NANOTECHNOLOGY APPROACH FOR TARGETED TREATMENT OF TRIPLE NEGATIVE BREAST CANCER

By

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ABSTRACT OF THE THESIS

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Breast cancer is one of the most devastating diseases worldwide. Triple negative breast cancer cells (TNBCs) are defined by the lack of progesterone receptor (PR), estrogen receptor (ER), and epidermal growth factor receptor 2 (EGFR2) expressions. TNBCs account for 10%- 20% of all breast carcinomas. The study is aimed at examining the efficacy of gefitinib and EGFR-targeted siRNA delivered by liposomes for treating triple negative breast cancer (TNBC). The experiments were carried out using two types of human breast cancer (BC) cell lines MCF-7 (estrogen positive BC, EPBC) and MDA-MB 231(TNBC). EGFR-targeted siRNA and gefitinib were delivered by cationic and neutral liposomes, respectively. A fluorescence microscope was used to study cellular internalization of labeled liposomes and siRNA. The expression of the targeted mRNA

was performed using quantitative reverse transcription PCR. Finally, cytotoxicity of liposomal siRNA and gefitinib alone or in combination was measured using the modified MTT assay with appropriate controls. It was found that liposomes effectively delivered siRNA into both types of BC cells and suppressed the expression of targeted EGFR mRNA. However, formulations without gefitinib did not influence significantly on the viability of BC cells. Free drug demonstrated the ability to kill both types of cancer cells. Nevertheless, toxicity of gefitinib in TNBC was 2.5 times lower when compared with EPBC cells. The delivery of the drug by liposomes significantly enhanced its toxicity (1.2 and 2.5 times in EPBC and TNBC, respectively). The combination of liposomal siRNA and liposomal gefitinib demonstrated exceptionally high cytotoxicity when compared with the free drug (143 and 62 times higher in EPBC and TNBC, respectively). Suppression of EGFR mRNA effectively suppressed resistance of TNBC cells to gefitinib. The data obtained support the proposed approach and showed high potential of liposomal EGFR siRNA in combination with liposomal gefitinib in treatment of TNBC.

DEDICATION

To my wonderful parents

Abid A. Al-Mahmood and Eehan A. Al-Sharefee

To my lovely husband

Jalal Nazar Abdulbaqi

To my amazing kids

Ibrahim, Mohammed, Abdullah

To my brothers and sisters

Asia, Aaeshah, Abdulrahman, Osama, Sara, Hamza

To my amazing advisor

Dr.Tamara Minko

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1 INTRODUCTION

Cancers can be solid tumors or hematologic and different approaches are needed for each kind of cancer. Solid tumors show a heterogeneous and dynamic biology that keeps changing with time. Breast cancer is a heterogeneous and solid tumor. Genetic factors can be responsible about the occurance of 5%-10% of breast cancer cases [1,2]. Many risk factors can affect the development of breast cancer disease such as status of lymph node, size of the tumor, age of the patient, histological grade and type or status of human epidermal growth factor receptor 2 (HER-2), estrogen receptor (ER) and progesterone receptor [3]. Early diagnosis can play an important role in decreasing the progression of disease and reducing the rate of death [4]. Traditional therapy of breast cancer which includes radiothaerapy, surgical resection combining with chemotherapy can affect both cancer and non-cancer cells [5–7]. In addition, about 95% of anticancer thaerapies have poor biopharmaceutical and pharmacokinetics properties such as very short circulation half-life or poor water soluble drugs. Innovative technologies such as nanotechnology can apply to improve the diagnosis, imaging and efficacy of breast cancer treatment [8–10].

Nanotechnology concerns any devices whose dimensions are within the range of 1–1, 00 nm [11–14]. Nanotechnology can include nanomedicines, nanomanufacturing, nanocarriers, nanomaterials, nanoscale devices and societal studies of nanotechnology benefits and risks [8]. Many nanotechnology products are approved by Food and Drug Administration (FDA) for clinical use, and more are in clinical trials [12]. Polymers, liposomes, dendrimers and metals such as gold and ion oxide are examples of nanoparticles that are using in the treatment of cancer [12,15].

The multifunctionality and unique characteristics of nanotechnology encourage their use in the cancer field. The size of the molecule can be considered as an important part in controlling the kinetics of tumor accumulation and preventing the diffusion again to the systemic vascular bed. The effect of the size of nanoparticles is more complex in blood circulation but does not follow the same rules for protein based chemotherapeutics or small molecules [8,11,16–18]. Payload density, duration of effect and properties of the surface can also be considered as important parameters of using nanoparticles in cancer rather than small molecules or nucleic acide treatment [17,18].

Nanoparticles can be used to target the drug to specific point at the site of disease and improve the bioavailability and uptake of poor water soluble drugs, however taking into accounts the microenvironment of tumor cells , biology of tumor cells, and tumor cells growth patterns [14].

2 BACKGROUND AND SIGNIFICANCE

2.1 Introduction

Breast cancer is a heterogeneous and a complex disease [19–23]. It is composed of different biological subtypes, which are human epithelial growth receptor type 2 (HER-2), luminal A, luminal B, claudin-low and basal-like. These five subtypes have different abilities to metastasize to distant organs, specific pathways with the preferred metastatic sites and different survival response after relapse [24]. Patients who have the luminal subtypes of breast cancer frequently for example have bone relapses; however, breast cancer of basal subtype often metastasizes to the lungs and brain, and cannot reach

statistical significance in patients with liver relapse [20,22]. The biological subtypes of breast tumor can be defined by immunoistochemical (IHC) biomarkers or gene expression profiles [20,25]. In general, the standard prognostic and predictive factors for breast cancer disease are human epidermal growth factor receptor 2 (HER2), progesterone receptor (PR), estrogen receptor (ER), and proliferation (Ki-67) status [22,26]. The choice of local or systemic treatment can vary related to these different subtypes of breast cancer [25]. Breast cancer can spread to other sites of the body resulting in metastatic breast cancer (MBC) [3]. Between 6%-60% of patients with breast cancer were diagnosed early with MBC [19,20,24,27–29]. MBC is the second leading cause of death among women in the United States [30]. Age, race, ethnicity, endogenous hormones, menopause, histological status of cells, smoking, first degree relative, number of metastatic sites, duration of breast feeding, mutation and the underlying biology of the tumor such as grade and size of the primary tumor can increase the chance of MBC occurrence [31–41]. The main sites of breast cancer to spread are lungs, bones, liver, brain, soft tissue, and adrenal glands [22,29,42,43].

2.2 Occurrence of metastasis breast cancer

Metastatic breast cancer (MBC) process is a complex multistep process that includes many steps of dynamic interactions between cells of the tumor and the host resulting in leaving of tumor cells from their primary site and metastasis to a distant area. Figure 2.1 shows the different physiological activities of MBC from the primary tumor to the secondary site [44–47]. Metastasis process can also know as non-passive or nonlinear process because it likes loops between cells of the tumor and cells of the host in the tumor microenvironment. When the tumor is formed, it grew and proliferated

overcoming the cellular restrictions that leading to disrupt the local homeostasis and affected hypoxia, acidosis as well as systemic and tissue pressures. During the initial phases of tumor proliferation, the host activates tissue repair mechanisms by providing the neoplasm with a supply of nutrients vascularization, removing of waste and escaping route for the prospective metastatic cell in an attempt to compensate changes in the primary site. At the same time, the physical stress of the growing lesion initiates an inflammatory response that mobilizes bone marrow-derived cells (BMDCs) and other leukocytes to the primary and potential secondary sites. This uncommon and unnatural mixture of cells resulting in a reactive microenvironment as well as a suitable environment of cytokines, growth factors and extracellular matrix (ECM) proteins. The re-modeling of ECM proteins within the interstitial space is a marker of highly invasive tumors. In case of tumor, the inflammation fails to resolve and stimulate the occurring involvement of the immune-regulatory cells leading to decrease the response of antitumor immune system [44,48–51]. Later, these tumor cells acquire more mutant alleles that enable them to spread and seed new colonies at different anatomical sites that are distant from the primary tumor mass. Activation of oxidoreductase enzymes, latent proteases alter topology of ECM and improve the invasion of tumor cell by the exposure of cryptic adhesive sites and the release of pro-migratory peptides. Therefore, the host cells can develop genetic changes that enable them to carry these mutant alleles to offspring of people within the primary tumor mass. Ligation adhesion receptors of tumor cell to this modified ECM simulating intracellular pathways that induce invasion through the stroma and finally into the lymphatics or bloodstream [48,52,53].

MBC occurs primarily through the lymphatic system. The spread of cancer cells by lymphatic vessels to lymph nodes sites is an important predictive of tumor aggressiveness for most human tumors [54,55]. On the other hand, the tumor cell must resist the physical stress caused by loss of vascular turbulence and adhesion before its arrest in a distant capillary bed in circulation. During transit, tumor cells can form a bolus with platelets, which protects them from the stresses of shear flow and enhances their sensitivity to chemokine gradients. Among combination of physical obstruction, attractive chemokine gradients and the complementary adhesive contacts, the cancer bolus is attracted and became surrounded by capillaries of the secondary site. As a result, lodged cancer cells may grow as an intravascular metastasis or may extravasate into the secondary tissue [44,48,56].

In the secondary sites, cancer cells are arranged in small capillaries and deformed to fit the vasculature in the new sites according to the blood pressure in the new organ and the size restrictions. Cancer cells can occur in the secondary sites as small pre-angiogenic metastases, solitary cells, or large vascularized metastases. Only a subset of these cells can persist and the remainder of cells (micrometastases) might either go into a state of dormancy (dormant solitary cells are cells that are undergoing neither apoptosis nor proliferation) or die during every step of the metastatic process. In general, micrometastases and solitary cells are clinically undetectable and only a proportion of vascularized metastases are clinically detectable [44,52].

2.3 Predictive and prognostic factors of MBC

Most deaths of women with breast cancer arise due to the metastatic behavior of breast cancer and not as a result of the primary tumor growth. Consequently, prognostic factors can be successfully used to identify patients at high risk of metastatic breast cancer and to select a most effective treatment individually for each cancer patient. Prognostic factors can be derived from the specific environment of the host and from the tumor itself [23]. These prognostic factors can be pathological factors such as histological grade of the tumor, size of the primary tumor and deposit of the tumor in the draining lymph nodes of the primary breast cancer. Specific genes and corresponding proteins related to the development of breast cancer have been discovered recently. These genes/proteins involved, *inter alia*, in controlling cell proliferation (such as c-erbB-2 and c-erbB-3), cell death (such as p53), cell differentiation (such as pS2, ERα, and PgR) and cell invasion (such as cathepsin D) in tissue-cultured systems. However, these molecular markers have more limited use than the pathological factors in predicting death of patient from metastatic disease because they can relate more to the growth of the primary tumor and not necessarily to the development of distant metastases [57,58]. Table 2.1 shows the main prognosis and predictive factors of MBC which will be briefly discussed below.

2.3.1 Axillary lymph nodal involvement

Axillary lymph nodal involvement is an important factor to recognize the staging, prognosis, and treatment of PFS and OS of breast cancer. The common methods for determine the lymph node involvement in breast cancer are sentinel node biopsy (SLNB), clinical assessment, axillary dissection, and evaluation of imaging methods. The predictor

of axillary lymph node metastasis in general should be easy reproducible, cost-effective, high accurate and induces minimum side effects on patients. If lymph-node metastasis is present, there is high risk of metastasis while if there is no lymph-node involvement, a patient has a low risk of metastasis. In addition, the presence of more than 4 lymph-node metastasis is associated with very high risk of metastasis and generally predicts a poor prognosis [23,29,59–61].

