.

On the other hand, invasive carcinomas are a heterogeneous group of tumours differentiated into histological subtypes. The major invasive tumour types include infiltrating ductal, invasive lobular, ductal/lobular, mucinous (colloid), tubular, medullary and papillary carcinomas. Among them, infiltrating ductal carcinoma (IDC) is, by far, the most common subtype accounting for 70–80% of all invasive lesions[12].

IDC is further sub-classified into three categories, comprising well-differentiated (grade 1), moderately differentiated (grade 2) and poorly differentiated (grade 3). These classifications are based on the levels of nuclear pleomorphism, glandular/tubule formation and mitotic index[13]. This classification scheme has been a valuable tool for several decades; it relies solely on histology without utilizing newer molecular markers with a proven prognostic significance.

1.1.3 Molecular classifications of BC subtypes

Perou and Sorlie proposed "Molecular Classification" terminology in BC for the first time with a comprehensive study showing the differences in gene expression in 2000[14]. The studies have identified several intrinsic molecular subtypes of BC and classified as: "Luminal subtype A and Luminal subtype B, HER2+, basal-like, normal breast-like and claudin-low[15] (**Figure 3**). Different molecular subtypes of BC emphasized biological heterogeneity of the disease, which has been histopathologically defined by the pathologist for long time[16]. Notably, molecular classification had a significantly improved ability to predict relapse risk compared to a model utilizing only clinical variables (tumour size, node status and histologic grade)[17].

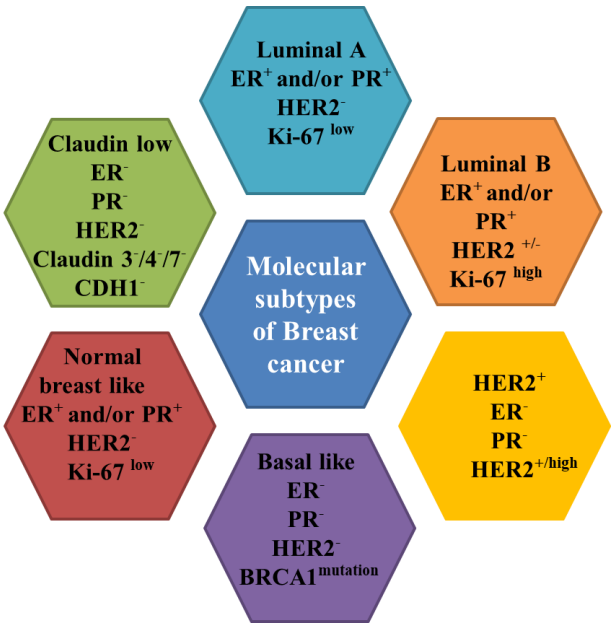


Figure 3: Molecular classification of BC: These schematic classifications are purely based on the intrinsic molecular subtypes of breast cancer, identified by microarray analysis of tumour patients' samples.

1.1.4 Risk factors of BC

The "established" risk factors for BC are female sexual orientation, age, benign breast disease, hereditary factors, early age at menarche, late age at menopause, late age at to begin with a full-term pregnancy, post-menopausal corpulence, low physical activity, race/ethnicity and high-dose exposure to ionizing radiation early in life.

The "speculated" risk factors for BC including never being pregnant, having only one pregnancy in a lifetime, not breastfeeding after pregnancy, utilize of postmenopausal hormone (estrogen/progesterone) substitution treatment, use of oral contraceptives, certain specific dietary practices (high intake of fat and low intakes of fibres, fruits, and vegetables, low intake of phytoestrogens), alcohol consumption, tobacco smoking, and abortion[18]. About 5-10% of BCs are hereditary. Germline mutations cause most inherited cases of BC in the BRCA1 and BRCA2 genes. These two genes are mostly involved in cell growth, cell division, and DNA damage repair. Mutations in the BRCA genes results in unrepaired DNA damage, which increases the chance to develop certain types of cancer. In general, people with BRCA 1 mutation have an increased risk of BC at an early age, promoting BC in both breasts, or developing more than one type of cancer in their lifetime[19]. Although men can and do develop BC, the disease is 100 times more likely to occur in a woman than in a man as women have much more breast tissue than men do.

1.1.5 Signs and symptoms of BC

Early stages of BC are generally asymptomatic As the tumour stage advances at least one of these clinical signs and manifestations emerge:: new lump in the breast or underarm, thickening or swelling of part of the breast, irritation or dimpling of breast skin, redness or flaky skin in the nipple area or the breast, pulling in of the nipple or pain in the nipple area, nipple discharge other than breast milk; including blood, any change in the size or the shape of the breast or discomfort in any area of the breast[20].

1.1.6 Diagnosis of BC

Diagnosis of BC is based on the clinical, radiological and pathological examinations. Clinical examination includes bi-manual palpation of the breasts and local-regional lymph node. Radiological examinations include bilateral mammography of the breasts and ultrasound of the breasts and local-regional lymph node. Pathological diagnosis is

based on a breast biopsy (core needle biopsy/ fine needle aspiration) obtained manually or by ultrasound guidance. The final pathological diagnosis gives information on the tumour type, the TNM staging, Nottingham classification, hormone receptor status, etc., all of which are vital for designing the treatment regimen.

Few other tests performed post confirmation of BC include chest X-ray (to detect any metastasis to lungs), bone scan (to provide information on bone involvement), MRI (to detect any metastasis to the brain) and blood tests (to plan surgery and adjuvant therapy). Also, genetic analysis methods such as Prediction Analysis of Microarray 50 (PAM 50 test) and MammaPrint test of the tumour give insight into the interplay of genes and enable dividing the tumours into good prognostic and deficient prognostic tumours for enabling individualized treatment.

1.1.7 Treatment modalities for BC

Treatment options for BC usually depend on the following key criteria's: tumour type, tumour size, resectable/unresectable tumour, hormone receptor status, symptomatic/asymptomatic metastasis, solitary/multiple metastases, pre/post-menopausal status, hereditary cancer involving BRCA gene mutations and any other medical conditions[21].

However, treatment within BC subtypes greatly simplifies therapy indications, since the subtypes themselves incorporate the risk and predictive factors. A broad recommendation for systemic treatment of various subtypes is: FDA approved endocrine therapy alone or chemo-endocrine therapy or anti-HER2 therapy for patients clinicopathologically classified as "Luminal A" or "Luminal B" or "Triple-negative" respectively, and sole reliance on standard chemotherapy along with antibody therapy for most patients classified as "HER2+"[22] thereby designating HER2+ cancer as an aggressive subtype with lack of targeted therapeutic options and inferior prognosis[23].

1.1.8 The invasion-metastasis cascade

A multi-step process forms metastasis. Cancer cells have acquired the ability to disseminate from the primary tumour through breaking the basement membrane and invade the nearby stroma is termed localized invasion. The step when cancer cells enter the blood and lymphatic vessels are called intravasation. The migrating cells may die

from anoikis, a form of apoptosis triggered by detachment from the extracellular matrix (ECM). They can also survive for long periods and in some instances extravasate into the surrounding tissue, leading to the formation of micrometastasis. If the foreign tissue microenvironment is favourable, the cancer cells may begin to proliferate and form a secondary tumour; a process termed colonization (**Figure 4**). Carcinomas are benign as long as the tumour cells do not break through the basement membrane and they are in the form of in situ carcinoma and malignant when they have acquired the ability to metastasize which is the leading cause of death in cancer patients or resistance to drugs[24]. Understanding the biological mechanisms behind the formation of metastasis is of utmost importance to prevent and eradicate the metastatic disease. In this thesis, one of the most important mechanisms involved in the process of metastasis formation named "Epithelial to Mesenchymal transition- EMT" is discussed in detail.

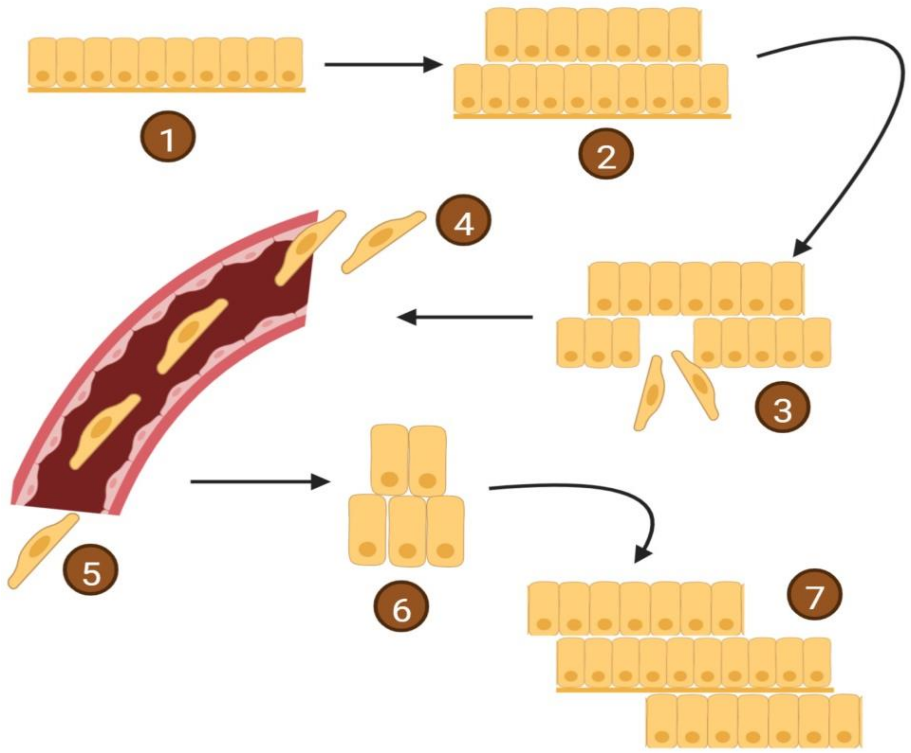


Figure 4: Schematic picture of cancer cell invasion and metastasis: 1. Normal epithelium. 2. Carcinoma in situ, 3. Invasive carcinoma. 4. Intravasation. 5. Extravasation. 6. Micrometastasis. 7. Colonization.

1.1.9 Role of EMT in BC

Clinical-histologic studies of basal-like BCs show most aggressive and deadly phenotype characters, displaying a high metastatic ability associated with mesenchymal

features[25], which is one of the key processes of EMT pathway. It is a biological process. Polarised epithelial cells undergo multiple biochemical changes such as loss of cellular polarity, destabilization of cell-cell junctions, remodelling and replacement of cytoskeletal components, to acquire the mesenchymal cell phenotype. This phenotype of the cells enhanced migratory capacity, invasiveness, elevated resistance to apoptosis and greatly increased ECM components[25]. Epithelial cell plasticity can generate distinct cellular subpopulations that contribute to the intratumoral heterogeneity in BC. Early studies verified that the BC cell lines with the increased invasiveness *in vitro* and displaying metastatic potential *in vivo* exhibited the mesenchymal intermediate filament protein and marker vimentin and less expression epithelial marker named E-cadherin[26]. EMT processes can be induced by a plethora of signalling pathways such as transforming growth factor-β (TGF-β), Wnt, Notch, tumour necrosis factor-α (TNF-α/NF-kB) and P13K/AKT pathways. All the mentioned pathways have a direct and indirect relation with ERBB family members[27]. EMT process is also influenced by the effects of tumour microenvironment such as hypoxia and differential expression of microRNAs (miRNAs)[28]. Several transcription factors, including the twist, EF1/ZEB1, SIP1/ZEB2, snail/slug family and E12/E47 function as master regulators of the EMT program.

Apart from the signalling pathways and transcription factors, miRNAs are also a major player in regulating EMT program. When the EMT program is induced, the expressions of several miRNAs (miR-15b, miR-30a, miR-33b, miR-200 family and miR-205,) are drastically reduced[29]. MiR-200 family directly regulates ZEB1 and ZEB2 mRNA expression, thereby increasing the E-cadherin expression leading to epithelial phenotype[29]. On the other hand, the miR-10b expression is increased during the EMT process, induced by the twist and limiting the expression of HOXD10, which facilitates BC cells' metastasis. TGFβ upregulates other miRs such as miR-155, miR-29a, and miR-21 induced EMT. Also, their expression levels are higher in mesenchymal-like cell lines compared to epithelial-like cells[29]. Overall, the differentially regulated miRs might be critical for EMT and cancer metastasis. Finally, EMT can also be regulated at the genetic and epigenetic level. For example, a gene mutation and hypermethylation at the promoter region of E-cadherin can inactivate this gene.

Induction of EMT is closely associated with “stemness” in the development process and carcinogenesis. During the gastrulation process, embryonic stem (ES) cells in the

blastocyst's inner mass have an epithelial phenotype that ingresses to form the primary mesoderm via induction of EMT process, illustrating the importance of EMT during the embryonic development[30]. EMT also regulates the stemness properties of the cells in different types of cancers. Expression of snail and twist in mammary epithelial cells induces EMT, leading to a CD24-/44+ phenotype associated with BC stem cells[31]. TGFβ signalling seems to be associated with EMT and cancer stem cells (CSC) formation in cancer. Mammary CSCs express high amounts of TGFβ1 and TβRII than the more differentiated epithelial counterparts, and inhibiting TGFβ signalling in CSCs can re-establish the epithelial phenotype. Apart from TGFβ signalling, Notch and Wnt signalling also contribute to CSCs generation in colon and pancreatic cancers, which is also known to induce EMT process[32]. Recently, a core EMT gene signature was identified, and it correlated with claudin-low and metaplastic BC subtypes[33]. This evidence suggests that induction of EMT and the gain of CSC-like properties are closely linked, which may be crucial for metastasis. Changing phenotype from epithelial to the mesenchymal state might be crucial for acquiring invasive abilities and survival benefits during systemic circulation for metastatic seeding (**Figure 5**).

Furthermore, receptor tyrosine kinases (RTKs) are associated with the chemoresistance and correlated to CSC markers suggesting another crosslink in BC's heterogeneity. It is believed that CSCs are the subset of the cell population retain in the tumour that is resistant to drugs and possesses characteristic of stem cell. For instance, HER2, an RTK responsible for tumour progression, metastasis and chemoresistance, is related to CSC markers as ALDH1 and NOTCH1[34]. In another study, it was found that HER2 overexpressed in MDA-MB-231 BC cells, co-operated with TGF-β to induce an enhanced pro-invasion, angiogenesis, and EMT signature correlating HER2 over-expression with EMT[35]. There are various signalling pathways other than stemness-related developmentally conserved signalling pathways involved in the crosstalk between HER2 and CSCs, leading to HER2 therapy resistance. The PI3K/AKT signalling pathway plays an essential role in cancer progression, drug resistance and EMT activation. Therefore, targeting one population in the tumour won’t eradicate cancer; until the specific treatment, the regimen has not been developed to target the tumour's root.

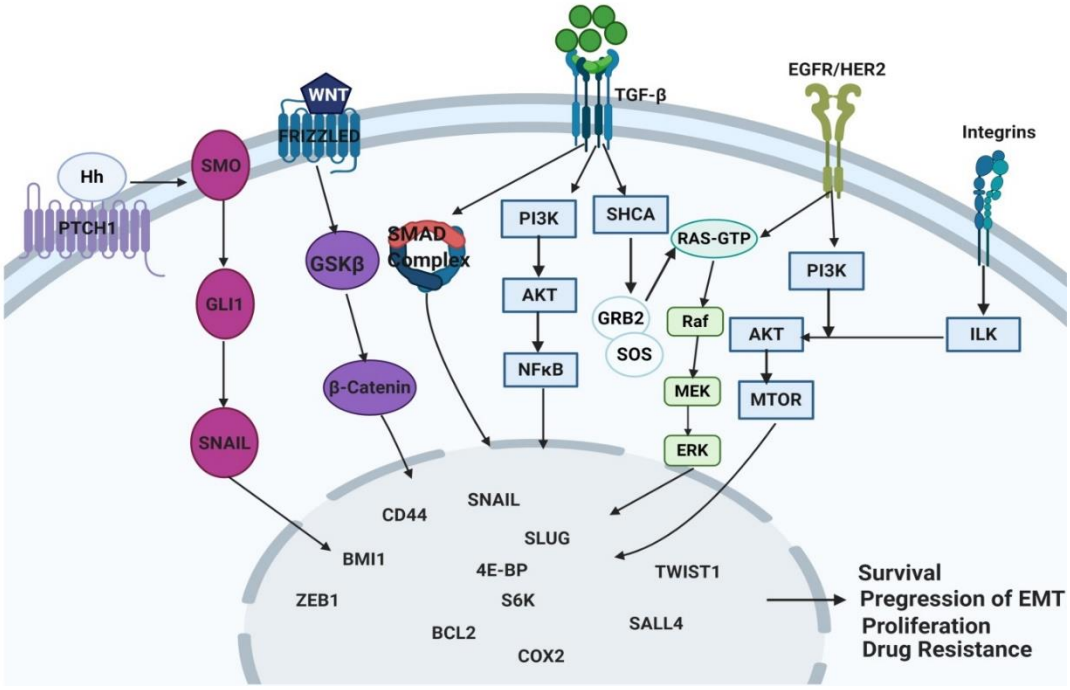


Figure 5: Targeting the CSC/EMT signalling pathways in BC: The schematic representation showed the involvement of different molecular pathways, which lead to activation of EMT and maintenance of stemness markers, that helps BC progression.

1.1.10 Protein Kinases involved in BC

Human protein kinases (PK) constitute a large family of enzymes, known as the human kinome, encoded by about 1.7% of all human genes[36]. The kinase helps regulate its target by covalently attaching a phosphate group known as phosphorylation of the protein, which leads to regulating particular biological reaction. The kinase superfamily has been classified into two main groups: Serine-Threonine kinases, which phosphorylate serine or threonine amino acids, and Tyrosine kinases (TKs), which phosphorylate tyrosine amino acids. A third group, consisting of dual-specificity protein kinases has also been proposed, as they can phosphorylate both tyrosine and serine/threonine residues[37]. According to the literature, tyrosine kinases are the first protein kinases to be identified. According to their location in the cell, tyrosine kinases are classified into:

Transmembrane receptor kinases consist of a ligand-binding extracellular domain and a catalytic intracellular kinase domain. Non-receptor tyrosine kinases, lacking the transmembrane domains and located in the cytosol, nucleus, or the inner surface of plasma membrane[38].

BC emerges as a consequence of dysregulation of different signalling pathways in mammary epithelial cells. Growth factors and chemokines activate various signalling cascades which crosstalk in tumour microenvironment leading to cancer progression. Hundreds of kinases play overlapping and intricate roles in cell transformation, tumour initiation, survival and proliferation. However, to understand and discuss their oncogenic undertakings; they can be vaguely categorized based on their cancer hallmark roles. Cytoplasmic tyrosine kinases are critical conveyers of extracellular signals, and mutations in these kinases have been reported to occur in various oncogenic conditions, mostly in HER2 positive BC.

1.1.11 Receptor tyrosine kinase (RTKs)

The discovery of the first receptor tyrosine kinase (RTK) was more than a quarter of a century ago. Many cell surface receptors have emerged as key regulators of critical cellular processes, such as proliferation, differentiation, cell survival, metabolism, cell migration and cell cycle control[39]. There are 58 known RTKs in humans and furthered divided into 20 subfamilies[40]. All RTKs share a similar protein structure comprised of an extracellular ligand-binding domain, a single transmembrane helix, and an intracellular region that contains a juxtamembrane regulatory region, a tyrosine kinase domain (TKD) and a carboxyl (C) terminal tail[41]. The role of the intracellular region is catalyzing the gamma phosphate groups of ATPs transferring to tyrosine residues. Phosphorylation activates the protein kinases (active form), then signal transduction started[42]. Deregulation of RTK signalling leads to many human diseases, especially cancer. The advancement of the genomic era and the implementation of next-generation sequencing (NGS) revealed several different types of alteration present in the genes encoding RTKs such as EGFR, HER2/ErbB2, MET, amongst many others[43].

1.1.12 RTKs families

As mentioned before, in cellular processes, the RTKs families include several families of receptors (**Figure 6**).

1. **Epidermal growth factor receptor (EGFR):** This receptor family plays an important role in regulating cell proliferation, survival, differentiation and migration[44].

2. **Insulin and Insulin-like receptor (IGFR):** This receptor family has two members: Insulin-like growth factor 1 receptor (IGF-1R) and insulin-like growth factor 2 receptor (IGF-2R). IGF1R signal transduction causes the activation of several intracellular signalling pathways, including MAPK and PI3K pathways[45]. IGF2R can induce activation of small G protein and its downstream pathways[46].
3. **Anaplastic lymphoma kinase (ALK):** This is a transmembrane RTK, initially identified in the nucleophosmin (NPM)–ALK chimaera of anaplastic large cell lymphoma, and has emerged as a novel tumourigenic player in several human cancers[47].
4. **Platelet-derived growth factor receptors (PDGFR):** These receptors family belongs to class III receptor tyrosine kinases. These receptors' activation is associated with many human diseases such as cancer, fibrosis, neurological conditions, and atherosclerosis[48].
5. **Vascular endothelial growth factor receptor (VEGFR):** This family receptor is mitogenic can regulate angiogenesis. They play an important role in mitosis and chemotaxis in vascular endothelial cells[49].
6. **Hepatocyte growth factor receptor (HGFR):** This receptor is well known as tyrosine-protein kinase Met or c-Met, encoded by the *MET* gene. It is overexpressed in some cases of human leukaemia and lymphoma and implicated in cellular proliferation, cell survival, cell invasion, cell motility, cancer metastasis and angiogenesis[50].
7. **Fibroblast growth factor receptor (FGFR):** The FGFR family is characterized by four receptors, binding to 18 ligands called fibroblast growth factors (FGFs), employing heparin as a co-factor[51]. These receptors have pivotal roles in embryogenesis and metabolism and play a critical role in developing the skeletal system[52].
8. **TAM family:** TAM family which includes Tyro-3, Axl, and Mer; these three receptors which share the vitamin K-dependent ligands Gas6 and Protein S.

   They have a conserved sequence within the kinase domain and adhesion molecule like extracellular domains signalling pathways employed by the TAM family. It has been recently elucidated and shown to mediate diverse cellular functions, including macrophage clearance of apoptotic cells, platelet aggregation, and natural killer (NK) cell differentiation[53].

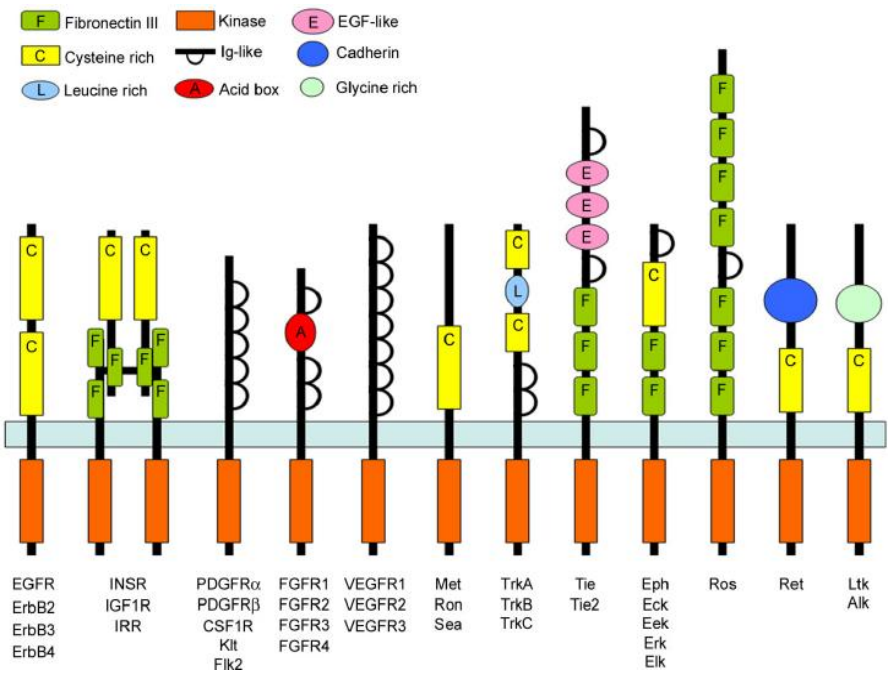


Figure 6: Receptor Tyrosine Kinase Families[54]**:** Schematic representation of receptor tyrosine kinase family involved in the regulation of biological properties.

1.1.13 ErbB/EGFR family

ErbB/HER family is well known as Type 1 RTKs, comprises four proteins, which bind growth factors of the EGF and Neuregulin families. The four ErbB/HER proteins are crucial in normal cellular functions and key players in pathological processes, such as malignant transformation. All the four proteins share a similar structure consisting of three parts: an extracellular domain, transmembrane and an intracellular domain[55]. The extracellular domain consists of 621 amino acids, which can bind to the corresponding ligand; the transmembrane region contains 23 amino acid residues, which forms the alpha helix hydrophobic domain, this region anchors the receptor to the cell membrane[56]. The intracellular region carries 542 amino acid residues and contains three sub-regions: Juxtamembrane region (JM), catalytic tyrosine kinase domain (TK) and carboxyl-terminal region (CT). Multiple mechanisms underlay the driver or supportive functions of ErbB/HER proteins in tumours, and they include receptor overexpression, receptor's point mutations, and internal deletions, as well as autocrine loops, meaning that a cell initiates its own proliferation through ligand secretion.

1.1.14 Family members

The epidermal growth factor RTK family consists of four members: EGFR (ErbB1, HER1), ErbB2 (HER2, neu in rodents), ErbB3 (HER3) and ErbB4 (HER4) as shown in (**Figure 7)**.

1. **EGFR (ErbB1, HER1):** The human EGFR gene is located in chromosome 7. The mRNA of EGFR consists of 28 exons and encodes 1186 amino acids[57]. The molecular weight of the EGFR protein is 170 kDa. It is mostly expressed in cellular differentiation, and its structural alteration leads to tumorigenic signalling pathways in different kinds of tumours. EGFR first need to homodimerize, which occurs when it binds with its ligand[58]. There are various kinds of ligands; those activate EGFR in cellular processes, which are EGF, TGFα, Neuregulin (NRG), heparin-binding epidermal growth factor (HB-EGF), Amphiregulin (AREG), Epiregulin (EREG), betacellulin (BTC)[59], shown in (**Figure 7)**. Alteration of EGFR pathways can be due to following reasons:
   a) EGFR can be activated independently of the presence of its ligand due to the mutation in EGFR VIII. This mutation was observed in glioblastoma and non-small cell lung cancer[60].
   b) Overexpression of its ligand, for example, some tumour cells can overexpress Epidermal growth factor (EGF) or transform growth factor-alpha (TGFα), which can modulate and activate the downstream of EGFR signalling pathway[61].
   c) EGFR overexpression has been observed in the neck and head cancer, lung cancer, skin cancer and oesophageal cancer. This overexpression promotes tumour cells proliferation, angiogenesis, invasion and metastasis. They can also inhibit apoptosis which can lead to poor prognosis[62].

2. **ErbB2 (HER2, neu):** Neu/Her2, also known as ErbB2, was first discovered as a potent oncogenic mutant when isolated from neuro glioblastoma or Schwannomas carcinogen-treated rats. As it was initially found in the neurological tumour, it is named "neu"[63]. The human HER2 is located in chromosome 17, and its codes for a 185-kDa. ERBB2 is amplified and overexpressed in 15–30% of BCs. Downstream signalling pathways are activated upon ErbB2 receptor activation through either heterodimerization with

ligand-bound EGFR, ErbB3, or ErbB4 family receptors, or in the presence of overexpression of ErbB2 due to gene amplification, by ligand-independent homodimerization[64]. The homo/heterodimerization promotes the receptor activation that, in turn, leads to tyrosine phosphorylation of the C-terminal residues. Numerous phosphorylation sites exist within the cytoplasmic domain of ErbB2; these sites are essential for protein-protein interactions and induction of the signalling cascades downstream to ErbB2 receptor activation. To this regard, the activation of the phosphoinositide 3-kinase (PI3K) and Ras/RAF/MEK/ERK1/2 pathways are hallmarks of ErbB2 activation[65]. HER2 is low expressed in adult human epithelial cells, and under physiological conditions, HER2 promotes cell growth and differentiation[66]. The overexpression HER2 is related to different types of tumour metastasis and progression. It is widely known that overexpression of HER2 is closely related to the occurrence of HER2+ BC. Also, significantly overexpression of the HER2 gene is associated with poor survival of breast and gastric cancer[67].

3. **ErbB3 (HER3):** HER3 is a unique member of the HER family as it has been considered an inactive receptor, although a recent study suggests that HER3 contains weak kinase activity[68].This gene is located in chromosome 12 and translated into 1342 amino acid residues. Its structure is very similar to HER2 and EGFR. According to the sequence comparison, HER3 receptors have certain amino acid residues, including Cys-721, His-740, and Asn815, non-conservative substitutions. These changes significantly reduce the kinase activity of HER3[69]. Thus, HER3 has to form dimers with other receptors and be phosphorylated by its interactive partners, with HER2 being the most important one[70] to activates in downstream signalling. It has shown widely that the HER2/HER3 heterodimer is the most biologically active and potent to activate the PI3K/AKT signalling cascade[71]. This also has a ligand called Neuregulin, which enables the downstream pathway and helps tumour progression and metastasis in different cancer[72].

4. **ErbB4 (HER4):** The human HER4 gene is located in chromosome 2. The molecular weight of HER4 protein is 180 kDa[73]. This receptor can be regulated by different ligands such as Neuregulin, Beta-cellulin and Heparin-binding EGF.

HER4 needs to bind other ErbB family receptors to have kinase activity and exert their biological activity[74]. HER4 is mostly expressed in normal embryonic and adult tissues. It is also reported that HER4 is highly expressed in thyroid and ovarian cancer[75]. However, its expression has been correlated with favourable prognostic factors, which positively affects patients with BC[73]. These results indicate that HER4 has a different function in cancer cells from other ErbB family receptors.

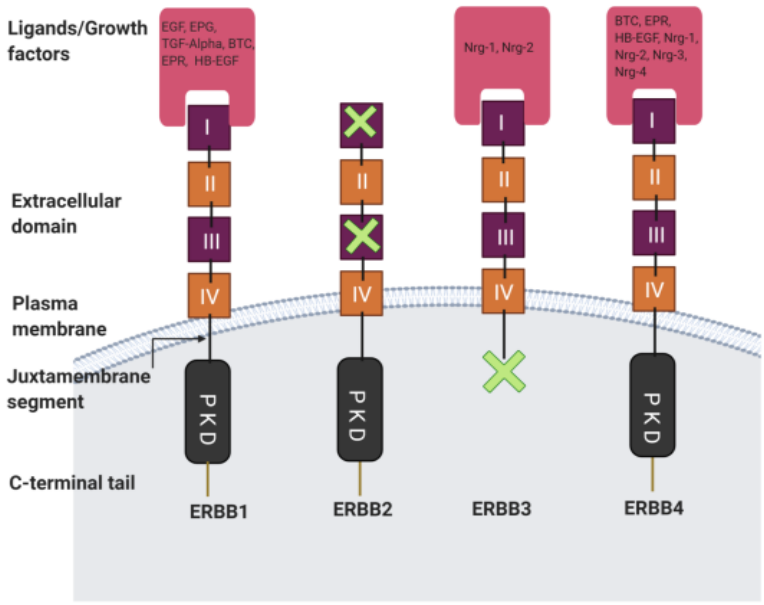


Figure 7: Structure, ligands, and ErbB family members: Picturized representation of the different subfamily members of the ERBB family with their widely viewed structure.

1.1.15 Significant Role of the HER2 signalling pathway in BC

HER receptors are expressed in various epithelial, mesenchymal, and neuronal origin tissues. They are involved in controlling diverse biological processes such as proliferation, differentiation, migration and apoptosis. Ligand binding to HER receptors results in dimerization and activation of their intrinsic kinase activity followed by phosphorylation of specific tyrosine residues in the receptor cytoplasmic tails as described before. In turn, this phosphorylated tyrosine provides recognition sites for intracellular signalling intermediates, which link receptor tyrosine kinases to downstream transduction cascades[76]. BC is one of the most common malignancies in women. Overexpression of HER2 is associated with a poorer prognosis in 20-25% of invasive BC cases[77]. Its overexpression in cell lines leads to transformation in the

absence of a ligand. None of the EGF family of ligands binds to HER2 directly. Therefore, in a technical sense, HER2 remains an orphan receptor. However, it appears due to HER2 being the preferred dimerization partner for all other HER receptors, which HER2 mainly functions as a shared co-receptor[78] (**Figure 8**). The phosphorylated HER dimers activate downstream cell proliferation (mitogen-activated protein kinase, MAPK pathway), cell survival (phosphoinositide 3-kinase pathway), and signal transducer and activator transcription pathways.

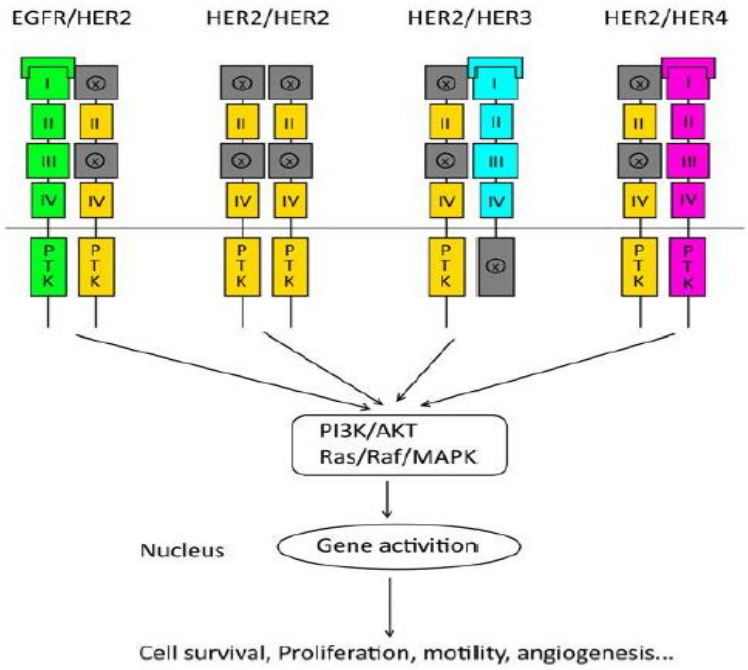


Figure 8: HER2 signalling pathway[78]**:** Diagrammatic representation showed the two important signalling cascades followed by ErbB family.

1.1.16 PI3K/AKT/mTOR (PAM) pathway in HER2+ BC

The phosphoinositide 3 kinases (PI3K)/AKT/mammalian (or mechanistic) target of rapamycin (mTOR) pathway is a complicated intracellular pathway, which leads to cell growth and tumour proliferation and plays a significant role in BC. This pathway is well established to play an essential role for providing nutrients, hormones and growth factor to stimulate tumour cell growth and proliferation. According to the substrate and structure specificity, PI3Ks can be divided into PI3K I, PI3K II and PI3K III[79]. PI3K I was most clearly studied and closely related to tumour development, further subdivided into two subtypes: PI3K IA and PI3K IB[80]. The PI3K IA has a central role in this pathway and which is a heterodimer composed and consists of two subunits, with the

regulatory subunit (p85) regulating the activation of the catalytic subunit (p110) in response to the absence or presence of upstream stimulation by growth factor receptor tyrosine kinases (RTKs)[81].

In the absence of an active signalling pathway, the regulatory subunit p85 interacts with the catalytic subunit p110 inhibiting its catalytic activity of p110[82] and bringing it close to the lipid substrates in the membrane. However, other mechanisms such as signalling through G-protein-coupled receptors (GPCRs) and the small GTPase Ras have also been suggested to initiate PI3K activation[83]. The PI3K phosphorylates the D3 position on phosphoinositides to yield biologically active phosphatidylinositol-3, 4, 5-trisphosphate (PI (3, 4, 5) P3), which can then interact with lipid-binding domains in PI3K effector proteins, changing their localization and/or activity[84]. Conversely, PI3K is negatively regulated by the tumour suppressor, phosphatase and tensin homologue deleted on chromosome 10 (PTEN), through the dephosphorylation of PI (3, 4, 5) P3 back to its inactive lipid state[85]. After active PI3K generates PI (3, 4, and 5) P3, the phosphatidylinositol recruits PDK-1 (3′phosphoinositide-dependent kinase 1) and the serine/threonine kinase AKT/PKB (protein kinase B) via binding of their plexin homology (PH) domains to the plasma membrane where they are subsequently phosphorylated and activated[86]. This event is often viewed as the central node of the pathway since the activity of AKT is responsible for pleiotropic effects on molecular functions within the cell, such as cell cycle progression, apoptosis, transcription, and translation. Three AKT isoforms exist (AKT-1, -2, -3), and each has very distinctive roles depending on the specific cell lineage. AKT activation, either by upstream signals or mutation, alters multiple downstream proteins, including the relief of mTOR repression by the tuberous sclerosis complex (TSC1/2) proteins. These two proteins (TSC1/2) form a heterodimeric complex that acts as a functional unit to suppress mTORC1 activity. TSC2 contains a GTPase-activating protein (GAP) domain that stimulates the intrinsic GTPase activity of the small G-protein Rheb, thereby enhancing the conversion of Rheb to its GDP-bound inactive state[87]. While the molecular mechanism is not fully understood, in its GTP-bound form, Rheb is a potent activator of mTORC1. Activation of mTOR results in phosphorylation and activation such as S6K1 and RhoA, leading to enhancement of translation, cell growth and proliferation, and cell survival. Phosphorylation of 4E-BP1 by mTOR leads to inhibition of interaction between 4E-BP1 and elF4E, resulting in activation of translation and increase in cells

proliferation[88] (**Figure 9**). It has recently been discovered that mTORC2 directly phosphorylates and activates AKT, indicating that mTOR has a complicated role both upstream and downstream of AKT[89].

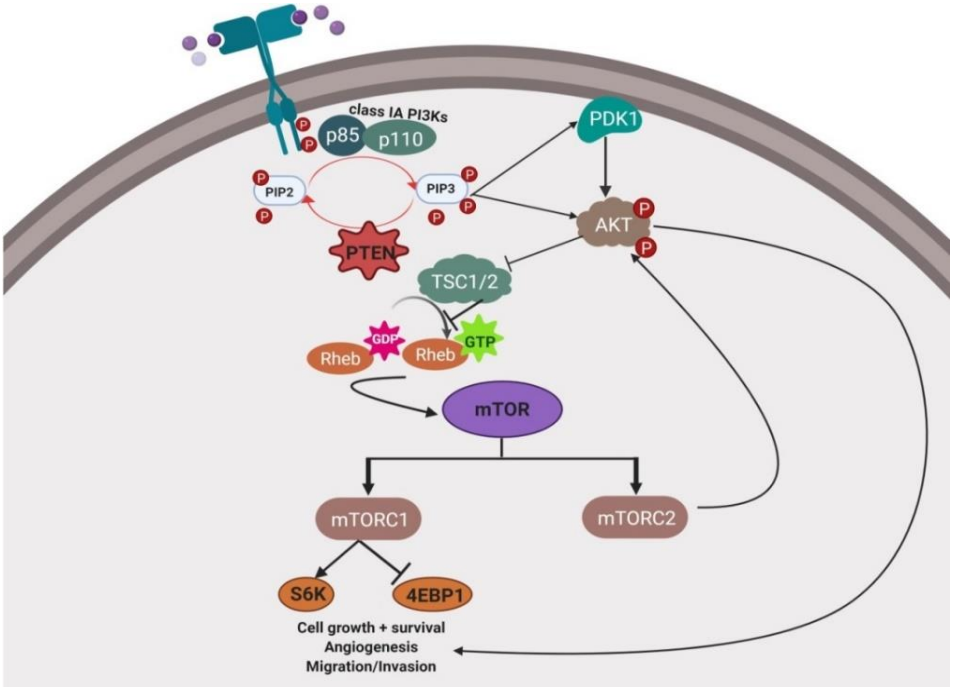


Figure 9: PI3K/AKT/mTOR (PAM) pathway: Elaborately viewed PI3K/AKT/mTOR pathway with their downstream targets, leading to cancer progression and aggressiveness.

1.1.17 MAPK/ERK Pathway

The RAS-RAF-MEK-ERK pathway is far more complex than other pathways from RTKs family. The pathway's general structure includes a small G protein (RAS) and three protein kinases (RAF, MEK, ERK). This pathway's starting point is the binding of the ligand to a transmembrane protein, a receptor tyrosine kinase (RTK). The resulting signalling cascade culminates with ERK (MAPK) translocation to the nucleus, where ERK activates transcription factors that result in gene expression. This pathway plays an important role in the initiation and developments of the HER2+ cancers[90] (**Figure 10**).

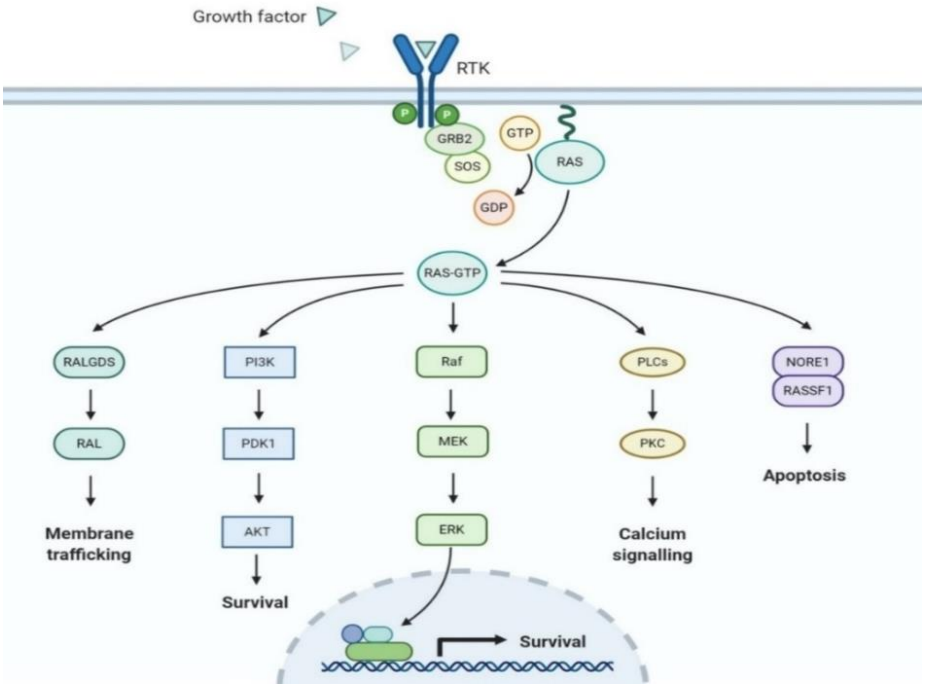


Figure 10: MAPK/ERK pathway: Schematic summary of the multiple MAPK pathways.

