

Update on Clinical Trials: Genetic Targets in Breast Cancer

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Abstract Breast cancer is the most commonly diagnosed cancer in women in United States. From data of American Cancer Society from 2007 reported total of 178,480 women diagnosed with breast cancer. The death rate from breast cancer has decreased in North America over time, but still accounts for second highest cancer death, following lung cancer. Breast cancer is staged based on tumor size, nodal involvement, and distant metastasis like any other solid tumors. However clinical staging is not the only important factor in management of breast cancer. Various molecular features divides breast cancer into many subgroups – that act differently, and respond differently from therapy. Thus the focus of breast cancer treatment has evolved focusing on specific targets. The most important biologic markers in subtyping of breast cancer so far are hormone receptor positivity and HER2/neu protein expression. Five molecular subtypes using intrinsic gene set include Basal mRNA, HER2+ mRNA, Luminal AmRNA, Luminal B mRNA, and Normal-like mRNA. In addition, better understanding of genetic target of breast cancer has given us arsenal of personalized, and more effective treatment approach.

This review will focus on examples that highlight several mechanism of tumorigenesis, giving us not just understanding of gene pathways and the molecular biology, that could lead us to therapeutic target. Several important molecular targets have been investigated in preclinical and clinical trials, others are yet to be explored. We will also describe genetic mechanisms discovery related to overcoming resistance to current targeted therapies in breast cancer, including hormone receptor expression and HER 2- neu amplification. We will also review other exciting

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developments in understanding of breast cancer, the tumor microenvironment and cancer stem cells, and targeting agents in that area.

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Cell Membrane Signaling

Growth Factor Receptors EGFR and HER2- neu as Molecular Targets in Breast Cancer

About 15–20% of breast cancers have over-expression of HER2 [1]. HER2(ErbB2) is a transmembrane glycoprotein with an intracellular receptor tyrosine kinase(TK) domain, and extracellular ligand binding domain. The HER family consists of four family members – HER1(ErbB1 = EGFR), 2, 3, and 4. Each different sub-type of HER protein shares similar intracellular TK domains, but expresses distinct ligand binding extracellular domains [2]. The HER receptor acts via dimerization of receptors, either homodimerization, or heterodimerization between different proteins [3, 4]. HER2 overexpression is also found in other types of cancers, for example, gastric cancer. However especially in breast cancer, HER2 overexpression is one of the most important carcinogenic features, as well as being a prognostic and predictive marker for treatment response [5].

The first HER2 targeting agent approved by the FDA in 1998 was trastuzumab (brand name Herceptin®). Trastuzumab is a monoclonal antibody that targets the juxtamembrane domain of HER2 [6]. Since trastuzumab was approved by FDA, it has become the cornerstone of treating HER2 overexpressing breast cancer patients in the neoadjuvant, adjuvant and metastatic settings. Combining trastuzumab with chemotherapy in HER2 overexpressing breast cancer increases the median survival and disease free survival by 25% [7, 8]. Trastuzumab not only inhibits HER2 by binding at the extracellular domain, but it also induces the activity of p21 or p27 which then cause transcription inhibition [9]. Other mechanisms of actions of this antibody including an immunologic basis are also possible.

However, just like with other biologic agents, HER2 positive breast cancers either develop resistance, or are natively resistant to trastuzumab (Fig. 1). There are two major mechanisms of resistance. One is activation of HER2 downstream pathways (PI3K-AKT-mTOR) either by existence of a HER2 form that lacks the trastuzumab binding site, or via a mutated pathways controlled not by HER2 but by intracellular activation that does require HER2 for its activation [10]. A second mechanism of resistance is activation of alternative signaling pathways via HER2