2.3.2 Tumor size

Size of the tumor plays an independent role in the prognosis of MBC especially in several cases like axillary lymph node and HER-2 statues. The large size of tumor generally means worse prognosis and higher risk of MBC than small size tumor. The size of breast cancer ≤ 2 cm in patients younger than 40 years old generally indicates a relatively low risk of metastasis correlated with the presence of negative estrogen receptor status and axillary lymph node status. However, tumors with the size within 2-5 cm have high risk of metastasis while tumors a size more than 5 cm, have very high risk of metastasis. About 80% of patients with tumors measuring ≤ 1 cm have better 20 years recurrence PSF when compared with 72% of patients with tumor size 1.1-2 cm [29,62–64].

2.3.3 Estrogen receptor (ER) and progesterone receptor (PR) status

Estrogen receptor (ER) and progesterone receptor (PR) are considered the most important prognosis factors even before the invention of hormonal therapy. ER positive patients with node-negative breast cancer who treated with local therapy showed higher PFS and OS within 5 years. Hormone receptor is strongly associated with hormonal/endocrine treatment; however, hormonal therapy is not useful in hormone receptor negative tumor

cases. Moreover, the loss of either PR or ER in recurrent breast cancer will be related with poor response to hormonal/endocrine therapy [23,58,64,65]. Table 2.2 shows the percentage distribution of estrogen and progesterone receptors.

2.3.4 Circulating tumor cells (CTCs)

Circulating tumor cells (CTCs) are rare malignant cells that resulted or originated from the primary site. These cells circulate in the peripheral blood and can work as independent predictive and prognosis factor of early and advanced stage of breast cancer. The presence of more than 5 CTCs/7.5 ml of blood in MBC patients or more than 1 CTCs/7.5 ml of blood in non-metastasis patients can be predictive of poor PFS and OSC. As a result, CTCs can give information about the efficacy of the treatment by drawing a blood sample from cancer patient multiple times during his/her illness [66–75]. Figure 2.2 shows how CTCs works as prognosis factor for metastasis cells, treatment and understanding drug resistance in breast cancer.

2.3.5 Lymphovascular invasion (LVI)

Lymphovascular invasion (LVI) involves both the lymphatic and blood vessel invasion lying within an endothelial-lined space in the area that surrounding the invasive tumor. LVI can be used as predictive factor for breast cancer patients. In addition, it is prognosis factor for lymph node, lymph node positive and triple negative breast cancer. About 23% of patients with early stage breast cancer showed vascular invasion. [23,64,76–83]

2.3.6 Age at diagnosis

A retrospective study showed that patients younger than 35 years old with early stage of breast cancer following both mastectomy and breast conserving surgery had a worse prognosis with higher risk for developing MBC and greater overall recurrence comparing to older patients. In addition, prediction of the age at diagnosis showed that patients who are older than 40 years can be more prone to have triple negative breast cancer [64,84–86].

2.3.7 Race and ethnicity

The rate of death due to breast cancer remains higher among African Americans than Caucasian in the USA and this may be associated with the nature of tumors. In addition, black women patients more likely have hormone receptor-negative tumors, positive axillary nodes and positive axillary nodes associated with smaller tumors comparing with white women patients. Moreover, black women who receiving neo-adjuvant chemotherapy, showed worse PFS than white or Caucasian women but the OS in these groups was similar [87–92].

2.3.8 Cathepsin D

According to their active site amino acid, the cathepsin family of lysosomal hydrolases can be divided into three sub-groups: cysteine (B, C, H, F, K, L, O, S, V, W and X/Z), aspartate (D and E) and serine (G) cathepsins. Cathepsin D can be used as predictive factor for breast cancer. When the cathepsin D protein level exceeds 70 pmol/mg in patients with node negative tumor, it is associated with poor prognosis [23,93,94].

2.3.9 Angiogenesis Markers

The occurrence of tumor emboli in more than 3 blood vessels is most probably is associated by metastases. Microvessel density (MVD) is a common standard method of measuring angiogenesis of cancer. The high score of MVD in tumors in most cases indicates an easy and aggressive metastasis of cancer, and also is associated with a poor prognosis [29,95–98].

2.3.10 Bone marrow micrometastasis

Bone marrow micrometastasis refers to a small metastasis of less than 0.2 cm in diameter and can also include the tumor cells found in the bone marrow. The tumor cells usually can be found in 31% of lymph node negative patients and 55% of lymph node positive patients. The metastasis cells in the bone marrow are generally associated with poor clinical outcome in patients with breast cancer [23,29,99–102].

2.3.11 Overexpression of the c-erb B-2 (HER2/neu) proto-oncogen

Human epidermal growth factor receptor 2 (HER2) is a member of epidermal growth factor receptor EGFR family. Overexpression of HER2 was found in 18-25% of breast cancer cases. In most cases, overexpression of HER2 is associated with high risk of nodal involvement, hormone receptor negativity, metastasis and poor survival. Despite some uncertainties, HER2 status could be monitored in every patient scheduled to undergo hormonal/endocrine treatment [23,29,103].

2.3.12 Urokinase plasminogen activator (uPA) and plasminogen activator inhibitor type I (PAI-1)

The urokinase plasminogen activator (uPA) system includes the serine protease uPA, its cell surface receptor uPAR, and its serine inhibitors: plasminogen activator inhibitor type 1 (PAI-1) and plasminogen activator inhibitor type 2 (PAI-2). uPA is an extracellular matrix-degrading protease and PAI-1 representing the inhibitor of uPA is originally known as a blood-derived endogenous fast-acting inhibitor of uPA. Both uPA and PAI-1 can be used as independent prognostic factors for breast cancer patients since uPA has a role in the progression and metastasis of the tumor. In addition, uPA and PAI-1 are also included in cell signaling and can affect migration, chemotaxis, adherence, cell growth, anoikis and survival. Moreover, uPA and/or PAI-1 can play a role in the physiological processes like blood clotting, wound healing, fibrinolysis, pregnancy, and tissue remodeling. Paradoxically, high protein levels of both these markers were related to high metastasis risk and poor PSF. In addition, uPA and PAI-1 are considered the best prognostic biomarkers for lymph node-negative breast cancer [23,29,104–107].

2.3.13 Mutation of p53

Tumor protein p53 is a tumor suppressor and plays an important role in the pathways of cellular stress response and regulation of the transcriptional programs which is important for suppressing the formation and progression of the tumor. The most common mutation of this gene involves the substitution of an arginine for a proline at codon position 72. The high rate of mutant p53 is related with cancer metastasis, tumor proliferation and early death in node-negative breast cancer. Tumors with mutant p53 was also related with

high local failure rate and poor response to systematic treatment such as tamoxifen [23,108–112].

2.3.14 Expression of topisomerase II-alpha (topo IIα)

Topisomerase II-alpha (topo IIα) is located adjacent to the HER2 oncogene at chromosome 17q12-q21 therefore it can predict HER-2 positive breast cancer, lymph node metastasis, and advanced stage of cancer. In addition, the status of topo IIα gene in the primary breast cancer is correlated with its status in the metastases [23,113,114].

2.3.15 Proliferation markers

2.3.15.1 S-phase fraction (SPF)

It was shown that the SPF value can predict the proliferation of the tumor to metastasis. The high level of SPF is associated with larger tumor size, worse tumor grades and adversely with PFS and OS [23,115–120].

2.3.15.2 Thymidine labeling index (TLI) and mitotic activity index (MAI)

The high level of TLI is inversely correlates with the prognosis of node-negative tumors patients. In addition, low level of TLI in patients with early stage node positive breast cancer is associated with better survival. Moreover, when the value of MAI greater than 10 in patients with lymph node negative breast cancer, it is correlate with greater rate of recurrence and mortality [23,121–123].

2.3.15.3 Ki-67 nuclear antigen and proliferating cell nuclear antigen (PCNA)

Ki-67 antigen is expressed in the nucleus of cycling cells and used as independent factor to measure the rate of proliferation. The high level of Ki-67 is associated with

overexpression of HER2/neu, more lymph node involvement and larger tumor size in patients with breast cancer. In addition, higher PCNA was correlated with shorter relapse free and OS [108,124–129].

2.3.16 Gene expression profiling

Because of the variation in the predictive markers of patient's outcome that determined by IHC, the analysis of gene patterns can be considered as an alternative method to define the treatment efficacy and its outcome. Assessment of gene array can be assessed by a DNA microarray, which can be best done on fresh frozen tissue. In addition, method of real-time reverse transcriptase polymerase chain reaction (RT-PCR) can be used to assess the pre-selected specific number of genes or confirm expression of selected genes. The pre-select gene arrays determine about 21 predefined genes (included in multigene array) to predict response and recurrence to hormonal and drug therapies. On the other hand, the risk groups in gene pattern array can be classified more by using DNA microarrays into different groups according to gene expression: luminal A, luminal B, normal-like (mainly ER positive), basal-like (mostly ER negative) and HER2 positive (Mostly ER negative). These subtypes showed different prognosis and response to treatment; however, basal-like, luminal B and HER2 positive group showed worse outcomes. In addition, a good signature of 70 genes are related with low risk of metastasis while a poor signature of 70 genes are related with high risk of metastasis [23,29,130].

2.4 Models of breast cancer

The metastasis nature of tumor cells was discovered during the period 1970s - 1980s by methods of "experimental metastasis" assays. Fidler et al. concluded that cells derived

from outgrowths of metastatic cells have a higher metastatic activity than cells derived from the original cell line according to study of injecting intravenous metastatic cultured B16 melanoma cells into mice. Weigelt et al. described three different models of MBC, which are the traditional metastasis process, new models of MBC and the integrative model of MBC [29,46].

The first model of MBC cascade suggests that MBC occurs as either most cells of primary tumor have a low metastatic activity but acquire metastatic activity through additional somatic mutations during later stages of tumorigenesis, or spontaneous metastasis (all cancer cells have the capacity to develop metastasis); or the subpopulation of metastatic cells grow rapidly in the primary tumor and these variants are unstable leading to dynamic equilibrium between generation and loss of metastasis variants (Dynamic heterogeneity model), or subclone of metastasis grow rapidly with the primary tumor (clonal dominance therapy); or susceptible cell's transfection in distant organs with dominant and plasma-circulating oncogenes that are derived from the primary tumor (genometastasis hypothesis). On the other hand, the traditional model of MBC displayed the genetic behavior of the original cells that seed the cancer can affect the ability of mouse mammary cancer cells to metastasis; therefore, these observations encourage the role of genetic make-up of host cells to metastasis [29,131–136].

The second model, which is a genetic expression analysis of breast cancer suggested that MBC can occur due to the ability of cancer cells to acquire metastasis during the early stages of tumorigenesis; or more tissue specific expression profile predicting the site of metastasis as lung, bone and liver; or metastasis cancer cells can occur separately from the primary cancer cells during the early stages of oncogenesis (parallel evolution

model); or only breast cancer stem cells have the ability to metastasis to distant areas of the body [21,29,132,137–141].

The third model of MBC, which is the integrative model, predicted that the accumulation of somatic mutation and factors of tumor microenvironment such as fibroblasts, ECM, inflammatory cells and blood vasculature can be responsible for metastasis of cancer. However, mutation can occur from non-metastatic breast cancer. Then, these mutations which occur at different stages of tumor differentiation can control the capacity of cancer to disseminate. Moreover, breast cancer cells may differ in their tendency to disseminate to a specific organ or tissue. Furthermore, the breast cancer stem cells would induce the formation of new blood vessels at the site of metastasis and also induce a stromal response similar to that of their primary breast cancer. This model is based on studies of the fibroblast serum-response signature and prognostic markers like uPA/PAI1 and gene expression profile [21,29,142–144]. Figure 2.3 shows different models of MBC occurrence.