1.1.18 Targeted therapies of HER2+ BC

It has been described before that HER2 does not have specific ligands to bind with and activate the downstream pathway. As a result, it dimerizes with HER1, HER3, and HER4. When the HER2 gene is mutated, amplified or overexpressed, it allows multidisciplinary cellular processes such as cell growth, cell survival, cell differentiation. These changes followed signal transduction cascade mediated by PI3K/AKT activation and the RAS/RAF/MEK/MAPK pathways, which leads to drug resistance and tumour progression. As HER2 is overexpressed in HER2 positive BC subtypes in about 25-30% cancer patients, it has become a biological marker for BC's effective treatment. HER2 targeted therapy for this specific type of BC has been widely used in the clinics and has a good outcome[91].

There are two different types of targeted therapies that vary in how they target the cancer cells:

1) Monoclonal antibodies used for BC include trastuzumab, Pertuzumab, trastuzumab deruxtecan, TDM-1, and a new anti-HER2 humanized monoclonal antibody 19H6-Hu[92].

2) Small molecule inhibitors used for BC include Lapatinib, Palbociclib, Neratinib, Afatinib.

1.1.19 Trastuzumab: mechanisms of antitumour effects

Briefly, trastuzumab (Trade name: Herceptin) is a humanized monoclonal antibody directed against the extracellular domain of the HER2 receptor which prevents ligand-independent HER2 signalling. The FDA initially approved it for metastatic BC in 1998[93]. As it was first ever targeted therapy approved, it dramatically changed the treatment of HER2+ BC patients. Trastuzumab (or Herceptin) was developed by Genentech Inc (San Francisco, CA, USA) as a recombinant humanized monoclonal antibody directed against the extracellular domain IV HER2[94]. Clinical studies have shown that the combination of trastuzumab with standard chemotherapy produces far better response rates than chemotherapy alone[95] (**Figure 11**).

The anti-tumour mechanism of trastuzumab may be related to the following aspects:

1) Trastuzumab can recognize the extracellular domain of HER2 and prevents the dimerization of HER2 and its activation[96].
2) Trastuzumab has been proposed to trigger HER2 internalization and degradation by promoting tyrosine kinase activity – ubiquitin ligase c-Cbl[97].
3) One of the effective trastuzumab mechanisms is to attract immune cells (NK cells) to tumour sites that overexpress HER2, by a mechanism called antibody-dependent cellular cytotoxicity (ADCC)[98].
4) The most well-known effect of trastuzumab is the inhibition of the MAPK and PI3K/AKT pathway, which leads to an increase in cell cycle arrest, and the suppression of cell growth and proliferation. It is widely accepted that by interfering with the dimerization of HER2, trastuzumab inhibits HER2 activation and suppresses AKT phosphorylation[99].
5) Trastuzumab can arrest the G1 phase of the cell cycle by restoring p27 and suppressing CDK2 activity and decrease cell proliferation in cancer[100].
6) Some researchers showed that trastuzumab binding to HER2 could block tyrosine kinase Src signalling and increase PTEN level and activity. This also results in the suppression of PI3K/AKT signalling and reduction in cell growth and survival[101].

7) *Molina et al.* demonstrated that trastuzumab could block the shedding of the extracellular domain of HER2 by inhibiting metalloproteinase activity[102].
8) It has been observed that trastuzumab induces normalization and regression of the vasculature by reducing vascular endothelial growth factor (VEGF) production in cancer cells and modulate different regulators of the complex machinery of angiogenesis[103].
9) Trastuzumab can mediate HER2 endocytosis, after that degradation of HER2 will occur in the lysosomes[104].
10) It also can inhibit non-programmed DNA repair of tumour cells[105].

Additionally, trastuzumab works successfully in HER2+ BCs; its efficacy administered as a single agent is 12%-34%. Therefore, trastuzumab is currently used clinically in combination with other chemotherapies such as Taxanes[106], Capecitabine[107], Cisplatin[108], Doxorubicine[109]. The combination of these drugs treatment can significantly reduce the recurrence rate and prolong the survival of patients. The combination uses of tailored, dose-dense adjuvant chemotherapy and trastuzumab (Herceptin) were found to decrease the relative risk of relapse by 32% for patients with HER2+ BC.

Even though trastuzumab has many advantages, 70% of HER2+ patients will undergo resistance to this treatment after one year[95]. Besides, using trastuzumab for a long time may show some side effects such as cardiotoxicity[110] and nephrotoxicity[111].

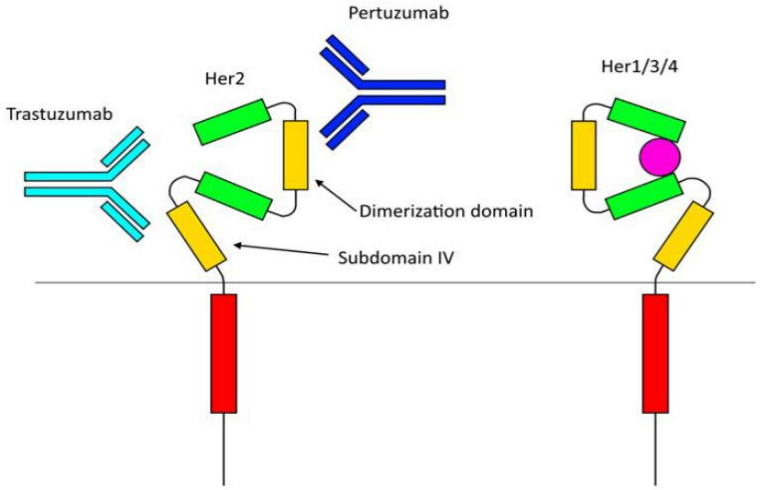


Figure 11: Binding of trastuzumab and Pertuzumab to HER2[78]. Trastuzumab binds HER2 subdomain IV and inhibits activation of HER2, whereas Pertuzumab binds HER2 subdomain II and inhibits activation of HER2 by interacting with HER1/3/4 molecule.

1.1.20 Mechanism of resistance to trastuzumab

Although trastuzumab is used for therapies against HER2 metastatic BC cases, employment of this drug on its own demonstrated to be successful only up to 35% of these cases. However, when combined with first-line chemotherapy, the success of therapy can range from 50%-84%. The disastrous results may be caused by the failure of trastuzumab to block the dimerization when high ligands are present. It is not clear what contributes to trastuzumab resistance; however, some additional mechanisms may influence the final therapy results.

There are different mechanisms involved in trastuzumab resistance:

1.1.20.1 Truncated HER2 (p95 ^{HER2})

Since trastuzumab links to the extracellular domain of the HER2 receptor, it was proposed the possible mechanism of resistance involving $p95^{HER2}$. This molecule acts as a truncated form of the HER2 receptor, which does not carry the external domain; therefore, Trastuzumab does not attach to $p95^{HER2}$ and does not promote its degradation. Moreover, proteolytic cleavage of the ectodomain results in a generation of a phosphorylated tyrosine kinase HER2 $p95^{HER2}$[102,112]. This molecule's activation might trigger mitogenic signalling cascades in the downstream process, influencing the cancer cells for the more aggressive or invasive[113]. In HER2 overexpression BC cell lines $p95^{HER2}$ is phosphorylated by heregulin (HRG) ligand, promoting HER3 transphosphorylation that is not inhibited by trastuzumab, while with lapatinib treatment the transphosphorylation is repressed[114]. Some resistance mechanisms are associated with $p95^{HER2}$ as the expansion of downstream pathways activated from other HER family members such as through IGFR and consequently PI3K activation[115]. The incidence of PI3K over activation can reach 70% of these breast carcinomas, reducing the efficacy of trastuzumab and lapatinib[116]. The heterodimer HER2/HER3 is a PI3K activator; however, the dimer resulting from $p95^{HER2}$ is more operative than the full-length $p185^{HER2}$ and TGFα expression increases in HER2 overexpression BC due to presence of $p95^{HER2}$ [117].

Mucin 4 Epitope masking has also been investigated as a mechanism of resistance to trastuzumab. It is large, highly O-glycosylated membrane-associated glycoprotein which may interfere with trastuzumab binding to the HER2 receptor through surface-epitope masking and decreased the antibody binding capacity[118] (**Figure 12**).

On the other hand, CD44 is a transmembrane receptor for hyaluronan. When CD44 binds with hyaluronan, it activates CD44 and its mediated signal transduction pathways, including RAS and PI3K in ovary cancer cells. CD44 and hyaluronan may also hinder the access of trastuzumab to HER2 receptor by masking its cognate epitope, and lead to treatment resistance[119].

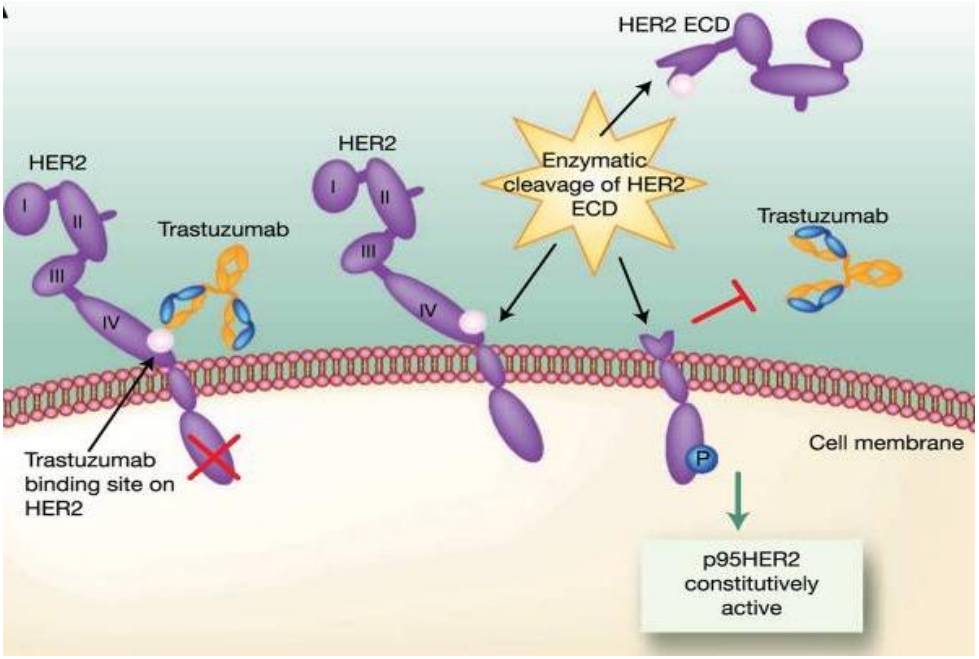


Figure 12: Obstacles for trastuzumab binding to HER2[120]. A constitutively active truncated form of HER2 receptor that has kinase activity but lacks the extracellular domain and the binding site of trastuzumab is originated from metalloprotease-dependent cleavage of the full-length HER2 receptor. Trastuzumab does not bind to $p95^{HER2}$ and therefore, does not affect it.

1.1.20.2 PI3K/AKT pathway & PTEN expression

When HER2/HER3 heterodimerization happens for any other reason the membrane-associated phosphatidylinositol 4, 5 – biphosphate (PtdIns(4,5)P2 or PIP2) is converted into Phosphatidylinositol 3,4,5 – triphosphate (PIP3) to achieve a phosphorylated AKT[84]. The PI3K/AKT pathway, highly activated in HER2 overexpressing cancer cells, stimulates inhibition of cell cycle arrest and/or an anti-apoptotic incident[88]. In contrast, PTEN is a crucial anti-tumour gene on chromosome 10 that is positively correlated with trastuzumab efficacy (**Figure 13**). As this protein is a phosphatase, it has an important role in converting the reactive PIP3 to PIP2, a natural antagonist of PI3K activation[76]. Furthermore, cancer cells expressing small amounts of PTEN exhibit the high activity of PI3K and therefore develop resistance to trastuzumab. The use of PI3K inhibitors is a possible strategy to achieve higher sensitivity to trastuzumab[101]. The molecular

mechanisms of trastuzumab identified comprise the inhibition of PI3Kand AKT pathway, [121]. PTEN, either through mutations or haploinsufficiency, is not expressed in standard levels within half of BC cases[122]. PTEN influences PI3K to promote PI3 and AKT activity production, inducing apoptosis and cell cycle arrest[85].

On the other hand, the tyrosine kinase Src possesses an important role as a pro-oncogenic factor. When associated with HER2, it turns on and inactivates the primary role of PTEN[123]. Therefore when trastuzumab interacts with HER2 in the same domain as Src, PTEN can perform its tumour suppressive functions, contributing to trastuzumab anti-cancer progression[101]. When trastuzumab blocks this oncogene's interaction with HER2, PTEN can contribute against the uncontrolled and high proliferated cancer cells. The lack of PTEN results in resistance to trastuzumab in HER2 overexpressing patients resulting in poor prognosis. Absence or low expression of PTEN is an excellent indicator of trastuzumab resistance[101].

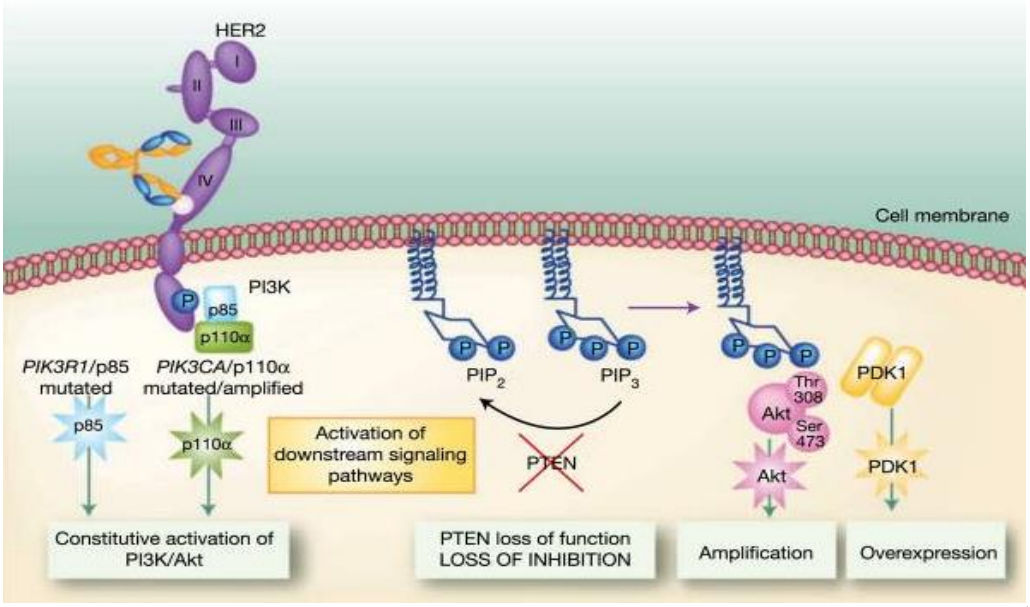


Figure 13: Schematic presentation PI3K/AKT pathways involved in trastuzumab resistance[120]. General mechanisms of resistance to trastuzumab: the presence of upregulation of HER2 downstream signalling pathways. PTEN is a tumour suppressor. Trastuzumab binding stabilizes and activates PTEN and consequently down-regulates the PI3K/Akt signalling pathway.

1.1.20.3 EGFR involved in trastuzumab resistance

EGFR might confer a trastuzumab resistance phenotype when it is expressed in high levels in HER2+ cell lines. Furthermore, this resistance is conducted by either the presence of EGFR/EGFR homodimers, EGFR/HER2 or EGFR/HER3 heterodimers,

which both trigger several downstream pathways, including MAPK and AKT[59]. The levels of HER2/EGFR reduced the trastuzumab therapeutic effects, and it is in part responsible for the trastuzumab resistance acquired by tumour cells[124]. Moreover, expression of HER ligands like EGF, TGF, betacellulin, and heregulin have also been associated with trastuzumab resistance[125,126]. Long periods of exposure to trastuzumab encourage EGFR expression creating resistance to this monoclonal antibody; however, further anti-EGFR agents, such as lapatinib, gefitinib or cetuximab, also increase sensitivity[127].

Moreover, lapatinib demonstrated to be more effective in antagonizing AKT activation. Additionally, two studies have implied that lapatinib's presence enhances HER2 levels while trastuzumab has an opposite effect[128]. Therefore, trastuzumab resistance might be partly explained by EGFR/EGFR homodimerization, which is present at high levels when cell lines were submitted to trastuzumab consecutively.

HER2/EGFR heterodimer levels are affected in different ways by the presence of lapatinib, increasing with low levels of lapatinib, however showing an opposite effect for high concentrations of this drug.

1.1.20.4 IGF-1R involved in trastuzumab resistance

The mechanisms by which IGF-IR promotes trastuzumab resistance remain mostly unknown. IGF-1R, which is not a part of the HER family, is a main PI3K upstream accelerator. However, this receptor might be associated with some of the HER family reactions. Cells that express both HER2 and IGF-1R level high they show good resistance against trastuzumab. IGF-I (ligand of IGF-1R) stimulation of cells with acquired trastuzumab resistance induced phosphorylation of IRS-1, HER2, AKT, and ERK1/2 with reduced expression of p27kip1[129]. Tyrosine kinase inhibition or antibody blockade of IGF-IR blocked phosphorylation of HER2, AKT, and ERK1/2. In MCF7/HER2 and SKBR3/IGF-IR stably transfected cells, IGF-I stimulation blocked trastuzumab-mediated inhibition of AKT and ERK1/2 phosphorylation[130]. On the other side, PI3K signalling, IGF-IR is likely to promote resistance via mTOR activation. Inhibition of mTOR has been effective in restoring sensitivity to trastuzumab in a variety of settings[131]. Downstream events resulting in altered expression and function of cell cycle regulators appear to mediate the ultimate increase in proliferation and cell survival propagated by increased IGF-IR signalling. Stable over-expression of IGF-IR

resulted in reduced cyclin-dependent kinase inhibitors p27kip1 and p21cip1 and increased cyclin E[132].

Similarly, trastuzumab-resistance cells have been reported to show reduced p27kip1[133]and increased cyclin E expression[134]. These results suggest that a potential mechanism by which IGF-IR promotes trastuzumab resistance is via increased ubiquitination and downregulation of p27kip1 protein, resulting in reduced growth arrest and increased proliferation. Reduced p27kip1 expression in resistance cells was associated with increased cdk2 activity and an increased fraction of cells in S phase (proliferation)[133]. Transfection of p27kip1 increased sensitivity to trastuzumab, suggesting that downregulation of this downstream protein is an important mechanism of resistance.

1.1.20.5 HER3 involved in treatment resistance

HER3 is a unique member of the HER family as it has been considered as an inactive receptor. Because of its weak kinase activity, HER3 is utterly dependent on any other family members of HER to activate and initiates its downstream pathway. So, for cancer therapies, HER3 is not a direct target to prevent its expression. There is some evidence that some HER2+ tumours associate HER3 as responsible for trastuzumab resistance. However, trastuzumab does not disturb HER2/HER3 downstream cascades in HER2 amplified BCs[135,136]. Of the four HER receptors, HER3 is best suited to induce activation of the PI3K/AKT pathway, a well-known survival signalling pathway in normal development and tumourigenesis137and one of the critical mechanisms of trastuzumab resistance.

HER2/HER3 heterodimerization activity may be inhibited to inactivate HER2 metabolic Role. However, this requires high concentrations of TKIs that would not be supportable *in vivo*[138]. HER3 plays a critical role in protecting HER2/HER3 dimerization function against TKIs action due to a high range of different mechanisms that can regulate HER3 expression and signalling. More precisely, the increase in HER3 levels is stimulated by the dramatic decrease of AKT signalling[138]. HER3 signalling is stimulated by different mechanisms to increase its expression, translation expansion, which involves the proliferation of the raptor complex of mTOR[139],[140]. Finally, HER2 interacted with both HER3 and IGF-1R to form a heterotrimeric complex in the trastuzumab-resistant BC

cells, creating the HER2/HER3/IGF-1R, not the heterodimer of HER2/HER3 or IGF-1R/HER2, which played a causal role leading to trastuzumab resistance[141].

1.1.20.6 Alternative signalling pathway

The c-Met receptor is frequently co-expressed in HER2 overexpression cell lines; the c-Met receptor may contribute to trastuzumab resistance through sustained AKT activation. HER2-overexpressing BC cells respond to trastuzumab with a rapid upregulation of c-Met receptor expression, and c-Met activation protects cells against trastuzumab. Loss of c-Met function produced through RNA interference improves the response of these cell lines to trastuzumab[103].

For obtained resistance to trastuzumab was related to CXCR4 upregulation and nuclear redistribution. Restriction in CXCR4 expression switched acquired trastuzumab resistance *in vitro*. *In vitro* tests recommend both α6β1 and α6β4 integrins may be involved in de novo and/or acquired resistance to targeted therapy against HER2[142] (**Figure 14**). Overexpression of PDK1 was found in approximately 20% of BCs. A preclinical model reported that the combined use of PDK-inhibitors with trastuzumab reversed the trastuzumab-resistant phenotype of SKBR3 human BC cells[143].

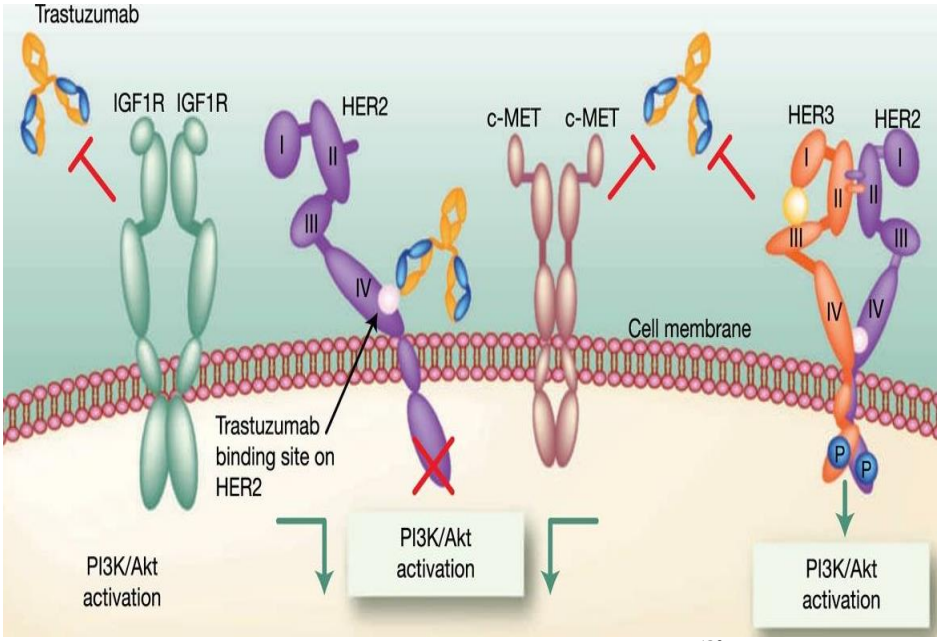


Figure 14: Involvement of other pathways in trastuzumab resistance[120]**.** General mechanisms of resistance to trastuzumab: the presence of signalling through an alternate receptor and/or pathway. Signalling may continue regardless of trastuzumab binding toHER2 when other receptors remain active on the tumour cell.

The rate of inherent resistance to single-agent trastuzumab in HER2-overexpressing metastatic breast carcinomas is impressive above 70%. Systemic treatment for BC, including conventional cytotoxic therapy (paclitaxel, doxorubicin, cyclophosphamide, fluorouracil, cis-platinum), endocrine treatment (tamoxifen, fulvestrant, letrozole, anastrozole), and targeted agents such as trastuzumab, plays an essential role in reducing mortality rate and prolonging survival time in patients with BC. However, resistance to therapeutic agents remains a consistent obstacle in treatment success, while drug resistance's underlying mechanism remains enigmatic.

A growing body of literature supports that EMT is closely linked to BC's progression, including enhanced migratory and invasive capacity, and elevated cancer cells' stemness. Now, emerging evidence suggests that EMT is also involved in treatment resistance in BC. Many signalling pathways which have significant regulating effects on EMT are intimately involved in drug resistance. Finding novel mechanisms of EMT regulation in HER2+ BC subtype would help design new treatments for future generations.

CHAPTER 2

EZH2-MYC loop is a master regulator of EMT process in HER2+ BC and negatively regulated by miR-33b

2.1 INTRODUCTION

HER2 is overexpressed in around 20–30% of BC tumours. It is associated with more aggressive disease, higher recurrence rate, and increased mortality[63,144]. Due to its high aggressiveness characteristics, high mortality rate, and unreasonable recurrence rate, this specific subtype of BC is a vulnerable subtype to study among researchers and scientists nowadays. HER2+ BC is a clinically and biologically heterogeneous disease. There are enormous genes, and miRs are involved in making this cancer subtype more aggressive and proliferative. Simultaneously, many genes and miRs acted as a tumour suppressive and became the patients' survivor. Therefore, it is essential to deeply explore the molecular mechanisms responsible for disease progression and therapy resistance to identify possible biomarkers guiding novel treatments.

MiRNAs are a family of evolutionarily conserved small, endogenous, single-stranded and non-protein-coding RNAs spanning 19 to 25 nucleotides in length[145]. The first miR, lin-4, was discovered in 1993 as a small RNA transcribed from the lin-4 locus of *Caenorhabditis elegans*[146]. In 2000 the let-7 gene and let-7 RNA were detected in humans, Drosophila, and other bilateral animals[147]. While the number of human miR candidates continuously increases, only a few are entirely characterized and experimentally validated. In 2019, researchers showed that a total of 2300 real human mature miRs are present in the human genome and from which 1115 are currently annotated in miRBase V22[148] (http://www.mirbase.org/). MiRs function as the vital post-transcriptional regulators of gene expression in different tissues and developmental stages via precise interactions and complex regulatory networks[149]. MiRs play a crucial role in regulating numerous metabolic and cellular pathways, notably those controlling cell proliferation, differentiation and survival[150,151]. Dysregulation of miR expression profiles has been demonstrated in most tumours examined. However, the specific classification of miR as oncogenes or tumour suppressors can be difficult because of miRs' intricate expression patterns. MiRs expression patterns differ for particular tissues, and differentiation states [152,153]. It is not always clear if altered miR patterns are the direct cause of cancer or an indirect effect of cellular phenotypes changes. Additionally, a single miR can regulate multiple targets and helps in cancer progression or cancer suppression[154].

2.1.1 MicroRNAs in BC

Different studies of the BC cell lines showed different miR expression, having distinct characteristics such as tumour suppressive or tumour initiative. Let-7b miR has been associated with high DNA repair capacity (DRC) levels in women with BC[155]. Several studies have been conducted to identify the miRs those are differentially expressed and regulate BC initiation and progression in different BC subtypes. Oncogenic miRs such as miR-10b-5p, miR-21, miR-23/27/24 cluster, miR-155, miR-125b, miR-181a/b cluster, miR-221/222 cluster are frequently upregulated in BC, and they act by repressing the expression of tumour suppressor gene/s mainly involved in apoptosis, cell proliferation, cell migration and invasion and metastasis[156,157]. Besides, numerous other miRs appear to be involved in the suppression of metastasis and invasion of BC *in vitro* and *in vivo*; these include the miRs like miR-33b, let-7, miR-200 family, miR-26a, miR-30b[158], miR-449 family[159] miR-497[160], miR-421[161], miR-193a[162], miR-211-5p[163], miR-335, miR-133a[164], and miR-124[165], which are proposed to suppress the expression of EZH2, EMT markers, cell cycle markers along with SMAD7[160], MTA1[161], WT1[162], SETBP1[163], EphA4, LASP1[164] and STAT3[165], respectively.

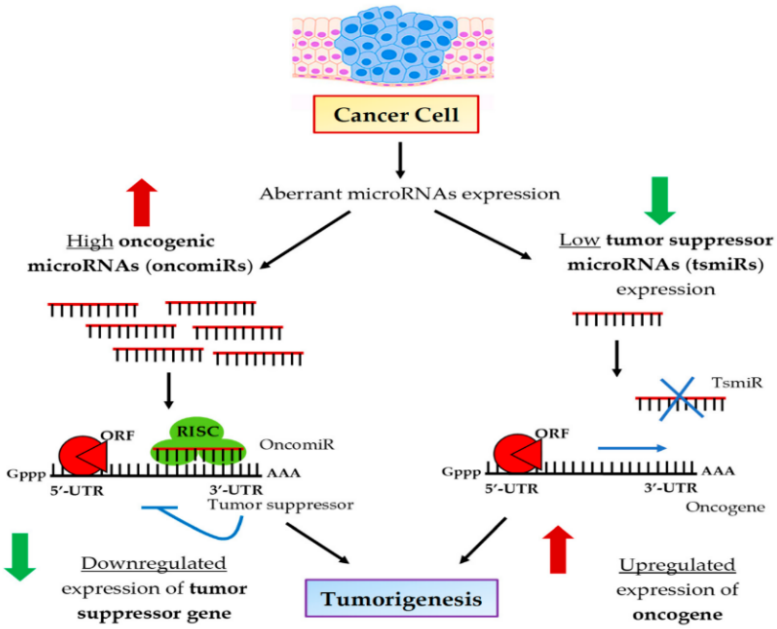


Figure 15: Regulatory mechanisms of oncogenic and tumour suppressor microRNAs in tumourigenic events.

This thesis has manifested a tumour suppressor miR named miR-33b belongs to a miR-33 family in HER2+ BC and found its molecular mechanism for regulating EMT through MYC-EZH2 loop.

2.1.2 MiR-33b in Cancer

MiR-33 is a family of miR precursors processed by the Dicer enzyme to give mature miRNAs and present within the intronic sequences of the SREBP genes in organisms ranging from Drosophila to humans. MiR-33 is found in several animal species, including humans[166]. The miR-33 family, which consists of miR-33a and miR-33b, is one of the most well-characterized miRs in disease world[167]. The hSREBF1 gene on chromosome 17 harbours miR-33b in intron 17, whereas hSREBF2 on chromosome 22 contains miR-33a in intron 16. Mature miR-33a and miR-33b only differ in 2 nucleotides and are predicted to have largely overlapping target gene sets[168]. miR-33 plays a role in lipid metabolism; it downregulates several ABC transporters, including ABCA1 and ABCG1, which regulate cholesterol and HDL generation.

Further related roles of miR-33 have been proposed in fatty acid degradation and macrophage response to low-density lipoprotein. It has been suggested that miR-33a and miR-33b regulate genes involved in fatty acid metabolism and insulin signalling[169]. For the first time, miR-33b, deletion, amplification or a mutation at the precursor miRNA was detected in 10% of medulloblastomas when miRNA-expression profiling was screened in 48 medulloblastomas in 2011[170]. This study showed that MYC mRNA has a potential target sequence within the 3′-untranslated regions for miR-33b binding. Additionally, they also showed that a point mutation in the miR-33b gene. The mutation was located in the sequence encoding precursor miR, indicating that the mutation is somatic, associated with MYC's overexpression in medulloblastomas[170]. In 2014, researchers showed that MYC was negatively regulated by miR-33b at the post-transcriptional level, via a specific target site within the 3′UTR. Overexpression of MYC impaired miR-33b-induced inhibition of proliferation and invasion in osteosarcoma cells. MYC's expression was frequently downregulated in osteosarcoma tumours and cell lines and was inversely correlated with miR-33b expression[171]. In 2015, it was observed that miR-33b is downregulated in lungs metastases[172]. Following, miR-33b acts as a tumour suppressor in different types of cancer such as melanoma cancer, lung cancer, colorectal cancer, gastric cancer, oesophageal squamous cell

carcinoma, nasopharyngeal carcinoma, gall bladder cancer and triple-negative BC. However, the role and the action mechanism of the miR-33b in HER2+ BC are still unclear. This thesis elaborates the molecular mechanism of miR-33b in HER2+ BC and how it regulates EMT pathway through EZH2-MYC loop.

2.1.3 The Role of EZH2 in BC Progression and Metastasis

Overexpression of the EZH2 in a wide range of malignancies has been established in cancer research. EZH2 was first associated with aggressiveness and metastatic characteristics of prostate cancer by analysing gene expression in human tumour microarrays. Through similar microarray profiling and other studies, EZH2 expression strongly correlates with BC aggressiveness acting as an independent predictor of recurrence and survival. EZH2 was also found to increase histologically normal breast epithelium with a higher risk of developing cancer, indicating that EZH2 may prove a valuable marker for detecting preneoplastic lesions. Elevated EZH2 expression has since been described in other types of cancers: bladder [173], liver[174], colon[175], lung[176], and many more. In all reported cancer studies, the common discovery is that EZH2 expression is increased in cancer compared to normal tissues, being the highest in the most advanced stages of the disease, and correlates with poor prognosis in patients.

Cao and colleagues have found in prostate cancer cells that EZH2 promotes EMT by repressing E-cadherin expression through interaction with Snail1. This influence on E-cadherin has since been demonstrated in many other types of cancer cells[177]. Ren and colleagues have reported that EZH2 directly represses the metastasis suppressor RKIP in breast and prostate cancer cells leading to increased invasion through interaction with Snail1[178]. Likewise, in hepatocarcinoma cells, EZH2 has been found to epigenetically repress several miRs characterized as tumour suppressors for their anti-tumour or anti-metastatic established roles[179]. Also, EZH2 has been implicated in promoting tumour angiogenesis, and ovarian cancer growth *in vivo* as VEGF-stimulated overexpression of EZH2 leads to the repression of VASH1, a negative regulator of angiogenesis[180]. Taken together, these studies confirm the essential roles EZH2 plays in tumour progression and suggest that blocking EZH2 expression or activity may have therapeutic implications. Although EZH2 primarily known as a gene silencer, this evidence has emerged indicating its activation in cancers. In genome-wide mapping ChIP experiments, 10-20% of polycomb-group (PcG) target genes were found actively

transcribed in embryonic stem cells, and RNA Polymerase II[199] also 2 % of genes bound by PcG proteins. Indeed, EZH2 has been demonstrated to be required in the expression of several genes essential in cell cycle regulation providing a proliferative advantage. Another study utilizing glioma CSCs and ChIP experiments also revealed that MYC is a positively regulated direct target of EZH2 as MYC expression was actively repressed upon EZH2 downregulation[181].

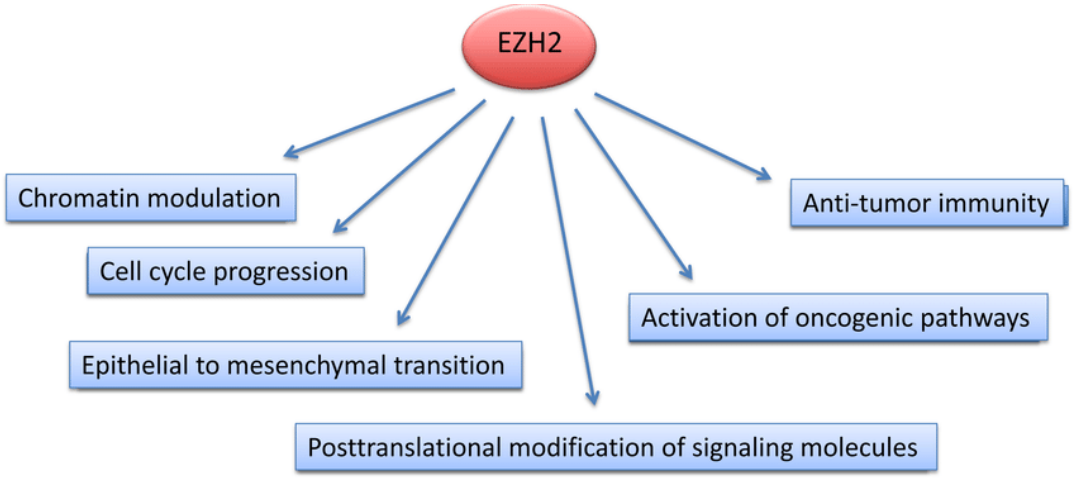


Figure 16: Mechanisms of EZH2-mediated implications in cancer

Moreover, EZH2 has been indicated to activate the different mechanisms such as cell cycle progression, anti-tumour immunity, EMT, and other oncogenic pathways in a different type of cancer[182] (**Figure 16**). This chapter explains how EZH2 plays an essential role in HER2+ BC progression through EMT and miR-33b act on this process negatively to repress EMT and cell proliferation.

2.2 Materials and Methods

2.2.1 Cell culture and reagents

Human BC cell lines BT474, SKBR3, MDA-MB-468, MCF7 and MCF-12A, MCF-10A non-tumourigenic human mammary epithelial cells were maintained in Dulbecco's modified Eagles medium (DMEM) (GIBCO) supplemented with 10% fetal bovine serum (FBS; Gibco), 10,000U/mL penicillin, 10,000 µg/ml streptomycin and 1% L-glutamine (200mM) (100x). All cells were cultured at 37 °C in 5% CO_2 atmosphere.

Cell lines	Properties	Subtype
BT474	Adherent epithelial-like cells, collected from invasive ductal carcinoma of the breast tumour tissue.	Luminal B/HER2+
SKBR3	Adherent epithelial cells. It is derived from the metastatic site.	HER2+
MCF7	Adherent epithelial cells. It is derived from the metastatic site.	Luminal A
MCF-10A	Adherent non-tumorigenic epithelial cell line.	Normal
MCF-12A	MCF-12A cell line is a non-tumorigenic epithelial cell line established from tissue taken at reduction mammoplasty from a nulliparous patient with fibrocystic breast disease.	Normal
MDA-MB-468	Adherent epithelial cells. It is derived from the metastatic site	Triple negative A

2.2.2 Transfection

Cell lines were transfected either with 100 nM hsa-miR-33b-5p mirVana mimic (assay ID MC12289, Ambion) or 100 nM inhibitor miRNAs (RNA oligonucleotides) (assay ID MH12289, Ambion) and 100 nM EZH2 siRNA (#s4916, #s4918, Thermofisher), as well as a negative control for the experiments. In-vitro transfections of the oligonucleotides were performed using Lipofectamine 2000 (Invitrogen; Thermo Fisher Scientific, Inc., Waltham, MA, USA) by the manufacturer's instructions. After six h of transfection, the

transfection media was replaced with complete medium. All the experiments were carried out at 48 h, and 72 h post-transfection.

2.2.3 RNA extraction and quantitative real-time RT-qPCR

To detect the expression of miRNA and mRNA, total RNA was extracted using TRIZOL reagent (Invitrogen, Carlsbad, CA) according to the manufacturer's instructions. cDNA was synthesized from 1µg of total RNA using a High-Capacity cDNA Reverse Transcription kit (Applied Biosystems) and a TaqMan® miRNA Reverse Transcription kit (Applied Biosystems, United States). RT-qRT was performed with a TaqMan® Universal Master Mix (Applied Biosystems) and TaqMan® 20× assay (Applied Biosystems) following the manufacturer's protocol on a quant-studio 3 and 5 real-time RT-qPCR system (Applied Biosystem, United States). Expression data were uniformly normalized to internal control. For miRNA expression, the endogenous control was RNU43, and for the gene expression, the endogenous control was GAPDH, and relative gene and mi-RNA expression was quantified using the $2^{-\Delta\Delta Ct}$ method.

2.2.4 Cell invasion and migration assays

For the migration assay, 5×10^4 cells (72 h post transfected) were seeded in 200 µl of the serum-free medium into the upper chamber of each insert (353097, Corning®) and 700 µl of medium supplemented with 10% FBS was added into the lower chamber. For the cell invasion assay, the polyester membranes of the upper surface of the insert (353097, Sigma) were pre-coated with a matrix gel (Corning® Matrigel® Basement Membrane Matrix, Ref: 356234) then incubated at 37°C. After 24 h, cells that invaded and migrated through the membrane were fixed and permeabilized with 70% chilled ethanol for two min and 100% methanol for 15 min respectively at room temperature. The invaded and migrated cells were further stained with 0.4% crystal violet for 10 min at room temperature. Cells were then imaged and counted from photographs of 5 randomly selected fields of the fixed cells.

2.2.5 Wound-healing assay

To check the motility capacities of the cells after miR-33b-5p transfection, the wound healing assay was performed. After 72 h of transfection, the cells were seeded in six-well plates to obtain 100% confluence in 24 hr. After 24 h wound was induced by

scratching the monolayer with a micropipette tip, and the dish was placed at 37 °C in a 5% CO_2 incubator chamber. Pictures were acquired at 0 h and after 24 h using a microscope.

2.2.6 WST-1 cell proliferation

After transfection, cell proliferation was assessed using the WST assay. 3×10^3 transfected cells and negative control cells were seeded in 96 well plates from 1 day to 7 days. In each mentioned day, cell proliferation was measured using WST-reagent (ab155902, Abcam). Seven per cent of the WST reagent was added to each well with phenol red-free media. The plate was incubated for 4 h at 37 °C. Following the absorbance was measured at 450 nm in a microplate reader with background correction at 650 nm. The significance of any differences was assessed using T-test.

2.2.7 Cell cycle analysis

To analyze cell cycle, 5×10^4 cells were seeded in six-well plates for each condition in triplicates. After 48 h of transfection, the cells were harvested by trypsin and washed with 1X PBS twice. The harvested cells were then fixed with chilled 70% ethanol and incubated at -20°C for 6-7 h. The cells were then centrifuged soon after washing with 1X PBS twice. The pellets were resuspended with propidium iodide staining buffer (PI/RNase, IMMUNOSTEP) stored at 4°C for the overnight. Stained cells were acquired for cell cycle analysis by flow cytometry using a FACSVerse™ flow cytometer (BD Bioscience, USA) and raw data was analyzed by FlowJo software.

2.2.8 Apoptosis analysis

According to the manufacture recommendation, apoptotic cells were determined by double staining using FITC Annexin V Apoptosis Detection Kit with PI (ANXVKF-100T, IMMUNOSTEP). In late apoptotic and necrotic cells, the plasma and nuclear membranes' integrity decreases, allowing PI to pass through the membranes, intercalate into nucleic acids, and display red fluorescence. During this process, the cell membrane's asymmetry distribution is disrupted, and phosphatidylserine becomes exposed on the plasma membrane's outside surface. Because the anticoagulant protein Annexin V binds with high affinity to phosphatidylserine, and detect apoptotic cells by flow cytometry. Briefly, 1×10^5 cells were seeded in a six-well plate. After 72 h post-

transfection, the supernatant media was taken in one tube. The attached cells were harvested by trypsinizing and collected into the same tube. The cells were washed with 1X PBS twice, and the pellet was resuspended with 1X Annexin binding buffer. Five µl of the Annexin V-FITC and five µl of PI were added to the resuspended cells and incubated for 15 min at room temperature in the dark. Further 400 µl of 1X binding buffer was added with DAPI (0.1mg/ml, 1-2 µl). The stained cells were acquired for cell cycle analysis by flow cytometry using a FACSVerse™ flow cytometer (BD Bioscience, USA) and raw data was analysed by FlowJo software.

