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REVIEW ARTICLE

Synopsis of new antiangiogenetic factors, mutation compensation agents, and monoclonal antibodies in target therapies of breast cancer

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Summary

Breast cancer is the most common type of cancer among females and the 5th most common cause of cancer death. About 5-10% of breast cancers occur due to gene mutations inherited from mother or father. The molecular basis of breast cancer has been extensively investigated, making gene therapy a potential new therapeutic alternative. Mutation compensation, molecular chemotherapy, proapoptotic gene therapy, antiangiogenic gene therapy, genetic immunopotentiation and genetic modulation of resistance/sensitivity are the main gene therapies used. The combination of gene therapy with chemotherapy or radiation is being investigated in ongoing trials. The purpose of this review was to make a synopsis of the currently existing target therapies in breast cancer.

Key words: antiangiogenetic therapies, breast cancer, gene therapy, mutation compensation

Introduction

The pathogenesis of breast cancer is associated with the interaction between environment and genetics. Cancer develops after the uncontrolled division of cells due to gene mutations. The mutations known to be involved in the pathogenesis of breast cancer occur in the gene repair mechanisms. Some of these genes are p53 and BRCA1-2. Normally, the cell death (apoptosis) is protected by protein clusters and pathways like the PI3K/AKT pathway or the RAS/MEK/ERK pathway. In addition, normally the PTEN gene turns off the PI3K/AKT pathway in case of an upcoming cell apoptosis. In case of PTEN mutations the pathway is not "turned off", leading to carcinogenesis. Furthermore, loss of heterozygosity (LOH) is recognized on several chromosomes: 1, 3, 4-11, 13, 16-18, 22, X [1-3]. Probably it is a result of loss or inactivation of tumor suppressor genes (TSGs) [3]. TSGs regulate cell growth, cell adhesion, or inhibition of proteolytic enzymes that are believed to be involved in breast cancer carcinogenesis.

Familial breast cancer has been investigated thoroughly in the last years, especially with regard to BRCA-1 and BRCA-2 genes. Defective DNA repair function is associated with these genes resulting in carcinogenesis. On the other hand the expression of BRCA-1 gene seems to be reduced in most sporadic cases [4]. As a matter of fact other factors and mechanisms might be involved in the malignant process, such as the nuclear phosphoprotein p53 which repairs DNA damage and inhibits the growth of abnormal cells [5,6].

Furthermore, the growth factor receptor c-erbB-2/HER-2 (neu) and the nuclear transcription factor C-myc are involved in the carcinogenesis of the breast. HER-2 gene encodes a tyrosine kinase receptor protein, a component of cell growth regulation [7,8]. TSG replacement, application of RNA and ribozymes, molecular chemotherapies, and antisense mechanisms provide some of the newly used methods in breast cancer therapy.

In addition, antiangiogenic, immunological and proapoptotic genetic therapeutic approaches have been investigated with promising results. Breast cancer cells are cells that have lost their ability to divide in a controlled fashion. Mutations allow cancer cells (or subpopulations of cancer cells within the tumor) to develop drug resistance and escape therapy. Tumors cannot grow beyond a certain size, generally 1-2 mm^3, due to lack of oxygen and other essential nutrients. Tumor cells induce blood vessel growth (angiogenesis) by secreting various growth factors (e.g. vascular endothelial growth

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factor/ VEGF). Growth factors such as bFGF (basic fibroblast growth factor) and VEGF can induce capillary growth into the tumor, necessary to supply the required nutrients, allowing for tumor expansion. In 2007 it was discovered that cancer cells stop producing the anti-VEGF enzyme PKG (cGMP-dependent protein kinase or protein kinase G is a serine/threonine-specific protein kinase that is activated by cGMP). It phosphorylates a number of biologically important targets and is implicated in the regulation of smooth muscle relaxation, platelet function, sperm metabolism, cell division, and nucleic acid synthesis. In normal cells (but not in cancer cells), PKG apparently limits beta-catenin (beta-catenin is a member of the armadillo family of proteins). These proteins have multiple copies of the so-called armadillo repeat domain which is specialized for protein-protein binding. An increase in beta-catenin production has been noted in people with basal cell carcinoma, leading to increased cell proliferation [9].