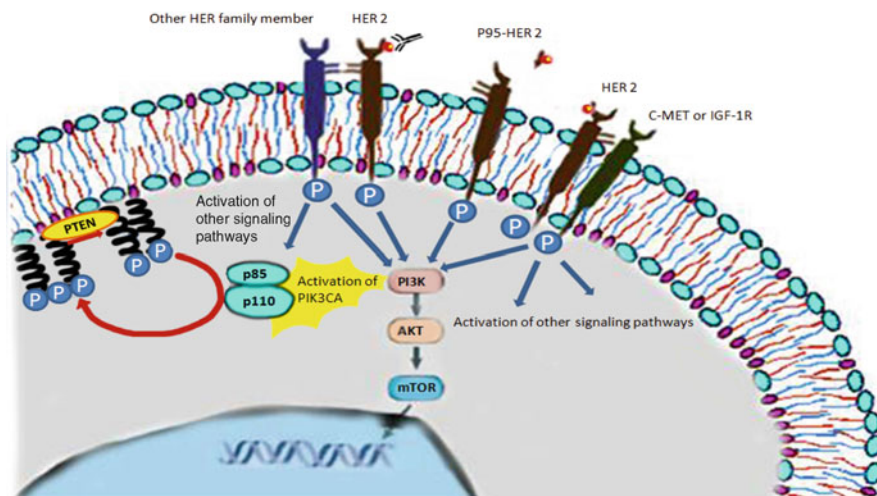


Fig. 1 Suggested trastuzumab resistance mechanisms. From the left: (1) Loss of PTEN, or mutations in PI3KCA result in constitutive activation of PI3K pathway (via activation of PI3K via dephosphorylation of PIP3 to PIP2). (2) Activation of PI3K pathway by HER2, can be activated also by other HER family members including HER3. (3) P95-HER2 can constitutively activate downstream, and escape trastuzumab. (4) Dimerization with other receptors, c-MET, IGF-1R

heterodimerization with other HER family members or non-HER family member dimerization partner proteins, (C-MET, IGF-1R for example) [10–12].

Existence of P95-HER2 is part of the first resistance mechanism. This is a form of truncated HER2, which lacks the binding domain of trastuzumab, thus it stays constitutively active. When there are a large numbers of p95-HER2 proteins, they will send downstream signals to activate PI3K-AKT-mTOR pathway despite presence of trastuzumab [13–15]. Also, if there is a PI3KCA mutation that does not require HER2 to be activated, but can be activated by PTEN intracellular activator, blocking HER2 by trastuzumab will not affect ultimate cell growth and proliferation. Improving the inhibition of HER2 using combinations of more than one biologic agents, is currently the direction of HER2 therapy [16].

HER2 activation of other HER family members by heterodimerization with other HER receptors also signal downstream activation so the cells can continue growth and proliferation. Thus, blocking other HER family members has been recognized as an important mechanism of overcoming resistance in HER2 overexpressing breast cancers. HER2 itself lacks a ligand binding domain, however other HER proteins have extracellular ligand binding domain, can form a dimer with HER2 and are activated by ligand binding. HER2 gains stable dimer activity and then activates intracellular signaling: Other receptor proteins that form dimer with HER2 also can send downstream signaling independently from HER2 [3, 17, 18]. HER3 for example, which is thought to be a signaling expert by having six different domains that can activate PI3K(p85 SH2 adaptor domain) HER3 can send signals to activate PI3K

pathway – via para/auto/intracrine expression even after HER2 is blocked [19, 20]. Thus, using inhibitors of HER2/3 coupling is emerging as a method of overcoming this form of resistance. Pertuzumab is a novel agent which inhibits this type of dimerization [21]. HER1 which can also form dimers with HER2, activates the MAPK downstream pathway, which is not affected by inhibition of HER2 [3].

Small molecule TKIs (tyrosine kinase inhibitors) have activity against HER2 overexpressing breast cancers. These small TKIs can bind to HER2 in different domains than trastuzumab; and some also interfere with HER2/3 coupling, which can escape from the resistance mechanism described above. Examples of these small molecules include lapatinib, pelitinib, erlotinib, gefitinib, afatinib, neratinib, and canertinib [22–24]. So far the most promising small molecules that showed activity is lapatinib. Lapatinib is an oral, small molecule TKI that has dual activity to HER1 and HER2. It also seems to inhibit insulin-like growth factor 1 signaling, another mechanism of trastuzumab resistance [25–27]. In the metastatic setting, lapatinib as first line therapy has similar efficacy to first line trastuzumab therapy in trastuzumab naïve patients [25–27]. Combinations of lapatinib and trastuzumab are also being investigated in early stage breast cancer, since these two agents target different sites of HER2. The ALTTO (Adjuvant Lapatinib Trastuzumab Treatment Optimization) study (NCT00490139) trial is looking at chemotherapy combined with Trastuzumab, compared with combination with Lapatinib arm, and chemotherapy combined with both Trastuzumab and Lapatinib [28]. Cleopatra trial (trastuzumab and pertuzumab with docetaxel) showed 6 months improvement in progression free survival in HER2 positive metastatic breast cancer. Based on promising data of this dual therapy combination, more dual targeted approaches using other agents are underway.