2.2.9 Western blot analysis

At the indicated time (72 h), the whole lysate of transfected cells was extracted using Thermo Scientific™ RIPA Lysis buffer (Ref: 89900). The lysates were transferred to a clean microfuge tube, placed on ice for 30 min and centrifuged for 30 min at 13,000 rpm. The supernatant was transferred to a fresh microfuge tube, and the protein concentration was determined using a BCA protein assay kit (Pierce™ BCA Protein Assay Kit, Ref: 23227). Protein lysates were separated on 10% SDS PAGE and transferred to nitrocellulose membranes (Ref: 1620115, Bio-Rad). The membranes were blocked in 5% BSA for 1 h and then incubated with antibodies of E-cadherin (BD Biosciences, #610181), β-Catenin (BD Biosciences, #610153), Vimentin (BD Biosciences, # 550513), EZH2 (Cell signalling, # 1674905S) and GAPDH (Thermo Scientific™, #MA5-15738) overnight at 4°C. The following day, membranes were washed and subsequently incubated with the appropriate HRP conjugated secondary antibodies for 1 h at room temperature. Following this incubation, the membranes were washed and briefly incubated with a Pierce™ ECL western blotting substrate reagent (Thermo Scientific™, Ref: 32106). All images were analysed as TIFF files with ImageJ for windows to build the figures. Graphs of signal intensity were obtained through band densitometry using Image J program.

2.2.10 Luciferase Assay methods

Luciferase reporter plasmid (pEZX-EZH2-3`UTR) and luciferase control plasmid were purchased from Tebu-bio (Portugal). According to the manufacturer, transient co-transfection of miR 33b mimics and luciferase plasmids was performed using Lipofectamine® 2000 (Invitrogen; Thermo Fisher Scientific, Inc.) ’s instructions in

HEK-293T cell line because of its transfection efficiency. Cells were seeded on 12 well plates (8×10^5) and were co-transfected with 1µg of Luciferase reporter plasmid with 100 nM miR-33b mimic or miR negative control (NC), according to the manufacturer's instructions. After 48 h, the luciferase activity was measured using the Luc-Pair Duo-Luciferase Assay Kits 2.0 (Tebu-Bio, Portugal) according to the manufacturer's instructions. Firefly luciferase activity was normalized to the corresponding Renilla luciferase activity.

2.2.11 Clinical samples and RNA isolations

Human BC tissues formalin-fixed, paraffin-embedded samples from different subtypes of BC patients and breast samples from healthy donors were selected to analyse the expression of miR-33b and EZH2 gene. The total RNA was isolated from tissue blocks using the Recover All Total Nucleic Acid Kit (Ambion) for standard mRNA/miRNA analysis. One µg of total RNA was retro-transcribed with random primers (for gene expression) and specific primers (for miRNA expression) using Reverse Transcription Kit (Applied Biosystems), and 5 ng of cDNA was used for quantitative RT-qPCR for both gene and miRNA expression analysis. The quantitative RT-qPCR analysis was performed, as mentioned above.

2.2.12 TCGA (The Cancer Genome Atlas) data analysis

The expression data for miRNA-33b was obtained from Xena browser database, (https://xenabrowser.net/) for TCGA BC (BRCA), which contained_cases of different BC subtypes solid tumours and normal. From there we were able to obtain 211 numbers of the specimen with clinical details including Luminal B (n= 49), Basal-like (n= 26), Luminal A (n= 92), HER2+ (n= 18) and healthy solid tissue (n= 26). For EZH2 expression we use the same database, which contained 1248 cases of different BC subtypes solid tumours and normal, wherefrom we obtained only 522 numbers of the specimen with clinical details including Luminal B (n= 127), Basal-like (n= 98), Luminal A (n= 231), HER2+ (n= 58) and normal solid tissue (n=8). The statistical analysis was done using the Shapiro-Wilk normality test and based on normality test results; the parametric and nonparametric test was applied to obtain a p-value of the analysis.

2.2.13 *In silico* survival analysis

Overall Survival associated with miRNA and gene expression was analysed using Kaplan-Meier plotter (KM plotter ©) tool (http://kmplot.com/analysis/). This tool works upon a database containing different BC Affymetrix microarray samples and associated survival information, with a median follow-up of 120 months. Based on the METABRIC dataset, by specifying the miRNA name and gene name on the search tool, and filtering down to "All BC subtypes and HER2+ subtype", the survival rates according to miRNA gene expression were obtained. The hazard ratio (HR) with 95% confidence intervals and log-rank p-value was calculated and showed. The obtained results were used to identify the prognostic value of miR-33b and EZH2 expressions on HER2+ BC.

2.2.14 Immunohistochemical (IHC) staining

Either breast core biopsy or surgery samples of BC patients were obtained from Hospital Clinico, Valencia. Immunohistochemistry (IHC) staining of specimens was carried out on formalin-fixed paraffin-embedded tissues using the polyclonal rabbit HER2 antibody (A0485; DAKO) at a dilution of 1:400, and a peroxidase-conjugated detection system (DAKO). Development was performed with diaminobenzidine, using hematoxylin counterstaining. HER2 IHC staining was scored as (0), weak positive (1+), moderate positive (2+) and strong positive (3+) based on the percentage of cells stained as positive and staining intensity following the standard of DAKO Hercept Test TM.

2.2.15 Immunocytochemistry (ICC) staining

The cells were fixed for 10 min at room temperature in 100% acetone and 50% methanol in PBS for the immunofluorescent labelling. After permeabilization in a 0.5% Triton X-100 solution and washing in 1x TBS for 3 times, the cells were incubated in the DAKO REAL Peroxidase-Blocking Solution (S202386-2) for 5-10 mins, subsequently, take out the blocking solutions and wash 3 times with 1x TBS. After several washes, the cells were incubated with specifically required antibodies for 1 hr in RT. After primary antibody incubation, cells were washed with 1x TBS for 3 times. The cells were incubated with Envision Flex Substrate Buffer (DAKO) for 1-2 mins in RT followed with 3 time washing with 1x TBS. Following DAKO EnVision FLEX

Hematoxylin buffer is used for counterstaining for 5 mins. The cells were hydrated and observed under the microscope.

2.2.16 Statistical analysis

The sample and control groups were compared using a two-tailed Student t-test. All data presented include the median and standard deviation (SD). P-values of less than 0.05 were considered to be statistically significant.

2.2.17 Ethics approval

The study was conducted under-recognized ethical guidelines (Declaration of Helsinki), and it was approved by the INCLIVA institutional review board (protocol number: 2018/077). All the participants in the study signed written informed consent.

2.3 Results

2.3.1 Characterization of HER2 in patients and cell lines

We examined the HER2 expression levels in the BT474, SKBR3 and MCF10a by immunocytochemistry staining method. BT474 and SKBR3 have HER2 amplification, which is considered HER2+ cell lines compared to MCF10a; an immortalized mammary epithelial cell line (**Figure 17 A-C**). Next, we examined the tissue samples, and those are obtained from the hospital clinic de Valencia, which showed a different pattern of HER2 expression such as moderate expression (2+) and with strong positive (3+), (**Figure 17 D-F**). This observation of the different pattern of expression of HER2 in cancer tissues showed a wide range of tumour differentiation from moderate to poor. Altogether, these data suggested that the cell lines and cancer tissue specimens are used for this study, are having high expression of HER2 and validated for this study and thesis.

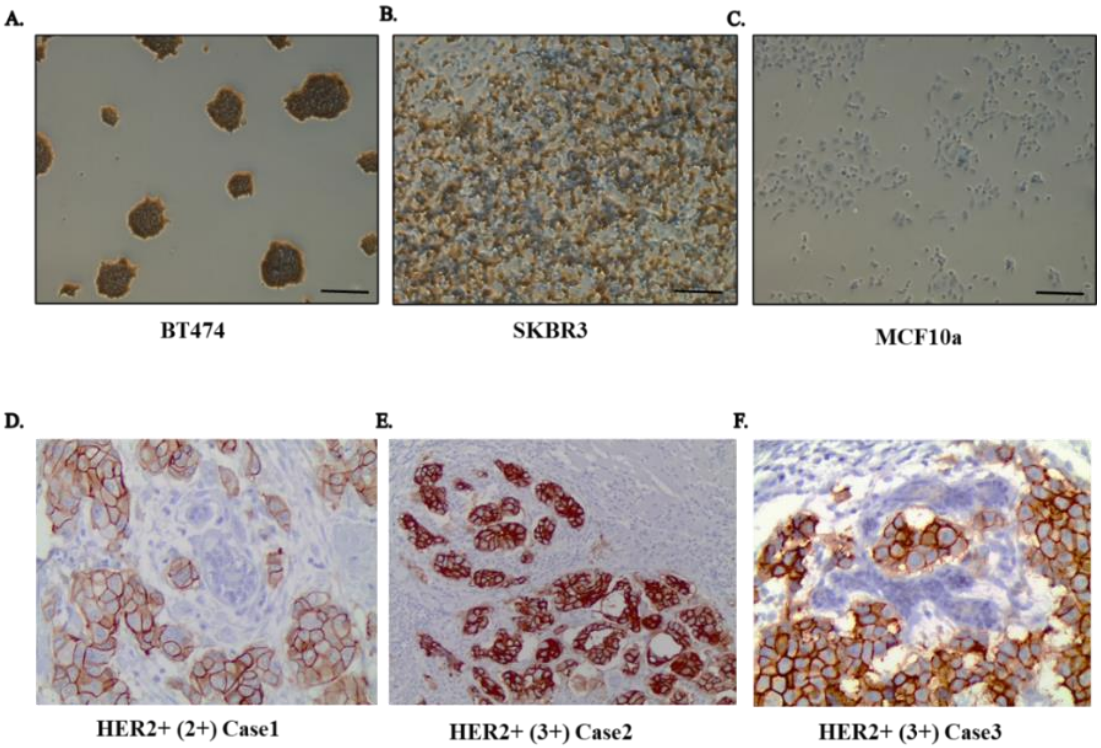


Figure 17: Immunocytochemical and immunohistochemical staining of HER2 in BC cell lines and primary tumours. (A-C) Expression levels of HER2 in BT474, SKBR3, and MCF10a are shown. BC tissues with HER2 IHC scores of 2+ (D), 3+ (E–F) were subjected to HER2 IHC assay.

2.3.2 EZH2 expressions in BC patients

EZH2 is aberrantly overexpressed in various malignant tumours, such as prostate cancer, colorectal cancer and ovarian cancer. Less has been explored in BC. So, EZH2 expression was determined in HER2+ and TNBC BC patient's primary tumour samples and breasts normal tissues collected from the Department of Oncology, Hospital Clinico de Valencia. In 26 HER2+ primary tumours, 24 TNBC primary tumour and 7 normal tissues were collected to obtain mRNA for *EZH2* expression analysis. Initially, RT-qPCR was performed to analyse the level of *EZH2* expression in BC tissues vs healthy tissues. The *EZH2* expression was significantly higher in HER2+, and triple-negative BC (TNBC) tissues samples vs healthy breast tissues, (**Figure 18 A-B**). These data collectively seemed to suggest that up-regulated *EZH2* may be implicated with aggressiveness and progression of HER2+ and TNBC subtype of BCs.

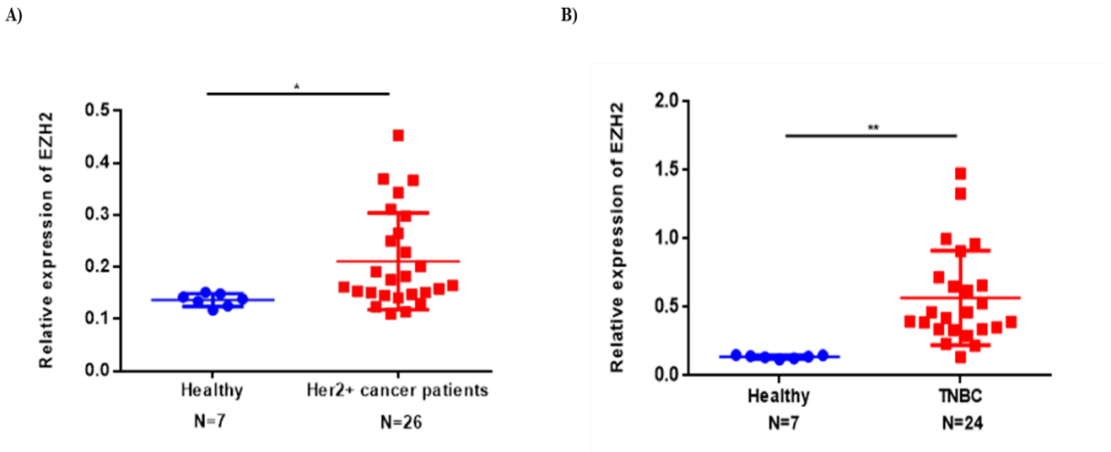


Figure 18: Expression of the *EZH2* in BC patients and normal tissues: The relative expression of *EZH2* was determined by RT-qPCR in HER2+ cancer tissue and non-tumorigenic tissue (A). *EZH2* gene expression was determined by RT-qPCR in TNBC tissue and non-tumorigenic tissue (B). Student's t-test was used to analyse the significant differences. ***P ≤ 0.05, **P ≤ 0.01.**

2.3.3 EZH2 expression levels in BC tumour or normal-like tissues from TCGA dataset

To further support our data on the expression of *EZH2* in different subtypes of BC tumours, we investigated the expression of *EZH2* in BC tissues from the TCGA database. The database contained 1248 cases of different BC subtypes. We obtained 522 numbers of specimen details; others had missing *EZH2* expression data, no follow-up data, or missing clinical information. Among them, there were Luminal B (n= 127), Basal-like (n= 98), Luminal A (n= 231), HER2+ (n= 58) and normal-like (n=8), (**Figure 19**). These samples mentioned above from the TCGA database were classified into five main intrinsic molecular subtypes of BCs. Therefore, while checking the expression of *EZH2* in those subtypes, we observed the high expression of *EZH2* in the basal-like subtype, a more aggressive subtype of BC, following *HER2* enriched and Luminal-B subtypes. *EZH2* expression is similar in Luminal-A and normal like tissues as compared to other subtypes. Jointly, these results suggested that *EZH2* expression positively corresponds to BC's high aggressiveness with poor prognosis contrasted to BC's good prognosis.

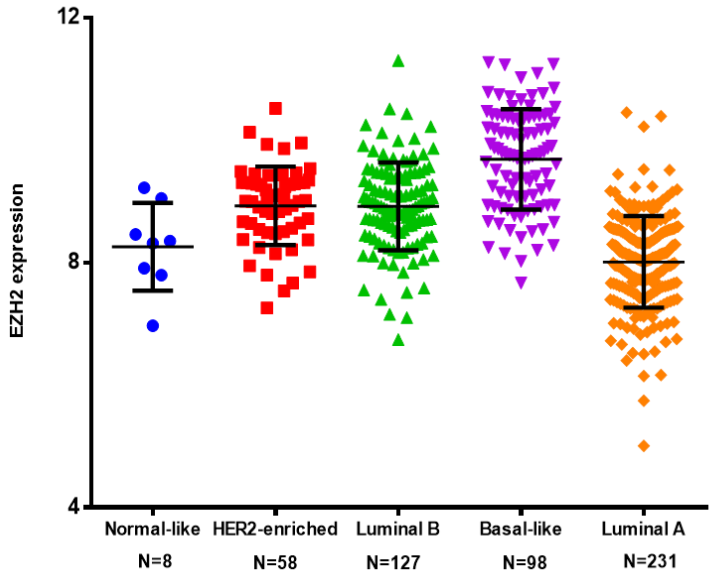


Figure 19: EZH2 expression is indicated in BC tumour or normal tissues from TCGA dataset: RT-qPCR analysis indicated the EZH2 expression levels in five main intrinsic or molecular subtypes of BCs tissues TCGA dataset.

2.3.4 EZH2 is overexpressed in the different subtype of cancer cell lines

To confirm EZH2 expression is high in BC, we analysed distinct subtype of cancer tissues and healthy breast tissues, as mentioned in result section **2.3.2**. This result section analysed EZH2 expression in different BC cell lines and immortalized normal human mammary epithelial cell lines. EZH2 expression was determined in four human BC cell lines including MDA-MB-468, MCF-7, BT474 and SKBR3 (HER2+) and the non-tumourigenic epithelial cell lines MCF12a and MCF10a as controls. Quantitative RT-qPCR data revealed that the EZH2 expression was significantly lower in the MCF10a than HER2+ BC cell lines, (**Figure 20A**) and protein level (**Figure 20B**). Additionally, the analysis showed that EZH2 is highly expressed in a triple-negative cancer cell line (MDA-MB-468) compared to luminal A (MCF 7) due to its high aggressiveness character. MCF10a and MCF12a normal cell lines were taken as a negative control for EZH2 expression (**Figure 20C**). The observation showed that EZH2 expression in BC cell lines was higher than normal mammary epithelial cell lines, which indicates that EZH2 may perform an essential role in the development of BC.

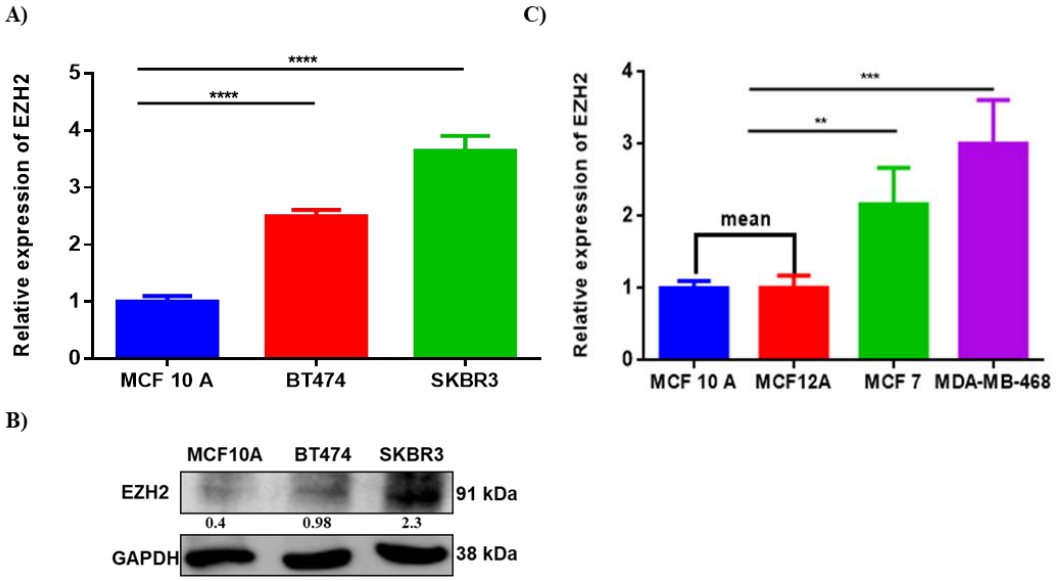


Figure 20: Expression of the EZH2 in cancer cell lines: The relative expression of the EZH2 was determined in HER2+ cancer cell lines and non-tumourigenic epithelial cell line (A). EZH2 protein expression was determined by western blot in mentioned cell lines (B). (C) EZH2 expression was determined in other subtypes of BC cell lines and non-tumourigenic epithelial cell lines by RT-qPCR and normalized to GAPDH. Student's t-test was used to analyse the significant differences. ****P ≤ 0.01, ***P ≤ 0.001, **** P ≤ 0.0001.** The quantification of the western bands is accomplished with ImageJ.

2.3.5 Knockdown of EZH2 gene using small RNA interference (siRNAs) molecules in two HER2+ BC cell lines

The above results showed the high expression of EZH2 might be involved in the development and progression of human HER2+ BC patients and *in vitro*. To explore the molecular mechanisms, it is crucial to know the gene's implications on cell proliferation and other phenotypic properties of human HER2+ cell lines. For this purpose, one of the strategies employed in this study was the silencing of siRNAs' EZH2 expression. In a previous result section, we demonstrated that EZH2 expression is upregulated in HER2+ BC tissues and two HER2+ BC cell lines BT474 and SKBR3 (HER2+), so they were selected further *in vitro* experiments. After transfection, the efficiency of silencing was checked by RT-qPCR, (**Figure 21 A, C**) and in protein level, (**Figure 21 B, D**). The results observed in the figure were obtained with two specific commercial siRNAs, named siEZH2 (si1) and (si2). Unitedly, these results suggested that the commercial silencers have been effectively silenced the mRNA and proteins of EZH2 in HER2+ BC cell lines so that it is sufficient to check the functional effect on cells.

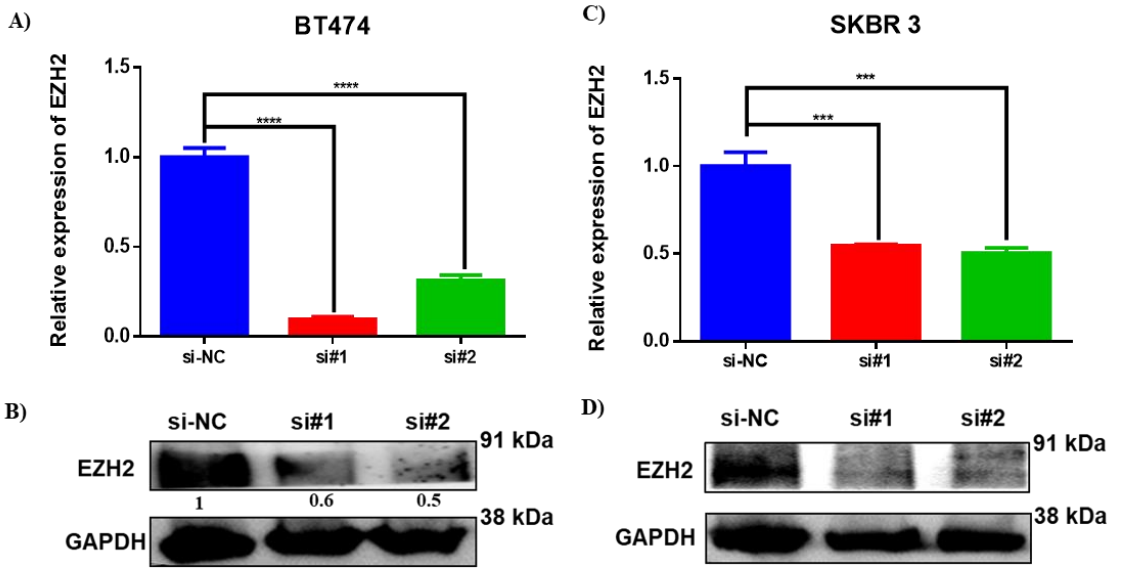


Figure 21: EZH2 expression after siRNAs transfection in HER2+ cell lines: RT-qPCR (A-C) and Western blot (B-D) results showing gene silencing efficiency siRNA sequences targeting EZH2 using two silencers. Student's t-test was used to analyse the significant differences**. ***P ≤ 0.001, **** P ≤ 0.0001**. The quantification of the western bands is accomplished with ImageJ.

2.3.6 Knockdown of EZH2 gene inhibits cell proliferation in two HER2+ BC cell lines

To evaluate the role of EZH2 in HER2+ BC cell lines on proliferation, BT474 and SKBR3 cells were transfected with two different siRNAs. RT-qPCR performed for confirmation of gene, and protein silencing in respective cell lines (shown above). WST assay showed that lower expression of EZH2 significantly decreased the cell proliferation in BT474 and SKBR3, and this inhibitory effect showed statistical significance until seven days, (**Figure 22 A-D**). These results indicated that EZH2 might act as a crucial gene for promoting tumour growth in HER2+ BC through modulating cell proliferation.

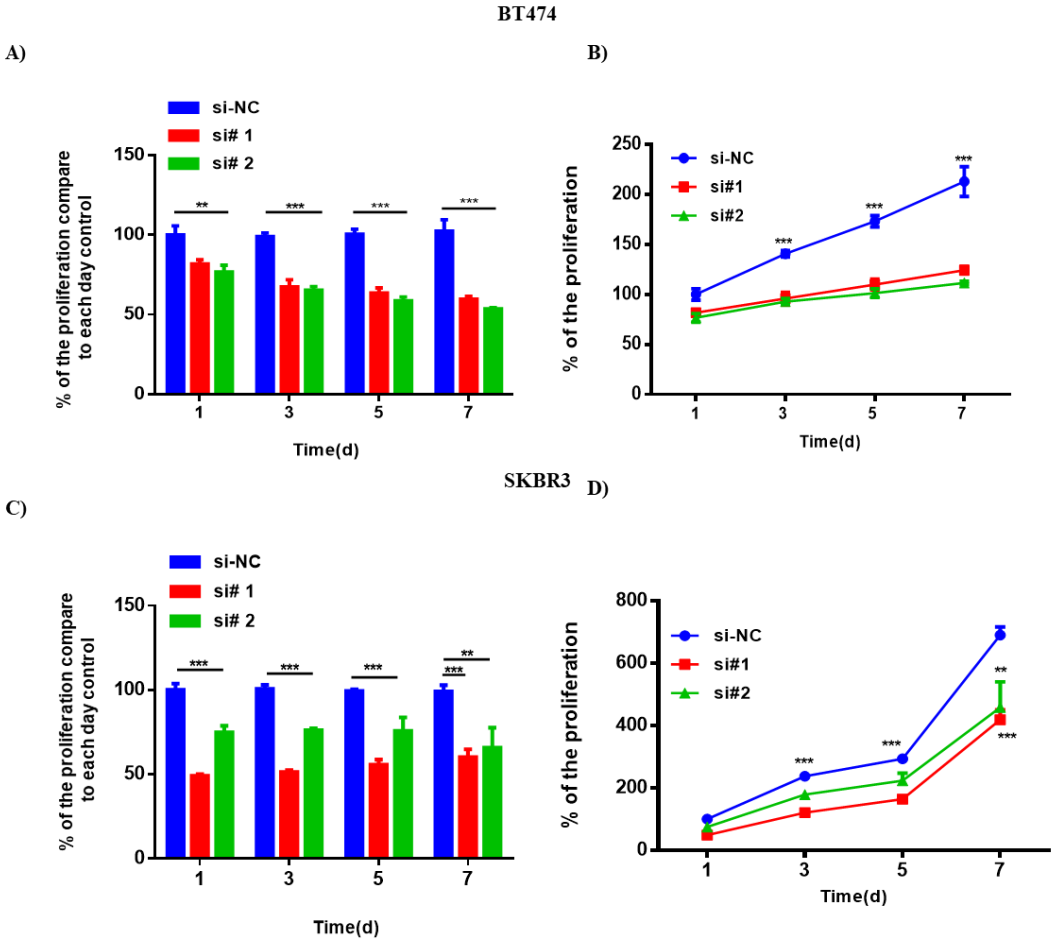


Figure 22: Effects of EZH2 silencing on cell proliferation: In two EZH2 expressing HER2+ BC cell lines, BT474 and SKBR3 were seeded. The proliferation of BT474 (A-B) and SKBR3 (C-D) was measure by WST assay after transfection with scrambled siRNA (blue line), EZH2 silencer si1(red lines) or EZH2 silencer si2 (green line). The graph shows the results of three independent experiments, run in triplicate. The experiments were carried out to 7 days. Student's t-test compared the results. ****P ≤ 0.01, ***P ≤ 0.001.**

2.3.7 Downregulation of the EZH2 inhibits invasion and migration in HER2+ BC cell lines

As we observed the expression of EZH2 is higher in HER2+ BC cell lines and patients than in controls, it might be the critical regulator of cancer progression. We shouted to explore the cells' invasion and migration capacity while silencing EZH2 expression to validate this assumption. We could transfect two different siRNAs into SKBR3; however, BT474 cell line is not a convincing model to carry out these experiments for its unique morphology. 72h post-transfected SKBR3 cells were seeded on matrigel coated transwell insert and followed the subsequent steps described in the material methods section. The counting of the cells capable of crossing the transwell membrane

with the matrigel layer, (**Figure 23A**) indicates that EZH2 silenced cells do not possess the same invasive ability than those frequently expressing EZH2 with a clear significant difference (p-value <0.001). Migration capacities of the cells were also studied by transwell assay, whose results indicate a considerable reduction (p-value <0.001) in the migration capacity through the pores of the inserts of the cell line SKBR3 when the EZH2 gene is silenced, (**Figure 23B**). These results indicated that the effect of EZH2 knockdown through siRNAs reduced the number of migrating and invading HER2+ BC cells compared to si-NC cells, describing EZH2 might be involved in the process of metastasis and EMT.

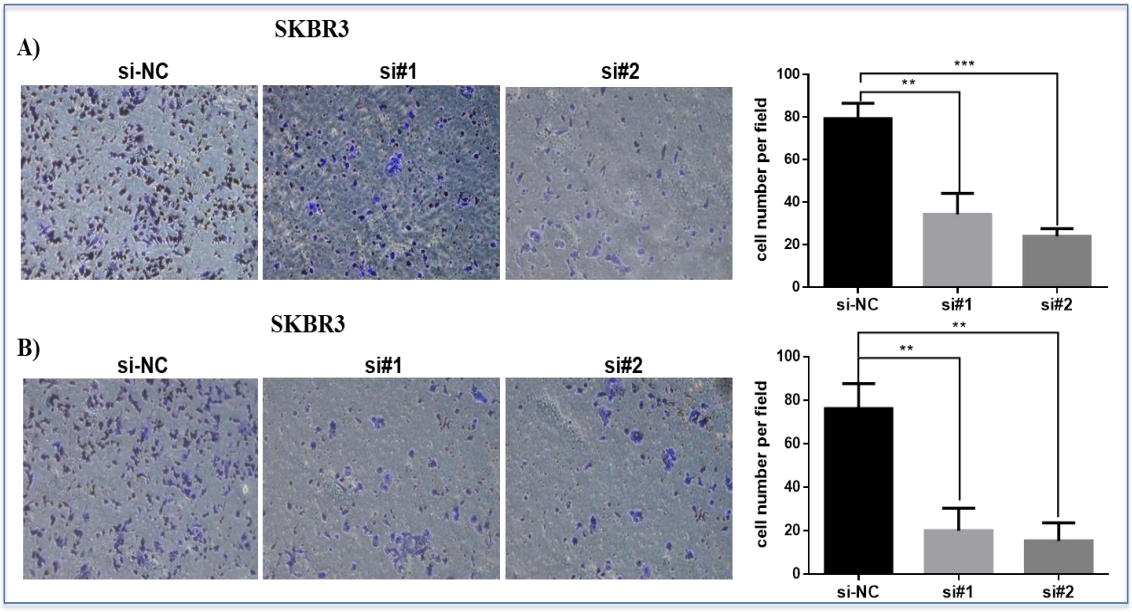


Figure 23: Effects of EZH2 silencing on cell invasion and migration in SKBR3 HER2+ BC cell: Reducing the expression of EZH2 inhibits the invasive (A) and migration (B) ability of SKBR3 cell. Student's t-test was used to analyse the significant differences. ****P ≤ 0.01, ***P ≤ 0.001.**

2.3.8 EZH2 induces epithelial-mesenchymal transition in HER2+ BC

The previous results suggested that EZH2 is involved in cancer cell migration and invasion. EMT is a complex process in which epithelial cells acquire the characteristics of invasive mesenchymal cells. EMT has been implicated in cancer progression and metastasis through invasion and migration properties. To evaluate the downregulation of EZH2 modifies EMT pathway in our model, we silenced the expression of EZH2 in BT474 and SKBR3 by two different siRNAs to analyse the gene set enrichment of

EMT. RT-qPCR confirmed the downregulation of the EMT genes and the protein by western blot in both cell lines compared to the control, (**Figure 24 A-D**).

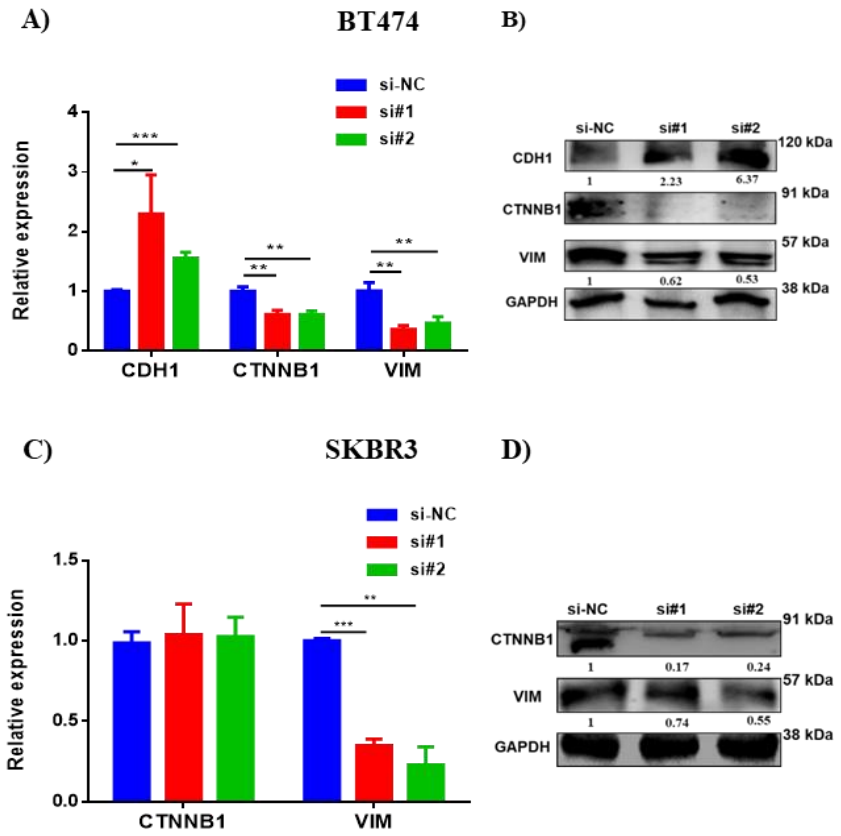


Figure 24: EZH2 promotes EMT in HER2+ BC cells: RT-qPCR and western blot results showing EMT marker levels after silencing EZH2 in BT474 (A-B) and SKBR3 (C-D). Student's t-test was used to analyse the significant differences. ***P ≤ 0.05, **P ≤ 0.01, ***P ≤ 0.001**.

The results showed that downregulation of the EZH2 expression induced a statistically significant increase of CDH1 and decreased β-catenin and vimentin in BT474 in both mRNA and protein level. There were no changes in β-catenin (CTNNB1) in mRNA level in SKBR3 with silencing of EZH2. However, there was a reduction in the expression of β-catenin and vimentin in protein level, but CDH1 expression could not be observed in this particular cell line due to its homozygous deletion of a large portion of the gene[183]. Taken together, these data suggest that EZH2 induces EMT to promote migration and invasion of HER2+ cancer epithelial cells.

2.3.9 Survival analyses for EZH2 expression in BC patients

From all the results explained above, it has been demonstrated that EZH2 is required for cancer cell proliferation, migration, invasion, and EMT, all of which are associated with cancer initiation, progression, and metastasis. As all the steps are involved in poor survival, we explored the correlation between EZH2 expression and patient survival using the Kaplan-Meier Plotter database (KM plotter ©) tool (http://kmplot.com/analysis/). Patients with high EZH2 expression showed significantly more reduced relapse-free survival (RFS), overall survival (OS) and distant metastasis-free survival (DMFS) in BC in general and also specifically in HER2+ BC subtypes, (**Figure 25 A-F**). These results suggested that EZH2 could act as an independent predictor of the development of metastases, relapse of the diseases and patient's survival in BC and HER2+ subtypes.

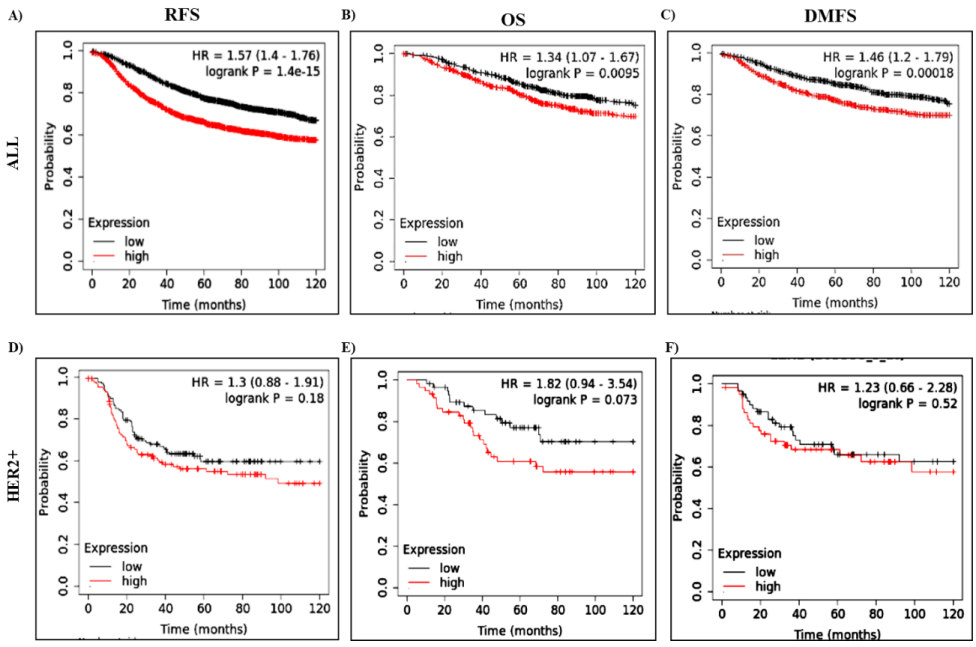


Figure 25: Prognostic value of EZH2 expression in BC patients: *In silico* relapse-free survival (RFS), overall survival (OS) and distant metastasis-free survival (DMFS) of EZH2 in BC patients (A-C) and HER2+ subtypes patients (D-F) with Kaplan-Meier Plotter.

2.3.10 Expression of the miR-33b in BC patients

MiR-33b was found to act as an anti-cancer miR inhibiting cell migration, proliferation and invasion in melanoma cancer, lung cancer, prostate cancer, osteosarcoma, gastric cancer and triple-negative BC. However, the role and the action mechanism of the miR-

33b in HER2+ BC subtype are still unclear. So, miR-33b expression was determined in different BC patient's samples and normal breast tissues collected from the Department of Oncology, Hospital Clinico de Valencia. RT-qPCR results revealed that miR-33b expression was significantly higher in healthy breast specimen vs HER2+. In TNBC BC tissues samples, the miR-33b expression is lower than healthy tissues but not significant due to a smaller number of patients, (**Figure 26 A-B**). Sum up; we considered that miR-33b might play an essential role in breast carcinoma inhibition. Still, many molecular studies and clinical-pathological studies are needed to explore more about this miRNA.

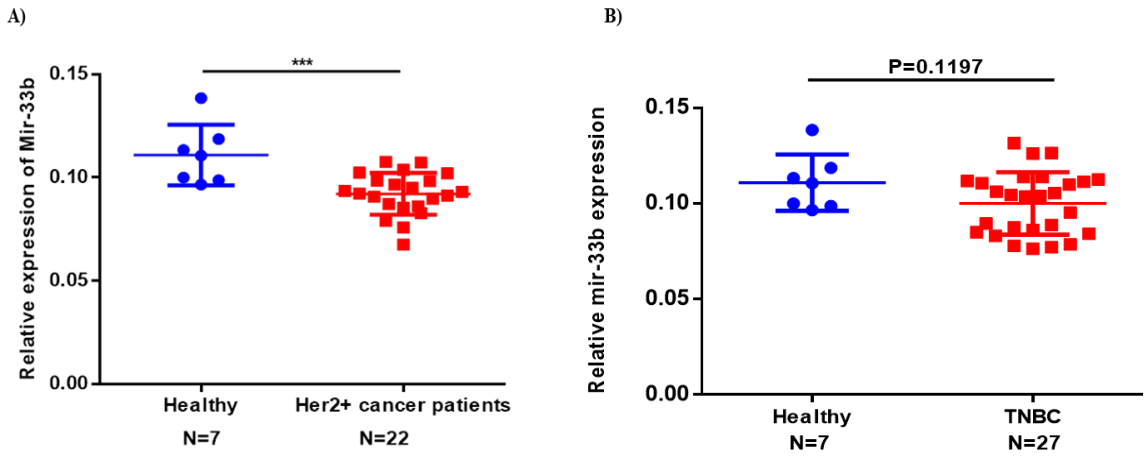


Figure 26: Expression of the miR-33b in BC patients and normal tissues: The relative expression of miR-33b was determined by RT-qPCR in HER2+ cancer tissue and non-tumorigenic tissue (A). MiR-33b gene expression was determined by RT-qPCR in TNBC tissue and non-tumorigenic tissue (B). Student's t-test was used to analyse the significant differences. ***** P ≤ 0.001**.

2.3.11 MiR-33b expression level in BC tumour or normal-like tissues from the TCGA database

As we mentioned above, miR-33b is downregulated in BC HER2+ and TNBC subtypes; we intended to analyse its expression in different molecular subtypes specimens, collected from the TCGA database. The database has contained 1285 cases of different BC subtypes solid tumours and normal like cancer tissues. We could obtain 211 numbers of specimen details; others had missing miR-33b expression data, no follow-up data, or missing clinical information. Among them there were Luminal B (n= 49), Basal-like (n= 26), Luminal A (n= 92), HER2+ (n= 18) and normal like solid tissue (n= 26). MiR33b expression was less in HER2+ following Luminal A, Luminal B and

basal-like subtypes, (**Figure 27**). This figure also demonstrated that miR-33b expression is higher in normal-like cancer, which has a good prognosis than other BC subtypes. Altogether, the data suggested that low expression of miR-33b in BC leading cancer progression and poor prognosis.