2.5 Treatments of metastasis breast cancer

The goal of metastasis treatment is to prolong survival, palliate symptoms and delay progression of the disease [23,145]. Treatment of MBC varies with certain factors such as risk for toxicity, preferences of the patient, burden of the tumor, characterization of the tumor itself such as HER2 status and hormone receptor status, age, history of prior therapy, co-morbidities, degree of tumor related symptoms and metastasis sites. In fact, treatment of MBC can fall into three categories surgery, chemotherapy and hormonal therapy [146–148]. Combination of two or more regimens of MBC therapy can improve

the quality of life and decrease the side effects associated with using single treatment. There are different types of MBC treatments:

2.5.1 Surgery

Surgery can precede either hormonotherapy or chemotherapy or follow induction therapy. It is one of the common treatment of MBC disease especially in nodal dissection for locoregional and sentinel lymph node cases. The use of surgery can vary according to the clinical situation and characteristics of the patient; therefore, it can be used as a single treatment or in combination with chemotherapy or hormonal therapy to enhance the efficiency of MBC treatment [149]. In addition, surgery can improve the overall survival and reduce breast cancer mortality by preventing the potentially disabling complications (medullary compression, pathologic fractures), resecting of metastases (lung, ovary, liver), providing a symptomatic treatment (infiltration of the chest wall, local recurrence, bone pain) and excluding of another tumor or non-tumor diseases [149–156]. On the other hand, surgery can cause an increase in the peripheral oxidative damage to macromolecules in the early postoperative period; therefore, perioperative antioxidant supplementation should be considered [157].

2.5.2 Radiation

Radiation therapy is used in breast cancer following the mastectomy and surgery. However, radiotherapy showed relapse about 7-12.6% among patients with five years [158]. In addition, resistance to this treatment can occur [159] and it is preferred to use combination of radiotherapy and hormonal treatment especially if the size of tumor is greater than 1 cm [160].

2.5.3 Hormonal Therapy

Hormonal or endocrinal therapy is an effective and a well-tolerated anti-cancer treatment. It is a systemic therapy and can be considers as the standard treatment in estrogen receptor positive tumors of the early and late stage of breast cancer [161]. Hormonal treatment is also used in order to minimize the toxicity associated with other treatments. In addition, it can be given pre-operatively (neoadjuvant) or post-operatively (adjuvant), or during the MBC disease setting (palliative treatment) [162,163]. However, sensitivity to hormonal treatment or resistant can occur among patients as side effects of this treatment [164,165].

2.5.3.1 Types of Hormonal Therapy

2.5.3.1.1 Ovarian Suppression

It is the first systemic therapy for any type of cancer and the oldest endocrinal therapy for hormone receptor–positive breast cancer that recently been replaced by ovarian irradiation. Ovarian Suppression is made by medical oophorectomy with the so-called luteinizing hormone-releasing hormone (LH-RH) analogues or agonists such as goserlin, leuprolide, buserelin and triptorelin [23,166]. Although, certain LH-RH receptors have been identified in breast cancer, LH-RH agonists alone did not diminish the recurrence or mortality. The uses of Ovarian suppression treatment is still controversially ; however, it is required in patients with MBC and receiving LH-RH agonist treatment and candidate for subsequently radiological or surgical ablation, as many subsequent therapy options involving aromatase inhibitors or subsequent second line treatment with aromatase inhibitors that need suppression of ovarian function [167,168].

2.5.3.1.2 Adrenalectomy and Hypophysectomy

Adrenalectomy and hypophysectomy surgery are considered the first line treatment in cases of postmenopausal women since adrenal gland is a source of steroid production in postmenopausal women. Both these treatments are used in the management of MBC but with limit effect on morbidity and mortality. Therefore, an advance stage of medical adrenalectomy is introduced. Glucocorticoids treatment (prednisone/prednisolone 5-10 mg) daily showed a low toxicity and response when used in the MBC treatment with moderate doses. Moreover, a major discovery had made with the introduction of amino glutethimide, which is adrenal blocker as treatment of MBC. Aminoglutethimide, which is unsuccessful antiepileptic drug, shows great antitumor effects due to its ability to inhibit aromatase enzymes [169,170].

2.5.3.1.3 Aromatase Inhibitors

Aromatase inhibitors can inhibit aromatase enzymes that are responsible for the synthesis of estrogens from androgenic substrates produced by the adrenal glands and therefore aromatase inhibitors drugs are used in the MBC treatment. These drugs are divided into: type 1 or steroidal inhibitors like exemestane; and type 2 or non-steroidal inhibitors like anastrozole. Steroidal inhibitors are analogues of adione and are irreversibly inhibitors of aromatase while non-steroidal inhibitors bind reversibly to the haem group of aromatase Although the first generation aromatase inhibitor, which is aminoglutethimide can suppress the estrogen and inhibit only aromatase enzyme therefore, the levels of circulating androgen were found to be not affected due to suppression of estrogens. In addition, because of the side effects and inconvenience of parenteral administration of the

first generation, second and third generation of the aromatase inhibitors such as anastrozole, formestane and letrozole were developed [169,171,172]. Moreover, the third generation of aromatase inhibitors showed a greater response than tamoxifen treatment alone [173,174].

2.5.3.1.4 Selective Estrogen Receptor Modifier (SERMS) and Selective Estrogen Receptor Downregulators (SERDS)

Tamoxifen is the most known drug of SERMS due to is antitumor activity and low toxicity. This drug is used as first line treatment in premenopausal as well as postmenopausal women with MBC [166,175,176]. Tamoxifen can interact with follicular maturation in premenopausal women leading to increase the plasma levels of estradiol 2 to 3 fold. The regular dose of tamoxifen is 5 mg daily. Droloxifene and Toremifene with high dose are other drugs of SERMS group. They show lower antitumor activity in premenopausal women but similar antitumor activity in postmenopausal women compared with tamoxifen [177,178]. SERDS is a novel group of drugs and fulvestrant is an example of this category of drugs. Fulvestrant is different from other SERMS drug in lacking any estrogen agonist activity and having a unique chemical structure. In addition, fulvestrant works by two mechanisms downregulation of the receptor or blocking of the receptor. Moreover, fulvestrant with dose of 500 mg has great antitumor activity similar to tamoxifen; however, it is required to administer parenterally [179,180].

2.5.3.1.5 Additive Hormone Therapy

Different treatments at high doses such as estrogens, androgens and progestins can be used in MBC. Androgens were used in the treatment of breast cancer before nowadays

treatments since most breast cancer receptors express androgen receptors at a level greater than 10 fmol/mg. However, androgen treatment shows a low response rate and is also associated with side effects such as hirsutism [181–183]. Considering estrogen, it is used with higher doses (diethylstilbestrol 15 mg daily) in premenopausal and postmenopausal women with breast cancer. Estrogen can work as antitumor drug due to its high concentration that is greater than the optimal concentration for cell growth and showed similar antitumor activity similar to tamoxifen [184]. Although progestin can suppress the estrogens therefore used as antitumor treatment but is associated with weight gain as side effect of its treatment. Both megestrol acetate with dose of 160 mg daily and medroxyprogestrone acetate with dose of 1000 mg daily showed similar antitumor activity similar to tamoxifen and aminoglutethimide [185,186].

2.5.3.2 Premenopausal Women with MBC

Premenopausal women accounts about one third of MBC cases. In general, because most of these women have estrogen and progesterone positive, endocrine treatment with ovarian suppression or ablation should be used. There are many approaches used to suppress the ovarian function such as ovarian ablation (surgical or radiological); or using of LH-RH agonists; or tamoxifen; or inhibitors such as hydrocortisone and aminoglutetimide; or additive such as glucocorticoids, androgens, estrogen and progestin [23,175]. However, tamoxifen combination with LH-RH treatment showed greater antitumor than using tamoxifen or LH-RH agonists treatment alone. Although, using of aromatase inhibitors is contraindicated in premenopausal women, aromatase inhibitors can be combined with ovarian ablation treatment. Furthermore, there is very limit data on using fulvestrant in premenopausal women [166,187].

2.5.3.3 Postmenopausal Women with MBC

Aromatase inhibitors either as monotherapy or in combination with tamoxifen can be considered as first line treatment of MBC among postmenopausal women with hormone receptor positive due to their high efficacy and low toxicity [164,188–190]. If relapse occurs within the treatment or below one year, therefore patients may be sensitive to this treatment. On the other hand, if relapse occurs after one year of the treatment, aromatase inhibitors or tamoxifen can be used as single treatment. In addition, fulvestrant which is a natural, selective estrogen receptor modifier (SERM) and estrogen blocker therefore it can also be used in the treatment MBC patients. In fact, fulvestrant is the drug of choice in MBC with patients resistant to aromatase inhibitors or tamoxifen and showed similar antitumor efficacy similar to aromatase inhibitors and tamoxifen [191,192]. Moreover, estrogen with high doses can be used in the treatment of MBC in combination with Fulvestrant and the side effects are acceptable [193].

2.5.4 Chemotherapy

The uses of chemotherapy treatment vary according to different cases of MBC. Chemotherapy is considered as the first choice of MBC treatment in women who rapidly develop progressive visceral metastasis chemotherapy and having symptomatic or having hormone receptor negative disease or having cancer resistant to endocrine therapy. In addition, chemotherapy is used as adjuvant treatment in patients with MBC who received local treatment and were at high risk of relapse it is more benefit in node positive patients than node negative patients were. However, systemic chemotherapy showed less impact with the age, severe side effects (nausea and vomiting), poor response and overall not

improve the survival benefits of patients. Cytotoxic drug can be administrated systemically (orally or intravenously) to kill cancer cells [194–197].

2.5.4.1. Common Chemotherapeutic Agents used in the Treatment of MBC

2.5.4.1.1 Anthracyclines

These drugs are the most common antitumor antibiotics used in the management of MBC. Epirubicin and doxorubicin antibiotics are examples of anthracyclines. They can work by different mechanisms such as impairing replication of DNA and mitochondrial function, generating oxygen free radicals, activating of apoptosis and matrix metalloproteinase as well as immune reactions [23,198]. About 30-40% of MBC patients with anthracycline treatment showed response of survival within 22 months [5]. The regimens containing anthracycline is better than regimens containing no anthracycline in time to progression but was associated with greater toxicity and there was no improvement in OS. The most common combinations of anthracyclines are CAF/CEF (cyclophosphamide 5-fluororacil plus epirubicin or doxorubicin) or AC/EC (doxorubicin/epirubicin plus cyclophosphamide). In addition, Myocet (liposome encapsulated doxorubicin) 75 mg/m2 every 3 weeks has shown to be less cardiotoxic and effective to the tradition doxorubicin in MBC [23]. The use of anthracycline is limited because they are associated with acute toxicity such as myelotoxicity, alopecia, nausea and vomiting and also due to their dose-dependent and irreversible cardio toxicity that is over 1000 mg/m^2 in case of epirubicin or 450 mg/m^2 in case of doxorubicin [198–200]. The combination of trastuzumab concurrently with anthracycline is a safe adjuvant regimen for breast cancer and does not increase cardiac events [201].

2.5.4.1.2 Taxanes

These drugs are microtubule inhibitors that inhibit tumor angiogenesis and are considered as the first line treatment in patients who resistant to anthracycline or cannot receive more anthracycline treatment. Docetaxel and paclitaxel are examples of taxanes, which showed high response rate in anthracycline resistant MBC cases [202,203]. Taxanes can be used as single agent or in combination with other treatments like the combination of anthracycline with taxanes that improve the quality of life better than anthracycline or taxanes treatment alone [23,204]. In addition, combination of taxanes plus biological drugs such as trastuzumab, trastuzumab showed improvement in overall survival in patients with MBC [205]. Moreover, combination of docetaxel plus thiotepa showed response that is more effective and less adverse effects in the treatment of MBC and can consider as an effective rescue and economical plan [206]. Furthermore, combination of lapatinib with docetaxel and trastuzumab can used as a first-line treatment of HER2-positive MBC [207]. However, dose limiting and neuropathy are common side effect of taxanes therapy, which can be managed by delays and reductions of the dose [202].