Another approach to HER2 targeted therapy, is the antibody-drug conjugate approach. This approach uses a targeted antibody not just for blocking the tumor regulating protein, but also uses them as carrier of toxin to targeted cells. T-DM1 is HER2 specific antibody-drug conjugate, that consists of trastuzumab and emtansine (DM1) [29]. Emtansine is cytotoxin mertansine, an anti microtubule maytansinoid derivative. This agent has shown activity in heavily pretreated and trastuzumab resistant patients [30]. The toxicity profile of this agent is more favorable than standard chemotherapy since the cytotoxic effect is limited to malignant HER2 overexpressing cells. The MARIANNE trial is a phase III registration trial currently accruing patients in the first line metastatic setting which will address the comparative efficacy of standard trastuzumab based therapy, versus T-DM1 and T-DM1 in combination with pertuzumab [31]. With the many new therapeutic options in her two therapy, the main issue will be how to optimally sequence and combine these therapies.

IGF in Breast Cancer

In vitro studies suggest that proliferation, migration and cell survival of breast cancer cell lines depends on activation of the insulin growth factor (IGF) type 1 receptor (IGF-1R). The IGF signaling pathway contributes to both cellular proliferation and apoptosis.

IGF receptor has been functionally linked with estrogen receptor signaling in breast cancer. Multiple polymorphisms of the IGF-1 pathway have been analyzed and studies have revealed certain functional polymorphisms as independent prognostic markers for tumor recurrence [32–35].

A U.K. study analyzed tissue samples of 222 patients with ER positive primary invasive breast cancer who had undergone surgery and adjuvant hormone treatment between 1981 and 2003. Six functional IGF-1 pathway polymorphisms were analyzed. Patients carrying one of these polymorphisms, IGF-1_rs2016347, had a significantly better disease free survival than patients without this polymorphism (5.3 years vs 7.6 years; $p=0.02$) [36].

IGF pathway signaling has also been linked to tamoxifen resistance. In a recent study, ligand stimulation of IGF-1R in ER-positive MCF7 human breast cancer cells rendered MCF7/IGF-1R cells highly resistant to the antiestrogens tamoxifen and fulvestrant. The downstream pathways of IGF-1R were found to act independently of ER signaling suggesting that the IGF-1/IGF signaling axis may play a crucial role in antiestrogen resistance despite continuous suppression of ER transcription by antiestrogens [37].

IGF insulin receptor kinase inhibitors are currently undergoing clinical trials. One such IGF-1R kinase inhibitor BMS-754807 shows promising results in pre-clinical evaluation. It showed synergistic in vitro anti-proliferative activity in combination with tamoxifen, letrozole or fulvestrant in the aromatase expressing breast cancer model, MCF-7/AC-1. Currently it is being evaluated in a phase II study in patients with HR positive breast cancer who have progressed with prior non-steroidal aromatase inhibitor treatment [38].

Anti-IGF-1R/InsR therapy has also been tested in triple-negative breast cancer. Litztenburger and colleagues tested the sensitivity of triple negative cell lines with IGF gene expression. These cell lines showed tumor growth inhibition and, in combination with docetaxel, resulted in tumor regression. Regression was associated with reduced proliferation, increased apoptosis, and mitotic catastrophe. This suggests rationale for further testing of IGF inhibitors in combination with chemotherapy in patients with triple negative breast cancer [39].

Impact of the Tumor Microenvironment and Cancer Stem Cells

Cancer Stem Cells and Stem Cell Niche in Breast Cancer

Stem cells are the cells with a capacity to self-renewal and to generate daughter cells that can differentiate into different cell lineages to form multiple differentiated cell types and mature tissue [40]. Stem cell biology has been the area of hematologic disease due to easy access to liquid biopsy of cancer cells. However recently, the importance of stem cell biology in solid cancers started to be elucidated [36]. Whether there is true ‘stem cells’ in solid cancer is ongoing debate. Also, the exact mechanism of stem cell activity and identification has been a challenge.

In solid cancers including breast, there are two main models suggested. In a transitory, or an induced model, a stem cell behavior can be gained or lost, and mammary cells can interact with microenvironment, or stem cell niche. On the other hand, in a permanent stem cell model, a fixed group of cells are the only ones with ability to function as stem cells. We know from the animal experiments, that mammary tree stem cell, or alveolar bud-only cells, or duct-only stem cells showed different level of ability to form structures, when the cells were engrafted in same clean fat pad. This could support the model of pre-existing selected stem cell population.

Whether stem cell in solid cancer is transitory, or permanent, many studies showed the importance of stem cell niche, or microenvironment in function of cancer stem cell. Stem cell niches in breast cancer are specialized locations within a tissue that have the ability to support stem cell function. In mice experiments, cancer cells injection only fail to generate full grown cancer, unless human fat pad microenvironment with proper vascular environment. There is also arguable evidence that when stem cells are depleted, nearby daughter cells can be induced to transform as stem cells [33, 41].