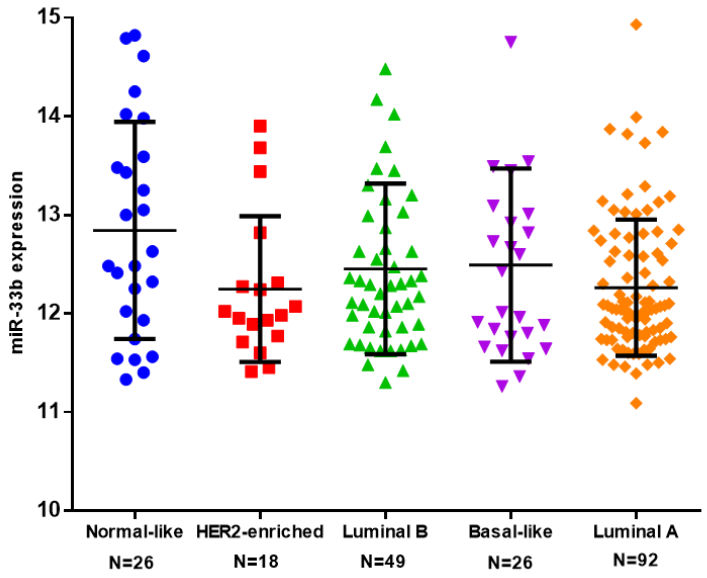


Figure 27: miR-33b expression is indicated in BC tumour or normal tissues from TCGA dataset: RT-qPCR analysis indicated the miR-33b expression levels in five main intrinsic or molecular subtypes of BCs tissues from TCGA dataset.

2.3.12 MiR-33b is downregulated in the different subtype of cancer cell lines

To confirm miR-33b expression is high in immortalized normal mammary epithelial cell lines, we analysed distinct subtypes of BC tissues and healthy breast tissues as mentioned in the result section **2.3.10**. This result section analysed the miR-33b expression in different subtypes of BC cell lines and immortalized normal epithelial cell lines. Mir-33b expression was determined in four human BC cell lines including MDA-MB-468, MCF-7, BT474 and SKBR3 (HER2+) and the non-tumourigenic mammary epithelial cell lines MCF12a and MCF10a as controls. RT-qPCR data revealed that the miR-33b expression was significantly higher in the MCF10a than HER2+ BC cell lines (**Figure 28A**). Additionally, the analysis showed, miR-33b is slightly expressed in triple-negative cancer cell line and luminal A due to its high aggressiveness character. MCF10a and MCF12a normal cell lines were taken as a positive control for miR-33b expression (**Figure 28B**). The observation showed that miR-33b expression in BC cell

lines was much lower than normal mammary epithelial cell lines. That indicates that miR-33b may perform an essential role in regulating BC and complement the results we observed in BC tissue.

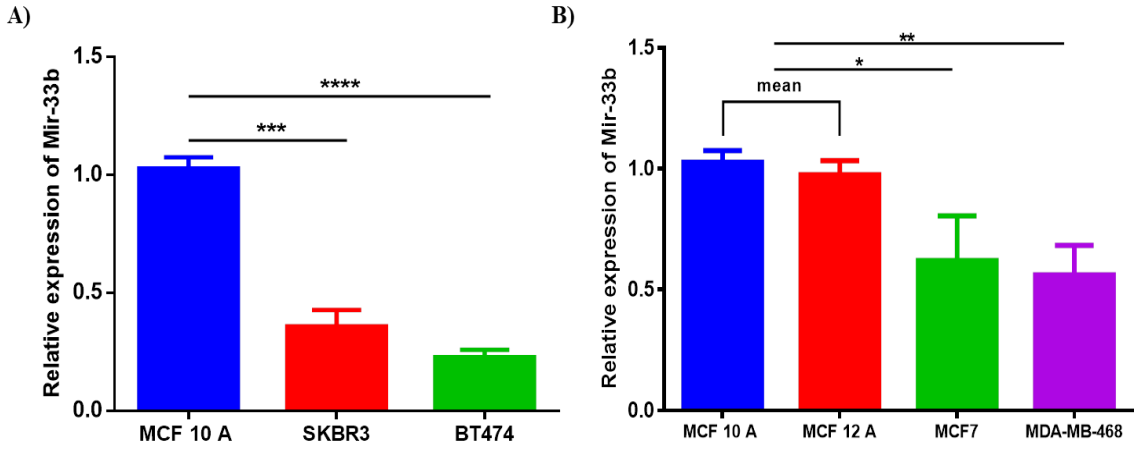


Figure 28: Expression of the miR-33b in HER2+ and other subtypes of BC cell lines: The relative expression of the miR-33b was determined in HER2+ cancer cell lines and non-tumourigenic epithelial cell line (A). miR-33b expression was determined in other subtypes such as luminal A and TNBC of BC cell lines and non-tumourigenic epithelial cell lines by RT-qPCR and normalized to RNU43(B). Student's t-test was used to analyse the significant differences. ***P ≤ 0.05, **P ≤ 0.01, ***P ≤ 0.001, **** P ≤ 0.0001.**

2.3.13 Ectopically over-expression of miR-33b in two HER2+ cell lines

In previous results, we observed that miR-33b is downregulated in BC, more specifically in HER2+. As this thesis focuses on HER2+ subtypes, we selected the same two cell lines that have been chosen to study EZH2 expression in previous experiments (BT474 and SKBR3). To evaluate the potential roles of miR-33b in HER2+ BC cells, we transfected miR-33b mimics or the mimic control into BT474 and SKBR3 cell lines to overexpressing its expression ectopically. The RT-qPCR data confirmed that the BT474 and SKBR3 cells transfected with miR-33b mimics had significantly higher expression levels of miR-33b than those transduced with the mimic control, (**Figure 29 A-B**).

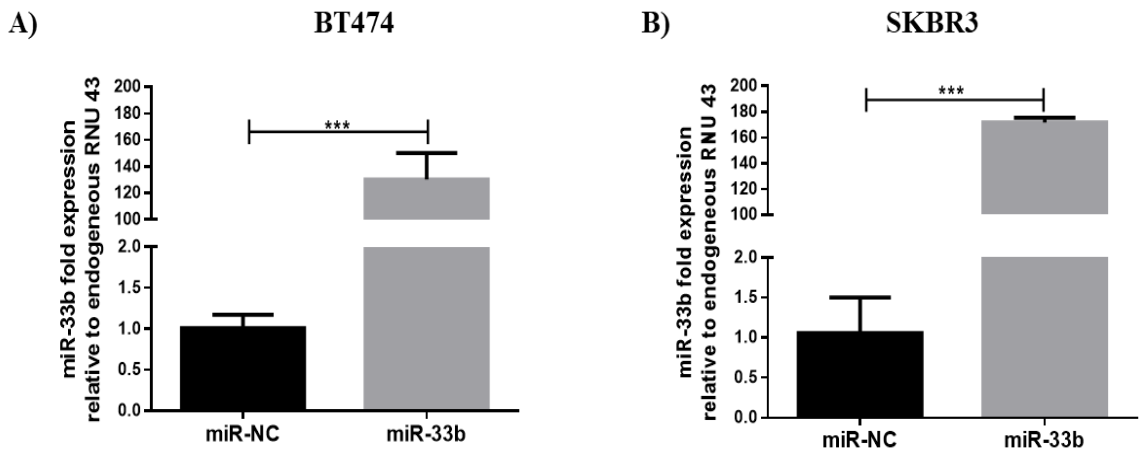


Figure 29: Ectopic expression of the miR-33b in HER2+ BC cell lines: Changes in the expression of miR-33b after transfection with miR-33b mimics or the mimic control. The relative expression levels of miR-33b were evaluated using RT-qPCR. The miR-33b mimics significantly up-regulated the expression levels of miR-33b in BT474 (A) and SKBR3 (B) cells. Student's t-test was used to analyse the significant differences. *****P ≤ 0.001.**

2.3.14 Over-expression of miR-33b suppresses the proliferation of HER2+ BC cells *in vitro*

To determine the potential effect of miR-33b on cell proliferation in HER2+ BC, cells were transfected with miR-33b mimic or mimic negative control (miR-NC). RT-qPCR checked the transfection in both cell lines (data are shown above). The WST cell proliferation assay was carried out to observe the proliferation effect, which showed that overexpression of miR-33b significantly decreased the cell proliferation compared to negative control in BT474 and SKBR3, (**Figure 30 A-B**), and the inhibitory effects showed statistical significance till seven days. Collectively, it showed that miR-33b has an anti-proliferative effect on HER2+ BC cell lines and might induce apoptosis and prohibit cancer cells growth.

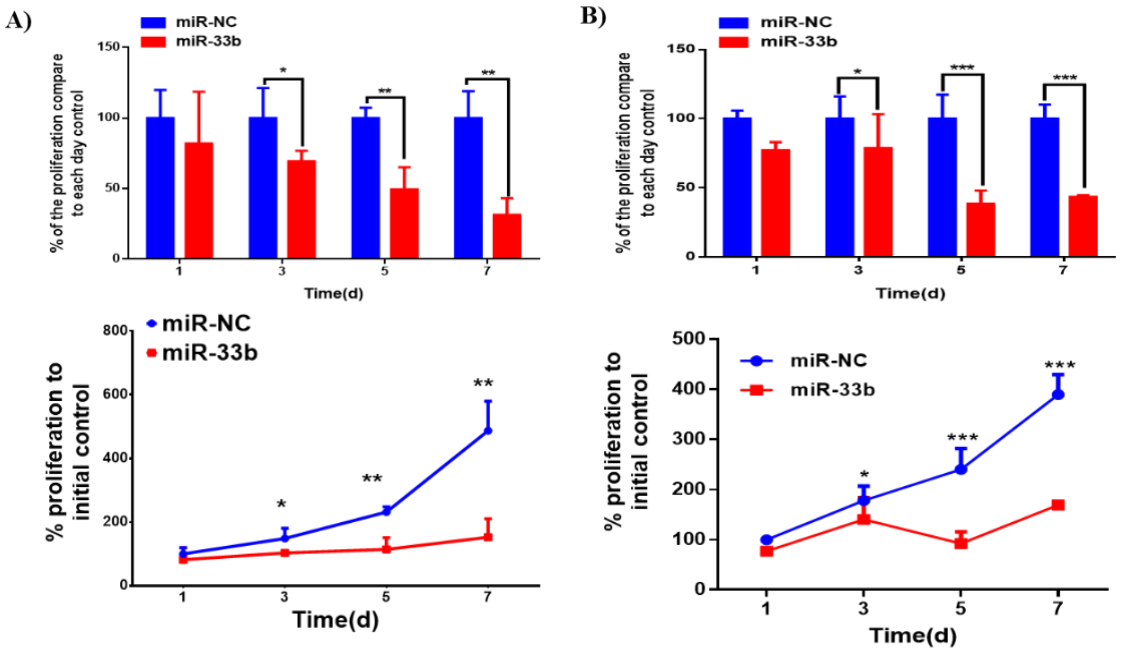


Figure 30: Over-expression of miR-33b suppresses the proliferation of HER+ BC cells: The WST assay determined cell proliferation after transfection with miR-33b mimics or the mimic control until seven days in BT474 (A) and SKBR3 (B). Student's t-test was used to analyse the significant differences. ***P ≤ 0.05, **P ≤ 0.01, ***P ≤ 0.001.**

2.3.15 MiR-33b induces apoptosis and arrest cells at subG0/G1 of HER2+ BC

Cell proliferation and cell death are essential, yet opposing cellular processes. Crosstalk between these processes promotes a balance between proliferation and death, limiting cells' growth and survival with oncogenic mutations. A recent study has shown that miR-33b regulates cell cycle and apoptosis[184]. So, taking all together these data and anti-proliferative properties of miR-33b, we evaluated apoptosis by annexin-V of BT474 and SKBR3 cells transfected with miR-33b mimic. Overexpression of miR-33b in BT474 and SKBR3, (**Figure 31A**), cells inducing early and late apoptosis through analysis with flow cytometry. To verify these results, we further investigated cell cycle by PI/RNAse of miR-33b transfected cell lines, which showed, a considerably increased of cells in sub- G0/G1 phase after transfection with miR-33b, compared to the control and a reduction almost in half in the number of cells in G1 and S phases (**Figure 31B**). Collectively, it showed that miR-33b has an antiproliferative effect on HER2+ BC cell lines and induced apoptosis with arresting the cells at sub- G0/G1 phase.

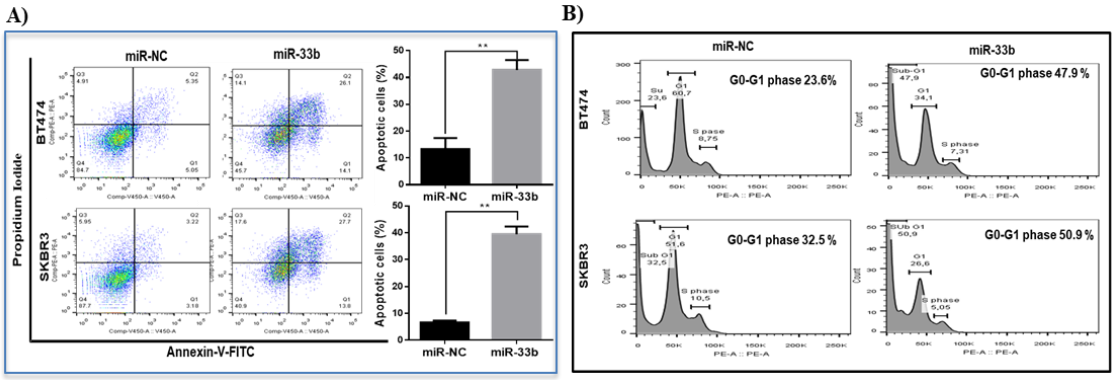


Figure 31:miR-33b induces cell apoptosis and regulates cell cycle in HER2+ BC: The apoptotic cell population was determined at 72h in both cell lines by flow cytometry through Annexin-V/PI staining (A). To confirm the apoptotic population cell cycle was analysed through PI-RNAse assay at 48h and percentage of subG0/G1 stage cells were shown (B). Student's t-test was used to analyse the significant differences. **$**P \leq 0.01$.**

2.3.16 MiR-33b inhibits migration and invasion in HER2+ BC cells

Tumour cell invasion and metastasis are tightly correlated with various processes, including EMT. Therefore, we studied its functional aspect, such as invasion and migration properties of the cells after transfection with miR-33b mimic. Additionally, migration and invasion assays were planned to explore the anti-metastatic effect of miR-33b. The SKBR3 cells were transfected with miR-33b mimic for 72h and seeded on matrigel-based transwells to check the invasion capacity within 24h. RT-qPCR confirmed the expression of the mature miR-33b in this cell line (data are not shown). The results showed that overexpression of miR-33b induced a decrease in SKBR3 invasion capability compared to controls (**Figure 32A**). The carried out the migration process of the HER2+ cancer cells. The results showed that the miR-33b overexpression reduced the migration properties of HER2+ cells significantly compared to the negative control (**Figure 32B**). Taken together, these results suggested that miR-33b inhibits cell invasion and migration and acts as a possible crucial regulator of the EMT process in HER2+ BC.

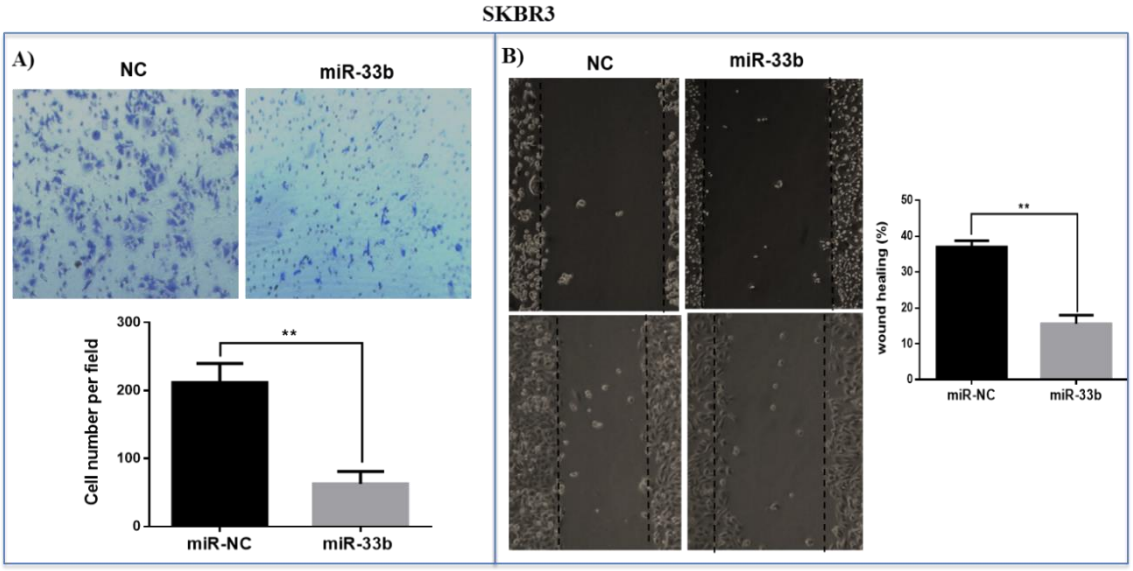


Figure 32: miR-33b inhibits cell migration and invasion in HER2+ cells: SKBR3 cells were transfected with miR-33b mimic and cells penetrating the membrane were fixed and 0.4% crystal violet staining after 24 h to evaluate the invasion capacity (A). A wound-healing assay was performed on SKBR3 transfected with miR-33b to explore the migration properties of the cells. Black arrows indicate the wound edge. The relative scratch gap was calculated as the percentage (%) of the remaining scratch gap at the given time point and the original gap at 0h (B). Student's t-test was used to analyse the significant differences. **$**P ≤ 0.01.**

2.3.17 Up-regulated miR-33b inhibits epithelial-mesenchymal transition in HER2+ BC

The above result sections showed that EZH2 is a master regulator of proliferation, invasion, migration, and EMT in HER2+ BC. EZH2 promotes aggressive breast carcinomas with metastatic potential. So, targeting EZH2 through small molecules or miRNAs is essential to combat cancer progression. Moreover, recent substantial data suggested that miR-33a could negatively regulate EZH2 in cancer progression by direct interaction in TNBC[185]. Several studies have demonstrated that the EMT process is vital for acquired drug resistance and initial step for metastasis in cancer cells, as we observed above. Thus, the protein expression levels of EMT-associated markers in the HER2+ BC cell lines were investigated through up-regulation of miR-33b. Thus, the HER2+ BC cells ectopically transfected with miR-33b and Overexpression of miR-33b in BT474 induced a statistically significant increase in the mRNA expression CDH1, and significant decreases of β-catenin, vimentin (**Figure 33A).** Consistent results were obtained with SKRB3 (**Figure 33C**). To confirm these data at the protein level, western

blot was performed (**Figure 33B, D**). CDH1 was unable to detect in SKBR3 because of its homozygous deletion of a large portion of the gene[183]. These findings concluded that miR-33b could effectively suppress EMT in HER2+ BC and act as a crucial tumour suppressor.

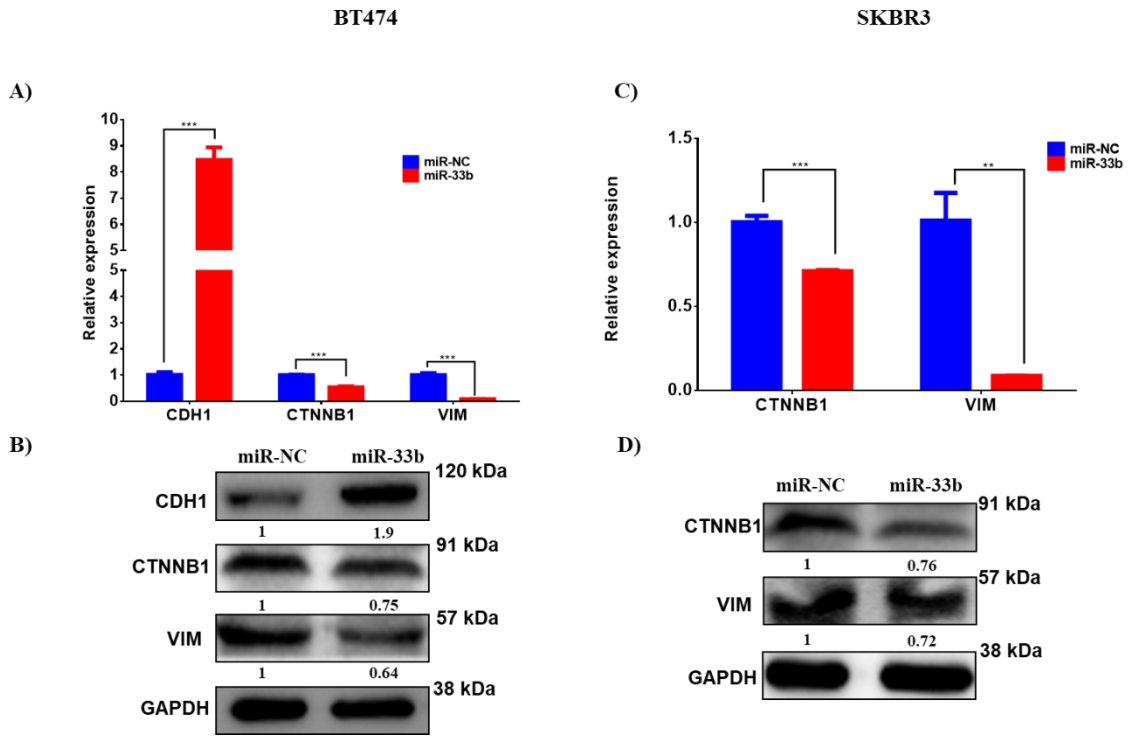


Figure 33: miR-33b inhibits the EMT in HER2+ cells: Relative expression of the EMT genes, and protein expression, was checked after transfection with miR-NC and miR-33b HER2+ cells (A-D). Student's t-test was used to analyse the significant differences. ****P ≤ 0.01, ***P ≤ 0.001.**

2.3.18 MiR-33b is downregulated in two control cell lines

Because of the overexpression of miR-33b reduced cell proliferation, invasion, migration and EMT in HER2+ BC cell, we wondered, if inhibition of this miRNA in control cells would have the opposite effect. To evaluate the potential inhibitory effect of miR-33b, we used two immortalized mammary epithelial normal cell lines named as MCF10a and MCF12a. We transfected miR-33b inhibitor and the inhibitor control into those mentioned cell lines to produce BC cells with miR-33b less-expression. The RT-qPCR data confirmed that the MCF10a and MCF12a cells transfected with miR-33b inhibitor had significantly lower expression levels of miR-33b than the negative control (**Figure 34 A-B**).

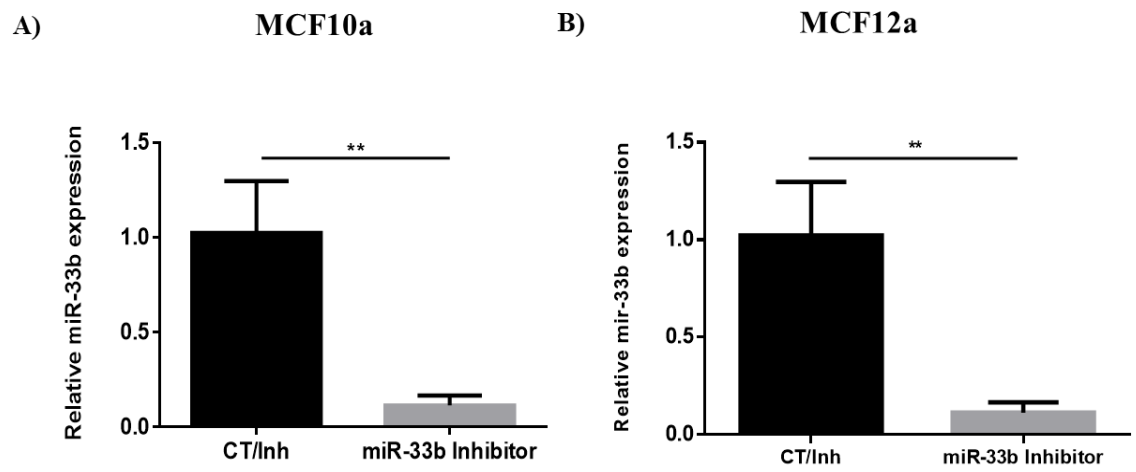


Figure 34: Ectopic expression of the miR-33b in control cell lines: Changes in the expression of miR-33b after transfection with miR-33b inhibitor or the inhibitor control. The relative expression levels of miR-33b were evaluated using RT-qPCR. The miR-33b inhibitor significantly down-regulated the expression levels of miR-33b in MCF10a (A) and MCF12a (B) cells. Student's t-test was used to analyse the significant differences. **$**P ≤ 0.01**.

2.3.19 Downregulation of miR-33b inhibits proliferation of control cell lines

Proliferation is an integral part of cancer development and progression. The cancer cell embodies characteristics that permit survival beyond its average life span and to proliferate abnormally. As in similar case we observed in our HER2+ cancer cells, miR-33b acted as anti-proliferative miRNA, so we believe that inhibitor of miR-33b in normal mammary epithelial cell line would have the opposite effect. To assess that results, we transfected miR-33b inhibitor in MCF10a and MCF12a. RT-qPCR checked the transfection in both cell lines (data not shown). The WST cell proliferation assay was carried out to observe the proliferation effect, which showed that the downregulation of miR-33b significantly increased cell proliferation compared to negative control in MCF10a and MCF12a results showed statistical significance five days (**Figure 35 A-B**). Collectively, it showed that miR-33b is required to control cell proliferation in cancer cells.

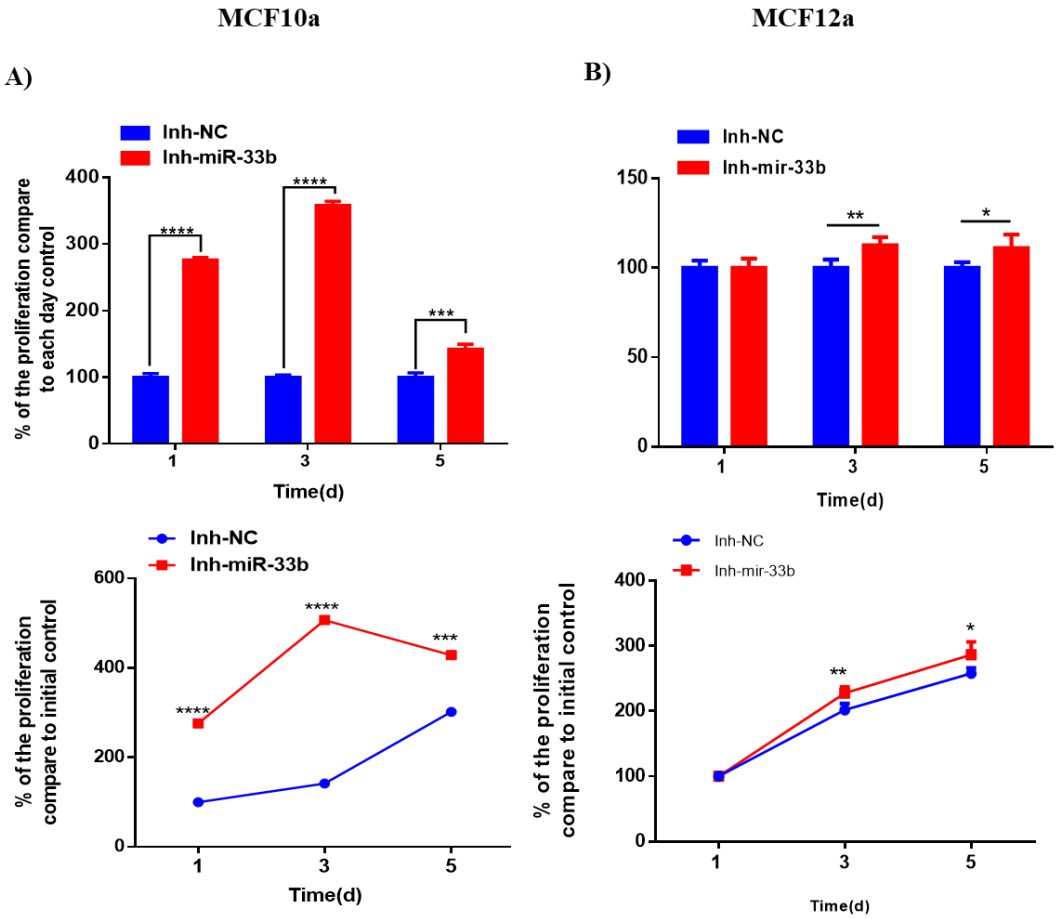


Figure 35: Repression of miR-33b promotes the proliferation of normal breast epithelial cells: Cell proliferation was determined by the WST assay after transfection with miR-33b inhibitors or the inhibitors control until five days. Student's t-test was used to analyse the significant differences. ***P ≤ 0.05, **P ≤ 0.01, ***P ≤ 0.001, ****P ≤ 0.0001**.

2.3.20 MiR-33b promotes migration and invasion of normal epithelial cell lines

To reveal the functional role of inhibition of miR-33b on migration and invasion of normal epithelial cell lines, both MCF10a and MCF12a cell lines were transfected with the miR-33b inhibitor/NC. The results showed MCF10a and MCF12a were transfected with miR-33b inhibitors both expressed at a relatively low level of miR-33b compared with cell lines transfected corresponding to the negative control (data not shown). Transwell assays without Matrigel were used to examine the miR-33b function on the migratory cell potential. Transwell assays with matrigel were used to detect the miR-33b effects on the invasive cell potential. Downregulation of miR-33b significantly increased the migration and invasion capacities in the MCF10a and MCF12a cells

(**Figure 36A-B**). In summary, these findings indicated that miR-33b might act as an anti-oncomiR and demote cell migration and invasion during HER2+ carcinoma progression.

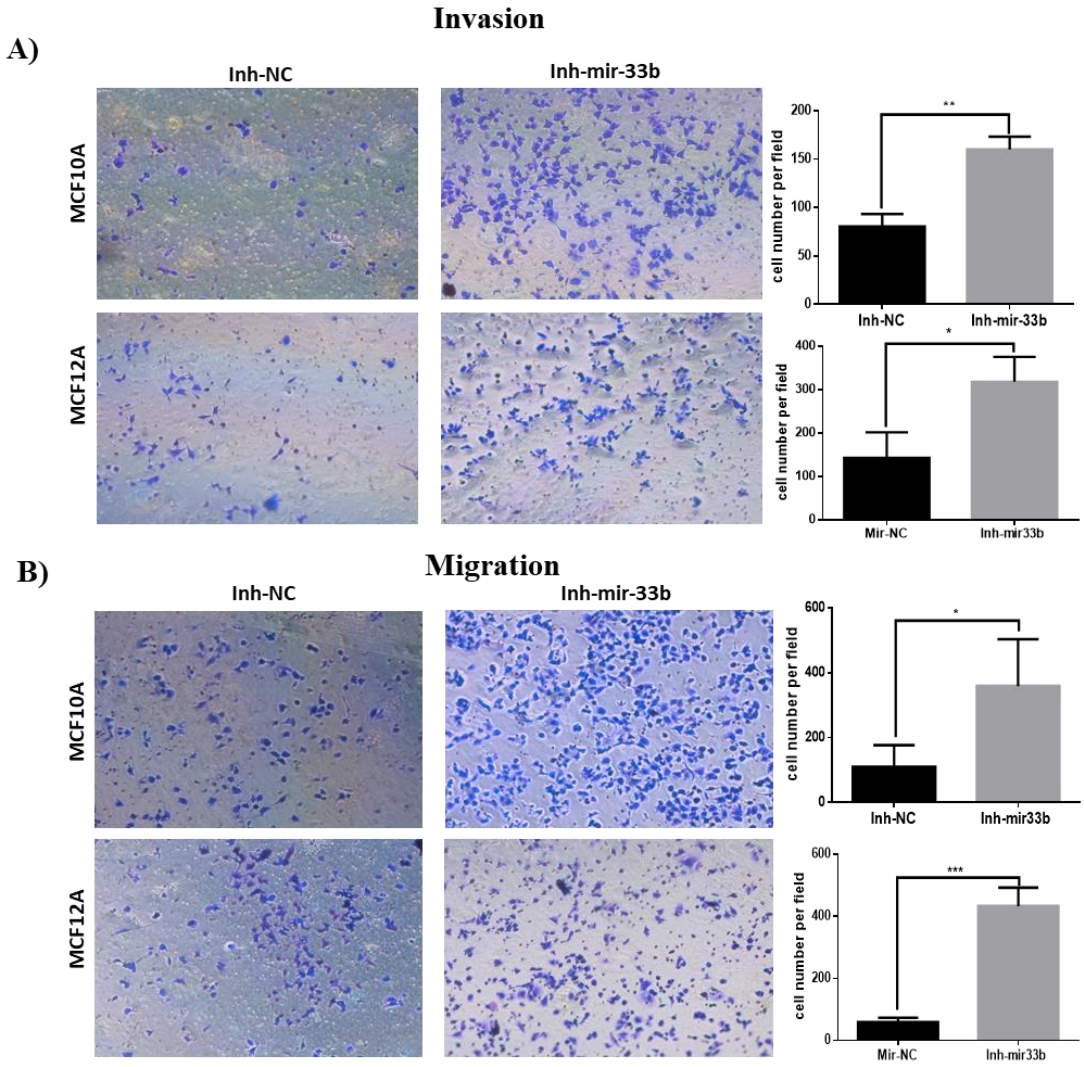


Figure 36: miR-33b inhibitor promotes invasion and migration of normal epithelial *in vitro*: (A and B) Transwell assay showed that inhibiting miR-33b expression promotes invasion and migration of immortalized normal human epithelial breast cells after transfection of miR-33b inhibitor or miR-ctrl. Student's t-test was used to analyse the significant differences. ***P ≤ 0.05, **P ≤ 0.01, ***P ≤ 0.001.**

2.3.21 MiR-33b could regulate EMT in normal epithelial cell lines

EMT has been identified to contribute to the cell invasion of different cancers by transforming polarized and adherent epithelial cells into motile and invasive mesenchymal cells. To determine whether miR-33b could regulate the molecular changes of EMT in normal breast epithelial cells, the expression of mesenchymal

markers, including β-catenin and Vimentin and the epithelial marker, E-cadherin, was examined in the cells by using western blot and RT-qPCR. Thus, MCF10a and MCF12a cells were transfected with inhibitor NC and miR-33b inhibitor. The results showed that with the inhibition of miR-33b, there is significantly diminished expression of CDH1 and an increase of β-catenin, vimentin and EZH2 in both mRNA and protein level (**Figure 37 A-D**). Thus, it supports that miR-33b can regulate EMT signalling in both control and cancer cell lines.

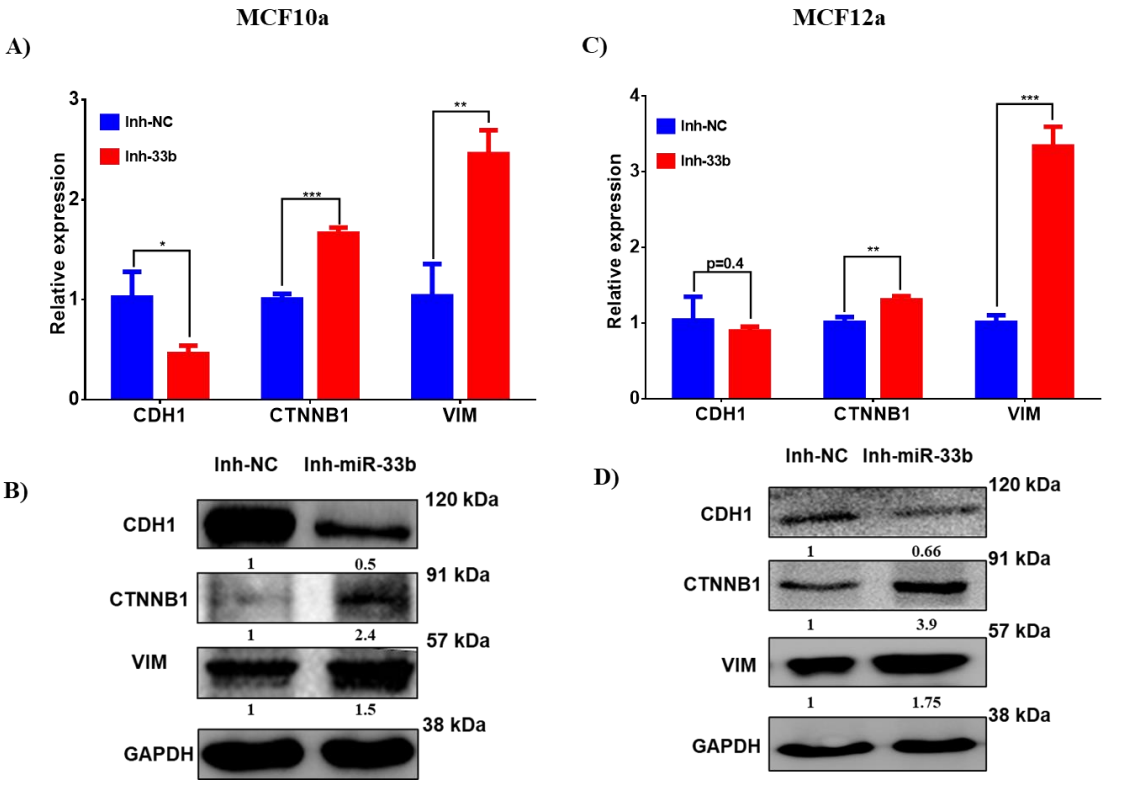


Figure 37: Inhibiting miR-33b induces EMT: (A-D) mRNA and protein expression of the EMT markers using RT-qPCR and western blot in MCF10a and MCF12a. Student's t-test was used to analyse the significant differences. ***P ≤ 0.05, **P ≤ 0.01, ***P ≤ 0.001.**

2.3.22 The low miR-33b expression is associated with the patient's overall survival

When the general BC patients (n=1262) of the database were evaluated with KM plotter (described in 2.3.8 results section), low miR-33b expression appeared to predict less overall survival (61.91 months) that the patients were having high expression of miR-33b (89 months, p=0.025, **Figure 38A**). Similarly, when only patients with HER2+ status (n=608) were analysed with the same database, it also showed the complementary

concordant results with general overall survival. Patients with low miR-33b expression were tended to have low overall survival (71.21 months), where high expression miR-33b showing patients have good survival (113.3 months, p=0.039, **Figure 38B**). In BC patients and specifically in HER2+ patients, high expression levels of miRNA improved patients' survival, stating that miR-33b expression is essential to stabilize BC patients' survival.

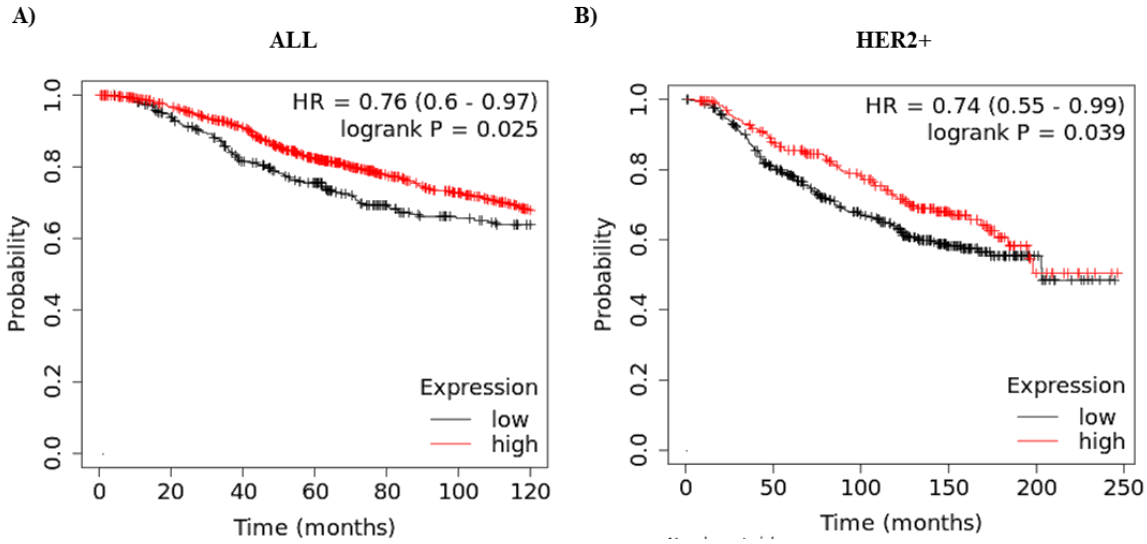


Figure 38: Overall survival in BC and HER2+positive cancer cases by the miR-33b expression: *In silico* overall survival (OS) miR-33b in BC patients (A) and HER2+ subtypes patients (B) with Kaplan-Meier Plotter analysis.

2.3.23 EZH2 is partially regulated through miR-33b

From the previous results, we demonstrated that EZH2 and miR-33b having contrary results in HER2+. Silencing of EZH2 and overexpression of miR-33b inhibits EMT, proliferation, invasion and migration in HER2+ cells. Taking these results in consideration, we tried to explore whether in this process, miR-33b is inhibiting EZH2 and as the consequences of reducing HER2+ cancer cells aggressiveness. We transfected mimic and inhibitors of miR-33b in respective cell lines which showed a reduction and overexpression of EZH2 at mRNA and protein level (**Figure 39 A-D**). Overall, these data suggested that miR-33b inhibits EZH2 expression due to suppressing HER2+ cancer cells' aggressiveness and proliferation. The physical interaction is analyzed in a later section.