Some clinicians believe that angiogenesis serves as a waste pathway, taking away the biological end products put out by rapidly dividing cancer cells. In either case, angiogenesis is a necessary and required step for transition from a small harmless cluster of cells, often said to be about the size of the metal ball at the end of a ball-point pen, to a large tumor. Angiogenesis is also required for the spread of a tumor or metastasis. Single cancer cells can break away from an established solid tumor, enter blood vessels, and be carried to a distant site, where they can implant and begin the growth of a secondary tumor. Evidence now suggests that the blood vessels in a given solid tumor may in fact be mosaic vessels composed of endothelial cells and tumor cells. This mosaicity allows for substantial shedding of tumor cells into the blood stream. The subsequent growth of such metastases will also require a supply of nutrients and oxygen or a waste disposal pathway.

Endothelial cells have long been considered genetically more stable than cancer cells. This genomic stability confers an advantage to targeting endothelial cells using antiangiogenic therapy compared to chemotherapy directed at cancer cells, which rapidly mutate and acquire "drug resistance" to treatment. For this reason, endothelial cells are thought to be an ideal target for therapies directed against them.

Formation of tumor blood vessels

Tumor blood vessels show perivascular detachment, vessel dilatation, and irregular shape. It is believed that tumor blood vessels are not smooth like in normal tissues and do not deliver oxygen to all of the tissues.

VEGF plays a crucial role in the formation of blood vessels that helps tumor growth. This is called sprouting angiogenesis [10-12]. Angiogenesis research is a cutting edge field in cancer research, and recent evidence also suggests that traditional therapies, such as radiation therapy, may actually work in part by targeting the genomically stable endothelial cell compartment, rather than the genomically unstable tumor cell compartment. New blood vessel formation is a relatively fragile process, subject to disruptive interference at several levels. In short, therapy is the selection of an agent which is being used to kill a cell compartment. Tumor cells develop resistance rapidly due to rapid generation time (days) and genomic instability (variation), whereas endothelial cells are a good target because of a long generation time (months) and genomic stability (low variation). This is an example of selection in action at the cellular level, using a selection pressure to target and differentiate between varying populations of cells. The end result is the extinction of one species or population of cells (endothelial cells), followed by collapse of the tumor ecosystem due either to nutrient deprivation or self-pollution from the destruction of necessary waste pathways. Angiogenesis-based tumor therapy relies on natural and synthetic angiogenesis inhibitors like angiostatin, endostatin and tumstatin. These are proteins that mainly originate as specific fragments of pre-existing structural proteins like collagen or plasminogen. Relatively recently, the first FDA-approved therapy targeting angiogenesis in cancer came on the market. This is a monoclonal antibody directed against an isoform of VEGF. The commercial name of this antibody is Avastin (Roche®), and the therapy has been approved for use in cancer in combination with established chemotherapy.

In the present article, data from the current literature have been reviewed in order to investigate whether the new gene therapies directed against breast cancer could present an alternative to the existing therapies.

Tumor suppressor gene therapy

The existing new therapeutic methods consist of genetic tumor growth suppression with TSGs replacement therapies or oncogenes ablation.

According to current evidence, replacement of p53 gene remains the most investigated topic. Antiangiogenetic factors, proapoptotic proteins and immune upregulation might be involved in the final therapeutic result after viral p53 introduction in human breast cancer cells [13]. It is important to point out that –concerning the existing trials– not only the p53 transduced tumor cells are

killed, but also the neighboring cells [14]. Also, other TSGs, like mda7, BRCA-1, BRCA-2, Rb, and p27 might serve as therapeutic tools [15-19].

Besides the viral TSGs insertion, antisense oligodeoxynucleotides block the transfer of genetic information. These nucleotides are short ssDNA molecules [20]. Other suspected mechanisms that lead to mRNA inhibition are translation arrest, inhibition of transcription or splicing. Concerning the current literature, efforts have been made to suppress oncogenetic genes which are protein kinase C-alpha (PKC-a) [21], Bcl-2 [22], insulin like growth factor receptor (IGF-IR) [23], and plasma membrane calcium ATPase [24].

Application of ribonucleic interference technology (RNAi) provides a possible specific downregulation mechanism of the c-myc gene [25]. Furthermore, RNA molecules, the ribozymes, are involved in the formation of covalent bonds in RNA strands. According to some studies, ribozymes might affect not only mRNAs in cancer cells but also those in normal cells. This could imply an important problem concerning the therapeutic targets. So, dimer minimized ribozymes (minizymes) have been investigated concerning their ability to suppress cyclin AD1 and hst-1 oncogenes of the breast [26].