2.5.4.1.3 Capecitabine

Capecitabine treatment is used in patients with disease resistant to anthracycline or taxanes treatment [208,209]. It is used as oral prodrug to generate 5FU in tumor tissue through activation pathway of thymidine phosphorylase. The oral solution of capecitabine was prepared to be similar to continuous infusion of 5FU [210–212]. Capecitabine therapy showed 15-26% response rate with a dose of 1250 mg/m^2 twice daily for 14 days [23]. The most common adverse effects of capecitabine therapy are

nausea, hand-foot syndrome, diarrhea and in very rare cases alopecia and Myelo-suppression [212–214]. Capecitabine has more toxic effects than gemcitabine and vinorelbine treatment, so it is not prefer to use alone [215]. Therefore, the combination of cpecitabine with other chemotherapy is used to prolong the duration of treatment, improve the efficacy, decrease the side effects and maintain the therapy for patients with MBC [212–214,216]. Cabazitaxel or docetaxel plus capecitabine combination can be used to improve survival in patients with MBC recurring after anthracycline treatment than docetaxel treatment alone [23,217]. Moreover, capecitabine plus trastuzumab combination treatment showed great effects when used as first or second line treatment in HER2 overexpressing MBC cases [218].

2.5.4.1.4 Gemcitabine

It is a deoxycytidine-analogue antimetabolite and a nucleotide analogue that inhibits the synthesis of DNA [23,204,219–221]. This drug is well tolerated in elderly patients. In addition, it is related with low incidence of alopecia, nausea and vomiting and the most common dose-limiting toxicities are thrombocytopenia and neutropenia [23]. Great efficacy, pharmacodynamics and limited toxicity of gemcitabine make it an ideal agent for polychemotherapy combinations, specifically with vinorelbine, taxanes and platinum derivates [220]. Gemcitabine plus paclitaxel combination showed 68% in overall response when used as first line treatment and as 48% when used as second line treatment [23,222]. In addition, gemcitabine plus transarterial chemoembolization can be used in the treatment of liver metastasis of breast cancer [223]. Moreover, Gemcitabine can be used with bisphosphonate in the treatment of bone metastases of breast cancer [224]. Furthermore, low dose of gemcitabine plus cisplatin combination weekly showed

efficacy and safety in the treatment of strongly pretreated MBC patients resistant to taxanes and anthracyclines treatments [225–228] and treatment of brain metastasis of breast cancer [229].

2.5.4.1.5 Vinorelbine

It is a semisynthetic and third generation of vinca alkaloid [230]. It is safe and can be used alone or in combination with other drugs in the treatment of MBC [231–233]. The oral dosage form of vinorelbine can be used alternatively to intravenous form in MBC treatment [234–236]. Vinorelbine treatment showed 35-50% response when used as first line treatment of MBC; however, their main adverse effects are superficial phlebitis, peripheral neuropathy, neutropenia, myelosuppression, leukopenia and gastrointestinal toxicities [237]. Vinorelbine plus epiribicin showed higher response rate (RR) and PFS but not OS [23]. The combination of oral vinflunine plus capecitabine treatment showed safe response and good anti-tumor activity in HER2/Neu-negative MBC patients who have failed to anthracyclines and taxanes [230,238–246]. Moreover, vinorelbine plus gemcitabine combination showed better progression free survival compared with vinorelbine treatment alone [247]. Furthermore, low dose of oral vinorelbine plus temozolomide combination showed safe and effective effects in the treatment of brain metastasis of breast cancer [248].

2.5.4.1.6 Carboplatin

It is an alkylating agent or platinum compound used in the management of MBC that failed to response to other treatments. Carboplatin treatment can produce 20-35% of objective response rate (ORR) [249]. Combination of Carboplatin to docetaxel/paclitaxel

showed higher efficacy than Carboplatin or taxane treatment alone. This combination showed higher efficacy in treating breast cancer that metastasis to brain tumor [250–254]. In addition, combination of carboplatin plus trastuzumab/paclitaxel treatment showed superior efficacy for patients with HER2 positive MBC than using trastuzumab/paclitaxel alone [255]. Moreover, the combination of carboplatin with gemcitabine showed an effective treatment option for pretreated MBC patients [249,256,257].

2.5.5. Immune Therapy

In most cancers, the immune microenvironment is a balance of immune cells between mediating and preventing the destruction of tissue. Type I immunity such as CD4+ T cells that secrete cytokines like TNF-α, IFN-γ and CD8+ and interleukin (IL)-2 cytotoxic T cells support the destruction of tissue environment. The IL-2 activation of T-cells induces a regression of MBC in renal cancer and melanoma. In addition, the abundance of tumor-infiltrating leukocytes, CD3+ and CD8+ T lymphocytes have been related with PSF and OS of breast cancer patients. Three immune metagenes that represent the tumor-infiltrating populations and strongly associated with high survival of MBC patients are (1) B cells/plasma B cells determined by the high expression of IgG antibody isotype-related genes, (2) a monocyte/dendritic cell population determined by the expression of myeloid specific markers and a host of major histocompatibility complex class II antigen-presenting molecules and (3)T cell/natural killer cell-specific population determined similarly. Furthermore, signal transducer and activator of transcription 3 (Stat3) controls genes that are involved in cell proliferation and in the production of angiogenic and antiapoptotic factors. Consequently, ablating Stat3 signaling in breast cancer cells may represent an effective approach to immunotherapy of breast cancer growth and metastasis

that can result in induction of a cellular senescence program. However, such approach requires extensive immunotherapy research. On the other hand, type II immune system composed of CD4+ T cells that secrete cytokines like IL-4, IL-6 and IL-10 which in turn decrease the acute inflammatory response and prevent the proliferation of cytotoxic T cells. Moreover, CD4+ T cells showed a strong relationship with the progression of the tumor and tumor-specific CD8+ T cells. It was shown that mutation in cytotoxic T cell epitopes within the tumor antigen resulted in the progression of the tumor. An interesting multipronged approach to cancer treatment combines NK cell and cytotoxic T cells-based autologous immune enhancement therapy (AIET) with conventional approaches of treatments such as surgery, chemotherapy and radiotherapy as well as other modalities like hyperthermia, proton beam therapy and also low dose chemotherapy. It seems that such complex approach can be effective in advanced cancers which are refractory to conventional simpler therapeutic approaches. Furthermore, treatment of breast cancer with biologic drugs can induce type I immunity microenvironment and improve the therapy or decrease the recurrence of breast cancer [258–262].

2.5.6 Gene Therapy

Genes that control metastasis of the cancer is divided into two groups: metastasis suppressor genes (MSGs) and metastasis promoter genes (MPGs). The normal function of MSGs is preventing cells from divisions or proliferation and inhibiting the spread and growth of cancer while MPGs do the opposite. In addition, the concept of metastasis related gene is known in 1970, but the search of MSGs started in the mid-1980. Since MBC is cascade of signals, targeting these signals of genes can potentially help to improve MBC therapy [23].

2.5.6.1 Targeting EGFR Family

The epidermal growth factor receptor (EGFR) is a transmembrane tyrosine kinase receptor which triggers the phosphatidylinositol 3-kinase (PI3K/Akt) pathway on activation. EGFR is also a member of the HER family that is membrane-bound receptor tyrosine kinases (RTKs) and composed of four structurally related receptors: EGFR, HER2, HER3/ErbB3, and HER4/ErbB4. EGFR has the ability to stimulate motility, proliferation of cells, angiogenesis and metastasis of breast cancer. About 50% – 75% of breast cancer cells and about 45% of MBC patients have been shown to be EGFR positive resulting in more aggressive tumor than cells lacking this factor. Consequently, inhibitors of EGFR (antibodies or small molecules) can be used in the treatment of MBC [51,263–270].

2.5.6.1.1 EGFR Inhibitors

2.5.6.1.1.1 Cetuximab

Cetuximab is a chimeric anti-EGFR monoclonal antibody. HER1 receptor has a role in mediated cell signaling which is related to proliferation of the tumor, angiogenesis, metastasis, and apoptosis. In addition, overexpression of HER1 receptor and its ligand is noticed in multiple human malignancies such as lung cancer, pancreatic cancer, colorectal cancer and breast cancer. Cetuximab has a synergistic effect with radiotherapy and chemotherapy and can be used in the treatment of triple negative breast cancer cells cells that are overexpressed EGFR. In addition, weekly combination of cetuximab with taxane can be used for patients with triple negative breast cancer [271–276].

2.5.6.1.1.2 Gefitinib

Gefitinib represents a small molecule that irreversibly inhibit EGFR receptor (tyrosine kinase inhibitor) [246, 247]. The major problems associated with gefitinib treatment are the development of resistance. Combination of gefitinib with other drugs is used to overcome this problem [264]. Gefitinib can be used in HER2 MBC patients in combination with trastuzumab and docetaxel to reduce the resistance and overcome toxicities associated with trastuzumab and docetaxel [277].

2.5.6.1.1.3 Vandetanib

Vandetanib is an oral active antagonist of epidermal growth factor receptor (EGFR or ErbB1or HER1), vascular endothelial growth factor receptor-2 (VEGFR-2), and RET kinase. Vandetanib can be used in the treatment of thyroid cancer, prostate cancer, non-small cell lung cancer, breast cancer and colorectal cancer. This drug received its first global approval for the treatment of metastatic medullary thyroid cancer in the USA on 6 April 2011. In MBC, vandetanib with docetaxel combination showed greater efficacy than placebo combined with docetaxel only. However vandetanib with 100 or 300 mg/day did not show a good response in the treatment of patients with previously treated MBC. Diarrhea, nausea, fatigue, abnormal hepatic function and hyperglycemia are side effects associated with using vandetanib therapy in breast cancer [278–280].

2.5.6.1.1.4 Erlotinib

Erlotinib is an oral and potent EGFR inhibitor. It is used for the treatment of pancreatic cancer and non-small cell lung cancer. However, it showed less activity in MBC women therapy. In addition, using erlotinibcan with bendamustine in metastatic triple negative

breast cancer produced prolonged and sever lymphopenia. Furthermore, combination of erlotinib with docetaxel/capecitabine can be used in MBC treatment [281–284].

2.5.6.1.2 HER2 Inhibitors

2.5.6.1.2.1 Trastuzumab

It is a humanized monoclonal antibody directed against HER2 glycoprotein (anti-HER2/neu trearment). HER2 is overexpressed in 20-25% of human breast cancers leading to increase the aggressiveness of the tumor and decrease OS. Trastuzumab showed about 35% of response in the treatment of MBC [127, 267–270]. In addition, trastuzumab recently have been used alone or in combination with chemotherapy in the treatment of MBC in patients that overexpress HER2 protein consequently. Trastuzumab showed a good effect in women with HER2/neu-positive disease compared with women with HER2/neu-negative disease [269, 271–278]. Trastuzumab plus paclitaxel combination showed higher TTP, RR and OS in MBC patients pretreated with an anthracycline [297]. In addition, combination of trastuzumab and docetaxel can be used for treating patients with HER2 positive or HER2– overexpressing metastatic breast cancer. This combination showed good results in fields with little additional toxicity, time to treatment failure, time to progression, rate and duration's response and overall survival [298,299]. Moreover, combination of trastuzumab with other cytotoxic agents such as anthracycline, carboplatin, taxanes, vinorelbine and gemcitabine were effective when used as first or second line treatment especially in HER2 positive MBC patients [153,201,300–304].

2.5.6.1.2.2 Ado-trastuzumab emtansine (T-DM1)

Ado-trastuzumab emtansine is a conjugate of the antibody (trastuzumab) with the drug (emtansine, anti-microtubule agent). Trastuzumab considered the backbone that attached to emtansine by stable linker to deliver chemotherapy agent to cancerous tissues that are overexpressed HER2 without adversely side effects on normal cells. T-DM1 has the ability to combine the cytotoxic effects of emtansine with the antitumor activity of trastuzumab (HER2 inhibitor). In addition, T-DM1 has been shown to improve PFS and OS in Her2 positive MBC. Moreover, T-DM1 can be used effectively in treatment of HER2-positive MBC patients that are previously received trastuzumab, taxane and lapatinib. Cardiotoxicity, thrombocytopenia and increased liver enzymes are the main adverse side effects associated with T-DM1 [305–309].