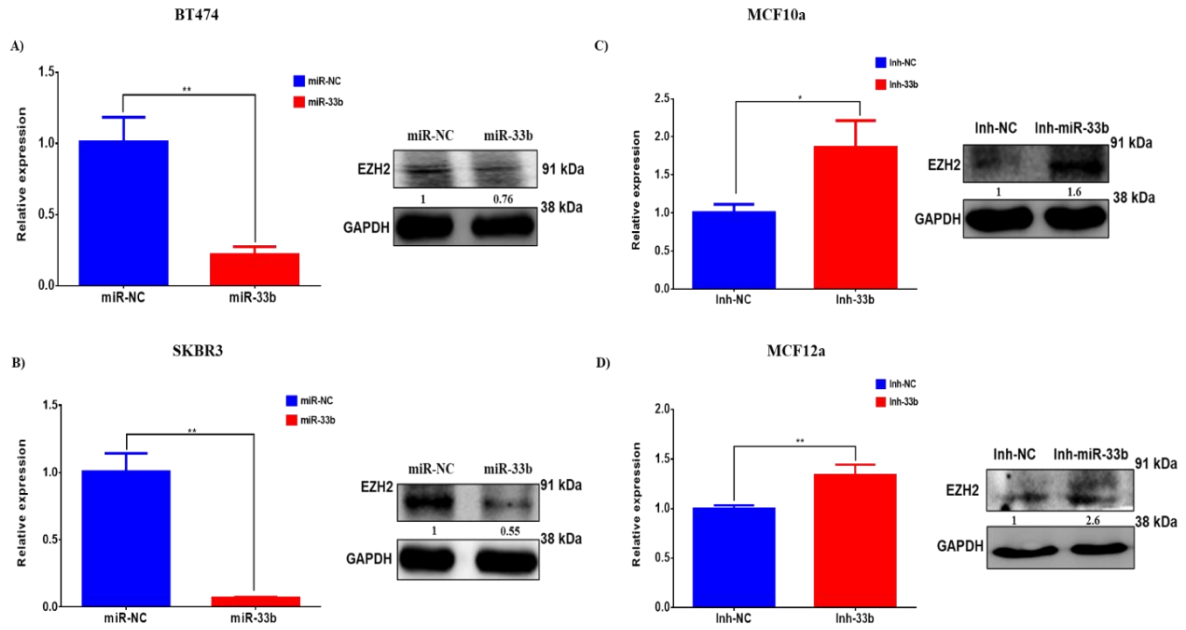


Figure 39: Regulation of EZH2 by miR-33b: (A-B) RT-qPCR and western blot results showing EZH2 expression level after transfection with a mimic in BT474 and SKBR3. (C-D) RT-qPCR and western blot results showing EZH2 expression level after transfection with an inhibitor in MCF10a and MCF12a. Student's t-test was used to analyse the significant differences. ***P ≤ 0.05, **P ≤ 0.01.**

2.3.24 EZH2 is not a direct target of miR-33b

The physical interaction of miR-33b and EZH2 has been checked in Targetscan, but no such evidence found in the database. We even checked the interaction in other databases such as miRDB - MicroRNA Target Prediction Database, miRNet, miRTarBase, and miRanda. We did not find a seed sequence interact with 3' or 5' UTR region of the EZH2. We used the Freiburg RNA tools to predict the interactions between miR-33b and EZH2 (http://rna.informatik.uni-freiburg.de), where we found the interaction but the yield of energy was very low (-0.3) (**Figure 40 A**). To confirm EZH2 is a direct target of miR-33b, luciferase activity was measured in-between EZH2 3'UTR containing reporter plasmid and control plasmid. This assay revealed no such difference between luciferase activity in 293-T cells transfected with plasmids containing the 3'UTR of EZH2 and cells transfected with the control plasmid (**Figure 40 B**). Collectively, we confirmed that there is no direct interaction between the 3'UTR region and miR-33b and EZH2 is not a direct target of miR-33b.

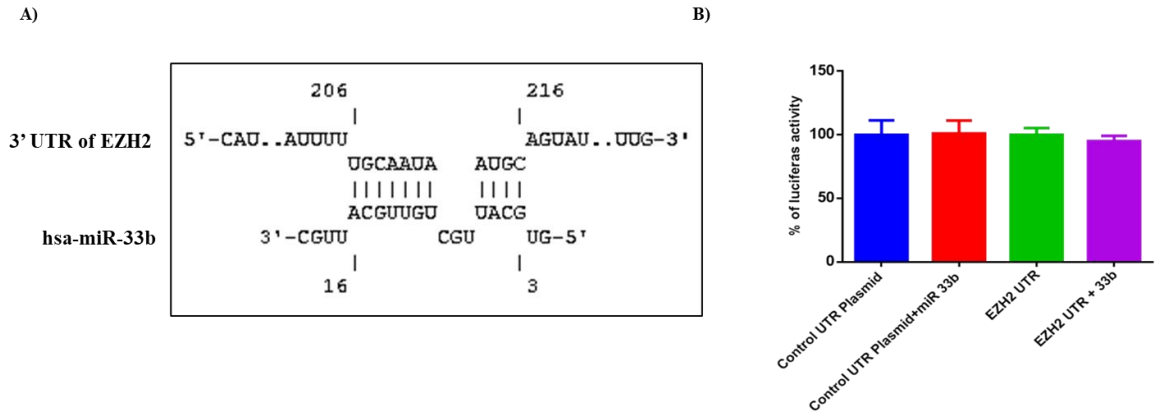


Figure 40: No direct interaction of miR-33b and EZH2: Schematic description of the hypothesized interaction between the EZH2 3´UTR binding site in the position from 206-216 with miRNA-33b (A). (B) Percentage of luciferase activity in between control UTR/miR-33b and EZH2 UTR/miRNA-33b mimic. As there were no such significant differences in the graph, **t-test was not done.**

2.3.25 MYC- is an intermediator of miR-33b and EZH2

From all the results described above, it is confirmed that EZH2 acts as an oncogene in HER2+ BC subtypes, and it is negatively regulated by miR-33b. But as we mentioned above, there was no direct interaction between miR-33b and EZH2, so we seek to explore this interaction's laydown molecular mechanism. In 2004, researchers showed that MYC was negatively regulated by miR-33b at the posttranscriptional level, via a specific target site within the 3′UTR. Overexpression of MYC impaired miR-33b-induced inhibition of proliferation and invasion in osteosarcoma cells[171]. Additionally, MYC also regulated EZH2 by direct binding[181] or by negatively regulates miR-26a[186] a repressor of EZH2. Stand on this hypothesis; we checked the physical interaction of miR-33b and MYC in targetscan, where we found, the MYC has a seed sequence in its 3'-UTR to bind with miR-33b (**Figure 41A**). Thus, we transfected miR-33b mimic and inhibitor in respective cell lines, with NC. The transfection efficiency was checked by RT-qPCR (data not shown). Thereupon, we saw a reduced MYC expression with transfection of mimics in BT474 and SKBR3 at mRNA and protein level (**Figure 41 B-C**). At the same time, we also checked an increased MYC expression with inhibitors of miR-33b in MCF10a and MCF12a at mRNA and protein level (**Figure 41 D-E**). Overall, these data suggested that miR-33b inhibits MYC expression through its direct interaction; thus, the downstream gene of the MYC pathway, EZH2 is downregulating in an eventual process.

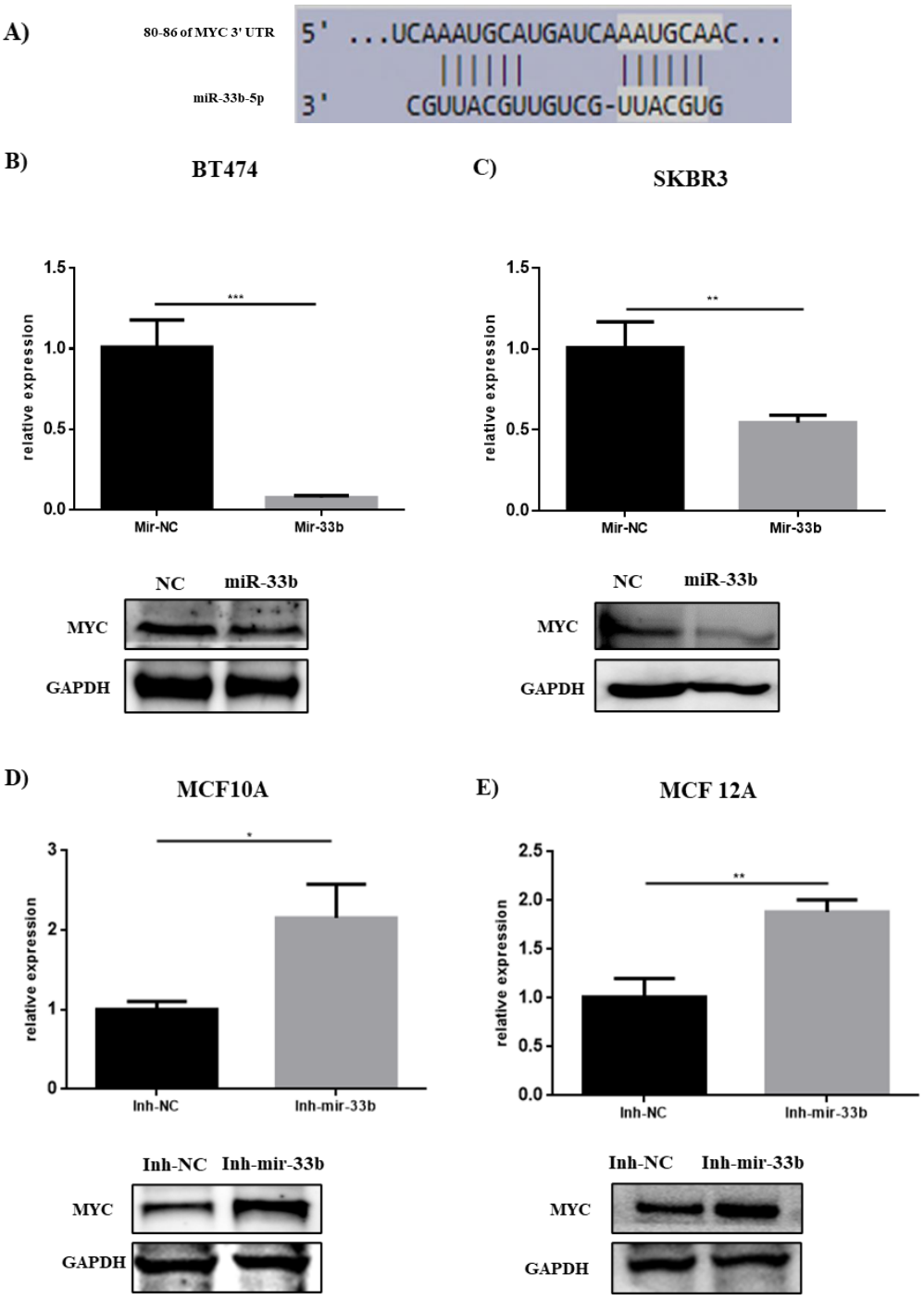


Figure 41: miR-33b inhibits EZH2 through MYC inhibition: (A) The sequences of miR-33b binding sites within the human MYC 3′UTRs. (B-C) RT-qPCR and western blot results showing MYC expression level after transfection with a mimic in BT474 and SKBR3. (C-D) RT-qPCR and western blot results showing MYC expression level after transfection with an inhibitor in MCF10a and MCF12a. Student's t-test was used to analyse the significant differences. ***P ≤ 0.05, **P ≤ 0.01, ***P ≤ 0.001.**

2.4 Discussion

Many accumulated data have pointed out that several miRNAs drive tumorigenesis, drug resistance and suppress cancer progression by targeting different oncogenes[187]. Although multiple studies have been carried to study miRNAs' roles in BC, most of them have focused on BC in general and not on specific subtypes. HER2+ BC subtype is one of the worse prognosis cancer and is associated with inferior outcomes in survival[188]and is an entity with a substantial heterogeneity at multiple levels[189]. In recent studies, miRs are critical regulators to uncover the heterogeneity's molecular mechanisms in HER2+ BC. MiR-33 family is one of the highest conserved miR families, consisting of miR-33a and miR-33b[169]. They both act as a tumour suppressor in different cancers such as non-small cell lung cancer[190], triple-negative BC[191], oesophagal squamous cell carcinoma[192], and colorectal cancer[193] via targeting EMT and proliferation. For the first time in this study, we reported that miR-33b was downregulated in breast HER2+ tumour samples compared to normal breast tissues. The under-expression of miR-33b is related to poor prognosis in HER2+ patients. We also found that miR-33b expression was higher in normal breast epithelial cell line than in HER2+ BC cell lines. It has also been described that in triple-negative BC, miR-33b represses cancer progression and metastasis by targeting oncogenes like SALL4, TWIST1, and HMGA2[194]. These data indicate that miR-33b acts as an onco-suppressive miRNA in BC progression. To investigate the specific role of miR-33b in HER2+ BC progression, miR-33b was ectopically overexpressed in different HER2+ cell lines, showing that upregulation of this miR inhibits cancer cells invasion and migration. As it has been already reported that, this miR regulates EMT[195], we here demonstrated that the overexpression of the miR-33b inhibits EMT process in HER2+ subtype of BC by regulating EZH2 expression.

Furthermore, we reported that the overexpression of the miR-33b has an impact on cell proliferation and induces apoptosis in this BC subtype. Besides, this miRNA also arrests the cell cycle in the sub-G0/G1 phase as compared with other stages in concordance with previous results in lung cancer[196]. Recently, some authors suggested that miR-33a can regulate EZH2 by their direct interaction[185]. We checked *in silico* the physical interaction between miR-33b and EZH2 in Targetscan, miRDB - MicroRNA Target Prediction Database, miRNet, miRTarBase, miRanda databases and the Freiburg RNA tools. In the later, we found interaction with a yield of shallow energy. Based on this

information, we performed the luciferase assay. We found no such direct interaction between miR-33b and EZH2 (data are not shown), which clarified that although miR-33a and miR-33b belong to the same family, they regulate the same gene differently. It has been previously demonstrated that MYC binds to EZH2 promoter and directly activates its transcription[181]. Besides, EZH2 expression is positively correlated with MYC expression in prostate cancer[197].

Moreover, it has been already described that MYC is a direct target of miR-33b[171]. Thus, in our present study, we showed that ectopically overexpression of miR-33b regulates MYC in our models (**Figure 41 B, C**). The sequences of miR-33b have binding sites within the human MYC 3'UTRs (Targetscan, **Figure 41 A**). Considering all these, we suggest EZH2 as a target of miR-33b via regulating MYC (**Figure 42**).

EMT is a fundamental process during the development of tumorigenesis and metastasis. Enormous pieces of evidence indicate that EMT is responsible for cancer cells invasion and migration, and an initial step of metastasis. EZH2 is reported to be upregulated in aggressive BC [198]and involved in epigenetic, post-translational modifications and EMT program by suppressing CDH1 expression[177]. In nasopharyngeal carcinoma, miR-142-3p was downregulated by DNA methylation due to EZH2 recruited DNMT1 occupied the upstream region of the miR-142 and determined ZEB2 activation, which leads to EMT and metastasis[199]. Furthermore, EZH2 is a direct target of miR-26a in docetaxel resistance cells, which could significantly suppress the proliferation, facilitate the apoptosis, inhibit the metastasis ability and reverse EMT- MET in lung adenocarcinoma cells[200]. In oral tongue squamous cell carcinoma, miR-101 inhibits the expression of EZH2 via two transcription factors Snail and Slug[201]. In BC, miR-92b may negatively regulate the expression of EZH2, promote autophagy, and decrease tumour cell viability, migration, and invasion[202]. Additionally, miR-139-5p transcription is inhibited by EZH2 through up-regulating H3K27me3, thereby downregulation of EZH2 and up-regulation of miR-139-5p impedes EMT in lymph node metastasis pancreatic cancer[203]. Accumulating all these results summarized, that expression of EZH2 is upregulated in a different type of cancer, and different miRNAs and drugs require for its inhibition to reduce cancer progression.

Given that the behaviour of EZH2 is context-dependent, in this study, we investigated the role of EZH2, specifically in HER2+ BC. Our research determined that EZH2 is

highly expressed in HER2+ BC cell lines and solid tumours compared to normal epithelial cell line and healthy breast tissue showing an inverse correlation. To dig more of the molecular mechanisms of the EZH2, it has been silenced through two different silencers in BC cell lines, which resulted in inhibition of cell proliferation, migration, invasion and EMT in HER2+ BC cells. That confirms that EZH2 expression has a crucial role in HER2+ BC progression (**Figure 42**). Future *in vivo* experiments to evaluate the role of miR-33b in HER2+ BC metastasis is needed.

In summary, EZH2 might be an essential factor of HER2+ BC progression and associated with a decrease in the patients' overall survival since EMT has been critically discussed as the critical process in tumour aggressiveness metastasis[204]. Our findings in the present study demonstrate for the first time that miR-33b acts as a suppressive miRNA in HER2+ BC that could inhibit tumour migration and invasion partly by impeding EMT through repression of MYC-EZH2 loop. This study suggests a novel miR-33b/C-MYC/EZH2 axis that modulates breast cells' growth and progression and could be clinically useful to design new drugs against HER2+ subtype cancer.

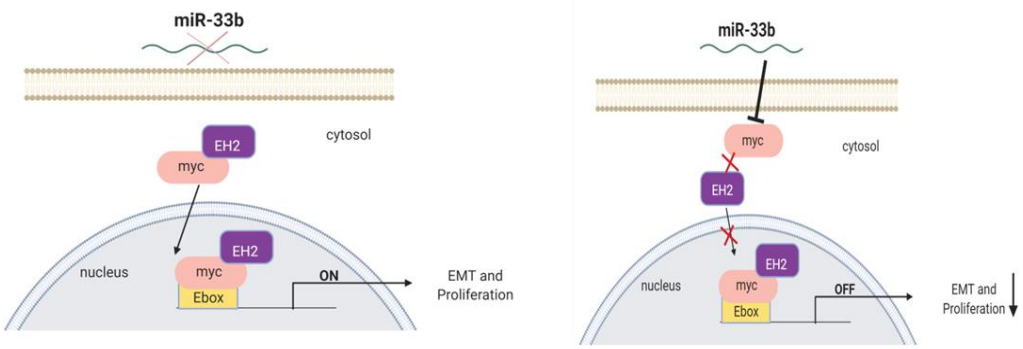


Figure 42: Proposed model of miR-33b inhibited EMT via targeting EZH2-mediated MYC signalling pathway in HER2+ BC cells.

CHAPTER 3

Oncofoetal Gene SALL4: As a potential marker for T resistance HER2+ BC

3.1 INTRODUCTION

In the chapter1 and chapter 2 have adequately described the known mechanisms of resistance to trastuzumab in HER2+ BC. HER2 plays a critical role in tumorigenesis and is associated with poor prognosis of patients with HER2+ BC. HER2 expression is elevated within a defined group of cancer stem cells believed to be the real oncogenic population in the heterogeneous BC and confer resistance to therapies: hormonal and targeted therapies[102]. However, drug resistance often develops de novo, which hinders therapy. Thus, identifying novel therapeutic targets critical for HER2 driving tumour development and resistance to treatment is still needed. The most commonly recognized anti-cancer mechanism of trastuzumab is targeting the extracellular domain of the HER2 receptor and the inhibition of the downstream phosphoinositide 3-kinase (PI3K)/AKT pathway. Therefore, PIK3CA, a mutation of the PI3K gene, was considered an important reason for trastuzumab resistance[115]. Knowing about the AKT pathway regulations through different molecular mechanisms is essential to keep track of resistance mechanisms. This chapter is more focused on exploring the mechanisms of resistance towards trastuzumab via SALL4.

3.1.1 The Embryonic Stem Cell (ESC) Factor SALL4

The human homologue of Drosophila spalt (sal) homeotic gene, *SALL4*, encodes a C2H2 zinc finger transcription factor[205]. Drosophila spalt was first identified in a mutational study as a novel homeotic gene in 1988[206]. Spalt acts in both the posterior head and the anterior tail regions of *Drosophila melanogaster* embryo instead of the trunk region. Mutations of spalt lead to the transformation of the posterior head segments to the anterior thoracic region, and the transformation of the anterior tail region to the posterior abdominal segments of the embryo[206]. These data are among the first evidence that supports an essential role for the spalt family of genes during early development.

In humans, there are four members in the SALL protein family: *SALL1–4*. **Figure 43** illustrates the conserved protein domains present in SALL1–4 proteins. Phylogenetic studies reveal that *SALL4* was produced due to duplication of *SALL2* gene derived from the divergence of ancestral spalt. *SALL4* underwent further duplication to produce *SALL1* and *SALL3*, which are the most closely related SALL members[207]. This chapter focuses on the study of *SALL4* involved in resistance to trastuzumab in HER2+ BC.

Homo sapiens SALL4 shows 24% and 75% protein sequence identity with *Drosophila spalt* and *Mus musculus SALL4*, respectively. The zinc finger domains are conserved evolutionarily. Interestingly, the N-terminal first 12 amino acids of SALL4 that are important for interaction with epigenetic complexes are also conserved in mice and human[208].

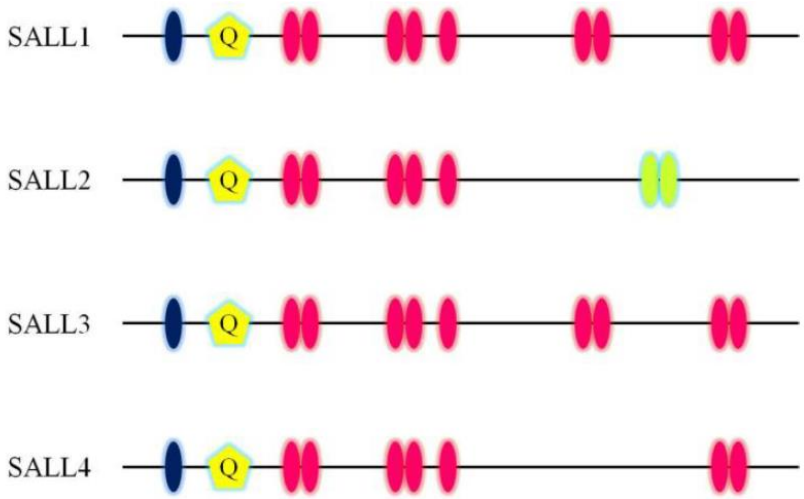


Figure 43: Schematic representation of the main conserved domains in vertebrate SALL proteins. There are four members in the vertebrate SALL family, SALL1–4. Two main conserved characteristics of SALL transcription factors are several zinc finger domains and a glutamine-rich region (PolyQ) in the proteins. Ovals represent zinc finger domains, and yellow pentagon represents glutamine (Q) rich region. Blue ovals represent C2HC zinc fingers, and pink ovals represent C2H2 zinc fingers and green ovals in SALL2 represent the C terminal zinc fingers that are not homologous to those in other SALL proteins[207].

SALL4 is implicated in various processes during embryonic development, including organogenesis, limb formation and neural development. Specifically, SALL4 co-expressed with Oct4 from the one-cell zygotic stage onwards and can activate Oct4 expression and be regulated by Oct4. SALL4 also interacts physically with Nanog. The function of SALL4 as a potent stem cell factor is further highlighted by its ability to enhance reprogramming of somatic cells to pluripotent cells. Being an important transcription factor implicated in early development, SALL4 is tightly regulated at different levels to ensure normal development. Any aberrations in SALL4 regulation or the presence of mutations in the SALL4 gene that affects its functions and regulations are expected to cause various human cancers and developmental disorders[209].

3.1.2 SALL4 in cancer

The stem-like phenotype in cancer results from epigenetic and genetic alterations leading to the expression of genes involved in cell migration, invasion, angiogenesis, self-renewal, anti-apoptosis, and immune-escape, which also are fundamental for the embryo-fetal development. A stem-like phenotype's expression seems to play a central role in defining different cancers' malignant potential. Several stemness-related genes have been proposed as diagnostic markers for cancer during the last decades, sometimes with prognostic significance. In particular, SALL4 has recently emerged as a potential prognostic marker in many tumours. Analyses of SALL4 expression and its epigenetic status, as well as studies on cellular models, have shown its oncogenic role in several tumours, such as precursor B-cell lymphoblastic lymphoma, acute and chronic myeloid leukaemia, gastrointestinal, breast, and lung cancers[210]. SALL4 expression is generally assessed by immunohistochemistry (IHC) on the whole section or tissue microarray (TMA) or molecular testing, such as real-time PCR and methylation analysis promoter region[210]. SALL4 is involved in the cells' self-renewal and anti-apoptosis behaviour. It has a higher probability of helping the cells escape from drugs or assist cells for being resistant to drugs treatments. This chapter mainly trams the novel molecular mechanism of the resistance towards trastuzumab and regulated by SALL4 and how microenvironment is a torch holder of SALL4 in this process.

3.1.3 SALL4 in BC

In 2006, a research group of Harvard medical school for the first time found that SALL4 is constitutively expressed in human primary acute myeloid leukaemia (AML). They also demonstrated that constitutive expression of SALL4 in mice is sufficient to induce Myelodysplastic Syndromes (MDS) like symptoms and transformation to AML that is transplantable. They also provided SALL4 can bind β-catenin to activate the Wnt/β-catenin signalling pathway and showed similar expression patterns at different phases of chronic myeloid leukaemia (CML)[211]. Following that year, many research articles stated that SALL4 plays an essential role in various cancer progression and drug resistance. Recently, emerging evidence has shown that after birth, SALL4 expression is downregulated and absent in adult tissues; however, it is re-expressed in a subset (30 %) of various solid tumours, such as breast, cervical, ovarian, gastric, hepatocellular carcinoma, and germ cell tumours[212]. In a recent study, SALL4 expression was detected

in 86.1 % of BC cases[213]. Another study reported that high cytoplasmic expression of SALL4 was found in BC tissues associated with normal adjacent tissues.

In contrast, *Yue et al.* [214] reported that high SALL4 levels were related to enhanced tumour invasion and lymph node stage in ER, PR, HER2, and TNBC. *Itou et al.* indicated that SALL4 represses E-cadherin gene (CDH1) expression and maintains cell dispersion in basal-like BC[215]. Besides, SALL4 presents a binding site for the TNF-α gene, which regulates cell death[216]. Another study reported that miR-33b played a role as an onco suppressive miRNA in BC progression by inhibiting BC cells' stemness and metastasis. According to these findings, miR-33b suppressed the stemness, migration, and invasion of BC cells by targeting *HMGA2*, *SALL4*, and *Twist1*[217].

In summary, SALL4 is generally upregulated in BC carcinogenesis. Therefore, SALL4 has several important functions in the development or progression of BC. SALL4 expression is related to cell proliferation, suppressing intercellular adhesion, and maintaining motility in BC cells. Moreover, SALL4 induces oncogenic transformation. When SALL4 expression is forced, the number of cells in the G1 phase is reduced. Research has also shown that SALL4 positively regulates the expression of *CCND1* and *CCND2*, which encode the cell-cycle progression factors Cyclin D1 and D2, respectively. SALL4 knockdown reduces the proliferative ability of BC cells[218]. SALL4 positively regulates the expressions of Bmi1, a member of the polycomb group of proteins initially identified in *Drosophila melanogaster* as a repressor of homeotic genes (in BC cells). In humans, the polycomb gene Bmi-1 plays an essential role in regulating adult, self-renewing hematopoietic stem cells (HSCs) and leukaemia stem cells[219]. It can be concluded that SALL4 is involved in the positive regulation of cell proliferation. Although there is not much in the literature on the role of SALL4 in BC, published studies suggest that it may be necessary, and this should guide future cancer treatment research (**Figure 44**).

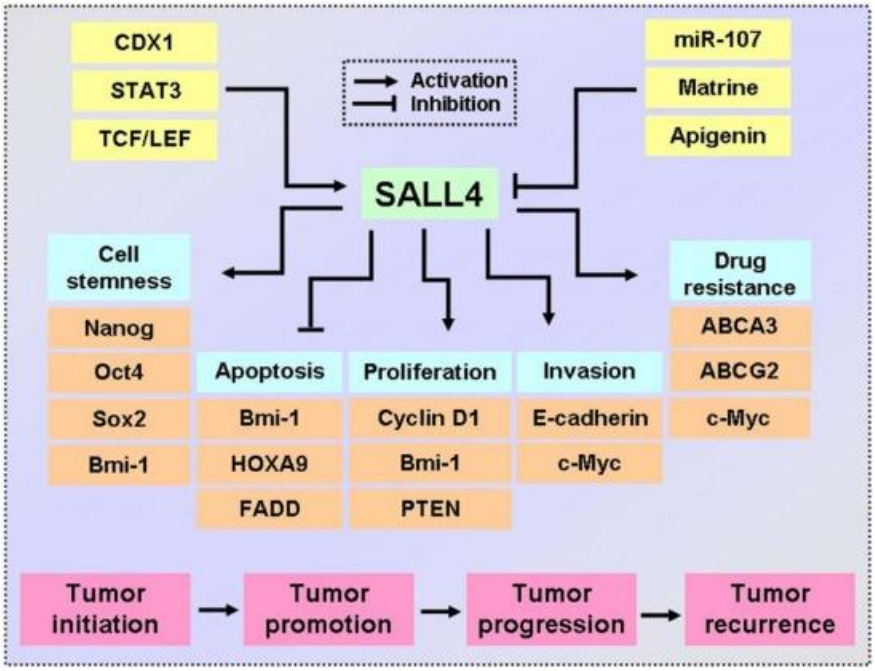


Figure 44: Proposed model for SALL4 regulatory network in cancer[220]

3.1.4 SALL4 in drug resistance

SALL4, being a stem cell marker, which makes the belief that it can be involved in drug resistance in cancer. In 2010, a research group of Johns Hopkins Medical Institutions found that NOTCH3 is overexpressed in ovarian cancer post-chemotherapy. It is an advantage for the cancer cells to survive under the selection pressure of chemotherapy. This study also showed that the Carboplatin resistance cell line has a high expression of NOTCH3 and other embryonic stem cell markers, including SALL4. That suggested SALL4 involvement in drug resistance[221]. It involved in the drug resistance was proved in 2011, in myeloid leukaemia patients. They reported that SALL4 expression is higher in drug resistance to Doxorubicin in primary acute myeloid patients than drug responding patients. They further demonstrated that SALL4 was able to bind to the promoter region of ABCA3 and activate its expression while regulating the expression of ABCG2 indirectly, which suggested a novel role for SALL4 in drug resistance in cancer[222,223]. Afterwards from 2014, some researchers group started taking SALL4 as an important target for chemoresistance in different cancers. In endometrial cancer, SALL4 overexpressed in Carboplatin drug resistance and induced EMT pathway by binds explicitly to the MYC promoter region and initiated its overexpression in this cancer type[224,225]. Fluorouracil and Oxaliplatin were two representative chemotherapy drugs used in colorectal treatment. However, certain patients showed high expression of

SALL4 in severe resistance against these drugs with mechanisms undefined, through regulating P-gp and MRP1 expression[226].

SALL4 expression could be a resistance factor against anticancer drugs such as Cisplatin, Carboplatin, and Paclitaxel in lung cancer. It has been reported that STAT3 pathway may be involved in drug resistance and may a positive-feedback loop exist between SALL4 and IL-6 via STAT3[227]. Another research group showed on the same cancer type is knockdown of SALL4 by siRNA in Cisplatin-resistant cells reduced the IC50 compared with the parental cells through AKT/mTOR signalling[228]. In 2016, for the very first time a group from Shanghai Jiao Tong University, School of Medicine, China demonstrated that SALL4 is overexpressed in BC cell line acquired resistance to doxorubicin hydrochloride. This study also suggested that knockdown of SALL4 inhibits proliferation of MCF-7/ADR (Adriamycin) cells through arresting the cell cycle in the G1 phase and that down-regulation of SALL4 reverses the drug resistance of BC by reducing the expression of ABCG2 and MYC[229]. So, in this chapter, we have explored for the first time the involvement of the SALL4 in acquired resistance to trastuzumab in HER2+ BC cells.

3.1.5 SALL4 in the microenvironment of HER2+ BC

Tumour cells exist near non-malignant cells. Extensive and multi-layered crosstalk between tumour cells and stromal cells tailors the tumour microenvironment (TME) to support survival, growth, and metastasis. The TME consists of different cell populations such as proliferating tumour cells, the tumour stroma, blood vessels, infiltrating inflammatory cells, and various associated tissue cells[230]. The tumour stroma consisted of the basement membrane, fibroblasts, extracellular matrix, immune cells, and vasculature. Fibroblasts are one of the largest populations of non-malignant host cells that can be found within the TME of the breast, pancreatic, and prostate tumours (**Figure 45**). Previous studies have identified cancer-associated fibroblasts (CAFs) originating from various cells types, including fibroblasts, endothelial cells, and vascular mural cells. Multiple independent studies have recently also implicated bone marrow-derived cells, most likely mesenchymal stem cells, as a considerable source of CAFs[231]. CAFs have a significant impact on cancer cells' behaviour, including proliferation, invasion, metastasis and chemoresistance in many ways. However, the underlying mechanism had not been fully elucidated.

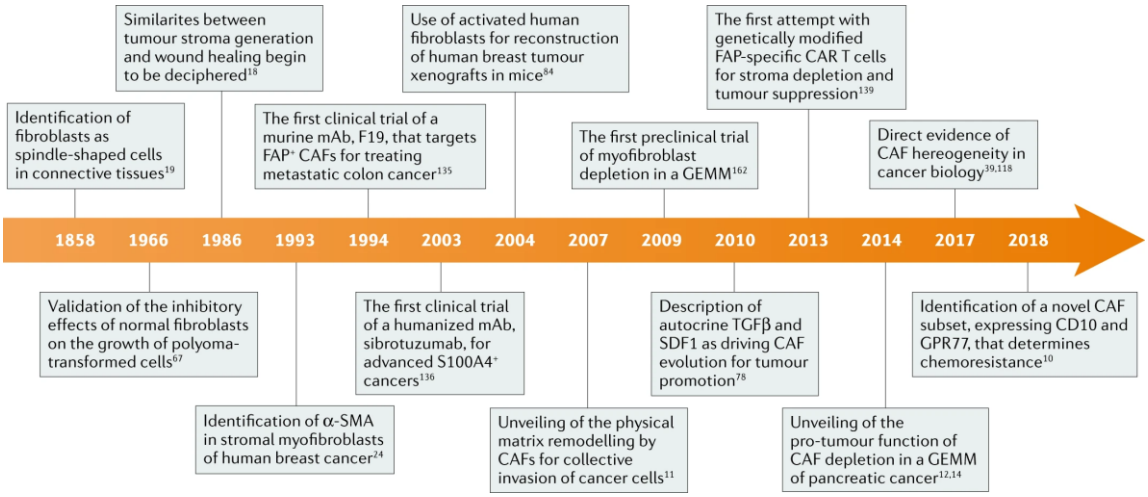


Figure 45: This timeline shows key developments and the changing focus of fibroblast research, particularly in association with cancers.

In recent years, it has been observed that SALL4 has a link with TME to facilitate tumour growth, progression and resistance to drugs. In 2018, for the first time, a research group from China stated that SALL4 and PDL1 are negatively correlated with miR-200c. Furthermore, they suggested that the hepatitis B virus (HBV) stimulates SALL4 production by activating STAT3. SALL4 directly suppresses miR-200c transcription by its transcriptional repressor function, subsequently leading to high PD-L1 expression due to destroying the CD8+T cell functions and helping in hepatocellular cancer (HCC) progression[232]. Additionally, there is evidence that in HCC, SALL4 was critical for regulating miR-146a-5p in exosomes and M2-polarization. Mechanistically, SALL4 could bind to the promoter of miR-146a-5p, and directly controlled its expression in exosomes. Blocking the SALL4/miR-146a-5p interaction in HCC reduced the expression of inhibitory receptors on T cells, reversed T cell exhaustion, and delayed HCC progression[220]. Also, in the same cancer type, another mechanism is that the inflammatory micro-environment promotes the stemness properties and metastatic potential of tumour cells. The researchers discovered that miR-497 directly targets *SALL4*, negatively regulates its expression, and further inhibits the self-renewal and metastasis of HCC. More importantly, inflammatory factor TNF-a inhibits the expression of miR-497 via NF-kB-mediated negative transcriptional regulation and simultaneously upregulates the expression of SALL4 and promotes the self-renewal and metastasis phenotypes of HCC cells[233].

So, taking all together above references, in this chapter, we have explored how SALL4 is also regulated by CAF isolated from human primary breast tumour and helps in acquired resistance.

3.2 Materials and Methods

Details of the material and methods have been described in Chapter 2, section 2.2. The remain methods have been elaborated below.

3.2.1 SALL4 overexpression plasmid transfection of BT474 cells and SKBR3 cells

BT474 and SKBR3 cells were grown to 70-90% confluence, and SALL4 plasmid and empty vector pcDNA3.1 (Addgene) were transfected with one ug/ml in the DMEM medium. Cells in the plasmid control group were transfected with vector only. Lipofectamine® 2000 reagent was diluted at 1:0.5 ratio in Opti-MEM medium (Gibco; Thermo Fisher Scientific, Inc.) and mixed well. The opti-MEM medium was used to dilute the DNA and to prepare a DNA master mix. Both mixture solutions were incubated for 5 mins in room temperature. Subsequently, Diluted DNA (1:1 ratio) was added to each tube of diluted Lipofectamine® 2000 reagent and incubated for 15 min at room temperature. Then, DNA-liposome complexes were added to the cells. Cells were incubated at 37˚C for 72 h, followed by extraction of cellular proteins and mRNA.

3.2.2 DRUGS assay

The WST assay is a colorimetric assay for assessing cell viability. We used this assay to evaluate the cell proliferation of FFW (peptide) in BT474 WT, BT474R and SKBR3 WT, SKBR3 R cells. We seeded cells in a 96-well plate at five $\times 10^3$ cells/well and incubated these for 24 h in 37 °C with 5% CO_2. We treated the cells with drugs in various concentrations and incubated the cells for a further 48, 72, 96, 120 and 168 h. For FFW, we used concentrations ranging from 1uM to 15 μM. After the designated duration of drug exposure, we aspirated the cell culture medium. We incubated the cells with 7%WST reagents diluted with phenol red-free DMEM for 3 h at 37 °C in 5% CO_2. We calculated cell viability by measuring the absorbance at 450-650 nm using a microplate reader (Infinite® M1000 Pro, Tecan Group, Switzerland) and comparing them with the control cells (treated with drugs carrier DMSO).

3.2.3 The patient's derived primary cell culture

HER2+ BC patient's samples were obtained from Hospital Clinico Universitario de Valencia, Valencia, Spain. Tissue samples were collected in a 50 ml falcon tube containing 15 ml media (DMEM-F12) with 1% antibiotics. Tissues were washed extensively with 1X PBS without centrifugation with 1% penicillin/streptomycin (Life Technologies, Carlsbad, CA, USA), for 3-4 times for every 10 mins. Tissue specimens were mechanically dissociated using a scalpel and transferred to an enzymatic solution of 3mg/ml collagenase 5ml (Thermofisher) and 5 U/mL Dispase (STEMCELL) 5ml, in a 37 °C water bath for at least 2-4 h, agitating every 10–20 mins. At the end of incubation, the same amount of DMEM-F12 supplemented with 10% FBS was added to stop the enzymatic reaction. The tumour cells were centrifuged at 1500 rpm for 5 mins to pellet down all the cells and tissue. Then the cells were cultured with DT-culture media (DMEM-F12 media, Gibco by life technologies) supplemented with 10% FBS, penicillin/streptomycin (1%), L- Glutamine (1%)) in t25 flask and incubated with 5% CO_2 at 37 °C. Viable tumour cells attached to the flask within 12–24 h. At the first medium change, rather than discarding medium containing unattached cells that may grow and provide a backup culture, put this into a new flask as some of them contain organoids. Cultures at 75% to 100% confluence were selected for subculture by trypsinization with 0.05% trypsin-EDTA. The culture medium was changed twice a week, and cellular morphology evaluated microscopically every 24–48 h. When possible, early passage and late passage, primary cultures were frozen in 90% FBS and 10% DMSO and stored in liquid nitrogen for further experiments.

3.2.4 Characterization of Primary cell lines

The monolayer primary cells are needed to characterize to obtain a different population of the cells. Cells growing in 6-well plates were trypsinized and collected in a tube. After counting the cells, 100,000 cells were put in another small Eppendorf, and 500 ul of PBS was added. Cells were then centrifuged at 1500 rpm for 5 mins. Pellet down cells were resuspended with Flow Cytometry Staining Buffer (00-4222-57, Thermofisher). Soon after, different antibody types were labelled with cells (CD36 for mesenchymal stem cells, CD326 for epithelial cells, CD140a for cancer-associated fibroblast, CD24 and CD44 for BC stem cells) and incubated for 30 mins in 4 °C in the dark. Following that additionally, 500 ul of Flow Cytometry Staining Buffer was added

to the Eppendorf and acquired and the data were analysed by FACSVerse™ flow cytometer (BD Biosciences). Cells were initially gated based on forward versus side scatter to exclude small debris, and ten thousand events from this population were collected. Control cells were treated with appropriate isotype conjugated IgG (BD Biosciences).

3.2.5 Collection of Conditioned Medium

For the experiments with conditioned media, HER2+ breast primary cells were plated in 6 well plates until sub confluence; fresh complete culture medium was added, and cells were incubated with 2 ml of medium for 72 h. The supernatant was recovered, filtered through a stericup (pore size 0.45 μm; Millipore; Billerica, MA, USA), and centrifuged (1,200 rpm for 5 minutes) to discard unattached cells and cell debris. The conditioned medium was aliquoted and stored at -80 °C for later experiments in future.

3.2.6 Cell viability studies with condition medium

To determine the concentration of condition medium for experiments, the HER2+ differentiated cells such as BT474, SKBR3 were seeded on 96 well plates (5000 cells per well). After 24 h of the different seeding concentration (5%-100%) of the cm diluted with 2%, FBS media were treated with the cells for different periods. 2% FBS with media was taken as a control for these experiments.

3.3 Results

3.3.1 Assessment of the efficacy of trastuzumab in HER2-positive BC cell lines

The trastuzumab-conditioned BT-474R and SKBR3R cell lines were established by culturing the BT-474 and SKBR3 cell lines in the appropriate medium supplemented with 15 μg/ml of recombinant humanized monoclonal HER2 antibody, trastuzumab (Herceptin, Genentech, USA). Trastuzumab was dissolved in sterile water at a stock concentration of 20 mg/ml. Simultaneously, the parental lines were grown without treatment to maintain their sensitivity to the drug intact to be employed as procedural controls. Once the establishment of resistance was confirmed, the cells were kept at a 15 μg/ml maintenance dose. These results are adopted from a group of Department of Pathology, IIS-Fundación Jiménez Díaz, Madrid, Spain, and they have given us the resistance cell lines for our experiments[234]. Two trastuzumab-resistant populations were

generated from each sensitive cell lines such as BT474-BT474R and SKBR3-SKBR3R. Sensitivity and resistance to trastuzumab were assessed in both cell lines by testing cell proliferation in the presence and absence of 15 µg/ml trastuzumab for seven days (**Figure 46 A-B**). Both the medium and the drug were replenished every three days. On the other hand, the cells' morphological characteristics remained identical between the parental and the equivalent resistant cell lines (**Figure 46 C-D**).