Whether mammary stem cells are related to actual breast cancer tumorigenesis, is different question. The cells expressing CD44/CD24, lacking expression of epithelial markers were suggested as putative breast cancer stem cells. These population of cells showed up to 50 fold increased ability to form tumor in xenograft model. Assuming these stem cell population will always grow regardless of targeting bulk of tumor cells by conventional therapy, a search for the method to better recognize this group of cells, and development of targeting method is highly mandated. Detection of putative genes, or epigenetic mechanism that cause the 'stemness' of breast cells, can lead us to anti-stem-cell therapy by silencing those targets. However this stem cell targeting therapy is at its beginning step, and long years of researches are to come.

Angiogenic Targets

Tumorigenesis is a complicated, well orchestrated, multi-step process. As researchers reach a better understanding of tumorigenesis, focus has turned to the microenvironment of the tumor. Tumors are analogous to small organisms that grow and survive, based on constant interactions with their microenvironment. A key component of this process is blood vessel transport function of crucial nutrients and waste products. For tumors to grow bigger than 1–2 mm, new vessel formation is mandatory [42–44]. Without a new viable vasculature supply, tumors can't grow further, and face cell death. Angiogenesis can happen in various steps of tumorigenesis – during maintenance of survival, and later during invasion and metastasis [45–47]. This vessel formation and activity can be measured as microvascular density (MVD), and considered as important prognostic factor to assess a cancer patient's prognosis [48–50].

An important factor involved in angiogenesis is hypoxia of tumor cells, either via VEGF or via direct increases of HIF (Hypoxia induced factor). When the tumor cells

get crowded enough to be in hypoxic environment, they secrete lymphangiogenic factors, like VEGF, PDGF, bFGF. Among these factors, VEGF is the most well studied, and known to be the key activator of angiogenesis [51–53]. Hypoxia upregulates VEGF levels by stabilizing messenger RNA, and increases VEGF expression by facilitating transcription of genes mediated by hypoxia inducible transcription factors (HIF) [54]. VEGF is heparin binding glycoprotein family, consisting of six isoforms. Isoforms include VEGF-A to E, and placental growth factor [55]. Normally, VEGF is produced by endothelial cells, however in tumors, it is produced by tumor cells or stromal cells via paracrine signaling. VEGF is essential in the induction, growth and maintenance of vascular endothelial cells by direct and indirect actions. Direct actions are via stimulation of endothelial mitogenesis, This Akt dependent pathway promotes endothelial cell survival, and endothelial cell migration by degradation of the extracellular matrix and synthesis of erythropoietin [56–59]. In actively growing and metastasizing tumors, VEGF levels are elevated. Up regulation of VEGF factors have been observed in many different tumor types, including breast, colorectal, gastric, cervical cancers [60–66]. Based on these findings, it has been suggested that VEGF is prognostic in various cancers, including breast cancer [67–72].

The other mechanism of enhancing angiogenesis is via mutation of oncogenes that increase growth factor signaling and which can result in the activation of MAPK, PI3K, or PKC. Activation of these pathways can then increase HIF-1 α activity or synthesis, even in a normoxic environment [51, 52]. However angiogenesis is not a simple straightforward process, and it requires interactions with the extracellular matrix, and involvement of pericytes, etc. [73–76].

Since angiogenesis is a key mechanism of tumor survival and growth, targeting angiogenesis in cancers stimulated active investigation. However, studies have shown that antiangiogenic drug alone is not sufficient, and sometimes can cause accelerated tumor growth. For instance, when a VEGF inhibitor was used as a single agent, growth of tumor itself is inhibited, but the ability of cells to become more invasive and metastatic can be enhanced. This can be explained by ‘paradoxical normalization of tumor vasculature’, which improves blood supply to the tumor, by selecting for healthy vessels. Since VEGF also acts as vascular permeability factor, inhibiting VEGF caused an increase in tumor interstitial pressure, and limited penetration of drugs [77]. Thus, these agents are currently being used in combination with other agents, and more often in the setting of treatment failure or metastasis, rather than as first line.

The agent which has been most widely tested in breast cancer is thus far bevacizumab. It is monoclonal antibody that binds and inactivates all isoforms of VEGF [78, 79]. In 2007, bevacizumab was given accelerated approval by the FDA for use in combination with paclitaxel for first line therapy in locally recurrent or metastatic HER2 negative breast cancer. This approval was based on improved progression free survival rather than overall survival data. After initial approval, the FDA requested further study to define clinical benefit. Three years later, the follow up study (NCT00028990) still did show significant progression free survival (11.3 months vs 5.8 months), but no overall survival benefit emerged. The marginal benefit of progression free survival was not felt to outweigh treatment related adverse

events. After review of these results, ODAC (oncologic drugs advisory committee) recommended withdrawal of the approval of bevacizumab for use in breast cancer [80]. The decision has been appealed, and bevacizumab is currently still under FDA consideration for use in metastatic disease. The agent continues to be investigated in trials for treatment of early breast cancer.