2.5.6.1.2.3 Pertuzumab

Pertuzumab is a humanized monoclonal antibody that block the dimerization of HER receptors leading to decrease the intracellular signaling of HER2 receptor. Pertuzumab is different from trastuzumab in that it binds to a different domain of HER2. Pertuzumab is used alone or in combination with trastuzumab and docetaxel in the treatment of HER2 MBC patients and showed prolonged PFS and improved OS. Furthermore, Pertuzumab showed acceptable tolerability and no evidence of increasing the risk of cardiotoxicity [310–317].

2.5.6.1.2.4 Ertumaxomab

Ertumaxomab represents a monoclonal antibody targeting HER2/neu and CD3 on T cells. It is able to stimulate the recognition and destruction of cancer cells by different

immunologic mechanisms such as dendritic cells (DC), dendritic cell cytokine 1 (DC-CK1), leukocyte function associated antigen (LFA), antibody-dependent cellular cytotoxicity (ADCC) and tumor necrosis factor-α (TNF-α) and cluster of differentiation (CD). Ertumaxomab in the treatment of breast cancer showed a strong immunologic response. The most common adverse effects of ertumaxomab are vomiting, fever, elevated liver enzymes and lymphocytopenia [288,318–320].

2.5.6.1.3 Dual inhibitors of EGFR and HER2

2.5.6.1.3.1 Lapatinib

Lapatinib is an oral inhibitor for both HER2 and EGFR1. It can be used alone or in combination with other pharmaceuticals in the treatment of HER2 positive MBC [265,288,321–323]. The combination of lapatinib with carboplatin represents an effective therapy for brain metastasis of HER2-positive breast cancer and especially for cases when trastuzumab has no effect [324]. Combination of lapatinib with capecitabine is more effective in patients, who received less than two regimens for metastatic breast cancer and are naive to capecitabine [325–330]. The oral combination of these therapies can be used in HER2 positive metastatic brain cancer form [327,331]. Moreover, the combination of lapatinib plus vinorelbine showed moderate efficacy among treated before MBC patients with overexpression of HER2 [332,333]. Furthermore, combination of lapatinib plus trastuzumab showed higher efficacy especially in metastasis brain cancer when compared with a single treatment alone [287,334].

2.5.6.1.4 Inhibitors of more than one receptor of EGFR family

2.5.6.1.4.1 Neratinib

Neratinib is an irreversible pan-tyrosine kinase inhibitor and that also demonstrates the activity against HER1, HER2, and HER4. Neratinib is a low molecular weight, orally administrated antitumor drug that used in patients with advanced HER2-positive breast cancer which early have been exposed to trastuzumab or are resistant to EGFR inhibitors. The most common adverse effects associated with neratinib treatment alone are nausea, diarrhea, vomiting and fatigue [288,335–337]. Neratinib is about 12- to 16-fold more potent than lapatinib n inhibiting proliferation of HER2 positive breast cancer cells [336]. Combination of neratinib with vinorelbine showed a great antitumor activity in HER2-positive MBC patients [231].

2.5.6.1.4.2 Afatinib

Afatinib is an oral, small molcule anilinoquinazoline compound which is highly selective inhibitor of EGFR/HER1, HER2, and HER4 tyrosine kinase activity. This drug can be used alone or in combination with other treatment in HER2 positive breast cancer. However, it demonstrates a limited effect in HER2 negative breast cancer patients. Afatinib can be combined with vinorelbine or trastuzumab in the treatment of HER2 positive MBC. Moreover, afatinib can be added to the standard neoadjuvant therapy that includes anthracycline/taxane and trastuzumab the treatment of HER2-positive operable or locally advanced breast cancer. The adverse effects of afatinib is mainly associated with gastrointestinal toxicities [288,338–341].

2.5.6.2 Targeting Metastasis and Invasion

2.5.6.2.1 Inhibition of the uPA system

uPA and its receptor uPAR have a role in the angiogenesis, invasion and metastasis of the tumor. uPA is a member of the serine protease family which catalyzes the conversion of inactive zymogen plasminogen to its active form plasmin. When uPAR stimulate direct plasmin mediated proteolysis, the plasmin degrades most components of the ECM like fibronectin, laminin, and collagen that are produced by tumor surrounding stroma and tumor cells. Binding of uPA to its receptor stimulates activation of other proteinases like metalloproteases (MMPs). Moreover, uPA is associated with chemotaxis, cell proliferation, and angiogenesis elevated in malignant tumor. Therefore, inhibition of uPA and its receptor uPAR represents an attractive approach for MBC treatment. The drug candidate WX-UK1 is a 3-amidinophenylalanine-based inhibitor of the uPA system that is used to inhibit the metastasis capacity of tumor cells in vitro. Combination of WX-UK1 with capecitabine can also be used in MBC treatment [107,342–345].

2.5.6.2.2 Matrix Metalloproteinases (MMPs) Inhibitors

Matrix Metalloproteinases (MMPs) especially MMP-2 and MMP-9 have been involved in several types of cancer and their metastasis such as ovarian, colorectal, ovarian and breast cancers. High MMPs content in the model of human osteosarcoma cell destroy ECM; therefore the level of MMPs is related with metastasis of the tumor. In addition, MMPs stimulate the migration of endothelial cells and facilitate the formation of new blood vessels. Moreover, MMPs showed strong correlation with u-PA and negative correlation between u-PA/MMPs with inhibitors of metalloproteinases (TIMPs). BAY

12-9566 is an inhibitor of MMP-2, MMP-9 and MMP-3 showed no musculoskeletal effects and well tolerated in patients with solid cancer. In addition, combination of BAY 12-9566 with etoposide, doxorubicin, carboplatin, 5-fluorouracil and leucovorin can be used in cancer therapy. Moreover, other MMP inhibitors, such asmarimastat, solimastat, metastat, prinomastat, BMS 275291 and neovastat are currently under the clinical trials [342,346–350]. Fig. 2.4 shows the role of MMPs in carcinogenesis.

2.5.6.3 Histone Deacetylase Inhibitors (HDACi)

Histone acetyl transferases (HATs) and histone deacetylases (HDACs) play an important role in maintaining the balance between the acetylated and deacetylated states of histones, gene expression and modification of chromatin structure. In addition, inactivation of HATs is related with tumorigenesis. Histone deacetylase inhibitors (HDACi) are new class of anticancer agents that stimulate differentiation/apoptosis and inhibit the proliferation of cancer cells by inhibiting the function of HDACs. HDACi sensitizes tumor cells to topoisomerase inhibitors by increasing their access and binding to DNA. In addition, HDACi have been related with a transcriptional down regulation of ER in ER positive tumor cells. The combination of HDACi vorinostat with doxorubicin showed a significant antitumor activity in prostate, melanoma, and breast cancer. Furthermore, combination of another HDACi - valporic acid, with epirubicin improved their antitumor activity in patients pre-treated with anthracyclines [342,351–353].

2.5.6.4 Insulin-like Growth Factor Inhibitors (IGF-IR)

Insulin-like Growth Factor Inhibitors (IGF-IR) plays a major role in the proliferation and metastasis in different types of cancer like pancreatic, colon, prostate, and breast cancer.

IGF-IR consists of an intracellular β subunit responsible for signal transduction and an extracellular α ligand-binding subunit and binds to IGF-1 and IGF-2 ligand-activated IGF-IR. High levels of IGF-I are strongly related with high risk of breast cancer. The overexpression of IGF-I leads to improved survival, proliferation signals for the breast tumor and develop resistance to cancer treatment. In contrast to normal tissues, IGF-IR is overexpressed in about 50% of primary breast cancer tissues. Therefore, inactivation of IGF-IR results in decreased growth and metastasis of breast tumor *in vivo*. IMC-A12 is a human monoclonal antibodies that bind with high affinity to IGF-IR and prevent the activation of ligand dependent receptor and downstream signaling. BMS-554417 is novel IGF-IR that has a pronounced proapoptotic and antiproliferative activity *in vitro* and *in vivo*. In addition, IGF-IR can be used in the treatment of breast cancer in combination with cytotoxic drugs (e.g. aromatase drugs) or hormonal treatment. Furthermore, IGF-IR can be used in combination with EGRF inhibitors like leptin, lapatinib and erlotinib to improve treatment of MBC [342,354–358].

2.5.6.5. Targeting Vascular Endothelial Growth Factor Family (VEGF)

The vascular endothelial growth factor is a potent inducer of cell invasion, migration, vascular permeability and vessel formation. There are five glycoproteins VEGFA, VEGFB, VEGFD and placental growth factor that act by three receptor tyrosine kinases VEGFR-1, VEGFR-2 and VEGFR-3 [342]. Consequently, drugs targeted VEGF can potentially be used for treatment of different cancers including the MBC.

3 SPECIFIC AIMS

Specific Aim 1: To determine the ability of lipoplex (liposome with siRNA) to penetrate breast cancer cells (MDA-MB 231 and MCF-7) *in vitro*

The ability of lipoplex penetration inside breast cancer cells should be determined to ensure the possibility of using it as co-treatment or in formulation with chemotherapeutic drug.

Specific Aim 2: To determine the efficacy of EGFR-targeted siRNA delivered by liposomes with gefitinib for treating triple negative breast cancer (TNBC).

Triple negative breast cancer cells (TNBCs) have resistance to chemotherapeutic drugs such as gefitinib. Therefore, using lipoplex (siRNA targeted to EGFR) can help in improve the efficacy of gefitinb. Suppression of EGFR expression by siRNA targeted to EGFR delivered by liposomes can effectively help to suppress resistance of TNBC cells to gefitinib and improve the breast cancer therapy.

4 MATERIALS AND METHODS

4.1 Materials

DOPC (1,2-Dioleoyl-sn-glycero-3-phosphocholine), DOPE (1,2-Dioleoyl-sn-glycero-3-phosphoethanolamine) and DOTAP (1,2-di-(9Z-octadecenoyl)-3-trimethylammonium-propane (chloride salt)) were purchased from Avanti Polar Lipids, (Alabaster, Alabama), Fetal bovine serum (FBS) was purchased from Sigma- Aldrich (Louis, MO), DPBS was purchased from Lonza (Allendale, NJ), Trypsin, Streptomycin and Penicillin were

purchased from Thermo Fisher Scientific (Waltham, MA), SYBR® green PCR master was purchased from Applied Biosystems (Warrington, UK). The sequence of siRNA targeted to EGFR mRNA (custom synthesized by Life Technology, Carlsbad, CA) was 5'-CACAGUGGAGCGAAUUCCUtt-3' (sense strand) and antisense 5'-AGGAAUUCGCUCCACUGUCtt-3' (antisense strand). ACTB and GAPDH genes were purchased from Qiagen (Valencia, CA).

4.2 Cell Lines

MDA-MB-231 and MCF-7 cell lines were purchased from the ATCC (Manassas, VA). Cells were cultured according to the method provided by ATCC [359]. Briefly, MCF-7 and MDA-MB-231 cells were cultured in RPMI 1640 medium (Sigma, St. Louis, MO). In both cases, media contained L-Glutamine (Lonza, Allendale, NJ) supplemented with 10% fetal bovine serum (FBS, Fisher Chemicals, Fairlawn, NJ), 6 ml of antibiotics (100 μg/ml streptomycin and 100 U/ml penicillin G, Sigma, St. Louis, MO) and 12 ml of sodium bicarbonate (Fisher Chemicals, Fairlawn, NJ). Cells were grown at 37 °C in a humidified atmosphere of 5% CO_2 (v/v) in air. The culture medium was changed every other day.