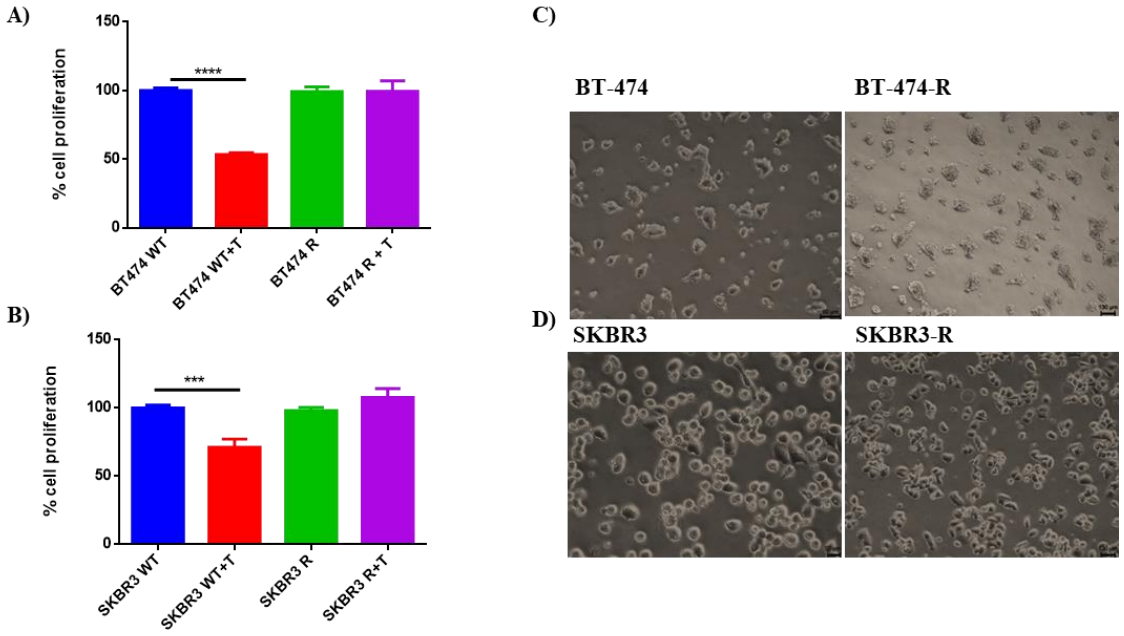


Figure 46: The effect of trastuzumab in cell proliferation and morphology: (A, B) The effect of trastuzumab on the BT-474 and SKBR3 (parental and acquired resistance) cell lines for seven days exposure to trastuzumab 15 µg/ml. Every trastuzumab-treated condition was compared to its corresponding non-treated one (parental/wild type) phase-contrast images showing cultured monolayers of parental and trastuzumab-resistant cells for both BT474-BT474R and SKBR3-SKBR3R. Morphological characteristics did not differ between sensitive and resistant cells of the same line. Student's t-test was used to analyse the significant differences. *****P ≤ 0.001, **** P ≤ 0.0001.**

3.3.2 SALL4 expression in sensitive vs resistance HER2+ BC cell lines

The quantitative RT-qPCR data revealed that the *SALL4* expression was significantly lower in the BT474-WT as compared to BT474-R at mRNA and the protein level (**Figure 47 A-B**). Additionally, the same patterns were observed in SKBR3-WT compared SKBR3-R protein level. Still, in mRNA level, the expression of *SALL4* was not significantly different (**Figure 47 C-D**). The observation showed that SALL4 expression in acquired resistance cell lines was much higher than the wild type, which

concluded that SALL4 might involve in the molecular mechanism of acquired resistance in HER2+ BC cell lines.

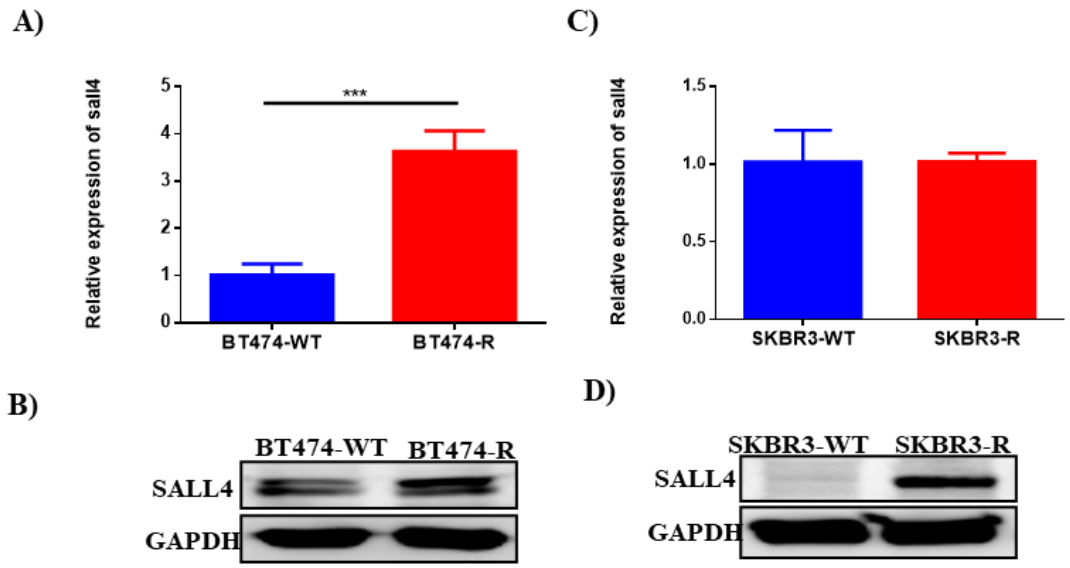


Figure 47: Expression of SALL4 in sensitive and acquired resistance HER2+ breast cell lines: The relative expression of the SALL4 was determined in HER2+ cancer cell lines both in parental and acquired resistance cell lines of BT474 and SKBR3 (A, C). SALL4 protein expression was determined by western blot in mentioned cell lines (B, D). Student's t-test was used to analyse the significant differences. *****P ≤ 0.001.**

3.3.3 Expression of SALL4 in HER2+ BC patients and PDX samples

SALL4 is aberrantly overexpressed in various malignant tumours, such as gastric cancer, lung cancer and AML. Less has been explored in BC and its relation to resistance. So, SALL4 expression was determined in different BC patients comprised 18 clinical primary tissue samples, of which 7 were responder, and 11 were non-responded to treatment, i.e., trastuzumab. It showed that the appearance of SALL4 is higher in non-responding patients to trastuzumab /metastatic compared to the responder (**Figure 48A**). To support these results, we obtained PDX (Patients derived xenograft) samples from trastuzumab non-responder patients. A total of 6 PDX samples originated from one non-responder patient, denominated as PDX1, PDX2, PDX3, PDX4, PDX5, and PDX6 respectively. PDX1 is only one PDX sample, of which the original tumour was derived from a responding patient to trastuzumab. When we analysed the expression of SALL4 in different PDX tumour sample derived from one responding patient and one non-responding patient to trastuzumab, we found that SALL4

expression was higher in 5 of the PDX samples, especially (PDX2, PDX3, PDX5) as compared to responder PDX sample (PDX1) (**Figure 48B**). These data collectively seemed to suggest that up-regulated SALL4 may be implicated on aggressiveness and a key regulator of trastuzumab resistance in HER2+ subtype.

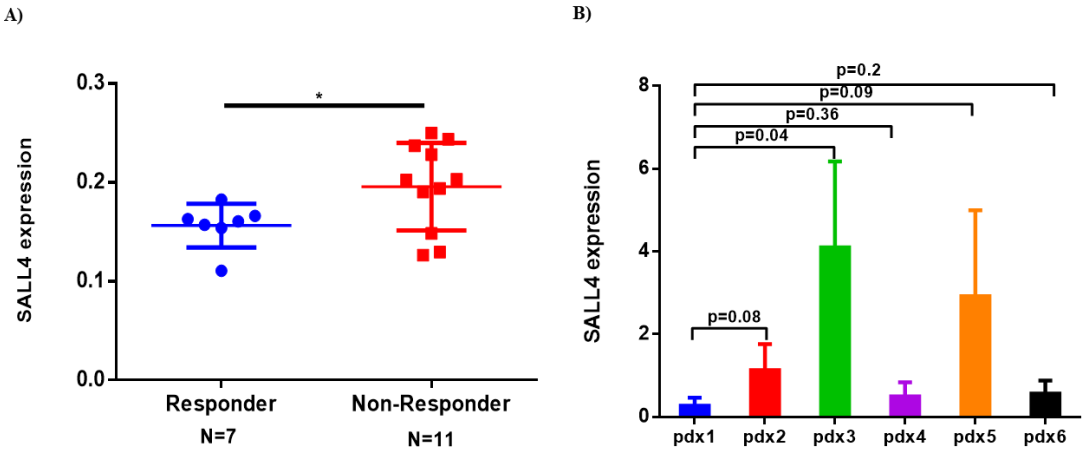


Figure 48: Expression of the SALL4 in BC patients and PDX samples: The relative expression of SALL4 was determined by RT-qPCR in HER2+ cancer tissue responding to trastuzumab, non-responding to trastuzumab (A). Relative SALL4 expression was determined in different PDX samples compared to one responder control. Student's t-test was used to analyse the significant differences (B). ***P ≤ 0.05.**

3.3.4 SALL4 expression mRNA analysis in a public database (TCGA)

As it observed that SALL4 expression is related to trastuzumab resistance, we aimed to know the expression of SALL4 in different molecular subtypes of BC in basal level. From the TCGA database, we obtained 523 numbers of specimen details; others had missing SALL4 expression data, no follow-up data, or missing clinical information. Among them, there were Luminal B (n= 127), Basal-like (n= 98), Luminal A (n= 231), HER2+ (n= 58) and normal-like tissue (n=9), which showed high expression of SALL4 in HER2-enriched tissue samples following Luminal-A, Luminal B and basal-like subtypes (**Figure 49**). The low-level expression was observed in normal-like subtypes, which suggested that high SALL4 expression is required to drive HER2+ cancer progression and induce resistance to treatments.

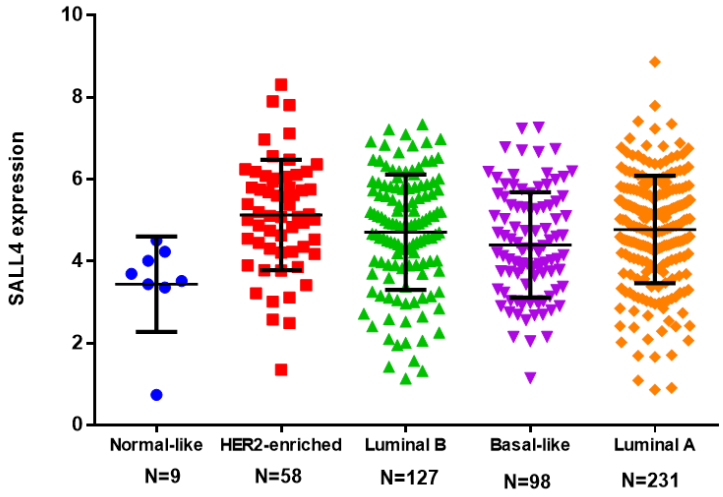


Figure 49: *SALL4* expression is indicated in BC tumour or normal like tissues from TCGA dataset: RT-qPCR analysis indicated the *SALL4* expression levels in five main intrinsic or molecular subtypes of BCs tissues from TCGA dataset.

3.3.5 Survival analyses for SALL4 expression in BC patients and HER2+ BC patients.

The overexpression of SALL4 in BC tissue was highly correlated with poor prognosis and disease relapse. As we observed the SALL4 expression is higher in resistance in in-vitro and in patient's samples, we analysed the OS, RFS and DMFS in all type of cancer and specifically in HER2+ subtype of cancer. RFS showed different results, suggesting that high expression of SALL4 extends disease-free survivability in ALL subtypes while in HER2+, it has opposite results (As expected) (**Figure 50 A, D**). When looking at the overall survival, we noted that in both ALL and HER2+, the more expression of SALL4 related to less survival significantly, which suggested that SALL4 might act as tumour oncogene, but its involvement in relapse-free survival is subtype-specific (**Figure 50 B, E**). DM-free survival (DMFS) was defined as the duration between primary local recurrence and distance metastasis. While analysing the DMFS in km plotter, we noticed that the SALL4 had followed the same like RFS (**Figure 50 C, F**). These results explained that SALL4 could act as an independent predictor of the development of metastases, relapse of the diseases and patient's survival in breast HER2+ subtype.

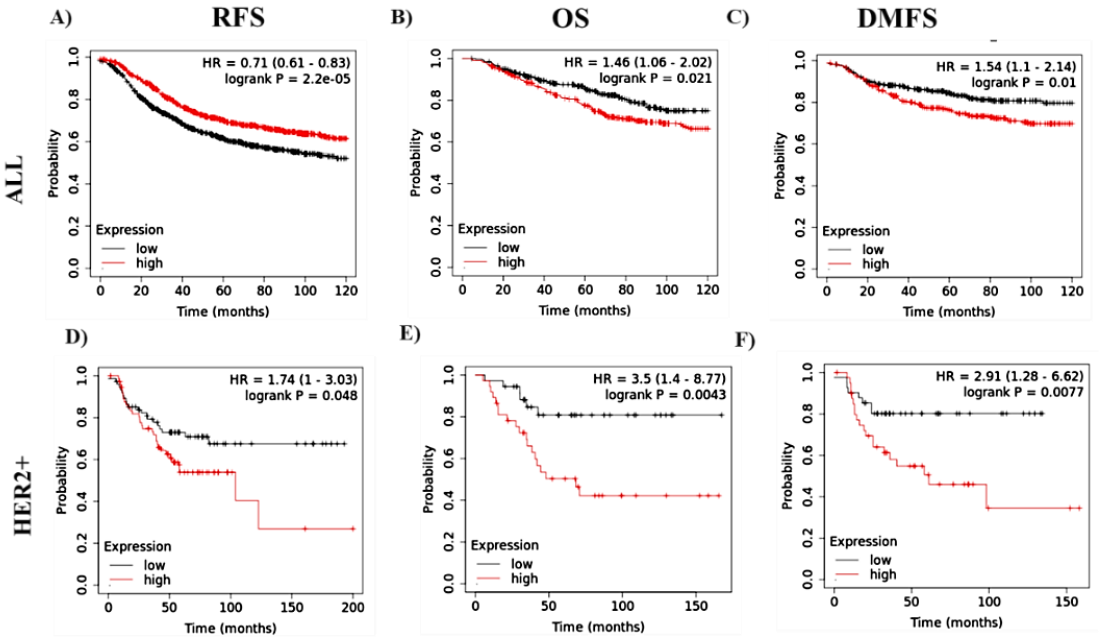


Figure 50: Prognostic value of SALL4 expression in BC patients: *In silico* relapse-free survival (RFS), overall survival (OS) and distant metastasis-free survival (DMFS) of SALL4 in BC patients (A-C) and HER2+ subtypes patients (D-F) with Kaplan-Meier Plotter.

3.3.6 Knockdown and overexpression of *SALL4* in HER2+ WT/R cell lines using silencers and plasmid.

Previous results showed that high expression of SALL4 is being involved in trastuzumab resistance and progression of human HER2+ BC. It is crucial to know the gene's implications in cell proliferation and other phenotypic properties in human HER2+ cell lines to explore the molecular mechanisms. For this purpose, one of the strategies employed in this study was the overexpressing SALL4 in sensitive cell lines by plasmid and silencing of the SALL4 in resistance cell lines by specific siRNA for the SALL4. After transfection with plasmid and silencer in respective cell lines with negative control for each condition, the efficiency of transfection was checked of mRNA by RT-qPCR from 48 h -96 h and in protein level at 72h (**Figure 51 A-H**). Unitedly, these results suggested that the commercially avail plasmid and silencer have been effectively overexpressed and silenced the mRNA and proteins of SALL4 in HER2+ BC cell lines so that it is sufficient to check the functional effect on cells.

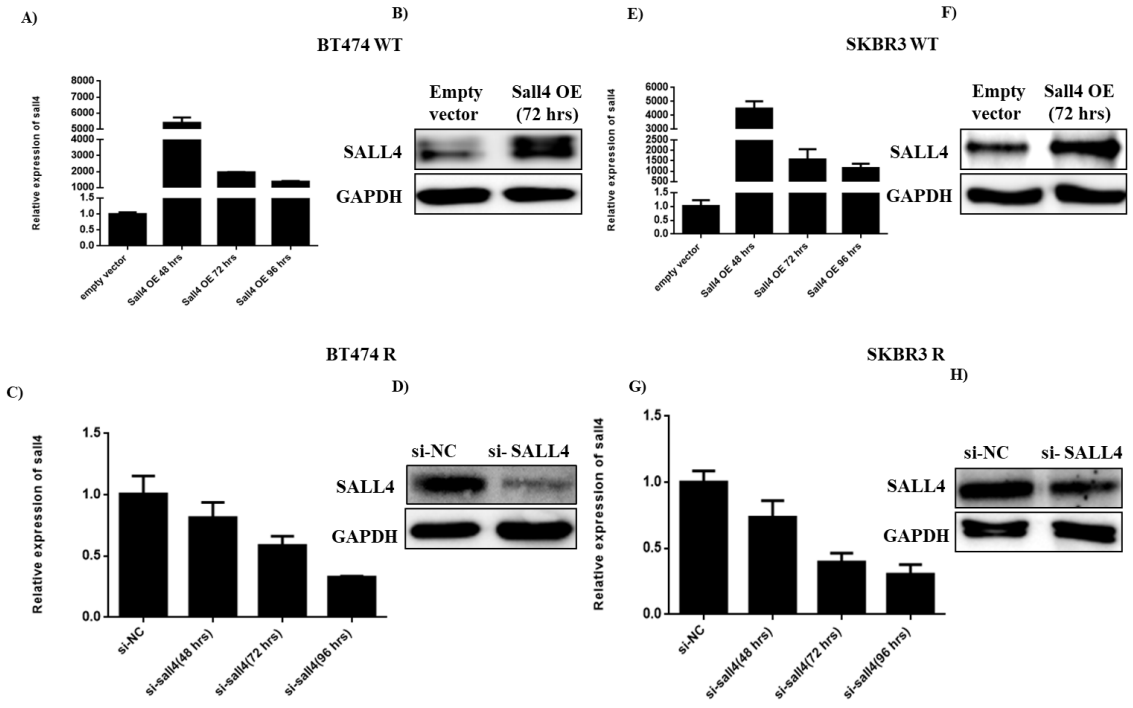


Figure 51: SALL4 expression after plasmid and siRNA transfection in HER2+ cell lines: RT-qPCR (A, E) and Western blot (B, F) results showing the overexpression efficiency of a plasmid (pcDNA3.1+/SALL4) for SALL4 in both sensitive cell lines such as BT474WT and SKBR3 WT. The relative expression of SALL4 using a specific silencer resulting in satisfied downregulation of the expression at mRNA (C, G) and protein (D, H) level in both acquired resistance cells lines such as BT-474R and SKBR3 R.

3.3.7 Silencing *SALL4* partially restores trastuzumab sensitivity in acquired resistance cell lines

Drug resistance is a significant obstacle that affects the overall survival rate for advanced and recurrent HER2+ BC patients. We sought to analyze whether SALL4 in BC cells participated in mediating cellular resistance to trastuzumab. It was revealed by cell viability assay that an ectopically increase expression of SALL4 in BT474 and SKBR3 parental cells make cells less sensitive to trastuzumab than control (empty vector) at seven days (**Figure 52 A-B**) due to high proliferation of the cells, as a primary effect of overexpression of SALL4. Meanwhile, downregulation of SALL4 in BT474-R and SKBR3-R through two different silencers showed a significant decrease in proliferation and more responding to trastuzumab treatment than the negative control of the silencer (**Figure 52 C-D**). In summary, these findings indicate that SALL4 is

involved partially in drug resistance in HER2+ BC cells and helps cells to escape from treatments.

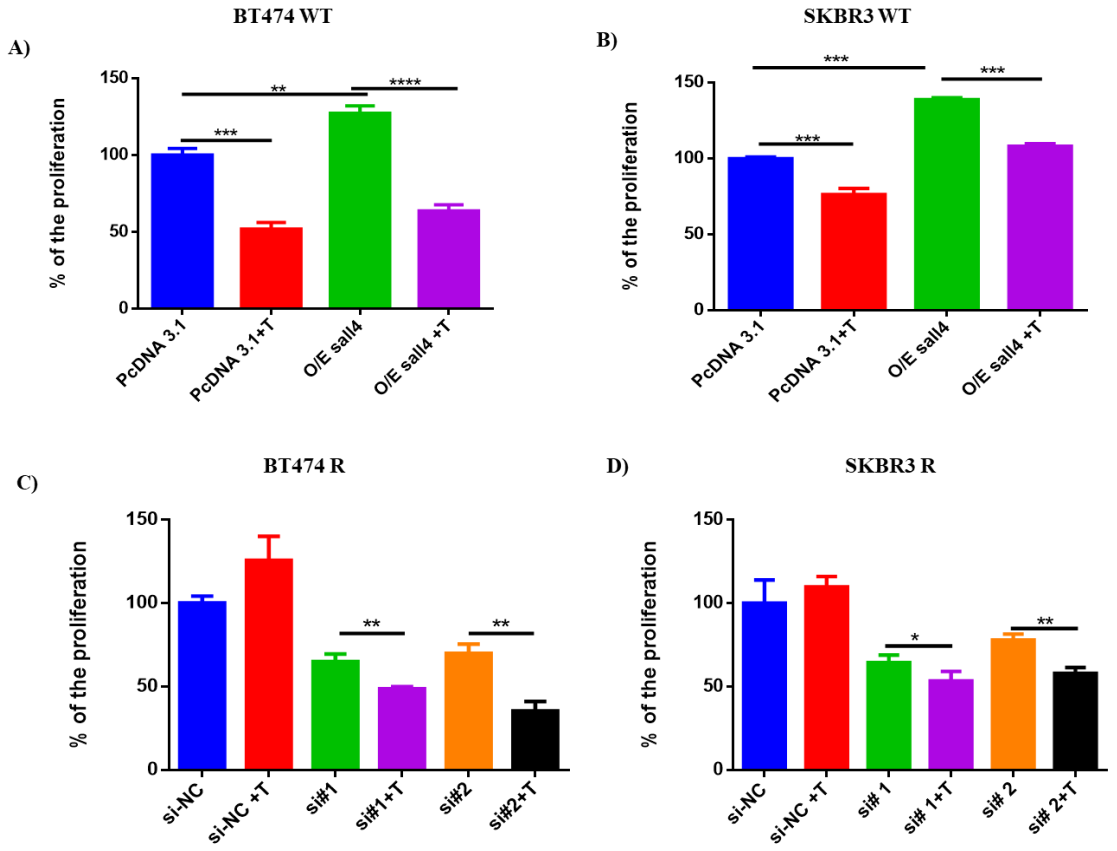


Figure 52: Assessment of trastuzumab efficacy through regulation of SALL4: The proliferation of BT474 and SKBR3 parental cells with overexpression of SALL4 and treatment with trastuzumab (A-B) was measure by WST assay. The proliferation with silencing of SALL4 by two different silencers in both acquired resistance cell lines; BT474-R and SKBR3-R was also determined with presence of trastuzumab by WST assay (C, D) The graph shows the results of three independent experiments, run in triplicate. The experiments were carried out to 7 days. Student's t-test compared the results. ***P ≤ 0.05, **P ≤ 0.01, ***P ≤ 0.001 and **** P ≤ 0.0001.**

3.3.8 SALL4 follows the PI3K/AKT/PTEN pathway for partially restore the trastuzumab effect.

One of the most potent signalling pathways promoted by HER2 overexpression is the phosphatidylinositol PI3K/AKT/PTEN signalling cascade, which affects cell cycle progression and can inhibit apoptosis. It has been reported that activated AKT can be an indicator of poor prognosis, possibly promoting cell survival. To regulate AKT activity, PI3K is opposed by PTEN, which converts PIP3 back to PIP2, thus preventing

phosphorylation and activation of AKT. As a result, cellular proliferation is inhibited, and tumour formation is suppressed. On the other hand, *Lu et al.* showed that SALL4, as a transcription factor, repressed PTEN expression by binding to the promoter regions in intrahepatic cholangiocarcinoma[235]. So, taking together, we hypothesized that SALL4 might play an essential role in regulating trastuzumab resistance through the PI3K/AKT/PTEN pathway. We overexpressed the SALL4 ectopically in parental cell lines of BT474 and SKBR3, which showed the results that, with the overexpression of SALL4 activates PI3K and AKT as an outcome of repression of PTEN expression (**Figure 53 A-B**). When AKT phosphorylates, it activates many downstream targets, such as BCL2, resulting in increased cell survival and proliferation. In contrast, in acquired resistance cell lines BT474R and SKBR3R ectopically down-regulation of SALL4 represses PI3K and AKT's activation and overexpress PTEN along with downregulation of BCL2 (**Figure 53 C-D**). These results suggested that SALL4 regulates HER2+ pathway through a complicated intracellular pathway PI3K/AKT/PTEN, which leads to cell growth, tumour proliferation, escape from trastuzumab and plays a significant role in BC progression and resistance.

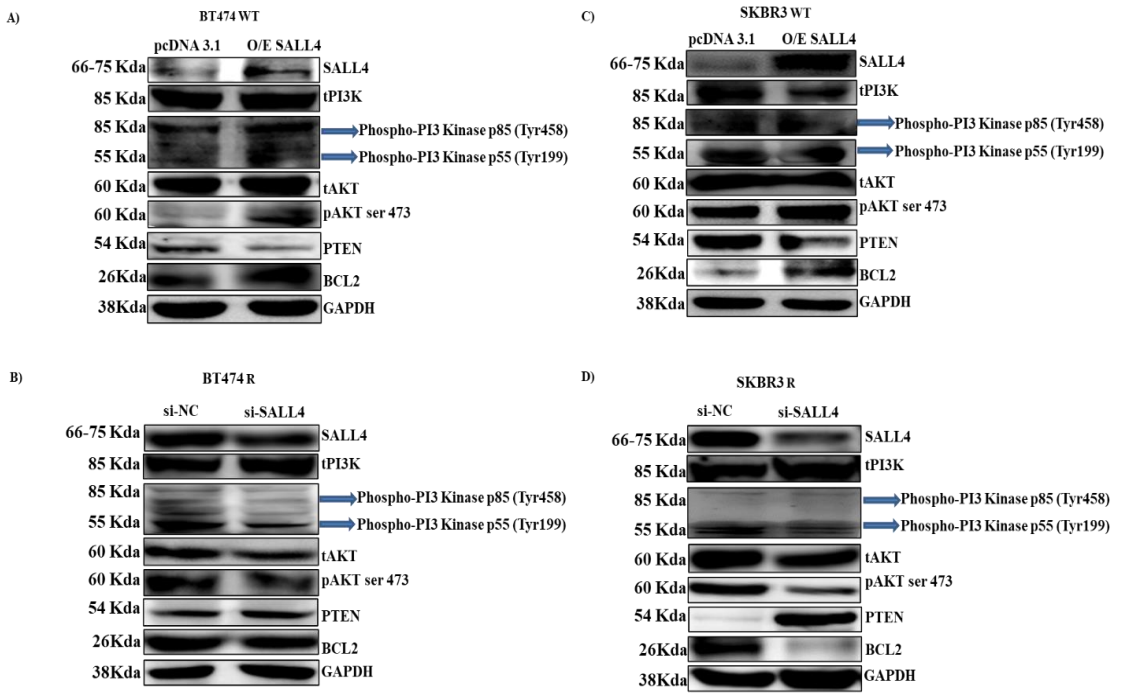


Figure 53: Western blotting of PTEN and PI3K/AKT pathway-associated proteins: Expression levels were analysed by western blotting following 72 h of transfection of plasmid for overexpression of SALL4 in parental cell lines and si-RNA for down-regulation of SALL4 in acquired resistance cell lines.

3.3.9 SALL4 promotes EMT: a crucial regulator of drug resistance

EMT transcription factors might induce enhanced phenotypic plasticity that would allow HER2+ BC cells to "enter" into and "exit" dynamically from trastuzumab-responsive stem cell-like states. Considering this hypothesis and being SALL4 as a transcription factor which plays an essential role in maintaining stem cell-like states, we hypothesised to investigate EMT markers in all cell lines with the gain-loss expression of SALL4. Further, we ectopically overexpressed and downregulated SALL4 in wild type and resistance cell lines. This results section has used two different siRNAs specifics to reducing SALL4 expression in transfected cell lines. While SALL4 is overexpressed in wile type cell lines, the EMT markers such as E cadherin (CDH1) have significantly reduced its expression compared to empty vector. Whereas mesenchymal markers expression like β-catenin (CTNNB1), Vimentin (VIM) and Fibronectin (FN1) and also MYC an essential transcription factor for essential to regulate EMT and/or CSCs, have increasing acceptably (**Figure 55 A, B, E, F**). Meanwhile, we observed the adverse effect in resistance cell lines, when we silenced the SALL4 expression by two different silencers (**Figure 55 C, D, G, H**). In summary, our results uncover that overexpressing of SALL4 promotes EMT, which is required for the cells to make them follow the resistance pathway in the future.

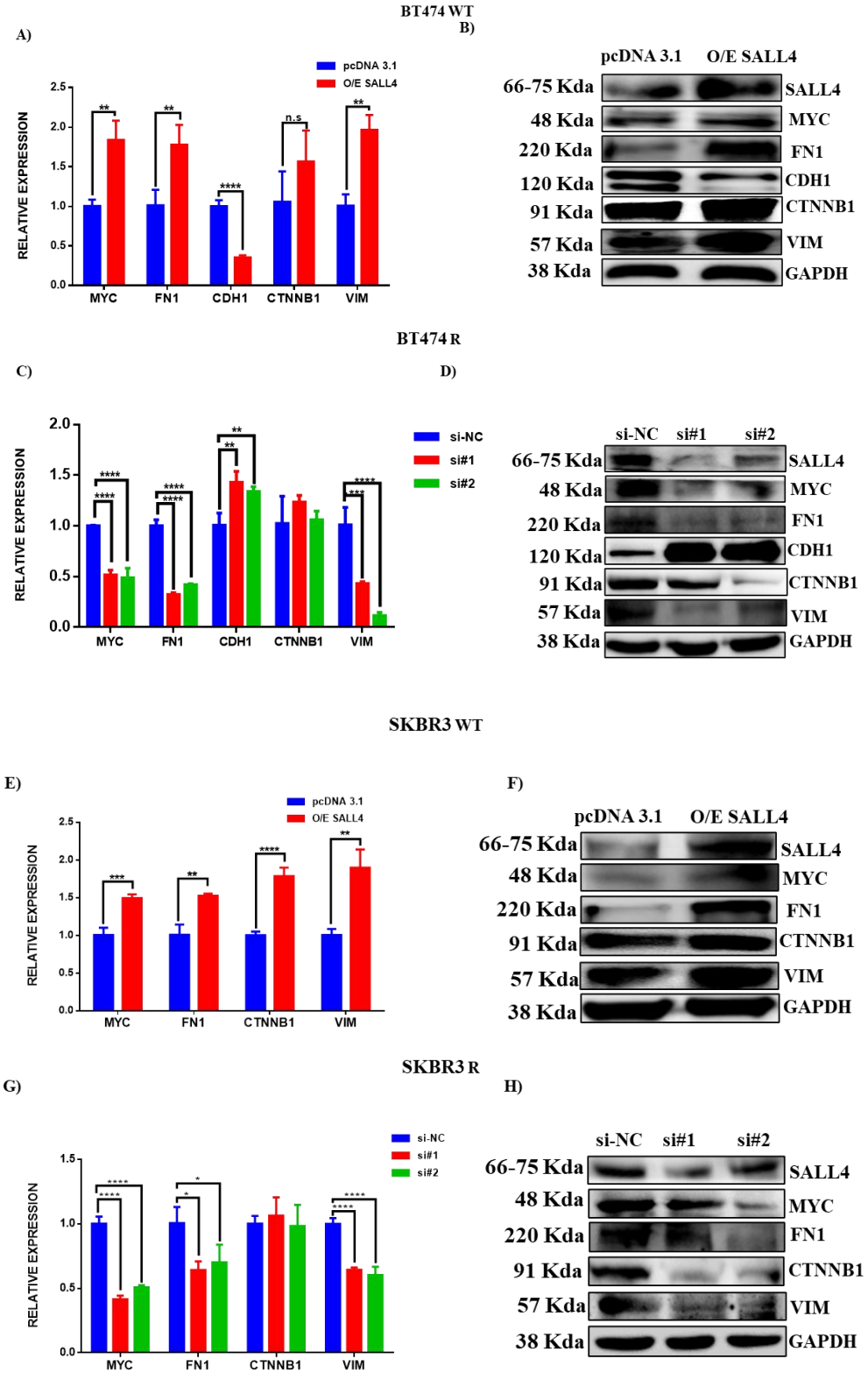


Figure 54: SALL4 regulates EMT in HER2+ BC cells: RT-qPCR and western blot results showing EMT marker levels after overexpressing and silencing sall4 in parental cells (A, B, E, F) and acquired resistance cells (C, D, G, H). Student's t-test was used to analyse the significant differences. ***P ≤ 0.05, **P ≤ 0.01, ***P ≤ 0.001 and **** P ≤ 0.0001.**

3.3.10 NURD complex can play an essential role in resistance.

In mammalian cells, there are two major histone deacetylation complexes, Mi-2/NuRD and Sin3. These two protein complexes share four common components, HDAC1, HDAC2, RbAp46 and RbAp48. Mi-2/NuRD complex additionally includes Mi-2, MTA-1, MTA-2, p66 and MBD3. Among these eight factors, HDAC1 and HDAC2 are histone deacetylases, RbAp46 and RbAp48 are histone binding proteins, and Mi-2 is a chromatin-remodelling ATPase. Therefore, histone deacetylation and chromatin-remodelling ATPase activities are uniquely linked in this single protein complex Mi-2/NuRD, whose function is to produce compactly packed, hypoacetylated nucleosomes that switch an active, hyperacetylate promoter to its inactivated state[236]. NuRD has also been implicated in regulating transcriptional events that are integral to oncogenesis and cancer progression. SALL4 has been reported to act as a repressor by interacting with the epigenetic HDAC / NuRD complex. It is suggested that the oncogenic role of SALL4 in cancer development may be due in part to its repressive role in the tumour suppressor PTEN. In hepatocarcinoma, SALL4 has been shown to physically interact with retinoblastoma 4-binding protein (RBBP4), part of the NuRD complex, and that a specifically designed peptide is capable of antagonizing the SALL4-NuRD interaction[237]. We emphasized that the NuRD complex might be involved in HER2+ BC progression and trastuzumab resistance based on the present hypothesis. All the NuRD complex members' protein expression shows different expression in sensitive vs resistance of BT474 and SKBR3 (**Figure 55 A-B**). The mediator, who plays an essential role between SALL4 and NuRD complex interaction and cancer progression in different types of cancer is RBBP4[237]. Therefore, when we tried to check the gene and protein expression of RBBP4 in both resistance and sensitive cell lines of HER2+ subtypes, we found that RBBP4 expression is higher in resistance as compared to sensitive in both levels (**Figure 55 C-D**). This result has also been confirmed by microarray (Dr Federico Rojo, laboratory, Molecular Pathology at the Jiménez Díaz Foundation, Madrid). This also suggested that the RBBP4 expression is higher in resistance cell lines than the parental cell line (**Figure 55E**). All together above data suggested that interaction of SALL4 and RBBP4 (NuRD complex) is required for trastuzumab resistance cell lines, where they can inhibit the expression of PTEN and activates PTEN/PI3K/AKT pathway to assist cancer cells in surviving.

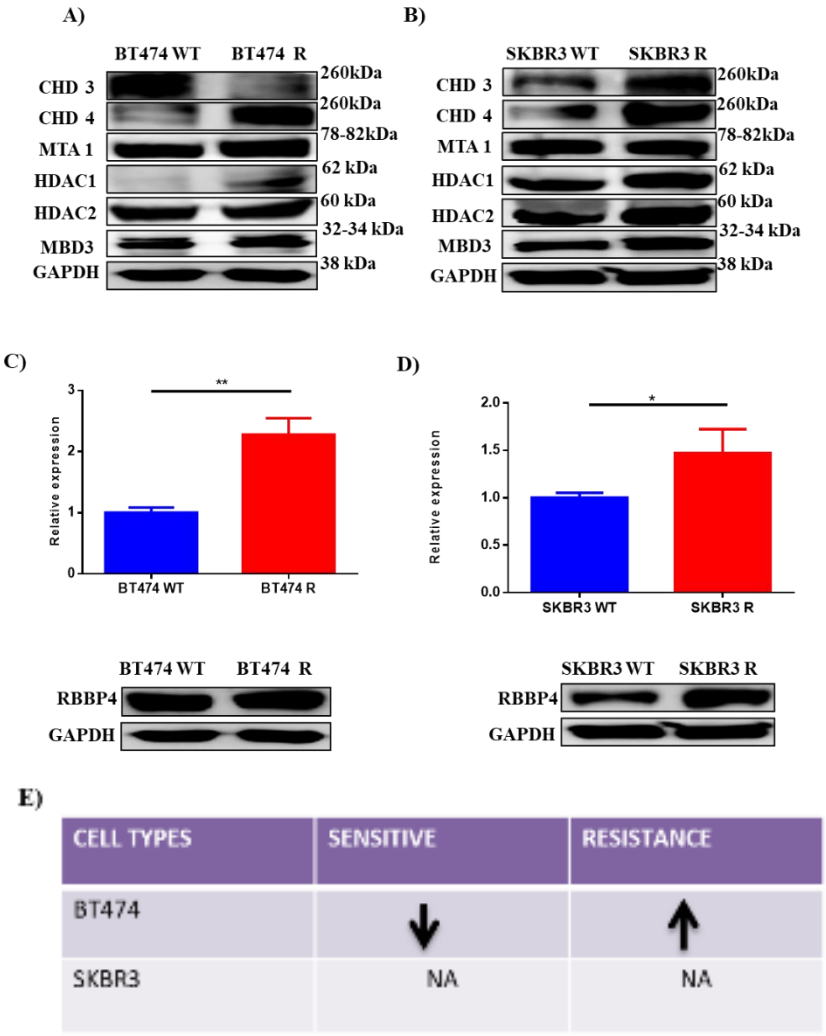


Figure 55: Expression of the NuRD complex in HER2+ cancer cell lines and BC patients: All the units of NuRD complex basal level expression were measured by western blot in BT474 WT and BT474 R (A, B). Basal level expression at mRNA and protein level of RBBP4 were measured by qRT-PCR and western blot in both parental cell line and acquired resistance cell lines (C, D). Microarray data were analysed of both sensitive and resistant cell lines to evaluate the expression of RBBP4 (E). Student's t-test was used to analyse the significant differences. ***P ≤ 0.05, **P ≤ 0.01.**

3.3.11 Analysis of RBBP4 mRNA expression in the TCGA database

After analysing the RBBP4 expression in HER2+ cancer cell lines, we intended to know the expression of RBBP4 in different molecular subtypes of BC in basal level collected from the TCGA database. We could obtain 976 numbers of specimen details; others had missing RBBP4 expression data, no follow-up data, or missing clinical information. Among them, there were Luminal B (n= 194), Basal-like (n= 142), Luminal A (n= 434), HER2+ (n= 87) and normal-like tissue (n=119), which showed high expression of RBBP4 in almost all molecular subtype of BC except in normal-like subtype (**Figure**

56). The low-level expression in normal-like subtype suggested that high RBBP4 expression is required to drive HER2+ cancer progression along with inducer of resistance to treatments and also helps SALL4 in the aggressiveness of the different BC subtypes.

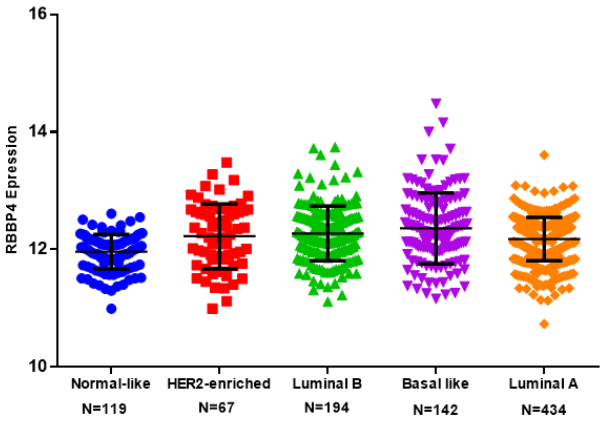


Figure 56:RBBP4 expression is indicated in BC tumour or normal tissues from TCGA dataset: RT-qPCR analysis indicated the SALL4 expression levels in five main intrinsic or molecular subtypes of BCs tissues TCGA dataset.

3.3.12 Positively regulation of the NuRD complex through SALL4 might play an essential role in HER2+ resistance cell lines.

According to the previous results, we observed NuRD complex is overexpressing in resistance cell lines, and it interacted with *SALL4*. Therefore, we shouted to know NuRD complex expression while regulating *SALL4* expression in wild type and resistance cell lines. While overexpressing SALL4 in parental cell lines and silencing SALL4 in resistance cell lines of BT474 and SKBR3, showed a modulation in NuRD complex proteins. It has been observed that expression of RBBP4, MTA1, MBD3 and HDAC1 has positively regulated by SALL4 gain-loss expression (**Figure 57 A-D**). It is already described before that, SALL4 acts as a repressor of PTEN by recruiting the NuRD complex. So, we also found that while there are changes in NuRD complex, it is also affecting the PTEN expression, which is a direct target of the NuRD complex and might have a secondary effect on PTEN/PI3K/AKT pathway. The vice versa is happening in the silencing of SALL4 in resistance cell lines. This novel hypothesis links interaction between SALL4 and NuRD complex, which is required to suppress PTEN expression and activate AKT pathway, which is one of the critical pathways in HER2

signalling trastuzumab mechanism of action as mentioned above. So, altogether this data showed a novel molecular mechanism of trastuzumab resistance in HER2+ subtype of cancer.