Molecular chemotherapy by using the so called "suicide genes" provides another method of breast cancer gene therapy (Figure 1) [27]. Vitral genes that express toxic molecules or activate enzymes of specific prodrugs are introduced in the cancer cells. Due to the so called bystander effect (effect also on nontransduced cancer cells) this method of enzyme activation is not limited only in cells which are genetically modified to express an activating enzyme [27,28]. The enzyme-activating prodrug therapy consists of genes of both human and non-human origin, e.g. cytochrome p450 isoforms and thymidine kinase.

More efficient might be the transfection of cancer cells with two different suicide genes, due to the activation of two prodrugs. In addition, combination of suicide and cytokines genes, such as IL-2 gene, is supposed to achieve better results according to relative experiments [29]. Promising results are published concerning the development of genetic prodrug activation which is affecting the same cancer transcription [30]. On the contrary, tumor regression is reported to be not significant. According to other trials the use of MetXia-p450, a recombinant retroviral vector which encodes the human cytochrome p450 type 2B6 gene, might present a safe and promising approach [30]. The cytochrome p450 enzymes are mainly located in the liver and convert cyclophosphamide to an active phosphoramide and acrolein [30,31].

Lastly, it has been postulated that intracellular single-chain antibodies could downregulate the cell surface erbB-2 levels. erbB-2 is a protooncogene which is overexpressed in breast cancer cells [32].

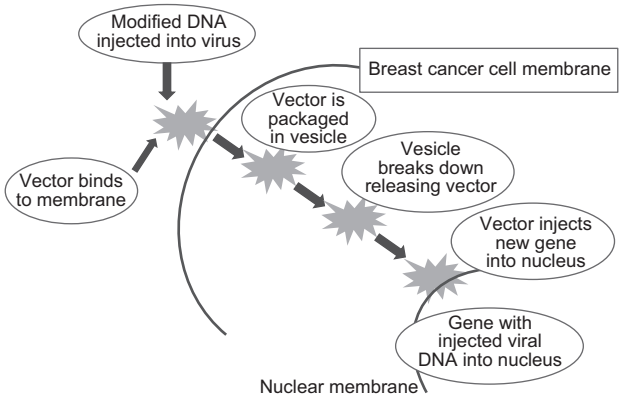

Figure 1. Gene therapy using a viral vector (adenovirus).

Antiangiogenetic therapies

Tumor growth, invasion and metastatic dissemination are dependent on the formation of new microvessels. The process of angiogenesis is regulated by a balance between proangiogenic and antiangiogenic factors, and the shift to an angiogenic phenotype (the "angiogenic switch") is a key event in tumor progression. The use of antiangiogenic agents to restore this balance represents a promising approach to cancer treatment. Angiogenesis represents a mechanism which is being involved in the carcinogenesis of the breast. Tumor growth and metastasis are associated with angiogenesis and, according to this, it has been postulated that factors which could diminish or arrest this situation might represent an additional therapeutic approach in breast cancer. Furthermore, tumors with diameter < 3 mm show a decreased growth process if blood perfusion is limited. Moreover, metastatic spread of solid tumors depends on the vascularization of the primary mass, indicating that a blockage of tumor angiogenesis will also block tumor metastasis. A wide range of angiogenesis inhibitors has been tested, many of which showed efficacy against a variety of solid tumor types, in particular the endogenous inhibitors. Factors that might be involved in this antiangiogenetic strategies are endostatin and angiostatin. These therapeutic strategies are believed to have a better result if they are combined with hormonal and chemotherapeutic agents. Concerning existing studies, recombinant proteins might have promising results, but their use is limited because of the difficulty to produce proteins in large quantities, which are necessary in order to achieve the optimal therapeutic aim. On the other

hand, no sufficient data exist concerning the comparison between recombinant molecules and gene-delivered angiostatic factors. Furthermore, it still remains unknown which levels of antiangiogenetic factors should be reached within the tumor to attain a therapeutic effect. The administration of different angiogenesis inhibitors by gene transfer has been shown to result in inhibition of tumor growth in animal tumor models. However, the potency of these genes has been only partially evaluated in comparative studies to date.