4.3 Liposome Preparation and Characterization

4.3.1 Liposome Preparation

PEGylated liposomes were prepared using the procedure published in [10, 15, 125, 126]. Briefly, lipids: EPC, Cholesterol and 1,2-distearoyl-sn-glycero-3- phosphoethanolamine-N-aminopolyethelenglycol – Mw - 2000 ammonium salt (DSPE-PEG) were dissolved in chloroform, evaporated to a thin film layer using rotary evaporator Rotavapor® R-

210/R-215 (BUCHI Corp., New Castle, DE, USA) and rehydrated with 0.9 % NaCl to final lipid concentration 20 mM. In order to prepare fluorescently labeled liposomes, 5 mg of EPC labeled with Rhodamine were added to the lipids mixuture. The lipid mole ratio for this formulation was 51:44:5 EPC: Chol: DSPE-PEG respectively. Liposomes were stored at room temperature for an hour followed by extrusion through polycarbonate membranes 200 nm and 100 nm using the extruder device (Northern Lipids Inc., Vancuver, BC, Canada). Liposomal formulation of Gefitinib was performed using a mixture of egg phosphatidylcholin (EPC), cholesterol, 1,2,-distearoyl-sn-glycero-3-phosphoethanolamine-N- aminopolyethelenglycol – Mw - 2000 ammonium salt (mPEG-DSPE) in mole ratio 55/40/5 mol/mol respectively, and gefitinib at a lipid to drug weight ratio of 20:1 were dissolved in chloroform and subsequently evaporated at 35°C to form a thin film using rotary evaporator Rotavapor® R-210/R-215 (BUCHI Corp., New Castle, DE, USA). The resulting lipid film was rehydrated with PBS (pH 7.4) at room temperature and stored an hour followed by extrusion through polycarbonate membranes 200 nm and 100 nm using the extruder device (Northern Lipids Inc., Vancuver, BC, Canada). Canada). siRNA possess negative charges requiring positively charged (cationic) liposomes to form stable complexes. Cationic liposomes were prepared from positively charged DOTAP at concentration 5 mg/mL using thin layer procedure as previously described [10, 15], followed by extrusion through 100 nm polycarbonate membrane. The siRNA was dissolved in RNase free water at a concentration of 400 μM. To this solution, appropriate volume of DOTAP (5 mg/mL) was added, mixed by pipette and incubated for 30 min at room temperature. The molar ratio of siRNA/DOTAP was ~1:100. Resulting siRNA-cationic liposome complex was used in the studies. siGLO

Green was dissolved in RNAse free solution to the final concentration of 200 μM. DOTAP liposomes were mixed with siGLO in the ration 6:1v/v and incubated at room temperature for 15 min before use. Mean DOTAP/siGLO complex size was around 200 nm.

4.3.2 Liposome Particle Size Measurement

Particle size distribution was determined by dynamic light-scattering (DLS) at room temperature) using 90 Plus Particle Sizer Analyzer (Brookhaven Instruments Corp., New York, NY).

4.3.3 Zeta Potential Measurement

Zeta potential was determined on PALS Zeta Potential Analyzer (Brookhaven Instruments Corp, New York, NY) [123]. It measures the electrophoretic mobility that reflects the electric charge on the particle surface. The strength of the applied field was 20 V cm^{-1} and zeta potential values were recorded automatically by Zetasizer. All measurements were done in triplicate, and average values were calculated.

4.4 Cellular Internalization (Microscopic Technique)

MDA-MB 231 and MCF-7 cells were plated with density of 2 $x10^3$ cells/well in 6 well tissue culture plate for 24 hours. Then the media was removed and replaced by liposome-siRNA complex solution (the concentration of the liposome-fluoresence was 10 mg/ml and the concentration of siGLO was 20 μM and incubated for 24 hours. Then, the treatment was removed and washed with PBS buffer for 2-3 times and incubated with fluorescence stain DAPI (4,6-diamidino-2-phenylindole) 1:10000 (Invitrogen, Carlsbad,

CA) for 20 minutes. Later, the cells were washed with PBS buffer for 5 times, 1 ml of media was added to each well. Cellular internalization of siRNA-liposome complexes were analyzed by fluorescence (Olympus America Inc., Melville, NY) microscope. The resulted fluorescent images were digitally scanned and fluoresce inside cells (that reflect cellular accumulation of labeled siRNA) was expressed determined in arbitrary units.

4.5 Cytotoxicity Assay

A modified MTT (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide) assay was used to assess the cytotoxicity of different formulations of gefitinib, siRNA, liposome and lipoplex (liposomal formulation of siRNA targeted to EGFR mRNA). To measure cytotoxicity, human breast cancer (MCF-7 and MDA-MB231) cells were separately incubated in 96 microtiter plate at 37 °C in an incubator with different concentrations of each formulation. Control cells received an equivalent volume of fresh medium. The duration of incubation was 24 hours. After that, MTT reagent was added to each well and incubated for 3 hours. Then, the solubilization solution was added later and incubated overnight. An equivalent volume of fresh medium was added to the control cells. Absorbance was recorded on a microplate reader at 570 nm wavelength. The relative cell viability (%) was expressed as a percentage relative to the untreated control cells. A decrease in cellular viability and a decrease in the IC_{50} dose indicated a high toxicity.

4.6 RNA Extraction

MDA-MB231 and MCF-7 cell lines were grown on media containing flasks. Total RNA was extracted from confluent cells using the RNeasy mini kit (Qiagen, Valencia, CA).

The concentration and quantity of RNA were assessed using absorbance multi-mode microplate reader (Tecan Trading AG, Switzerland) with the absorbance of 260 nm. Then, the RNA was reverse-transcribed to cDNA using High Capacity cDNA Reverse Transcription Kit (Applied Biosystems, Warrington, UK). Three cDNA samples from separate RNA extraction were used for each cell line.

4.7 Gene Expression (Real-Time Quantitative Polymerase Chain Reaction)

The mRNA was reversed transcribed with High-Capacity cDNA Reverse Transcription Kit (Applied Biosystems, Warrington, UK). Quantitative RT-PCR was performed with an Applied Biosystems StepOne™ Real Time PCR system and SYBR master mix (Applied Biosystems, Warrington, UK). All samples were run in triplicate. The amplification was done as following: an initial step at 95°C for 10 minutes, followed by 40 cycles of 95°C for 15 seconds and 60°C for 60 seconds. The $2^{-\Delta\Delta CT}$ method was used to calculate the amount of gene expression. EGFR mRNA expression was normalized to the parallel measured endogenous controls ACTB and GAPDH in each cell line. The comparative Ct method was used to calculate the relative amounts of mRNA.

4.8 Statistical Analysis

Data were analyzed using appropriate a paired Student's t test or single –factor analysis of variance (ANOVA), and shown as mean values ± standard deviation (SD). The difference between variants was considered significant if $P<0.05$.

5 RESULTS

5.1 Cellular Internalization of Liposomes

In the fluorescence microscope, siRNA (siGLO, red fluorescence) and liposomes (green fluorescence) were efficiently internalized into MDA-MB 231 and MCF-7 cell lines. The quantitative evaluation of liposome internalization efficiency was based on the emission intensity. The nuclei of the cells were stained with DAPI (blue fluorescence). Figure 5.1 shows the superimposition of images (yellow fluorescence) allows for detecting of cytoplasmic localization of both siRNA and liposomes as well as enhances the ability of siRNA and liposomes to internalize the cells.

5.2 EGFR mRNA Expression by Real-Time Quantitative Polymerase Chain Reaction (RT-QPCR)

It was found that the expression of EGFR mRNA in MDA-MB 231 cells was more than 45 times higher when compared with MCF-7 cells (Figure 5.2A). Treatment of MDA-MB 231 cells with liposomes containing siRNA targeted to EGFR mRNA significantly suppressed the expression of this gene (Figure 5.2B).

5.3 Cytotoxicity Assay

MDA-231 and MCF-7 cells were incubated for 24 hours with different formulations (Liposomes Neutral, liposomes Cationic, naked siRNA targeted to EGFR mRNA, free Gefitinib, liposomal siRNA targeted to EGFR mRNA, liposomal Gefitinib, liposomal siRNA targeted to EGFR mRNA + Liposomal Gefitinib). Figure 5.3 shows the cytotoxicity of these formulations on viability of MDA-MB 231 and MCF-7 cells. A and

C showed cytotoxicity formulations that not contain gefitinib while B and D showed the cytotoxicity of formulations containing gefitinib for both types of cells (MDA-MB 231 and MCF-7).

6 DISCUSSIONS

Breast cancer is the most malignant disease among women. Over expression proteins of epidermal growth factor receptor (EGFR) family is strongly associated with the severity of breast cancer. About 20-70% of EGFR is over expressed in breast cancer. Gefitinib is a drug approved by FDA for treating non-small lung cancer with EGFR mutation [360].

Results from cellular internalization of liposomes showed a great internalization of liposomes-siRNA inside the cells of MDA-Mb 231 and MCF-7 breast cells lines that improve the idea of the liposome ability to penetrate cancer cells. EGFR gene expression in MCF-7 and MDA-MB-231 human breast cancer cells showed higher gene expression in MDA-MB 231 cell line than MCF-7. In addition, the level of gene expression of MDA-MB 231 cells lines was lower followin the treatment (lipoplex) than the control (untreated cells).

Moreover, data from *in vitro* cytotoxicity showed the variability of MDA-MB 231 and MCF-7 human breast cancer cell lines that incubated with 24 hours with different formulations: control (fresh media), liposomes neutral, liposomes cationic, naked siRNA targeted to EGFR mRNA, free gefitinb, liposomal siRNA targeted to EGFR mRNA, liposomal gefitinib and liposomal siRNA targeted to EGFR mRNA with liposomal gefitinib. Free drug demonstrated the ability to kill both types of cancer cells. Nevertheless, toxicity of gefitinib in TNBC was 2.5 times lower when compared with

EPBC cells. The delivery of the drug by liposomes significantly enhanced its toxicity (1.2 and 2.5 times in EPBC and TNBC, respectively). The combination of liposomal siRNA and liposomal gefitinib demonstrated exceptionally high cytotoxicity when compared with the free drug (143 and 62 times higher in EPBC and TNBC, respectively).

7 CONCLUSIONS

First, liposomes effectively delivered siRNA into both types of breast cancer cells: MCF-7 (estrogen positive, EPBC and MDA-MB 231(Triple Negative, TNBC). Second, siRNA targeted to EGFR mRNA delivered by liposomes successfully suppressed the expression of EGFR gene in the Triple Negative MDA-MB 231 breast cancer cells. Third, suppression of EGFR mRNA effectively reduced resistance of Triple Negative breast cancer cells to gefitinib and, consequently, decreased viability of MDA-MB 231 cells. Fourth, the data obtained support the proposed approach and demonstrated high potential of liposomal EGFR siRNA in combination with liposomal gefitinib for treatment of Triple Negative breast cancer.

8 FUTURE DIRECTIONS

1) Using animal models (*In vivo* experiments) to examine the effects of lipoplex with liposomal gefetinib co-treatment.
2) Comparative study with other chemotherapeutic drug.

9 ILLUSTRATIONS

TABLES

No.	Prognostic and Predictive Factors
1	Axillary Lymph Nodal Involvement
2	Tumor Size
3	Estrogen Receptor (ER) and Progesterone Receptor (PR) Status
4	Circulating Tumor Cells (CTCs)
5	Lymphatic and Vascular Invasion (LVI)
6	Age at Diagnosis
7	Race and Ethnicity
8	Cathepsin D
9	Angiogenesis Markers
10	Bone Marrow Micometastasis
11	Overexpression of the c-erb B-2 (HER2/neu) Proto-oncogen
12	Urokinase-Type Plasminogen Activator (uPA) and Plasminoge Activator Inhibitor type 1 (PAI-1)
13	Mutations of p53
14	Expression of Topisomerase II-alpha (topo Iiα)
15	Proliferation Markers
16	Gene Expression Profiling

Table 2.1 Prognosis and predictive factors of MBC

Hormone Receptor Status (n=155,890)	**ER+/PR+ (%)**	**ER+/PR- (%)**	**ER-/PR+ (%)**	**ER-/PR- (%)**	
	64%	13%	3%	20%	100%

Table 2.2 The distribution of estrogen and progesterone receptors in different groups of patients.