Other studies using bevacizumab are AVADO, RIBBON-1, and RIBBON-2, which also showed improved progression free survival, as well as increased tumor response rate; however the differences were less significant than those seen in the pivotal E2100 study. Progression free survival may be a more meaningful endpoint in metastatic breast cancer, (because most patients receive therapy following progression), and may replace overall survival in this setting. However more data is needed regarding quality of life to answer whether or not this increased progression free survival is with a quality of life compared to standard therapy. Other anti-angiogenic agents include small molecule tyrosine kinase inhibitors, like Sorafenib. Sorafenib has multiple target activities towards VEGFR2, VEGFR3, Raf, PDGFR and KIT, RET [81]. TKIs are of interest in both as anti-ras/raf agents in the breast cancer setting, as well as for their anti-angiogenic effect. These agents will be reviewed in next section.

Intracellular Signaling and Genetic, Epigenetic Targets

Intracellular Signaling; Ras, Raf, mTOR

Elucidating intracellular signaling pathways in breast cancer has proved challenging. Not only is there is extensive crosstalk between pathways; but there is also overlapping function of these pathways. This makes single targets, or pathways, difficult to block. A particularly important pathway in breast cancer is the ras/raf/MAPK pathway. Ras is a GTPase which has downstream effects on proliferation and cell regulation. Ras is activated extracellularly by estrogen, IGF and EGF ligands [82]. Ras mutations occur in 2% of breast cancers, but ras hyperactivation is frequent [83]. In HER2 -neu overexpressing and estrogen receptor positive breast cancers, ras signaling is dysregulated. In addition, ras has been found to be over expressed in other breast cancers [84].

One strategy for inhibiting ras signaling is the use of farnesyl transferase inhibitors (FTI). Ras has to be prenylated which renders it hydrophobic and causes the protein to be localized near the cell membrane. FTIs block this process which decreases ras. Several studies of a farnesyltransferase inhibitor, tipifarnib have been reported. A phase II study of 32 patients treated with tipifarnib, in addition to standard cytotoxic neoadjuvant therapy, has been reported. Interestingly complete pathologic responses were seen in hormone positive tumors, which is unusual in hormone positive tumors treated with neoadjuvant cytotoxic therapy. In metastatic breast cancer, tipifarnib, in combination with fulvestrant, had a 51% clinical

benefit rate [85]. A small study with tipifarnib and letrozole in patients unresponsive to one hormonal therapy did not show any improvement in response rate [86].

Sorafenib is a multikinase inhibitor which blocks angiogenic and ras pathways. A phase II trial of sorafenib was conducted in 23 patients, but was stopped when there were no responses. Only two patients achieved stable disease. Sorafenib was well tolerated. The investigators concluded that sorafenib was ineffective as a single agent but may be effective in combinations [82].

The PI3K-AKT-mTOR pathway is an important regulator of cell survival. This pathway has multiple stimulating and inhibiting molecules and is responsible for transition through the cell cycle, and transcription and response to angiogenic signals [81]. There is aberrant function of this pathway in breast cancer. Thus, mTOR inhibitors have been actively investigated in clinical trials - and showed improved survival in metastatic setting in combination with exemestane (Bolero trial).

Metformin, an insulin lowering medication, blocks multiple kinases. Cell death has been observed in vitro in breast cancer cell lines treated with metformin. Metformin can block the mTOR pathway indirectly. In addition, metformin also modifies IGF expression and binding. Retrospective data have shown higher neoadjuvant response rates in patients who are concurrently on metformin. At least two clinical trials are ongoing to address the efficacy of metformin. The NSABP is conducting a large adjuvant trial analyzing the effect of metformin on breast cancer recurrence. Others are prospectively adding metformin to neoadjuvant therapy in breast cancer [87, 88].

Urokinase plasminogen activator is a poor prognostic factor in breast cancer. This protease is important in disrupting the extracellular membrane which facilitates migration and invasion of breast cancer cells. Disabling this pathway may be a target for breast tumors which over-express urokinase plasminogen activator [89].