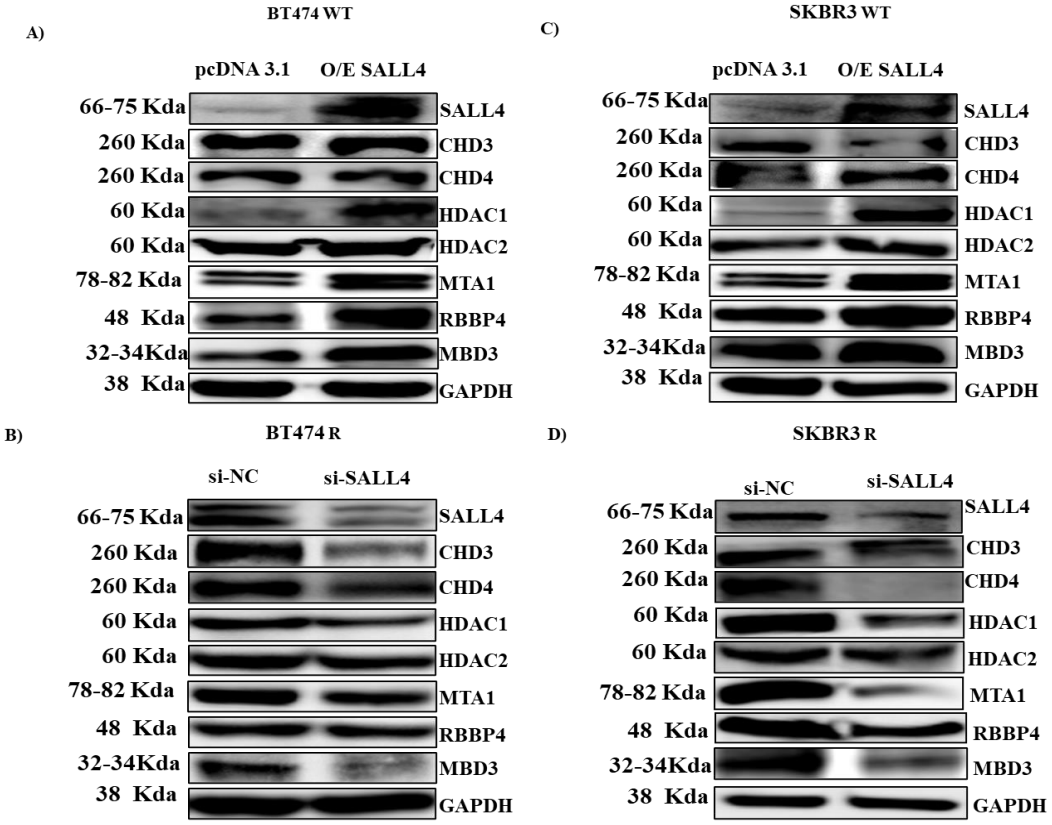


Figure 57: The modulation of the NuRD complex through SALL4: Overexpression and downregulation of the SALL4 in parental and acquired resistance cell lines showed the different expression pattern of NuRD complex core components through western blot (A, B, C, D).

3.3.13 The peptide FFW has an effect on cell proliferation on HER2+ BC both resistance and WT cell lines

From the above results, we found that SALL4 acts as a repressor of PTEN by recruiting the nucleosome remodelling deacetylase (NuRD) complex in both WT and resistant cell lines. The retinoblastoma binding protein 4 (RBBP4) is a subunit of NuRD. It is a WD40 repeat-containing protein, which consists of a seven-bladed β-propeller domain. In NuRD, RBBp4 acts as a chaperone in nucleosome assembly by bringing together histones H3 and H4 onto newly replicated DNA[238]. It has also been proved that SALL4-NuRD complex interaction happened for RBBP4. Therefore, there is an urgent need to explore alternative approaches for the treatment of this deadly disease. SALL4, however, falls into the class of what is termed as “undruggable” targets, as a nuclear

factor lacking a typical, druggable pocket for inhibitor binding. In this report, we discovered that the SALL4– NuRD interaction offers an intriguing potential therapeutic target by a novel peptide called "FFW" (PEN-FFW: RQIKIWFQNRRMKWKK-RRKFAKFQWI). Here we have shown that with the treatments of this FFW peptide, we were able to reduce the proliferation of HER2+ BC in both resistance and wile type of BT474 and SKBR3.

We found that IC50 of the drugs are changing mostly in BT474S-R compared to SKBR3 S-R, where BT474 is a Luminal B/HER2+ subtype, and SKBR3 is pure HER2+ subtype. There is different IC50 in BT474 wt (**13.4 um in 96 h**) vs r (**6 um in 96 h**). It is demonstrated that in resistance, the interaction between SALL4-RBBP4 is stronger than the wild type in BT474. In SKBR3 wt vs r, there are mild changes in IC50 (**Figure 58 A-D**). These results suggested that might this peptide increase the efficacy of the trastuzumab in acquired resistance cell lines. So synergetic study is required with trastuzumab in both parental and acquired resistance cell lines.

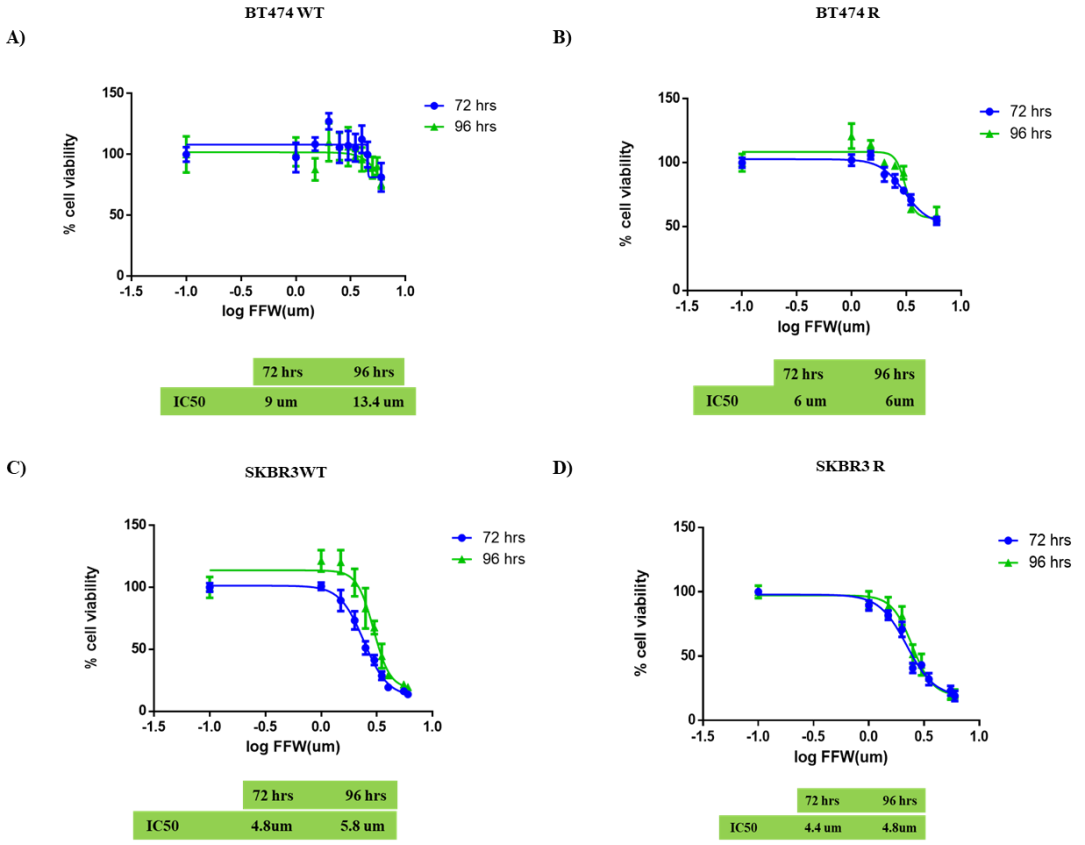


Figure 58: IC50 Values for the treatment of FFW on HER2+ cells lines: WST assay was carried out on both parental cells and acquired resistance cells. Different FFW concentrations were treated on both kinds of cell lines for 72 h and 96 h. All values are averages of replicates expressed relative to cell

viability values in untreated cells normalized to 100%. IC50 of the FFW was calculated through GraphPad prism.

3.3.14 Mir-33b targets SALL4 based on cell type-specific.

According to literature, miR-33b (results of its evaluation in HER2+ shown in chapter 2) can target SALL4 and reduces its expression, which resulted in the reduction of stemness and invasion of cancer cells[194]. When we checked in our HER2+ BC model, we found that with the ectopic overexpression of miR-33b has reduced the mRNA *SALL4* expression only in BT474 but not in SKBR3 (**Figure 59 A-B**). Nonetheless, we tried different bioinformatics tool to check the physical interaction of miR-33b and SALL4. Still, nowhere we did not find any convincing results but in the Freiburg RNA tools to predict the interactions between miR-33b and SALL4, which showed a weak physical interaction with less yield of energy -2.94329 kcal/mol (**Figure 58C**). Collectively, these results suggested miR-33b might target SALL4 but not in all types and subtypes of cancer. It acts on cell type-specific.

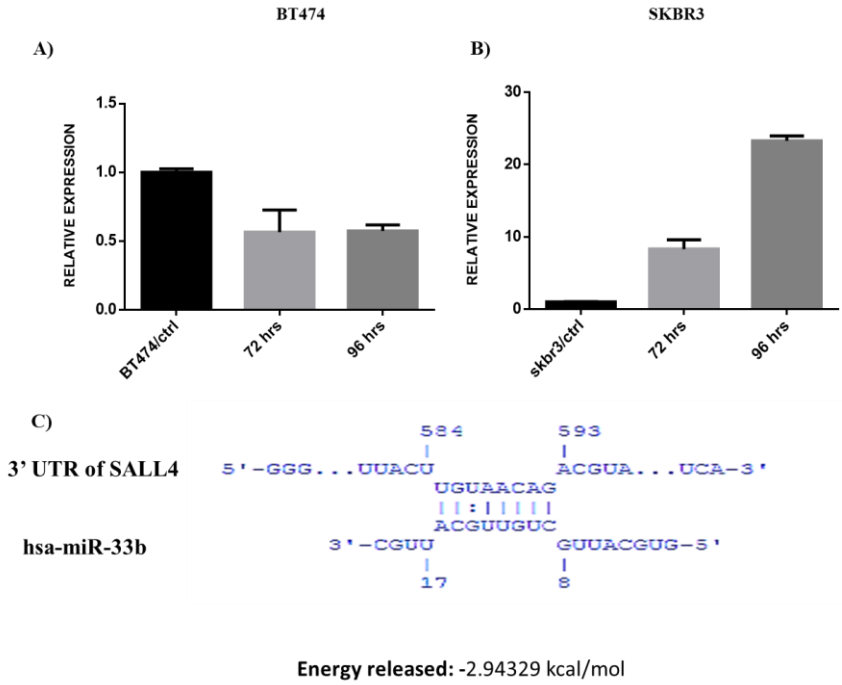


Figure 59: miR-33b and SALL4 interaction is cell type-specific: SALL4 expression was analysed at mRNA level by qRT-PCR in BT474 and SKBR3 parental cell lines post-transfection with miR-33b mimic (A-B). Schematic description of the hypothesized interaction between the SALL4 3´UTR binding site from 584-593 with miRNA-33b (C).

3.3.15 Patient-derived primary cell cultures establishment.

The heterogeneity of BC disease is a disadvantage for *in vitro* studies. Established cancer cell lines have been widely used with all the difficulties that come with it. The development of primary cultures provides an exceptional opportunity to reproduce cancer conditions and evaluate the proposed and new therapeutic approaches.

Too deep inside our study, more than 20 BC tissues were used to establish primary cultures. Three of them cancer tissue specimens were from the HER2+ subtypes patients. The protocol of preparation for patient-derived cells was optimized to obtain a high yield of viable primary cells. After enzymatically degradation of the tissue, we put then in complete DMEM-F12 medium, supplemented with 10% FBS, 1% P/S and 1% L-glutamine. Post 24 h of the seeding; we observed that the small tissue started attaching to the cells and some of the single cells and coming out from those tissues called the initiation process (**Figure 60A**).

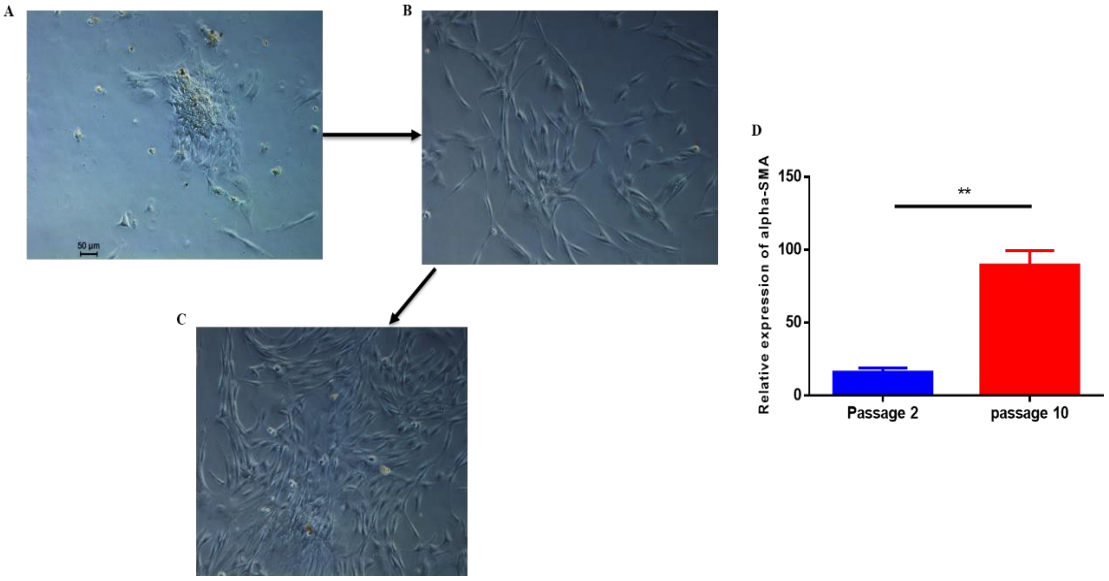


Figure 60: Establishment of Breast primary 2D cell line: Bright field microscopy shows the stages of (A) Initiation (B) purification and (C) wholly purified confluent cell line. Relative expression of alpha-SMA was determined in different passages of the primary cells (D). Student's t-test was used to analyse the significant differences. ****P ≤ 0.01**.

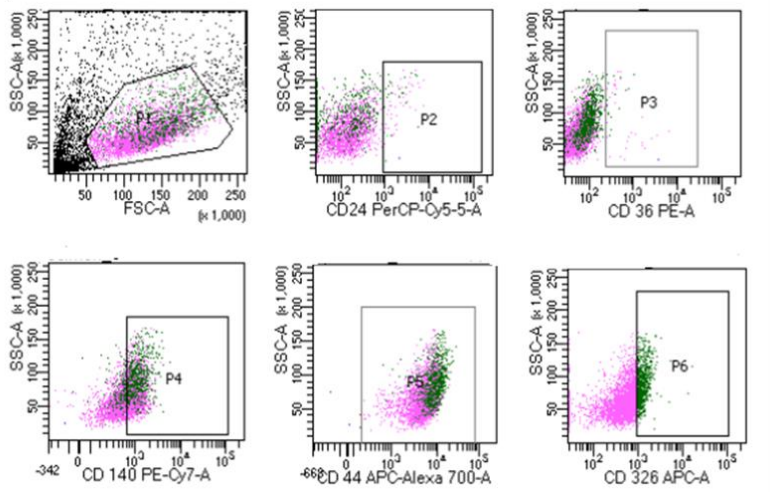
Then days after we noticed that those individual cells have a spindle-like structure, they started doubling and presenting morphological heterogeneity of cells, such as long, flattened mesenchymal-like cells epithelioid cells with the presence of the occasional multinucleated cell, which process is called purification (**Figure 60B**). After 10-15

days, we found a single layer of confluence primary cells with their appropriate morphology and reportedly no contamination, and this final process is called completely purified confluent cell line (**Figure 60C**). Subsequently, we analysed the alpha-SMA expression, a marker of activated cancer-associated fibroblasts (CAFs) in a different passage such as passage 2 and passage 10. We found that with the different passage and aggressiveness of the trypsinization, the cells are gradually becoming more CAFs than the early passage (**Figure 60D**).

3.3.16 Molecular characterization of primary cells.

Breast primary cells early passage is the mixture of a different cell population such as epithelial, adipose stem cell, BC stem cells and CAFs. We used different markers conjugated with varying fluorochromes in flow cytometry to demonstrate these populations in our established primary cell lines. The molecular markers, those have been used for identifying the population are for stem cell population cd 24- and cd 44+, for epithelial cells cd 326, for adipose-derived stem cell (ADSC) cd 36 and CAF cd 140a. We found that even though the primary cells are in early passage, in the mixture of population, the CAF population is higher in percentage than other population of the different cells (**Figure 61A**). A significantly higher population of cd 140a and cd 44 were determined (**Figure 61B**), known as a familiar marker of CAF population regulates various cellular functions including tumour heterogeneity, resistance to drugs, and aggressiveness of the tumour. Which is also considered the driver of current anti-tumour therapies' failure, involves both the transformed epithelial cells and the stromal cellular components.

A)



B)

Markers	Population (%)
CD 44	99.8 %
CD 140 a	59.4 %
CD 36	1.2 %
CD 24	2%
CD 326	19%

Figure 61: Characterization of the cell population using different markers by flow cytometer in breast primary cells. Passage number is 4 and subtype is HER2+. The percentage of the cell population was calculated based on positive staining of the specific markers.

3.3.17 The conditioned medium (CM) derived from the HER2+ primary BC cell lines induce cancer cell proliferation

The primary breast cell lines' molecular characterisation showed the highest population of CAFs having active fibroblasts with positive α-SMA expression and cd140a expression, so we considered the primary cells as CAFs cell lines. The stromal cells separated from primary BC tissues were a heterogeneous mixture of various cells in the primary culture, with fibroblasts being the main component. After different passaging of the primary cells, uniform fibroblasts started to grow. The stromal fibroblasts showed positive staining for the mesenchymal marker vimentin in all types of BC subtypes compared to MCF10a, a non-tumourigenic epithelial cell line (**Figure 62A**).

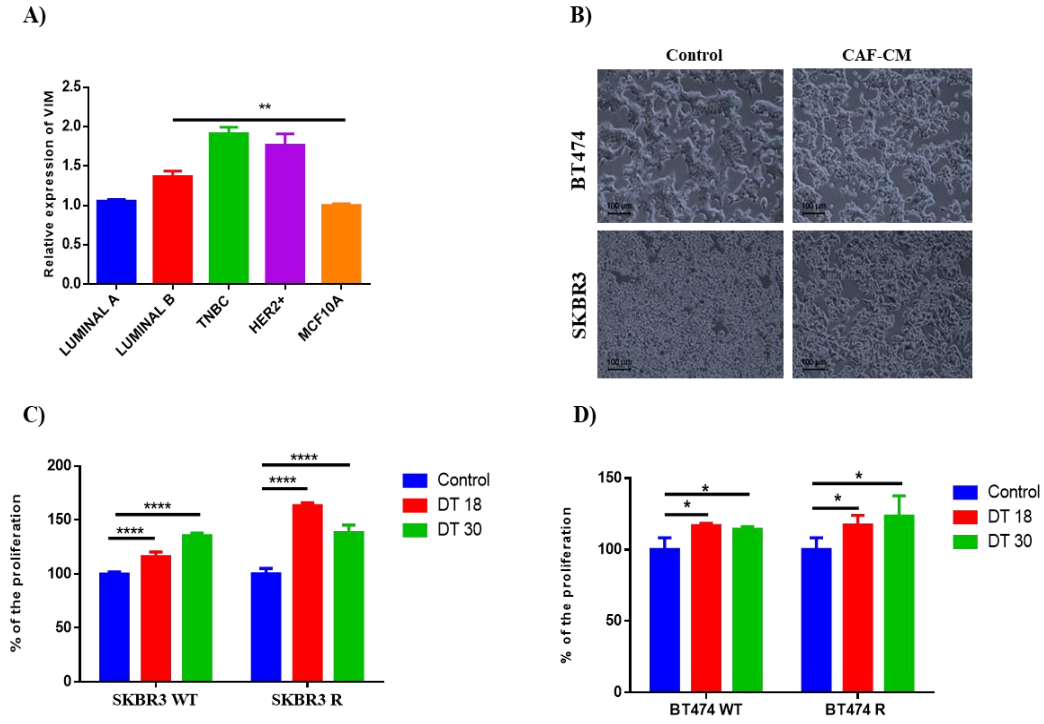


Figure 62: Effect of the CM on HER2+ cancer cells: Relative expression of vimentin, a marker of the CAF population was determined by qRT-PCR in different subtypes of primary BC cell lines (A). Bright-field microscopy of BT474 and SKBR3 showed the morphological effect induced by CM (B). The proliferation of BT474 and SKBR3, co-cultured with conditioned media for 72 h (C, D). Student's t-test was used to analyse the significant differences. ****P ≤ 0.01, **** P ≤ 0.0001**.

Even we observed that HER2+ and TNBC subtypes have more expression of VIM as compared to control. To investigate the effects of CAFs on BC cells proliferation, we selected two HER2+ subtypes primary cell lines. We named DT-18 and DT-30 (DT-dissecting tumour and number denoted the count of tumour tissue samples have been processed.), the CAF-CM was collected, and 50% of the CM used to culture with HER2+ BC cell lines BT474 and SKBR3. The cells cultured with CAF-CM showed more spindle-like shape and cell scattering in 72 h (**Figure 62B**) than controls, in addition to morphological changes, we checked the proliferation with 50% treatment of CM from both patients. It showed that at 72 h, the proliferation is significantly increased in both CM treated cells compared to control (control was taken 2% FBS with DMEM-F12 medium) (**Figure 62 C-D**). All the above results suggested that CAF-secreted proteins could stimulate these different BC cell lines to change their morphologies and phenotypes to have more metastatic potential and aggressiveness characteristics.

3.3.18 The condition media treated cells induce SALL4 expression

After analysing the effect of CM on cell proliferation, we shouted to explore the expression of SALL4 in mRNA and protein level. As earlier, we explained the SALL4 directly induces cell proliferation of HER2+ cancer. We treated BT474 and SKBR3 with DT 18 and DT 30 CM for 72 h to access the mRNA and protein expression. In both cell lines after treating with cm, the SALL4 mRNA expression was a 3-fold increase in SKBR3 and a 2-fold increase in BT474, respectively (**Figure 63 A-B**). The protein expression of SALL4 was also higher in both CM treated cells in 72 h (**Figure 63, C-D**). Together, these results suggested that the cytokines present in the conditioned medium instigating the expression of SALL4 higher and induced cell proliferation. So, it would be essential to characterise the CM for the reorganization of the cytokines, which involved high SALL4 expression, and would open a new link between SALL4 and microenvironment.

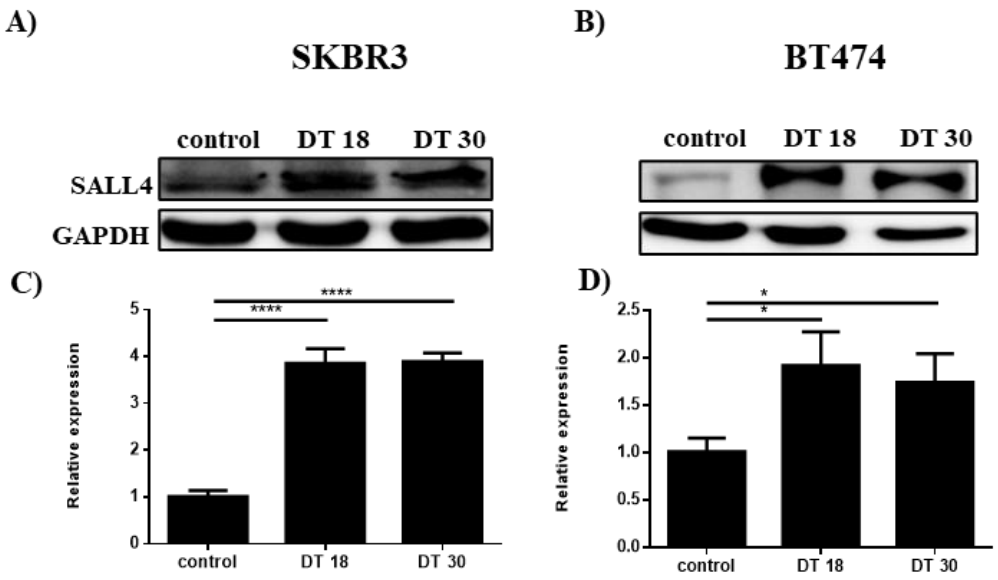


Figure 63: CM induces SALL4 expression in HER2+ cell lines: SALL4 expression was determined in mRNA (A, B) and protein level (C, D) with the treatment of CM collected from DT18, DT 30 by qRT-PCR and western blot. Student's t-test was used to analyse the significant differences. ***P ≤ 0.05, **** P ≤ 0.0001.**

3.4 Discussion

HER2+ BC is an aggressive disease that is more likely to reoccur than luminal A and B. Still, while HER2+ BC recurrence affects some patients, recent advancements in targeted therapies and long-term treatment approaches have made relapse less likely than ever before. To continue with this approach, it is essential to explore the different molecular mechanism, that will guide to advance the targeted therapies and prevent the recurrence more effectively. Among them, transcription factors play a vital role in controlling various direct mechanisms including chromosomal translocations, gene amplification or deletion, point mutations and alteration of expression, and indirectly through non-coding DNA mutations that affect transcription factor binding. Multiple approaches to target transcription factor activity have been demonstrated, preclinically and, in some cases, clinically, including inhibition of transcription factor–cofactor protein-protein interactions, inhibition of transcription factor–DNA binding and modulation of levels of transcription factor activity by altering levels of ubiquitylation and subsequent proteasome degradation or by inhibition of regulators of transcription factor expression. Besides, several new approaches to targeting transcription factors, it is not adequate to omit the disease completely. These innovations in drug development hold great promise to yield agents with unique properties that are likely to impact future cancer treatment.

It is known that SALL4 plays a vital role in stem cell self-renewal and pluripotency through different mechanisms, depletion of SALL4 results in early embryonic development defects[239]. Enhanced expression of SALL4 was first found to be associated with carcinogenesis in acute myeloid leukemia[211]. Subsequently, overexpression of SALL4 has been demonstrated to promote tumorigenesis, tumour growth, and tumour progression in various cancers. SALL4 is an important oncofoetal gene in a subset of different types of cancers with an aggressive phenotype. Blocking this gene's action with a short peptide could have therapeutic potential.

In recent studies, SALL4 expression was determined in 86.1 % of BC cases[213]. This high level of expression has been detected even in the early stages of tumours, but no significant correlation was found between the clinicopathological features and SALL4 expression levels[240]. Another study reported that high cytoplasmic expression of SALL4 is directly correlated with worse OS, and determining the expression level has excellent predictive value. SALL4 cytoplasmic expression is higher in invasive ductal carcinoma, showing that this gene may be an independent prognostic marker[214]. It has also been demonstrated that SALL4 holds a binding site for the TNF-α gene, which regulates cell death. It also might be related to PMS2, a mismatch repair protein indicator of poor prognosis in BC[241]. *Dimri et al.* revealed the Bmi-1 gene's overexpression, an oncogene stimulated by SALL4 in human mammary epithelial cells. SALL4 might also induce telomerase activity during cell transformation[242]. As SALL4 acts as a transcription factor, it has various roles in various types of cancers. It acts as a master regulator of EMT in different cancer types by targeting CDH1 and overexpressing Beta-catenin, Vimentin, and MYC. *Itou et al.* indicated that SALL4 represses E-cadherin gene (CDH1) expression and maintains cell dispersion in basal-like BC[215]. In the present study, we suggested that increased expression of SALL4 promoted metastasis by the EMT process in both BT474 and SKBR3 wild type cell lines in both protein and gene level.

In contrast, in resistance cell lines, the downregulation of SALL4 through two different silencers showed reduced expression of mesenchymal markers and high expression of E-cadherin in both gene and protein level. SALL4 plays important roles in multiple tumour-associated processes, including cell metastasis and drug resistance. Metastasis is not only the leading cause of cancer death but also the malignant properties of cancer. Cancer cells which undergo EMT process will acquire the invasiveness and metastasis

ability. The present research showed that SALL4 induced EMT-related proteins, including decreased cellular adhesion molecules E-cadherin and an increase in mesenchymal marker N-cadherin, Vimentin and Fibronectin. The expression of E-cadherin and N-cadherin was closely associated with cancer cells invasive and metastatic capacity. The recent researches have shown that SALL4 was involved in the metastasis and progression in colorectal cancer[243]. In addition, SALL4 overexpression induced EMT in gastric cancer cells, with increased expression of Twist1, N-cadherin and decreased expression of E-cadherin[244]. This evidence suggested that SALL4 could induce EMT and promote invasion in a variety of tumours. More importantly, we demonstrated that overexpression and downregulation of SALL4 regulated EMT and control metastasis in HER2+ BC cells.

In our research, we first time demonstrated that SALL4 has expression in HER2+ BC, but when it gets acquired resistance to trastuzumab, the expression of the SALL4 becomes higher compared to the basal level. Which indicated that targeting SALL4 in trastuzumab resistance cancer, would assist a novel targeted therapy for HER2+ cancer. Gain and loss function of SALL4 in wild types and acquired resistance cell lines showed that, while SALL4 expression is reduced in acquired resistance cell lines, it restored the trastuzumab's sensitivity to reducing the proliferation of the cells. The contradictory happened with overexpression of SALL4 in wild type cell lines. The above results demonstrated that, because of SALL4 involvement directly in cell proliferation, as justifying *Kobayashi et al.* theory, Nanog and SALL4 are vital factors for maintaining undifferentiated state and cell proliferation respectively[240]. So, since high SALL4 expression, cell proliferation is higher, which hinders the trastuzumab effect (with optimal concentration) on wild type cell lines. It suggested that SALL4 is holding back trastuzumab effect partially on cells due to high proliferation rate.

Further, to justify the mechanism of cell proliferation and less effective towards trastuzumab, we shouted to explore the PI3K/AKT/PTEN pathway, as it is one the most studied pathway precisely for HER2+ BC. This pathway responds to the availability of nutrients, hormones and growth factor stimulation and has been well established to play a very significant role in tumour cell growth and proliferation. Additionally, two of the significant mechanistic hypotheses behind trastuzumab resistance disease have been mutational activation of the PI3K/AKT pathway and changes in the HER2 molecule itself. In particular, mutational activation in PIK3CA, loss of PTEN, increased

expression of p95-HER2, and loss of expression of HER2 have been proposed to contribute to resistance to trastuzumab as mentioned in the introduction part. So, making this hypothesis as a link, and considering that SALL4 might have a particular role in this pathway, we started to look the complete pathway consisting of proteins which are upstream, downstream and regulator of this pathway through gain- and loss-of-function of SALL4 in HER2+ BC. In 2017 a research group from China suggested that silencing SALL4 in glioma cells reduced cellular growth and proliferation dramatically and resulted in an increase in PTEN expression, which depressed the activation of PI3K/ AKT pathway and leads to inhibit cancer development[245]. We mimicked the same mechanism in our model, which showed that induced overexpression of SALL4 in BT474WT and SKBR3 WT conferred phosphorylation of AKT at serine 473 residue and downregulation of PTEN. The inverse happened in the silencing of SALL4 in both resistant cell lines. In addition to the PI3K/AKT pathway's downstream protein, we demonstrated that with overexpression of SALL4, MYC and BCL2 expression were positively regulated in both wild type cell lines, and vice versa occurred in resistance cell lines. MYC function activates transcriptional programs that favour cell growth and proliferation, and suppress programs that cause cell growth arrest. MYC must favour the induction of crucial programs involved in the bioenergetics of growing cells[246].

SALL4 positively regulates MYC because SALL4 has a binding region on MYC promoter region[225] as described in the introduction part. Likewise, BCL2 is an anti-apoptotic protein belong to the BCL2 family. BCL2, as a protooncogene, contributes to malignancy by protecting cells from apoptosis. In 2008, a research group from Harvard Medical School invented that SALL4 directly binds with BCL2 and positively regulates leukemic cell growth[247]. In the other hand, it has been shown that the PI3K/AKT pathway can mediate cell-survival signals through the BCL2 family. The serine/threonine kinase mTOR, the major sensor of cell growth along the PI3K/AKT pathway, can be activated by agents acting on microtubules. Damaged microtubules induce phosphorylation of the BCL2 protein and lower the threshold of programmed cell death, which leads the cell to survive and escape from drugs. Altogether, the link between proteins mentioned above delivered a novel pathway justifying that, SALL4 is involved in HER2+ cancer cell proliferation, and makes resistance cells more aggressive through this novel pathway.

The NuRD complex is one of four major types of ATP-dependent chromatin remodelling complexes. Like other chromatin remodelling complexes, the NuRD complex has important roles in transcription, chromatin assembly, cell cycle progression and genomic stability in different types of cancers. RBBP4 is an essential subunit of the NuRD complex, which plays a key role in maintaining key regulators' silencing during embryonic development. A deregulated SALL4–RBBP4/NuRD pathway results in tumour suppressors' silencing, such as PTEN in HCC cell[237]. According to this hypothesis, we intended to dig into the NuRD complex expression between sensitive and resistance cell lines in HER2+ BC. The microarray data suggested that RBBP4 expression is significantly higher in the resistance cell line of HER2+ acquired resistance cell lines than sensitive cell lines. The qPCR and western blot results confirmed the microarray data, which suggested that SALL4 and RBBP4 interaction might happen in resistance cell lines instead of inhibiting the PTEN expression and activating BCL2 expression. This interaction might be essential for the resistance cell line to become a survivor from the drug treatment and worsens cancer prognosis. Therefore, targeting the SALL4–NuRD pathway in HER2+ BC, mostly in acquired resistance cell lines would be a promising therapeutic approach and a better treatment for this specific type of cancer in future. For the first time, *Daniel et al. and* his group designed a peptide named FFW with side chains of the SALL4 peptide intercalating into the grooves of RBBP4, providing an opportunity for an engineered peptide to inhibit the interaction competitively[237]. We likewise attempted a similar peptide in our cancer model, which demonstrated that the IC50 of this peptide in resistance cell lines is lesser than the sensitive cell lines. That explained the competency of the SALL4-RBBP4 interaction presence in resistance cell lines compared to sensitive cell lines. More future studies are needed for the complete endeavour of this mechanism in HER2+ acquired resistance cell lines. And a synergetic study with trastuzumab is needed to elucidate the peptide efficiency as a pharmacologic approach.

Taken together, we demonstrated that SALL4 expression was upregulated in HER2+ trastuzumab acquired resistance BC cell lines and positively correlated with poor prognosis and aggressive properties. We identified that SALL4 induced EMT and increased drug resistance through the PI3K/AKT/PTEN pathway by targeting their downstream genes such as MYC and BCL2. We further showed that SALL4 regulates NuRD complex. Blocking its physical interaction with RBBP4 (a member of the NuRD

complex) by a peptide can give an essential pharmacological approach to treat trastuzumab acquired resistance BC patients. In conclusion, our study provided a potential molecular mechanism related to SALL4-induced EMT and trastuzumab resistance in HER2+ BC cells. SALL4 and NuRD complex may be novel therapeutic targets for this specific subtype of BC.

CAFs are prominent components of the microenvironment in most types of solid tumours and were shown to facilitate cancer progression by supporting tumour cell growth, extracellular matrix remodelling, promoting angiogenesis, and mediating tumour-promoting inflammation. In recent years, CAFs are emerging as central players in immune regulation that shapes the tumour microenvironment. CAFs contribute to immune escape of tumours via multiple mechanisms, including secretion of multiple cytokines and chemokines and reciprocal interactions that mediate the recruitment and functional differentiation of innate and adaptive immune cells. Moreover, CAFs directly abolish the function of cytotoxic lymphocytes, thus inhibiting the killing of tumour cells. In this study, we have used two different HER2+ cancer patients, from where we collected the tissue and extracted the primary cells. The primary cells were characterized by qPCR, which indicated that they expressed a large amount of vimentin and alpha SMA being mainly CAFs. Based on their level of expression, we considered these cell lines as CAF cell lines. The CM is collected from these cell lines and treated them in BT474 and SKBR3. With the treatment with CM, the cells' proliferation rate was higher than control, and SALL4 expression was re-expressed in CM treatment. Altogether, it suggested that secretion of multiple cytokines and chemokines in the CM, triggering SALL4 expression in less SALL4 expressed cell lines. That concluded that the tumour microenvironment could regulate SALL4, and this link can shed light on a new molecular mechanism, that can lead a novel path for cancer treatment.

CHAPTER 4

4.1 MAIN CONCLUSIONS

1) Enhancer of Zeste Homolog 2 (EZH2) acts as an oncogene in HER2+ breast tumour type. EZH2 downregulation decelerates BC progression and metastasis through regulating proliferation and EMT process along with that the high expression of EZH2 is related to worse over survival in BC patients.

2) Mir-33b plays an essential role in inhibiting HER2+ cancer progression through regulating proliferation, apoptosis and EMT.

3) MiR-33b directly targets MYC, it inhibits the downstream protein EZH2, acting as a tumour suppressor in HER2+ BC cells. Altogether, we suggest a novel miR-33b/MYC/EZH2 axis implicated in HER2+ BC cell growth and progression.

4) The oncofoetal transcription factor SALL4, the target of miR33b, was overexpressed in trastuzumab resistance HER2+ BC cell lines. Highlights the partial restoration of trastuzumab sensitivity in resistance cell lines via PI3K/PTEN/AKT pathway and regulating EMT through MYC oncogene. Interestingly, high SALL4 expression levels were significantly associated with lower survival in HER2+ BC patients.

5) Considering another novel mechanism of trastuzumab resistance is recruiting the NuRD complex by SALL4. The interaction of SALL4 with RBBP4, a member of the NuRD complex, helps cells escape from the trastuzumab treatment by regulating the downstream proteins PTEN and BCL2.

6) The activated cancer-associated fibroblasts from primary BC patients induce an increment of SALL4 expression in BC cells lines. This evidence the role of

microenvironment in response to trastuzumab treatment and highlights the need for more in-depth evaluation.

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ANNEXES

5.1 ANNEX 1: List of Publications and Communications:

1) **Manuscript: Pattanayak B**, Garrido-cano I, Adam-artigues A, Tormo E, Pineda B, Cabello P, Alonso E, Bermejo B, Hernando C, Martínez MT, Rovira A, Albanell J, Rojo F, Burgués O, Cejalvo JM, Lluch A, Eroles P. 2020. MicroRNA-33b Suppresses Epithelial-Mesenchymal Transition Repressing the MYC – EZH2 Pathway in HER2 + Breast Carcinoma. Front Oncol 10:1–12.

2) Poster presentation entitled **"Micro-RNA 33b inhibits BC migration and invasion through regulating epithelial-mesenchymal transition in HER2 positive BC cell lines"** at MAP international conference, at the Swissôtel, Zurich, SWITZERLAND from October 13th to 14th, 2017.

3) Poster presentation entitled **"Micro-RNA 33b inhibits BC migration and invasion through regulating epithelial-mesenchymal transition in HER2 positive BC cell lines"** Desafío Oncológico 8, Valencia in November 29th and 30th, 2017.

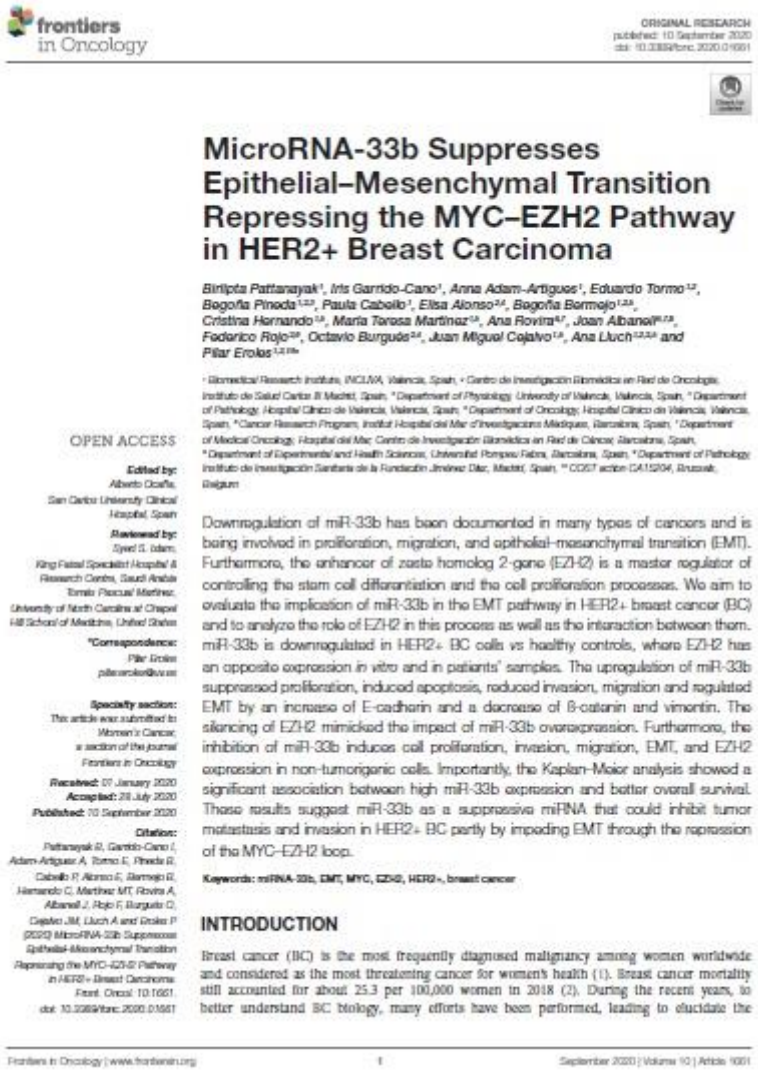
4) Poster presentation entitled **"Characterization of the different cell population in primary culture of breast tumour"** at MAP international conference, 14-15 September 2018, Paris, France.

5) A selected talk entitled **"Precision Medicine: From stratified therapy to personalized therapy"** COST Action: CA15204 Meeting Title: Anaesthesia and cancer workshop Location: Biomedical Research Institute INCLIVA, Valencia, Spain

6) A selected talk entitled "**Oncofoetal Gene SALL4: a potential marker for trastuzumab resistance HER2+ breast cancer",** III Young Researchers Meeting CIBERONC, Virtual meeting, Spain

7) The manuscript is in preparation **"Oncofetal Gene SALL4: As a potential marker for T Resistance HER2+ BC."**

5.2 ANNEX 2: MANUSCRIPT



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MicroRNA-33b Suppresses Epithelial–Mesenchymal Transition Repressing the MYC–EZH2 Pathway in HER2+ Breast Carcinoma

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Downregulation of miR-33b has been documented in many types of cancers and is being involved in proliferation, migration, and epithelial–mesenchymal transition (EMT). Furthermore, the enhancer of zeste homolog 2-gene (EZH2) is a master regulator of controlling the stem cell differentiation and the cell proliferation processes. We aim to evaluate the implication of miR-33b in the EMT pathway in HER2+ breast cancer (BC) and to analyze the role of EZH2 in this process as well as the interaction between them. miR-33b is downregulated in HER2+ BC cells *vs* healthy controls, where EZH2 has an opposite expression *in vitro* and in patients' samples. The upregulation of miR-33b suppressed proliferation, induced apoptosis, reduced invasion, migration and regulated EMT by an increase of E-cadherin and a decrease of ß-catenin and vimentin. The silencing of EZH2 mimicked the impact of miR-33b overexpression. Furthermore, the inhibition of miR-33b induces cell proliferation, invasion, migration, EMT, and EZH2 expression in non-tumorigenic cells. Importantly, the Kaplan-Meier analysis showed a significant association between high miR-33b expression and better overall survival. These results suggest miR-33b as a suppressive miRNA that could inhibit tumor metastasis and invasion in HER2+ BC partly by impeding EMT through the repression of the MYC–EZH2 loop.