Modified from [23].

FIGURES

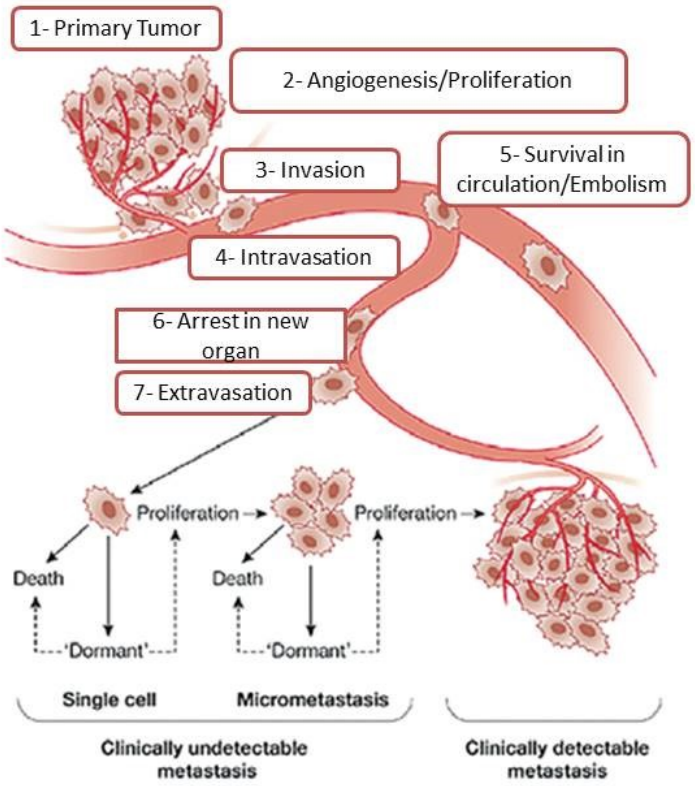

Figure 2.1 Major steps of metastasis formation

MBC is a complex and multifunctional process that involves different dynamic physiological activities from invading the local tissue then entry lymphatic or blood circulation that transport the metastatic cells to distant organs where they may extravasate and enter the microenvironment. Modified from [361].

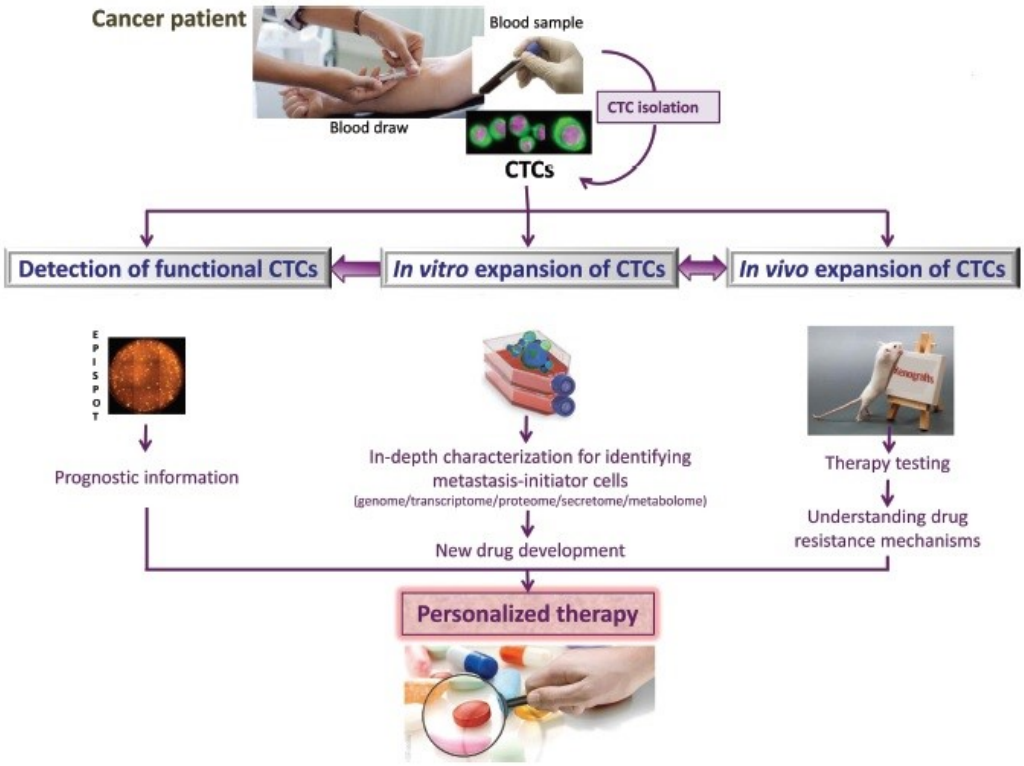

Figure 2.2 Role of CTCs in breast cancer in vitro and vivo.

After enrichment of CTCs from blood samples of breast cancer patient, viable CTCs can be enumerated with a functional assay or prognostic information, CTCs can be cultured in vitro and establishment of CTCs lines may indicate metastasis initiating cells or expanded in vivo for testing of therapy or understanding mechanisms of drug resistance. Modified and reproduced with permission from the American Association for Clinical Chemistry from [68].

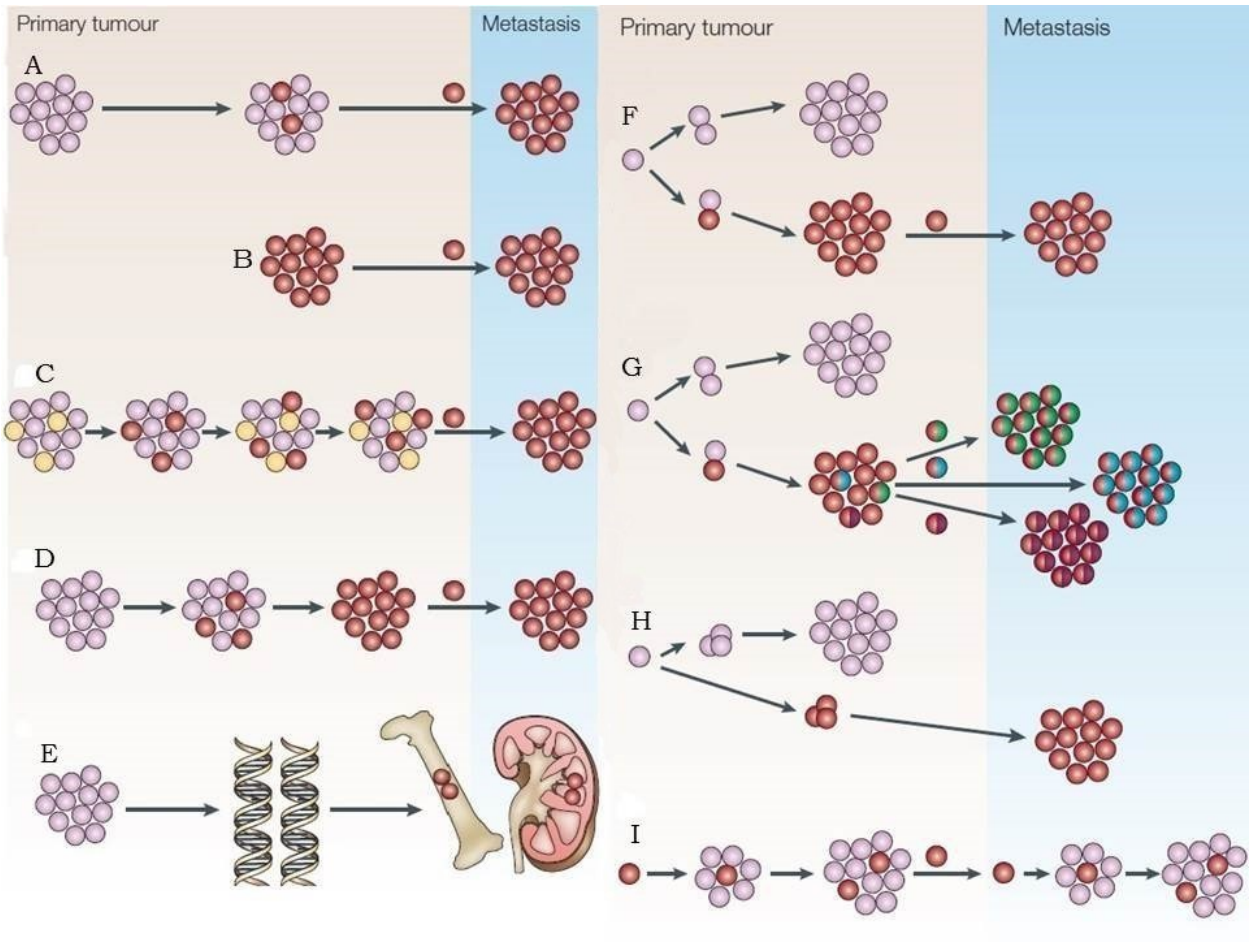

Figure 2.3 Models of MBC.

Modified from [29].

(A)Tradition model of MBC, (B) Spontaneous metastasis assays, (C) Dynamic heterogeneity model, (D) Clonal dominance model, (E) Genometastasis hypothesis, (F) Gene expression profile (G) models of metastasis to lung, bone and liver (H) Parallel evolution model (I) Breast cancer stem cells model. The pink-color represents non-metastasis breast tumor cells (good prognosis), red-color represents metastasis tumor cells (poor prognosis), yellow-color represents variant of tumor cells, green-

color represents metastasis to bone, blue-color represents metastasis to liver and purple-color represents metastasis to lung.

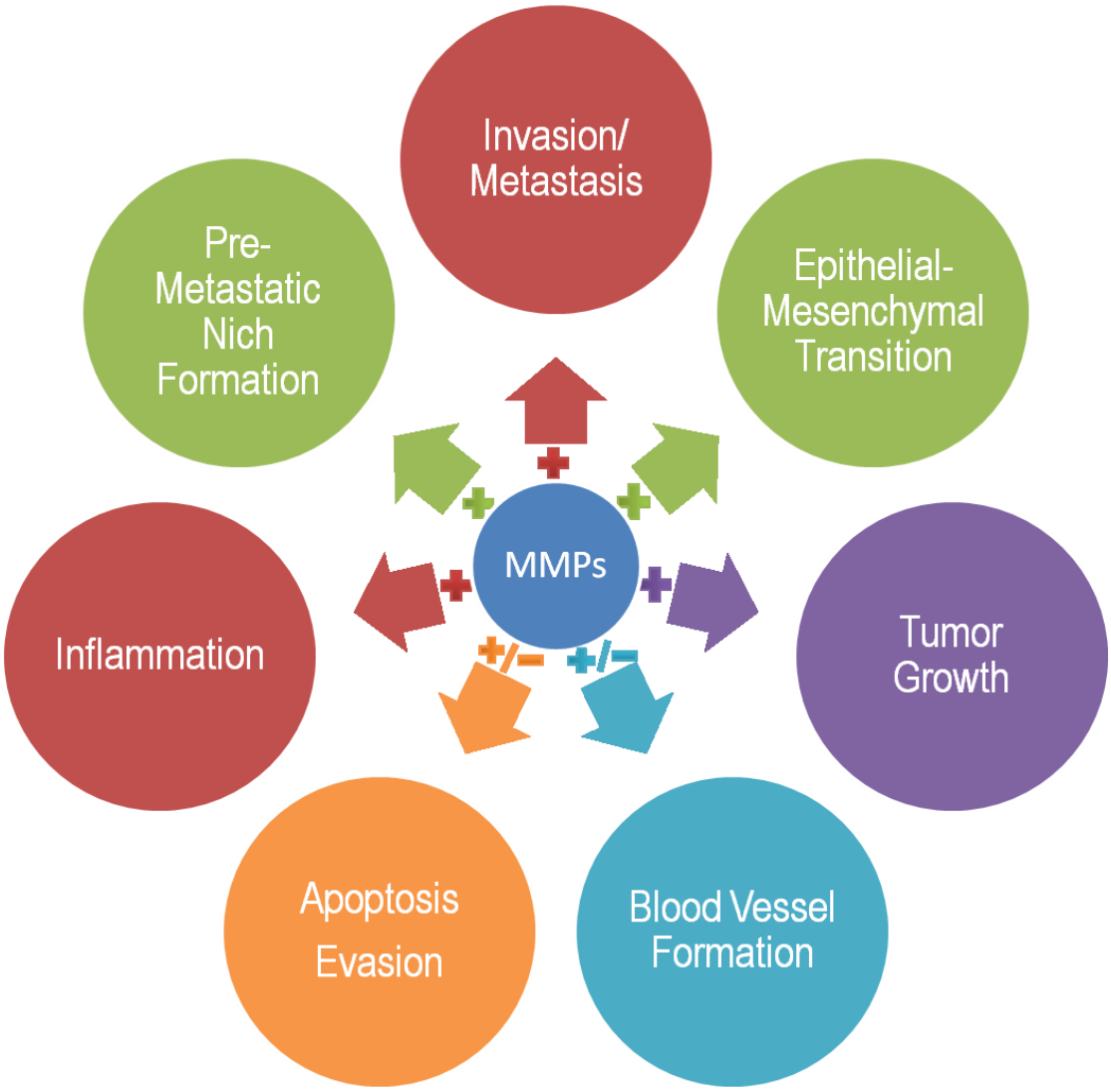

Figure 2.4 Role of MMPs in the progression and metastasis of cancer.

modified from [362].