Post-Translational Modification: Histone Deacetylase Inhibitors (HDACIs)

HDAC (histone deacetylases) are enzymes whose principal role is regulating gene transcription by counter activity of histone acetyl transferases (HATs). While HATs acetylate histone and non histone proteins, HDACs work by removing acetyl groups from lysine residues of histone tails of chromatin, and from non histone proteins. HDACs were initially found to contribute to tumorigenesis by regulating DNA transcription, as well in addition to another mechanisms [90, 91]. There are different groups of HDAC. There are total 18 HDACs that form four groups. Class I consists of HDAC 1,2,3, 8 and this class of HDAC family members are located in the nucleus. Class II families are located both in the nucleus and in the cytoplasm, and includes 4,5,6,7, and 9. Class III family is class of Sir2 homologues (sirtuin), and class IV includes HDAC 11. Class IV HDAC, 11 shows features of class I and II HDAC family, and are also structurally similar. Mainly class I, II, and IV are the focus of developing targeting agents, and class III has a distinct activity from the others [57, 58].

Each HDAC members shows expression in different types of cancers [92–95]. In breast cancer cells, HDAC 4,6,11s were found to be overexpressed [96]. HDACs play important role in epigenetic mechanism in cancers, both in steps of initial tumorigenesis and metastasis [97].

Interestingly, HDACs are involved in tumorigenesis not only through regulation of transcription, but also by several other mechanisms. When tumor cells activate angiogenesis responding to hypoxia, HDACs functionality is also affected. First, cells in a hypoxic environment express increased level of HDAC mRNA and proteins, which increase HDAC expression. The increased number of HDACs can interact directly with HIF-1 α via ODD domain, regulate stability and transcriptional activity of HIF-1 α . Different types of HDAC are related to different mechanisms of activity, and different cells are affected distinctively [92–94, 98]. In renal cell carcinoma, inhibition of HDAC4 and 6 showed that it induced HIF-1 α protein stability, via proteosome dependent pathway [99]. HDAC mediates also angiogenesis by indirect mechanism, by regulation of p53, pVHL expression. Both in vitro and in vivo, overexpression of HDAC1 reduced expression of p53 and pVHL, and subsequent overexpression of HIF-1 α , VEGF [100].

Another mechanism by which HDACs are involved in cancer biology is, via indirect and direct regulation of apoptosis [101]. Apoptosis is programmed cell death, a well regulated process that is important in tissue homeostasis and development. During this apoptosis, cells undergo typical morphologic changes. The cell's membrane forms a bleb, chromatin gets condensed, DNA gets degraded, and the cell fractionates into smaller vesicles. These fractionated vesicles get engulfed by phagocytes. Increased HDACs prevent DNA from degradation, and give cells a way to escape from apoptotic process [102–105].

HDACIs (HDAC inhibitors) were first found as antitumor agents by inhibiting HDAC function on the histone tail, however further studies found that they also affect gene transcription by inhibiting the interaction of HDACs with other proteins. Moreover, HDAC mutation or change in the level of expression was found to be correlated with HDACIs activity. HDACI seems to work both by transcription dependent and independent mechanisms, as suggested by studies about HDACs involvement in tumorigenesis [106–108]. It is not surprising to see that many HDAC inhibitors are under evaluation by researchers. There are many HDAC inhibitors undergoing phase I and II trials. HDAC inhibitors showed some benefit in animal and some early human data, however they primarily have more promising results when they are used in combination with other chemotherapy, or targeted therapies [109–111]. There are number of medications under way, however the main ones that has been and further studied include vorinostat(SAHA), romidepsin(FK228, FR901228), etinostat(SNDX-275, MS-275), and panobinostat(LBH-589).

Vorinostat, (brand name Zolinza) is Suberoylanilide hydroxamic acid (SAHA), is a member of a larger HDAC inhibitor compounds (HDACI). It is one of the most studied compounds among HDACI. Currently vorinostat is approved by the FDA for use in cutaneous T cell lymphoma (CTCL). This agent is undergoing phase I and II trials in many solid cancers including colon, lung, renal, and breast cancers. In breast cancer, vorinostat showed restored sensitivity of aromatase inhibition after primary or secondary resistance when it was used in combination for estrogen

receptor positive breast cancer patient [112]. Also, it is currently studied as part of advanced solid tumor studies including breast cancer, in combination with doxorubicin, docetaxel [113], and carboplatin-paclitaxel combination. Romidepsin, the other agent approved by FDA to treat lymphoma, is also studied in advanced solid cancers in phase I trial including breast cancer [114].

Entinostat currently studied in combination with the aromatase inhibitor, letrozole in phase II study in ER positive breast cancer patients [90]. This is based on mouse xenograft models with ER-negative breast cancer cells. Entinostat was found to upregulate ER α receptors and aromatase expression and subsequently sensitized ER-negative MDA-MB-231 cells to estrogen and letrozole [115]. Panobinostat and letrozole in aromatase-positive breast cancer inhibition. Panobinostat is 40 times more potent than SAHA in its ability to suppress aromatase expression. It selectively inhibits promoters I.3/II of the human aromatase gene. Panobinostat, the required concentration of letrozole was only one-fifth of that used in the absence of panobinostat, to achieve the same degree of aromatase inhibition [116].