Keywords: miRNA-33b, EMT, MYC, EZH2, HER2+, breast cancer

INTRODUCTION

Breast cancer (BC) is the most frequently diagnosed malignancy among women worldwide and considered as the most threatening cancer for women's health (1). Breast cancer mortality still accounted for about 25.3 per 100,000 women in 2018 (2). During the recent years, to better understand BC biology, many efforts have been performed, leading to elucidate the

heterogeneity of different subtypes [luminal A, luminal B, HER2-positive (HER2+), and triple-negative] susceptible for personalized approach and treatment. HER2 gene amplification occurs in 20–25% of BCs and is associated with disease relapse and poor prognosis. There are different targeted agents; both monoclonal antibody and tyrosine kinase inhibitors have radically changed the history of this disease (3). Nevertheless, after a neoadjuvant or an adjuvant approach, the rate of relapse remains substantially high (4). It is therefore essential to explore deeply the molecular mechanisms responsible for disease progression and therapy resistance to identify possible biomarkers that would guide novel treatments for this subtype of BC.

MicroRNAs are small molecules based on 20–22 nucleotides, having a main function in regulating gene expression post-transcriptionally by inhibiting protein translation or causing the degradation of the target mRNA (5). Different cancer types have a varied expression of miRNAs; in addition, some miRNAs may function as oncogenes or tumor suppressors. They have been directly implicated in cancer metastasis or the prevention of cancer progression by participating in the regulation of the epithelial–mesenchymal transition (EMT) pathway, stemness and targeting apoptosis pathway. In this scenario, miR-33b was found to act as an anti-cancer miRNA, inhibiting cell migration, proliferation, and invasion in melanoma cancer (6), lung cancer (7, 8), prostate cancer (9), osteosarcinoma (10, 11), gastric cancer (12), and triple-negative BC (TNBC) (13). However, the role and the action mechanism of miR-33b in HER2+ BC subtype are still unclear.

Furthermore, the enhancer of zeste homolog 2-gene (EZH2) has a master regulatory function in controlling processes such as stem cell differentiation, cell proliferation, early embryogenesis, and X chromosome inactivation (14). EZH2 is overexpressed in metastatic prostate cancer and promotes cell metastasis and proliferation by inhibiting apoptosis (15). It is also described as a master regulator of the EMT by overexpressing Snail, Slug, and vimentin and suppresses E-cadherin (CDH1) expression in endometrial cancer and gastric cancer (16, 17), but less has been explored in the HER2+ BC subtype. Moreover, the transcription factor MYC (18) has been suggested as a positive regulator of EZH2 by different mechanisms in several types of cancers. MYC might enhance EZH2 expression through inhibiting the microRNAs miR-26a and miR-26b (19) and also by the activation of the EZH2 expression through binding with E-box, a DNA binding site of MYC (20). Emerging shreds of evidence also showed that miR-33b negatively regulates MYC in osteosarcoma cancer (10) and prostate cancer progression (9) by directly binding with its 3′ UTR region. Moreover, there is recent substantial data which suggested that miR-33a could negatively regulate EZH2 in cancer progression by direct interaction in TNBC (21).

Taking all these information together, our paper aimed to explore more about miR-33b from miR-33 family in HER2+ BC. As HER2+ is an aggressive disease with significant mortality, it requires massive molecular mechanism studies to defeat its aggressiveness (22). Our results show, for the first time, that the under-expression of miR-33b is related to the poor prognosis and low survival in HER2+ BC, while a high expression of EZH2 is directly proportional to tumor aggressiveness and proliferation. As the miR-33b and EZH2 molecular mechanism functions have been less elucidated in this subtype of BC, we tried to fill the loophole between them. MiR-33b exerts its function by indirectly targeting EZH2 through directly inhibiting MYC to repress the migration, invasion, proliferation, and EMT development of HER2+ BC. Furthermore, we identified a novel 33b/MYC/ EZH2 axis implicated in proliferation and invasion in HER2+ BC.

MATERIALS AND METHODS

Cell Culture and Reagents

Human BC cell lines BT474, SKBR3, MDA-MB-468, MCF7 and MCF-12A, and MCF-10A non-tumorigenic epithelial cells were maintained in Dulbecco's modified Eagles medium (GIBCO) supplemented with 10% fetal bovine serum (FBS; Gibco), 10,000 U/ml penicillin, 10,000 µg/ml streptomycin, and 1% L-glutamine (200 mM) (×100). All cells were cultured at 37°C in 5%-CO_2 atmosphere.

Transfection

The cell lines were transfected either with 100 nM hsa-miR-33b-5p mirVana mimic (assay ID MC12289, Ambion) or inhibitor miRNAs (assay ID MH12289, Ambion) and 100 nM EZH2 siRNA (#s4916, #s4918, Thermofisher), as well as negative control for the experiments. *In vitro* transfections of the oligonucleotides were performed using Lipofectamine 2000 (Invitrogen; Thermo Fisher Scientific, Inc., Waltham, MA, United States) according to the manufacturer's instructions. After 6 h of transfection, the transfection medium was replaced with a complete medium. All the experiments were carried out at 48 and 72 h post-transfection.

RNA Extraction and Quantitative Real-Time PCR

To detect the expression of miRNA and mRNA total RNA was extracted using TRIZOL reagent (Invitrogen, Carlsbad, CA, United States) according to the manufacturer's instructions. cDNA was synthesized from 1 µg of total RNA using a High-Capacity cDNA Reverse Transcription kit (Applied Biosystems) and a TaqMan® MiRNA Reverse Transcription kit (Applied Biosystems, United States). Real-time-qPCR was performed with a TaqMan® Universal Master Mix (Applied Biosystems) and TaqMan® 20× assay (Applied Biosystems) by following the manufacturer's protocol on a quant-studio 3 and 5 real-time PCR system (Applied Biosystem, United States). The expression data were uniformly normalized to the internal control. For the miRNA expression, the endogenous control was RNU43, and for the gene expression, the endogenous control was GAPDH, and relative gene and mi-RNA expression was quantified using the 2-ΔΔ Ct method.

Cell Invasion and Migration Assays

For the migration assay, 5×10^4 cells (72 h post-transfection) were seeded in 200 µl of serum-free medium into the upper

chamber of each insert (353097, Corning®), and 700 µl of medium supplemented with 10% FBS was added into the lower chamber. For the cell invasion assay, the polyester membranes of the upper surface of the insert (353097, Sigma) were pre-coated with a matrix gel (Corning® Matrigel® Basement Membrane Matrix, Ref: 356234). Following equal amount of above mentioned transfected cells were seeded in 200 µL of serum-free medium on the pre coated insert. The lower chamber was supplemented with 700 µL of complete medium and incubated at 37°C. After 24 h, the cells that invaded and migrated through the membrane were fixed and permeabilized with 70% chilled ethanol for 2 min and with 100% methanol for 15 min, respectively, at room temperature. The invaded and migrated cells were further stained with 0.4% crystal violet for 10 min at room temperature. The cells were then imaged and counted from photographs of five randomly selected fields of the fixed cells.

Wound Healing Assay

To check the motility capacities of the cells after miR-33b transfection, wound healing assay was performed. At 72 h post-transfection, the cells were seeded in six-well plates to obtain 100% confluence in 24 h. After 24 h, the wound was induced by scratching the monolayer with a micropipette tip, and the dish was placed at 37°C in a 5%-CO_2 incubator chamber. Pictures were acquired at 0 h and after 24 h using a microscope.

WST-1 Cell Proliferation

After transfection, cell proliferation was assessed using the WST assay. A total of 3×10^3 transfected cells and negative control cells were seeded in 96-well plates from 1 to 7 days. On each of the mentioned days, cell proliferation was measured using WST-reagent (ab155902, Abcam). Seven percent of the WST reagent was added to each well with phenol red-free media. The plate was incubated for 4 h at 37°C. Then, absorbance was measured at 450 nm in a microplate reader with background correction at 650 nm. The significance of any differences were assessed using *t*-test.

Cell Cycle Analysis

To analyze cell cycle, 5×10^4 cells were seeded in six-well plates for each condition in triplicates. After 48 h of transfection, the cells were harvested by trypsin and washed with 1× phosphate-buffered saline (PBS) twice. Then, the harvested cells were fixed with chilled 70% ethanol and incubated at −20°C for 6–7 h. The cells were then centrifuged soon after washing with 1× PBS twice, and the pellets were resuspended with propidium iodide (PI) staining buffer (PI/RNase, IMMUNOSTEP) and stored at 4°C overnight. Stained cells were acquired for cell cycle analysis by flow cytometry using a FACSVerse™ flow cytometer (BD Bioscience, United States), and raw data were analyzed by FlowJo software.

Apoptosis Analysis

Apoptotic cells were determined by double staining using FITC Annexin V Apoptosis Detection Kit with PI (ANXVKF-100T, IMMUNOSTEP) according to the manufacturer's recommendation. Briefly, 1×10^5 cells were seeded in a six-well plate. After 72 h post-transfection, the supernatant medium was taken in one tube. The attached cells were harvested by trypsinizing and were collected into the same tube. The cells were washed with 1× PBS twice, and the pellet was resuspended with 1× annexin binding buffer. Five microliters of annexin V-FITC and 5 µl of PI were added to the resuspended cells and incubated for 15 min at room temperature in the dark. Furthermore, 400 µl of 1× binding buffer was added with DAPI (0.1 mg/ml, 1–2 µl). The stained cells were acquired for cell cycle analysis by flow cytometry using a FACSVerse™ flow cytometer (BD Bioscience, United States), and raw data were analyzed by FlowJo software.

Western Blot Analysis

At the indicated time (72 h), the whole lysate of transfected cells was extracted using Thermo Scientific™ RIPA lysis buffer (Ref: 89900). The lysates were transferred to a clean microfuge tube, placed on ice for 30 min, and centrifuged for 30 min at 13,000 rpm. The supernatant was transferred to a fresh microfuge tube, and the protein concentration was determined using a BCA protein assay kit (PierceTM BCA Protein Assay Kit, Ref: 23227). The protein lysates were separated on 10% SDS PAGE and transferred to nitrocellulose membranes (Ref: 1620115, Bio-Rad). The membranes were blocked in 5% BSA for 1 h and then incubated with antibodies of E-cadherin (BD Biosciences, #610181), ß-catenin (BD Biosciences, #610153), vimentin (BD Biosciences, #550513), EZH2 (Cell Signaling, #1674905S), and GAPDH (Thermo Scientific™, #MA5-15738) overnight at 4°C. On the following day, the membranes were washed and subsequently incubated with the appropriate HRP-conjugated secondary antibodies for 1 h at room temperature. Following this incubation, the membranes were washed and briefly incubated with a Pierce™ ECL Western Blotting Substrate western blotting detection reagent (Thermo Fisher Scientific™, Ref: 32106).

Clinical Samples and RNA Isolations

Formalin-fixed and paraffin-embedded samples of human BC tissues from different subtypes of BC patients and breast samples from healthy donors were selected to analyze the expression of miR-33b and EZH2 gene. The total RNA was isolated from tissue blocks using the RecoverAll Total Nucleic Acid Kit (Ambion) for standard mRNA/miRNA analysis. One microgram of total RNA was retro-transcribed with random primers (for gene expression) and specific primers (for miRNA expression) using Reverse Transcription Kit (Applied Biosystems), and 5 ng of cDNA was used for quantitative PCR for both gene and miRNA expression analysis. The quantitative PCR analysis was performed as mentioned above.

TCGA Data Analysis

The expression data for miRNA-33b were obtained from Xena browser database[1] for The Cancer Genome Atlas (TCGA) BC, which contained 1,285 cases of different BC subtypes solid tumors and normal. From there, we were able to obtain only 211 specimens with clinical details, including luminal B ($n = 49$), basal-like ($n = 26$), luminal A ($n = 92$), HER2+ ($n = 18$), and normal solid tissue ($n = 26$). For EZH2 expression, we used the same data base, which contained 1,248 cases of different BC

[1]https://xenabrowser.net/

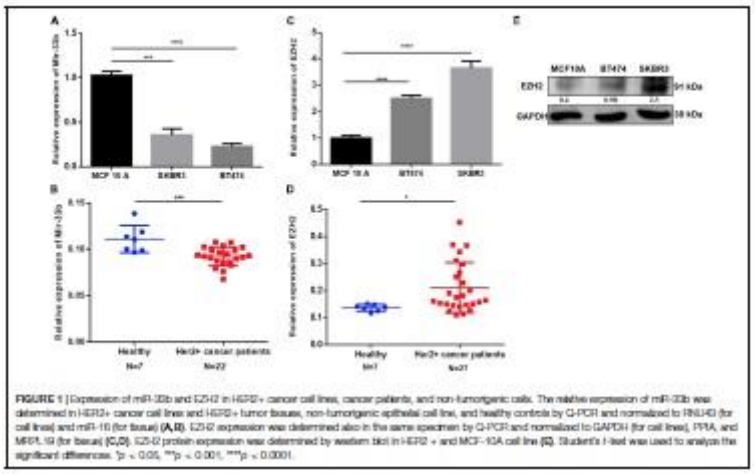

FIGURE 1 | Expression of miR-33b and EZH2 in HER2+ cancer cell lines, cancer patients, and non-tumorigenic cells. The relative expression of miR-33b was determined in HER2+ cancer cell lines and HER2+ tumor tissues, non-tumorigenic epithelial cell line, and healthy controls by Q-PCR and normalized to RNU48 (for cell lines) and miR-16 (for tissue) **(A,B)**. EZH2 expression was determined also in the same specimen by Q-PCR and normalized to GAPDH (for cell lines), PPIA, and MRPL19 (for tissue) **(C,D)**. EZH2 protein expression was determined by western blot in HER2 + and MCF-10A cell line **(E)**. Student's *t*-test was used to analyze the significant differences. $^{*}p < 0.05$, $^{**}p < 0.001$, $^{***}p < 0.0001$.

subtypes solid tumors and normal, wherefrom we only obtained 522 specimens with clinical details, including luminal B ($n = 127$), basal-like ($n = 98$), luminal A ($n = 231$), HER2 + ($n = 58$), and normal solid tissue ($n = 8$). The statistical analysis was done using Shapiro–Wilk normality test, and based on normality test results, parametric and non-parametric tests were applied to obtain the *p* value of the analysis.

In silico Survival Analysis

Overall survival associated with miRNA and gene expression was analyzed using Kaplan–Meier plotter (KM plotter) tool[2]. This tool works upon a database containing different subtypes of BC Affymetrix microarray samples and associated survival information, with a median follow-up of 120 months. Based on METABRIC dataset, by specifying the miRNA name and the gene name on the search tool and filtering down to "all breast cancer subtypes and HER2+ subtype," the survival rates according to miRNA or gene expression were obtained. The hazard ratio (HR) with 95% confidence intervals and log-rank *p*-value were calculated and shown. The obtained results were used to identify the prognostic value of miR-33b and EZH2 expressions on HER2+ BC.

Statistical Analysis

The sample and the control groups were compared using two-tailed Student's *t*-test. All data presented include median and standard deviation. *P*-values less than 0.05 were considered to be statistically significant.

[2]http://kmplot.com/analysis/

Ethical Approval

The study was conducted in accordance with recognized ethical guidelines (Declaration of Helsinki), and it was approved by the INCLIVA institutional review board (protocol number: 2018/077). All the participants in the study signed a written informed consent.

RESULTS

Expression of miR-33b and EZH2 in HER2+ Breast Cancer Cell Lines, Patient Samples, and Non-tumorigenic Cells

MiR-33b expression was determined in four human BC cell lines, including MDA-MB-468, MCF-7, BT474, and SKBR3 (HER2+), with the non-tumorigenic epithelial cell lines MCF12A and MCF-10A as controls. The quantitative PCR (Q-PCR) data revealed that miR-33b expression was significantly higher in MCF-10A as compared to that in HER2+ BC cell lines (**Figure 1A**). An analysis of tissue samples from Department of Oncology, Hospital Clínico de Valencia and the TCGA database for a HER2+ BC retrospective cohort confirmed a significantly lower miR-33b expression level than the breast control samples (**Figure 1B** and **Supplementary Figure 1E**). The EZH2 expression was

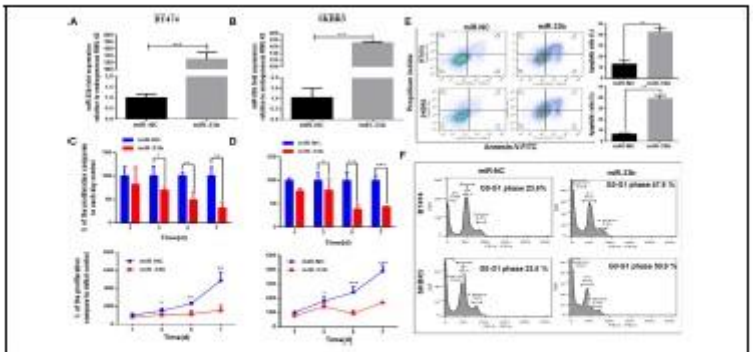

FIGURE 2 | MiR-33b reduces cell proliferation and induces early and late apoptosis. Satisfactory transfection efficiency of miR-33b mimic in both HER2 + cell lines BT474 (**A**) and SKBR3 (**B**). Cell proliferation was carried out through WST assay in both cell lines after transfection with miR-33b mimic until 7 days (**C,D**). The apoptotic cell population was determined at 72 h in both cell lines by flow cytometry through annexin-V/PI staining (**E**). To confirm the apoptotic population and the percentage of sub-G0/G1 population, cell cycle was analyzed through PI-RNAse assay at 48 h (**F**). Student's *t*-test was used to analyze the significant differences. $^{*}p < 0.05$, $^{**}p < 0.01$, $^{***}p < 0.001$.

significantly higher in HER2+ BC cell lines than in MCF-10A as determined at the mRNA level (**Figure 1C**) and at the protein level (**Figure 1E**). Similarly, a significantly higher EZH2 expression was found in HER2+ BC tissue samples vs. healthy breast tissues (**Figure 1D**) and also from the TCGA data portal (**Supplementary Figure 1F**). The analysis of miR-33b and EZH2 in other BC subtypes showed as well a significant higher expression of miR-33b in control cell lines vs cancer cell lines and an oppositely significant higher expression of EZH2 on cancer cell lines in comparison with those of the controls (**Supplementary Figures 1A,C**). MiR-33b and EZH2 expression in the TNBC patients' samples showed the same tendency than the HER2 + samples compared with those in healthy breast tissues (**Supplementary Figures 1B,D**). These data altogether suggested a downregulation of miR-33b and a high expression of EZH2 in HER2+ BC subtypes both *in vitro* and in BC tissues, being one of the important reasons for the high aggressiveness of this subtype.

Overexpression of miR-33b Reduces Proliferation and Induces Apoptosis in HER2+ BC Cell Lines

To determine the potential effect of miR-33b on cell proliferation and apoptosis in HER2 + BC, cells were transfected with miR-33b mimic or scramble miR (miR-NC). Its expression was confirmed by Q-PCR in both cell lines (**Figures 2A,B**). The WST cell proliferation assay was carried out to observe the proliferation effect, which showed that the overexpression of miR-33b significantly decreased cell proliferation as compared to scramble in BT474 and SKBR3, and the inhibitory effects showed a statistical significance after 7 days (**Figures 2C,D**). A recent study showed that miR-33b regulates cell cycle and apoptosis (23). To confirm this effect in our model, we evaluated apoptosis by annexin-V. As shown in **Figure 2E**, the ectopic expression of miR-33b induced early and late apoptosis in both HER2+ cell lines. To verify these results, we further investigated the cell cycle by PI/RNAse with miR-33b transfected cell lines, which showed a considerable increase of cells in the sub-G0/G1 phase compared to the control and a reduction almost by half in the number of cells in G1 and S phases (**Figure 2F**). Collectively, it showed that miR-33b has an anti-proliferative effect on HER2+ BC cell lines and induced apoptosis with arrest of the cells at sub-G0/G1 phase.

Overexpression of miR-33b Suppresses Invasion, Migration, EMT Process, and Expression of EZH2 in HER2+ BC Cell Lines

Tumor cell invasion and metastasis are tightly correlated with various processes, including EMT. During EMT, epithelial cells acquire mesenchymal characteristics with a high expression of vimentin and ß-catenin, whereas the epithelial protein marker CDH1 is downregulated. It has also been described that miR-33b is a key regulator of MYC pathway, and one of the downstream targets of this pathway is EZH2, which is a potential regulator of cell proliferation, EMT, invasion, migration, and drug resistance (24). The overexpression of miR-33b in BT474 induced a statistically significant increase of the expression of

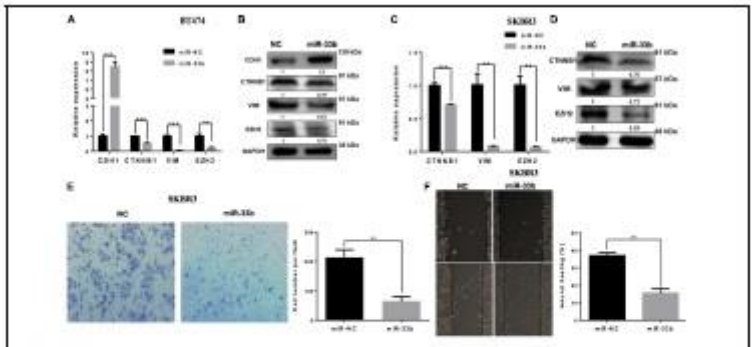

FIGURE 3 | miR-33b inhibits cell epithelial–mesenchymal transition, migration, and invasion in HER2+ cell lines. The relative expression of the EMT genes and protein expression were checked after transfection with miR-NC and miR-33b in HER2+ cell lines: BT474 **(A,B)** and SKBR3 **(C,D)**. SKBR3 cells were transfected with miR-33b mimic, and cells penetrating the membrane were fixed with 0.4% crystal violet stain after 24 h to evaluate the invasion capacity **(E)**. A wound healing assay was performed on SKBR3 transfected with miR-33b to explore the migration properties of the cells. Black arrows indicate the wound edge. The relative scratch gap was calculated as the percentage (%) of the remaining scratch gap at the given time point and the original gap at 0 h **(F)**. Student's *t*-test was used to analyze the significant differences. $^{*}p < 0.01$, $^{**}p < 0.001$.

CDH1 and significant decreases of ß-catenin, vimentin, and EZH2 (**Figure 3A**). Consistent results were obtained with SKBR3 (**Figure 3C**). To confirm these data at the protein level, western blot was performed (**Figures 3B,D**). However, CDH1 was unable to detect SKBR3 because of its homozygous deletion of a large portion of the gene in this cell line (25). Additionally, migration and invasion assays were planned to explore the anti-metastatic effect of miR-33b. The SKBR3 cells were transfected with miR-33b mimic for 72 h and seeded on matrigel-based transwells to check the invasion capacity within 24 h. The expression of the mature miR-33b was confirmed by Q-PCR in that cell line (data not shown). The results showed that the overexpression of miR-33b induced a decrease in SKBR3 invasion capability compared to the controls (**Figure 3E**). The migration process was carried out by the wound healing assay. The results showed that miR-33b overexpression significantly reduced the migration properties of HER2+ cells compared to the negative control (**Figure 3F**). Taken together, these results suggested that miR-33b inhibits cell invasion and migration and acts as a possible crucial regulator of the EMT process in HER2+ BC. Probably it can be an indirect tumour aggressiveness inhibitory effects through targeting EZH2 in the specific BC subtype.

Inhibiting miR-33b Expression Induces Cell Proliferation, Invasion, and Migration in Non-tumorigenic Cells

Because the overexpression of miR-33b reduced cell proliferation, invasion, migration, and EMT in HER2 + BC cell, we wondered if inhibition of this miRNA in control cells would have the opposite effect. To further investigate it, the miR-33b inhibitor was transfected in control cell lines. The efficiency of transfection was confirmed at 72 h post-transfection in both non-tumorigenic cell lines (**Figure 4A** and **Supplementary Figure 2A**). WST assay was carried out to evaluate the effect in proliferation, which showed that inhibition of miR-33b increases the proliferation at 3 days as well as at 5 days in both control cell lines (**Figure 4B** and **Supplementary Figure 2B**). After 72 h of transfection with the miR-33b inhibitor, the cells were seeded on transwells to evaluate the invasion (**Figure 4C** and **Supplementary Figure 2C**) and the migration (**Figure 4D** and **Supplementary Figure 2D**) properties of the cells. The results showed that inhibition of miR-33b significantly promoted cell migration and invasion in both non-tumorigenic cell lines. These data suggested that miR-33b is required to control cell migration, invasion, and proliferation.

Downregulation of miR-33b Induces EMT and EZH2 Expression

To better understand the molecular mechanism of action of miR-33b on cell migration and invasion, we opted to explore the regulation of EMT signaling and the regulation of EZH2. Control cell lines were transfected with an inhibitor of miR-33b, and at 72 h after transfection, EMT signaling pathway factors were checked on the level of mRNA and protein expression. The results showed that, with the inhibition of miR-33b, the expression of CDH1 was significantly diminished, and there was an increase of ß-catenin, vimentin, and EZH2 in both mRNA and protein

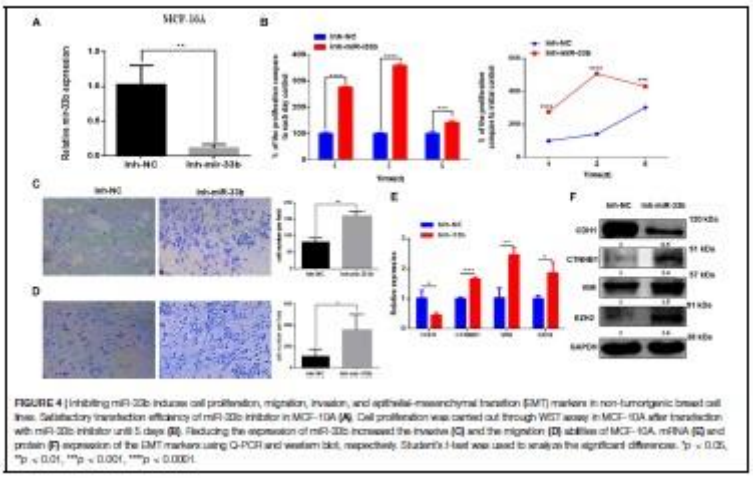

FIGURE 4 | Inhibiting miR-33b induces cell proliferation, migration, invasion, and epithelial–mesenchymal transition (EMT) markers in non-tumorigenic breast cell lines. Satisfactory transfection efficiency of miR-33b inhibitor in MCF-10A (**A**). Cell proliferation was carried out through WST assay in MCF-10A after transfection with miR-33b inhibitor until 5 days (**B**). Reducing the expression of miR-33b increased the invasive (**C**) and the migration (**D**) abilities of MCF-10A. mRNA (**E**) and protein (**F**) expression of the EMT markers using Q-PCR and western blot, respectively. Student's *t*-test was used to analyze the significant differences. $^{*}p < 0.05$, $^{**}p < 0.01$, $^{***}p < 0.001$, $^{****}p < 0.0001$.

level (**Figures 4E,F** and **Supplementary Figures 2E,F**). Thus, it supports that miR-33b can regulate EMT signaling in both control and cancer cell lines.

Downregulation of EZH2 Inhibits Proliferation in HER2+ BC Cell Lines

The previous results suggest that miR-33b is regulating EZH2. In order to evaluate the role of EZH2 in HER2+ BC cell lines, BT474 and SKBR3 cells were transfected with two different si-EZH2. Confirmation of gene and protein silencing was performed by Q-PCR and by western blot, respectively. Both siRNAs significantly inhibited EZH2 expression in BT474 and SKBR3 cells compared to controls (**Figures 5A,B**). To explore the effect of silencing of EZH2 on cancer cell proliferation, the WST assay showed that a lower expression of EZH2 significantly decreased the cell proliferation in BT474 and SKBR3, and this inhibitory effect showed statistical significance until 7 days (**Figures 5C,D**). These results indicated that EZH2 may act as a crucial gene for tumor aggressiveness in HER2+ BC through modulating cell proliferation.

Downregulation of EZH2 Inhibits EMT, Invasion, and Migration in HER2+ BC Cell Lines

High levels of EZH2 have been involved in BC progression by the regulation of the EMT process. To evaluate if the downregulation of EZH2 mediates the inhibition of the EMT pathway in our model, we silenced the EZH2 in BT474 and SKBR3 by two different siRNAs to analyze the gene set enrichment of EMT. Both cell lines were transfected with two specifically different siRNAs of EZH2. The downregulation of the EMT genes was confirmed by Q-PCR and the protein expression was evaluated by western blot in both cell lines compared to the control (**Figures 6A–D**). The results showed that the downregulation of EZH2 expression induced a statistically significant increase of CDH1 and a decrease of ß-catenin and vimentin in BT474 at both the mRNA and the protein levels. There were no changes in ß-catenin (CTNNB1) at the mRNA level in SKBR3 with the silencing of EZH2. However, at the protein level, there was a reduction in the expression of ß-catenin and vimentin at both the gene and the protein levels. In addition, the silencing of EZH2 affects invasion and migration, resulting in a decrease in the SKBR3 cell line (**Figures 6E,F**). Altogether these results showed that EZH2 induces EMT to promote invasion and migration in HER2 + BC cells.

High miR-33b Expression Levels Were Correlated With Favorable Overall Survival Outcome in HER2+ BC Patients

To assess the prognostic value of miR-33b and EZH2, we used an *in silico* survival analysis of BC patients with the Kaplan–Meier plotter. As a result, the BC patients with high miR-33b expression showed a statistically significant improvement in overall survival (OS) ($p = 0.0246$, HR = 0.76, 95% CI 0.60–0.97) (**Figure 7A**), suggesting a good prognostic role of this

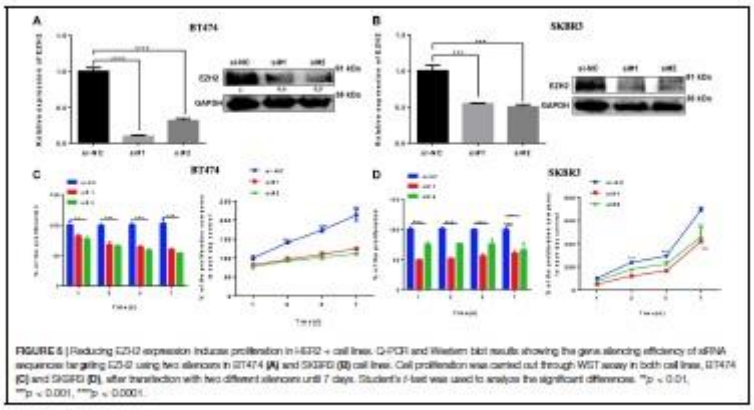

FIGURE 5 | Reducing EZH2 expression induces proliferation in HER2 + cell lines. Q-PCR and Western blot results showing the gene silencing efficiency of siRNA sequences targeting EZH2 using two silencers in BT474 **(A)** and SKBR3 **(B)** cell lines. Cell proliferation was carried out through WST assay in both cell lines, BT474 **(C)** and SKBR3 **(D)**, after transfection with two different silencers until 7 days. Student's *t*-test was used to analyze the significant differences. $^{**}p < 0.01$, $^{***}p < 0.001$, $^{****}p < 0.0001$.

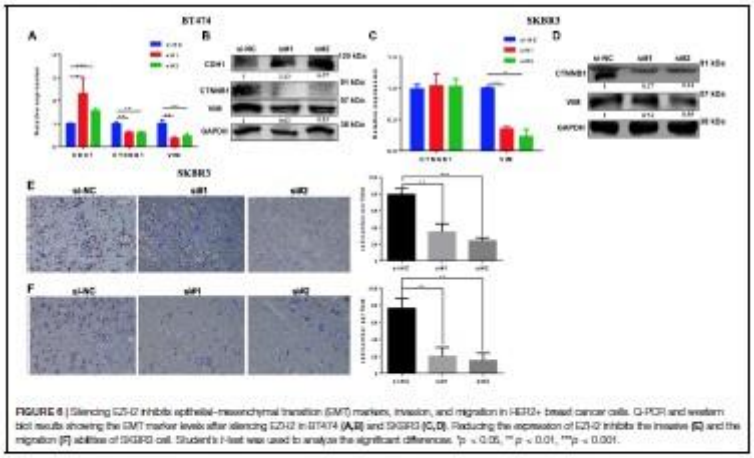

FIGURE 6 | Silencing EZH2 inhibits epithelial–mesenchymal transition (EMT) markers, invasion, and migration in HER2+ breast cancer cells. Q-PCR and western blot results showing the EMT marker levels after silencing EZH2 in BT474 **(A,B)** and SKBR3 **(C,D)**. Reducing the expression of EZH2 inhibits the invasive **(E)** and the migration **(F)** abilities of SKBR3 cell. Student's *t*-test was used to analyze the significant differences. $^{*}p < 0.05$, $^{**}p < 0.01$, $^{***}p < 0.001$.

miRNA. The, opposite results were found with EZH2 when it was evaluated among the same set of patients; it was observed that a high expression was associated with a worse OS ($p = 0.0095$, HR = 1.34, 95% CI 1.07–1.67) (**Figure 7B**). Similar results were obtained in a cohort of HER2 + BC patients. We found that a high miR-33b expression maintained a significant–good prognosis in terms of OS ($p = 0.039$, HR = 0.74, 95% CI 0.55–0.99) (**Figure 7C**). At the same time, high EZH2 expression showed

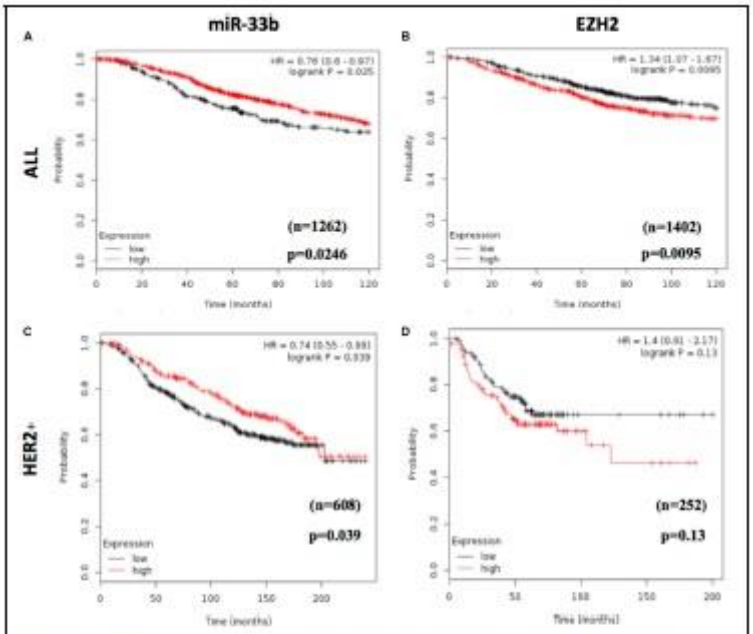

FIGURE 7 | Prognostic value of miR-33b and EZH2 expression in breast cancer patients. *In silico* overall survival analysis of miR-33b **(A)** or EZH2 **(B)** in breast cancer (BC) patients with Kaplan–Meier plotter. Survival analysis in a cohort of HER2+ BC patients: miR-33b **(C)** or EZH2 **(D)**.

a poor prognosis (HR = 1.4, p = 0.13). Nevertheless, it was not statistically significant, probably due to the low number of subjects (**Figure 7D**). These results corroborated the importance of this axis as a prognostic factor in HER2+ BC.

DISCUSSION

A lot of accumulated data have pointed out that several miRNAs drive tumorigenesis and drug resistance and suppress cancer progression by targeting different oncogenes (26). Although multiple studies have been carried to study the roles of miRNAs in BC, most of them have focused on BC in general and not on the specific subtypes. HER2+ BC subtype is one of the cancers with a worse prognosis and is associated with inferior outcomes in survival (27), being an entity with a large heterogeneity at multiple levels (28). In recent studies, miRNAs are being identified as one of the key regulators to uncover the molecular mechanisms of the heterogeneity in HER2 + BC.

The miR-33 family is one of the highly conserved miRNA families that consists of two members: miR-33a and miR-33b (29). They both act as a tumor suppressor in different cancers such as non-small cell lung cancer (30), TNBC (31), esophageal squamous cell carcinoma (32), and colorectal cancer (33) *via* targeting EMT and proliferation. For the first time in this study, we reported that miR-33b was downregulated in breast HER2+ tumor samples when compared to normal breast tissues and that the under-expression of miR-33b is related to a poor prognosis in HER2+ patients. We also found that miR-33b expression was higher in normal breast epithelial cell

lines than in HER2+ BC cell lines. It has been described as well that, in TNBC, miR-33b represses cancer progression and metastasis by targeting oncogenes like *SALL4*, *TWIST1*, and *HMGA2* (13). These data indicate that miR-33b acts as an onco-suppressive miRNA in BC progression. To investigate the specific mechanism of miR-33b in HER2+ BC progression, miR-33b was ectopically overexpressed in different HER2+ cell lines, where it was shown that the upregulation of this miRNA inhibits cancer cell invasion and migration. As it has been already reported that this miRNA regulates EMT (34), we here demonstrated that the overexpression of miR-33b inhibits the EMT process in HER2+ subtype of BC by regulating EZH2 expression. Furthermore, we reported that the overexpression of miR-33b has an impact on cell proliferation and induces apoptosis in this BC subtype. Besides that, this miRNA also arrests the cell cycle in the sub-G0/G1 phase as compared with the other phases, which is in concordance with previous results in lung cancer (8). Recently, some authors suggested that miR-33a can regulate EZH2 by their direct interaction (21). We checked *in silico* the physical interaction between miR-33b and EZH2 in Targetscan, miRDB-MicroRNA Target Prediction Database, miRNet, miRTarBase, miRanda databases, and the Freiburg RNA tools. In the latter case, we found an interaction with a yield of very low energy. Based on this information, we performed the luciferase assay and we found that there is no such direct interaction between miR-33b and EZH2 (data not shown), which clarified that although miR-33a and miR-33b belong to the same family, they regulate the same gene in a different way. It has been previously demonstrated that MYC binds to the EZH2 promoter and directly activates its transcription (20). Besides that, EZH2 expression is positively correlated with MYC expression in prostate cancer (35). Moreover, it has been already described that MYC is a direct target of miR-33b (10). Thus, in our present study, we showed that the ectopic overexpression of miR-33b regulates MYC in our models (**Supplementary Figures 3A–D**) and the sequences of miR-33b have binding sites within the human MYC 3′UTRs (TargetScan can, **Supplementary Figure 3E**). Considering all these, we suggest EZH2 as a target of miR-33b *via* regulating the MYC (**Supplementary Figure 3F**).

Epithelial–mesenchymal transition is a crucial process during the development of tumorigenesis and metastasis. Enormous evidences indicate that EMT is responsible for cancer cell invasion and migration and an initial step of metastasis. *EZH2* is reported to be upregulated in aggressive BC (36) and involved in epigenetic, post-translational modifications and EMT program by suppressing CDH1 expression (37). In nasopharyngeal carcinoma, miR-142-3p was downregulated by DNA methylation due to EZH2's recruitment of DNMT1 which occupied the upstream region of the miR-142 and determined ZEB2 activation, leading to EMT and metastasis (38). Furthermore, EZH2 is a direct target of miR-26a in docetaxel resistance cells, which could significantly suppress proliferation, facilitate apoptosis, inhibit the metastasis ability, and reverse EMT to mesenchymal–epithelial transition in lung adenocarcinoma cells (39). In oral tongue squamous cell carcinoma, miR-101 inhibits the expression of EZH2 *via* two transcription factors, Snail and Slug (40). In BC, miR-92b may negatively regulate the expression of EZH2, promoting autophagy and decreasing tumor cell viability, migration, and invasion (41). Additionally, miR-139-5p transcription is inhibited by EZH2 through upregulating H3K27me3; thereby, the downregulation of EZH2 and the upregulation of miR-139-5p impede EMT in lymph node metastasis pancreatic cancer (42). Accumulating all these summarized results, the expression of EZH2 is upregulated in different types of cancer, and its inhibition is required by different miRNAs and drugs to reduce cancer progression. Given that the behavior of EZH2 is context dependent, in this study we investigated the role of EZH2 specifically in HER2+ BC. In our study, we determined that EZH2 is highly expressed in HER2+ BC cell lines as well as in solid tumors in comparison with normal epithelial cell line and normal breast tissue which show an inverse correlation. To dig more on the molecular mechanisms of EZH2, it has been silenced through two different silencers in BC cell lines, which resulted in the inhibition of cell proliferation, migration, invasion, and EMT in HER2+ BC cells, confirming that EZH2 expression has a crucial role in HER2+ BC progression (**Supplementary Figure 3F**). Future *in vivo* experiments to evaluate the role of miR-33b in HER2+ BC metastasis are needed.

In summary, EZH2 might be an important factor of HER2+ BC progression and associated with a decrease in the overall survival of patients since EMT has been critically discussed as the key process in tumor aggressiveness and metastasis (43). Our findings in the present study demonstrate for the first time that miR-33b acts as a suppressive miRNA in HER2+ BC, which could inhibit tumor migration and invasion partly by impeding EMT through the repression of the MYC–EZH2 loop. This study suggests a novel miR-33b/MYC/EZH2 axis that modulates the growth and the progression of breast cells and could be clinically useful to design new drugs against HER2+ subtype cancer.

DATA AVAILABILITY STATEMENT

All datasets generated for this study are included in the article/**Supplementary Material**.

ETHICS STATEMENT

The studies involving human participants were reviewed and approved by INCLIVA institutional review board (protocol number: 2018/077). The patients/participants provided their written informed consent to participate in this study.

AUTHOR CONTRIBUTIONS

AI. and PE contributed to the conceptualization and design of the study. IIPa, IG-C, AA-A, and ET developed the methodology. BPt, PC, AA-A, and EA contributed to the acquisition of data. OB, ET, BB, JC, and PE contributed to the analysis and interpretation of data. PE, BPa, IG-C, JA, FR, AI., AR, and JC contributed to the writing, review, and/or revision of the manuscript. EA, BB, CH, MM, and OB provided administrative, technical, or

material support. JC, AL, and PE supervised the study. All authors contributed to the article and approved the submitted version.

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SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fonc.2020.01661/full#supplementary-material

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Conflict of Interest. JA and AL report being advisory board members from Roche. JA and AL report receiving other honoraria from Roche as speaker's bureau or travel grants.

The remaining authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.