MMPS are able to modulate the progression of the tumor in managing the epithelial-mesenchymal transition, invasion, metastasis and growth of the tumor; participate in pre-metastatic niche formation; inducing an inflammatory response. Also, MMPs can have a dual role during formation of the blood vessels and apoptosis evasion.

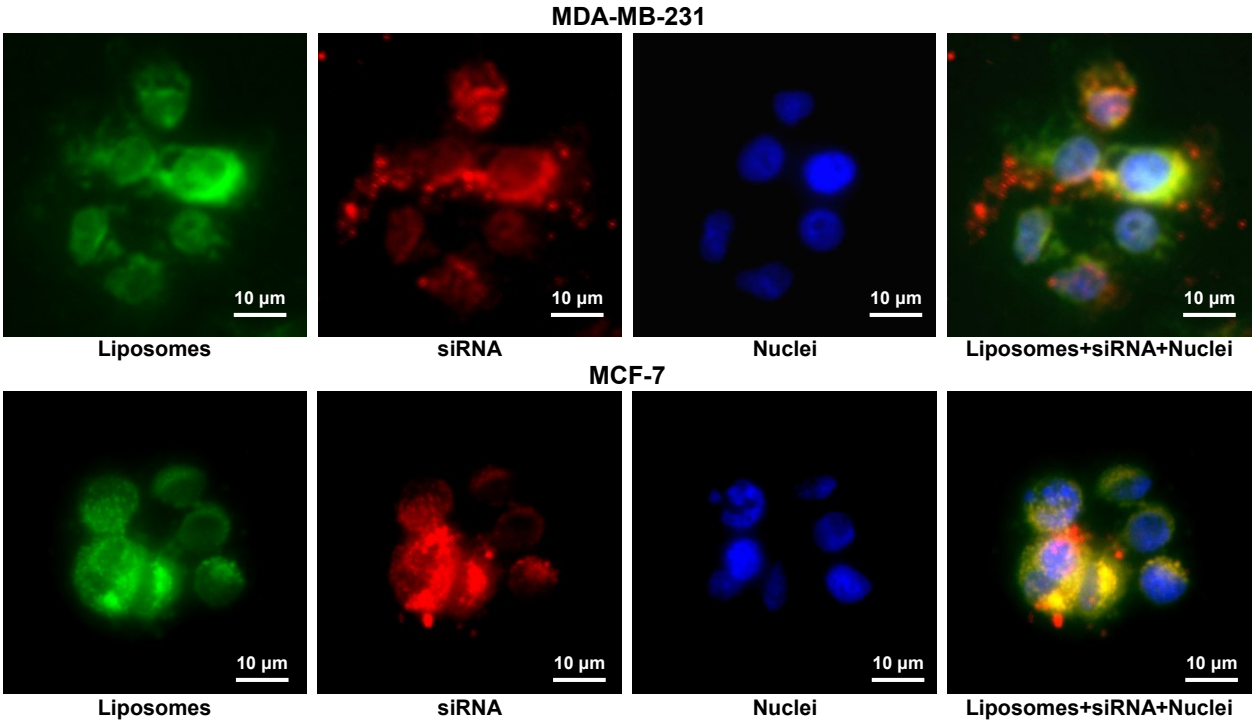

Figure 5.1 Cellular internalization of siRNA delivered by liposomes.

Representative images of human breast cancer (MDA-MB-231 and MCF-7) cells incubated within 24 h with liposomes (green fluorescence) containing siRNA (red fluorescence). Cell nuclei were stained with nuclear-specific dye (DAPI, blue fluorescence). Superimposition of red and green colors gives yellow color.

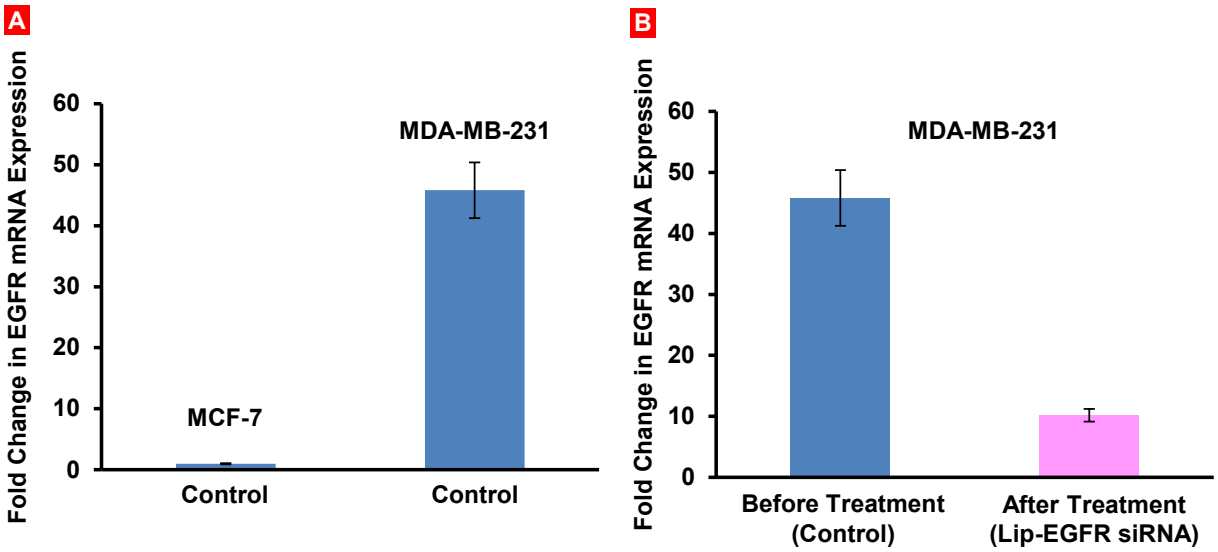

Figure 5.2 Expression of EGFR mRNA.

The relative quantity of EGFR gene expression in MCF-7 and MDA-MB-231 human breast cancer cells was calculated by the $2^{(DDCt)}$ method using quantitative PCR. The levels of gene expression were represented as a fold change. Means ± SD are shown. A – Expression of EGFR in MCF-7 and MDA-MB-231 cells incubated with media (control). B - MDA-MB-231 cells before and after treatment. Cells were incubated within 24 h with liposomal siRNA targeted to EGFR mRNA (Lip-EGFR siRNA).

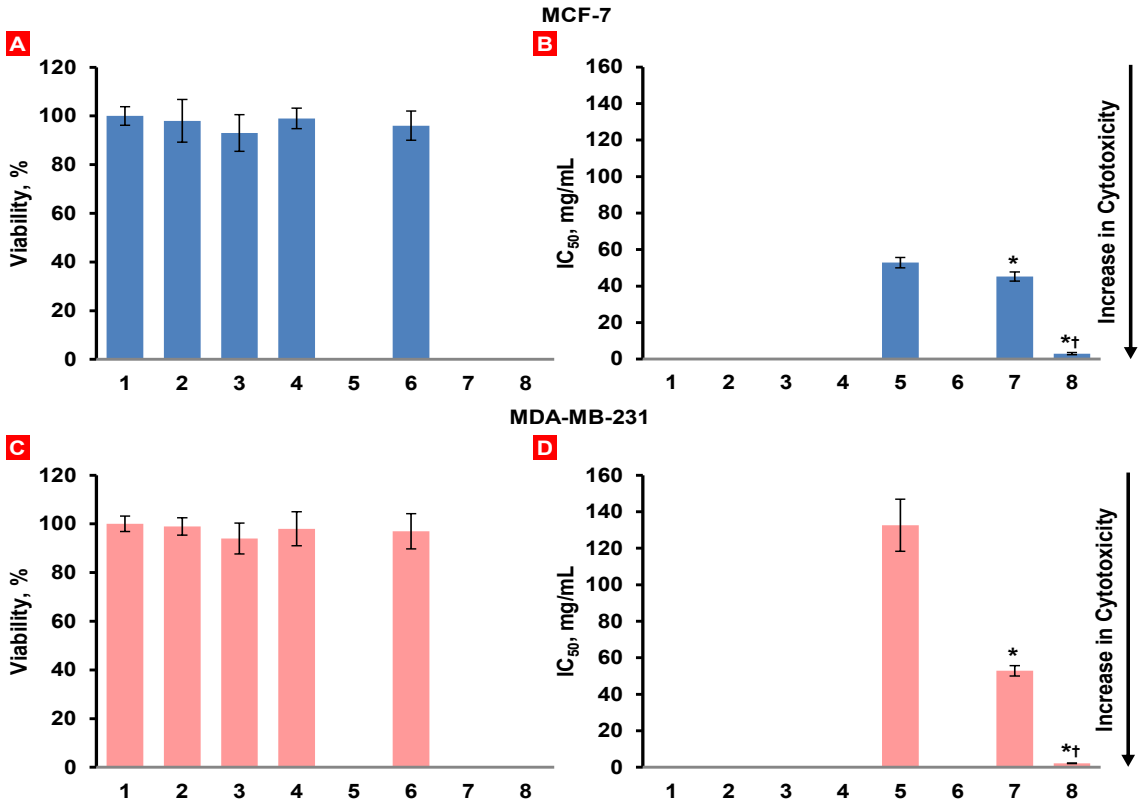

Figure 5.3 *In vitro* Cytotoxicity Assay

*Viability of MCF-7 and MDA-MB-231 human breast cancer cells incubated 24 h with the indicated formulations.*A, C – Cytotoxicity of formulations that do not contain Gefitinib in MCF-7 (A) and MDA-MB-231 (C) cells. B, D – Cytotoxicity of formulations that contain Gefitinib in MCF-7 (B) and MDA-MB-231 (D) cells. (1) Control (fresh media); (2) Liposomes Neutral; (3) Liposomes Cationic; (4) Naked siRNA targeted to EGFR mRNA; (5) Free Gefitinib; (6) Liposomal siRNA targeted to EGFR mRNA; (7) Liposomal Gefitinib; (8) Liposomal siRNA targeted to EGFR mRNA + Liposomal Gefitinib. Means ± SD are shown. $^{*}P < 0.05$ when compared with free Gefitinib; $^{\dagger}P < 0.05$ when compared with liposomal Gefitinib.

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