MicroRNAs and the Molecular Pathogenesis of Breast Cancer

In 1993, micro RNA (miRNA), small non coding sequences, was recognized as being responsible for degradation and silencing of messenger RNA. Multiple downstream affects of micro RNAs, via repression of transcription; on proliferation, apoptosis and differentiation have now been described in breast cancer. Thus far, almost 500 different microRNAs have been identified in human tissue, and more will likely be identified [117]. Of these known miRNAs, high through put sequencing has established differences in profiles between normal and malignant breast tissue. Using microarray and northern blot analysis of 76 human breast cancer samples, and ten normal breast tissue samples; Iorio et al., showed aberrant expression of *miR-10b*, *miR-125b*, *miR-145*, *miR-155* and *miR-21* in human breast cancer. Using, these miRNA expression profiles the investigators could predict, ER/PR status, tumor stage and proliferative index of breast tumors. The investigators also showed that down-regulation of *MiR-9-3* was associated with either high vascular invasion and metastatic potential [118].

Other miRNAs, such as *miR-21*, has been shown to have prognostic importance. Wang and colleagues revealed that upregulation of *miR-21* in doxorubicin resistant MCF-7 breast cancer cells was associated with downregulation of tumor suppressor gene (PTEN) protein. The half maximal inhibitory concentration (IC₅₀) of doxorubicin was 16.5 ± 0.08 $\mu\text{mol/L}$ for resistant MCF-7 cells with over-expression of PTEN versus $0.21 \pm \mu\text{mol/L}$ for parental MCF-7 cells [119]. Micro RNA 27a was investigated as a potential oncogene. Mertens-Talcott et al., showed an oncogenic effect of *miR-27a* by increasing expression of specificity proteins which in turn promoted cell survival and angiogenesis. This same micro RNA can suppress genes that cause G2-M arrest, thus promoting cell proliferation [120].

Micro RNAs are intriguing targets for development of novel therapy. Liang et al., reported on whether down regulation of microRNA would affect a tumor's metastatic potential via the CXCR4 pathway. They used artificial microRNA in transfected cell

lines to show an in vitro decrease of invasion and migration; and an in vivo decrease of lung tumor metastasis. Potentially, anti sense compounds could inactivate transcription, which could decrease tumor proliferation and enhance chemo-sensitivity of breast cancer cells. Alternatively, liposomal or viral delivery of other microRNA could slow tumor growth by preferential effects of apoptosis [121].

Telomerase Inhibitors in Breast Cancer

The telomerase enzyme in cancer cells provides an attractive new therapeutic target. Telomeres are repeating DNA sequences that cap the ends of chromosomes. Repeated cell division results in loss of telomere length and progressive shortening of chromosomes. Critical shortening of chromosomes triggers apoptosis. Telomerase (telomere terminal transferase) is an enzyme complex that elongates chromosomes by adding TTAGGG sequences to the ends of chromosomes. Activation of telomerase enables increased proliferation in cells. Since telomerase is several fold more active in malignant cells than in normal cells, effective inhibition of this activity has become a logical basis for investigation [122, 123]. Promising results from preclinical investigation [124] are leading to Phase I and Phase II clinical trials. Intriguing data suggest that in the appropriate setting, these agents might target cancer stem cells [125]. For example, imetelstat is a telomerase inhibitor that is now entering a trial in breast cancer where this agent will be combined with paclitaxel and possibly with bevacizumab as first or second line treatment for metastatic breast cancer [126].

PARP in Breast Cancer

PARP (poly ADP-ribose polymerase) is nuclear enzyme that catalyses the polymerization of ADP-ribose moieties into target proteins using NAD⁺. It was first found in 1963 by Chambon et al. Out of 17 members of the PARP superfamily [127, 128], PARP-1 and 2 are the ones well studied PARP-1 is recognized for its critical role in DNA repair. It is mainly integrated in base excision repair(BER). BER is a repair mechanism for single strand breaks (SSB) of DNA during cell growth and division. PARP-1 recognizes this single strand DNA breaks and catalyzes itself and activates PAR protein to repair this breakage [129–131]. PARP-1 also recruits other key BER proteins like XRCC1, and affects scaffolding by recruiting histones H1, H2B. Methylating agents and tomoisomerase I inhibitors attack tumor cells by causing SSB DNA damages [132]. Thus, by inhibiting PARP-1 activity, the damage to DNA caused by chemotherapy, or radiation will no longer be repaired, and the tumor cell will die more effectively by adding PARP-1 inhibitor [133].

Moreover, PARP-1 is involved not only in the repair of SSB, but also in that of double strand breaks(DSB). If there is deficient in PARP-1 activity, SSB repair failure (BER failure) leads to a collapsed replication fork. This collapsed fork now requires DSB repair by homologous repair(HR) by BRCA protein [134]. However if

there is BRCA mutation, defect, or down-regulation in the pathway, the cell can't have successful repair of its defective DNA, and undergo augmented cell death. This phenomenon, a double defect causing cell death, while only one deficiency does not, is called 'synthetic lethality'. The observation of synthetic lethality brought attention to PARP inhibitor as a single agent therapy for specific types of breast or ovarian cancer.

One of the biggest challenges in the treatment of breast cancer, has been developing a novel agent targeting 'basal like breast cancer'. This type of cancer expresses cytokeratin 5/6, similar to basal cells, and has low or nonexistent expression of hormonal receptors and HER2 protein. Basal like breast cancer is not the same as triple negative breast cancer, however there is large overlap. Basal like breast cancers, or triple negative cancers, share many characteristics of hereditary BRCA mutation related breast cancer. They both down regulate BRCA, not by mutations but other mechanisms. Many researchers paid attention to 'BRCAness' of this type of cancer. PARP inhibitors harboring the ability of 'synthetic lethality' in targeting this basal like breast cancer has shown some promising early results [135–137], and lead to development of various PARP inhibitor agents by many pharmaceutical companies.

Iniparib (BSI-201) by Sanofi-Aventis, showed a survival benefit in triple negative breast cancer (TNBC) in a phase 2 trial presented at 2009 ASCO. Unfortunately, the first phase III trial of a PARP inhibitor, reported by Joyce O'Shaunessy and colleagues, failed to show a benefit in progression free survival. Some postulate that this failure of efficacy may be because these patients were unselected. Biomarkers of PARP responsive tumors are needed.

Olaparib (AZD-2281) by AstraZeneca in TNBC showed PR as a single agent. Although some of phase 2 and 3 trial data were disappointing, there are still many other PARP inhibitors to come. At present, Veliparib (ABT-888) by Abbott, is in phase 2 trials in BRCA 1 and BRCA 2 deficient breast cancer and advanced breast cancer, as well as melanoma, colorectal cancer and hepatocellular carcinoma. PF-01367338 (AG-014699) is investigated by Pfizer, undergoing a phase 2 trial in triple negative breast cancer or BRCA1- and BRCA2- deficient breast or ovarian cancer.

We do not yet fully understand the role of PARP inhibitors in this breast cancer phenotype. We need to elucidate the role as single agent, and in combination with DNA damaging cytotoxins, in BRCA mutated tumors. Nevertheless, PARP inhibitors still hold promise and the possibility to open doors to the treatment of one of the most challenging subtypes of breast cancer.

Hormone Receptor Positive but Resistant to Hormone Therapy

Mechanisms of Hormone Resistance

The majority of breast cancers express estrogen receptor alpha. Unfortunately, while most metastatic breast carcinomas initially are responsive to endocrine therapies, most eventually develop resistance. Thus, there is great interest in restoring

endocrine sensitivity. A MCF-7 cell line which models acquired endocrine resistance was found to show differences in genomic expression in these cells compared to the cell line which was estrogen sensitive [138]. There was amplification of ESR1, a gene which upregulates ER alpha expression; suggesting that these cells require much less estrogen than they initially did prior to being deprived of estrogen. 11–20% of breast cancers show ESR1 amplification [139]. In addition, through signaling crosstalk, ER alpha can activate growth factor receptor pathways in a ligand independent manner. Tamoxifen resistance can also be facilitated by PI3K/AKT/mTOR and MAPK phosphorylation of ER alpha, in a hormone independent AF-1 area of ER alpha. Molecular activation of EGFR/ERBB/AKT in cells with acquired estrogen resistance supports the theory of growth factor signaling crosstalk as well. Unfortunately, to date, clinical trials aimed at blocking growth factor and ER alpha pathways have been unsuccessful. Subsets of AKT expression profiles by immunohistochemistry, in 402 breast carcinoma samples was able to predict prognosis. High pAKT and low AKT2 expression profiles were most likely to relapse. During tamoxifen treatment, AKT activation was associated with her two and ER alpha activation linking AKT to tamoxifen resistance [140]. Another mechanism of tamoxifen resistance is overexpression of FGFR1 (FGFR2 is overexpressed in triple negative breast tumors) [141]. FGFR is regulated by E2F1.

Conclusion

Although we have made substantial headway in understanding genetic targets in breast cancer therapy, many questions remain unanswered. There is little doubt, that successful treatment of breast cancer will require identification and manipulation of genetic targets.

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