Angiostatin

Angiostatin is a fragment of a larger protein, plasminogen, and is endogenously produced in the tumor stroma. Nowadays, it is accepted that endogenous angiostatin produced by the primary tumor inhibits the metastatic process. As a matter of fact, additional therapy with angiostatin might control the metastatic process. During tumor growth, hypoxia and acidosis of the tissue is established so that growth factors are produced in order to stimulate angiogenesis. The new vessels are characterized by blind ends or blood backflow of mixed arterial and venous blood. It has been shown that angiostatin can maintain metastases in a dormant state in laboratory animals when administered exogenously [33]. It is believed that possibly angiostatin promotes an anti-metastatic and anti-neovascularization action by binding the α/β subunits of ATP synthase [34,35].

Experimental models on angiostatin actions in breast cancer have shown that tumor cells are also able to express the nm23 gene [36-38]. Its translational product is actually a nucleoside diphosphate kinase called NDPK-B [39]. The NDPK-B isoform has been identified in the extracellular environment of a breast carcinoma cell line providing a mechanism for purine regeneration [40].

Due to this action it is believed that it might actually be a continuous source for localized production of extracellular ATP in a tumor environment, thus preserving the tumor and increasing its metastatic potential [41].

Recent studies in breast cancer show that angiostatin probably also inhibits the NDPK-B expression resulting to further reduction of available energy resources of tumor cells [42].

Additionally to this is has been proved that N-acetyl-cysteine promotes angiostatin production and vascular collapse in orthotopic models of breast cancer, resulting in endothelial apoptosis and vascular collapse of the tumor [43].

The half time of angiostatin is short, about 15 min, so that the exogenous administration is associated with rapid clearance and limited side effects. In addition, the co-administration of other factors, like endostatin, might prevent metastasis for a longer period of time. However, such studies are not yet well tested in humans. Such trials should focus on the possible most effective combination therapy, concerning the tumor type, as well as the side effects.

Plasminogen Kringle 5

The plasminogen Kringle 5 (K5) domain has also been identified to demonstrate an antiangiogenic action on breast cancer models [44]. K5 achieved a significant decrease in tumor neovessels length and density by inhibiting the tumor-derived endothelial cells and tumor-associated macrophages [45]. Besides promoting antiangiogenesis K5 was shown to exert a neutrophil chemotactic activation, contributing to the recruitment of tumor-associated neutrophils and NK T lymphocytes, thus leading to a possible antitumor immune response. This observation is in support of a proinflammatory mode that K5 has an inflammatory and microvascular suppression action role in breast cancer [45].

Matrix metalloprotease 12 (MMP12) and Pentraxin 3 (PTX3)

Two other significant factors that are believed to induce a limited angiogenesis are the matrix metalloproteases 12 (MMP12) and Pentaxin 3 (PTX3) which are the main inducers of antiangiogenesis in systemic sclerosis (SSc) [46-49].

In certain research models including infusion of these proteins on two breast cancer cell lines expressing low amounts of metalloproteases it was proved that this artificial overexpression led to reduced ability of tumor cells to stimulate angiogenesis both *in vivo* and *in vitro* [50]. Especially when both of MMP12 and PTX3 were expressed the tumor growth was significantly reduced without noticing any simultaneous angiostatin production.

According to these finding it is believed that further studies would strongly certify their antiangiogenic action leading to new forms of treatment.

Trastuzumab

The HER2 receptors are involved in the carcinogenesis of breast cancer. Molecular signals are transported through the cell membrane inside the cytoplasm

and the nucleus regulating the activation or inactivation of genes related to cell growth, survival, migration and differentiation. In breast cancer these receptors are overexpressed in 15-25% of the cases, owing to ErbB-2 gene amplification, which, in addition with other mechanisms, lead to loss of cell growth control [51]. According to this it has been postulated that antibodies against these receptors could decrease this cell growth malfunction. Trastuzumab is a humanized monoclonal antibody that selectively targets the extracellular domain of HER2. The extracellular effects include inhibition of cleavage of HER2 extracellular domain and formation of HER family receptors (Figure 2).

Trastuzumab has been shown to provide significant clinical benefits to patients with HER2-positive breast cancer when administered as monotherapy [52,53]. It has also offered better response rates when combined with chemotherapy than chemotherapy alone. Addition of trastuzumab for one year to adjuvant chemotherapy improves the disease free survival by 33-52% as well as the overall survival by 34-41%. Results from many studies have confirmed the increased incidence of symptomatic or asymptomatic cardiac arrhythmias. Risk factors associated with cardiac dysfunction include baseline left ventricular ejection fraction level, hypertension and older age. Concerning brain metastases studies suggest that trastuzumab is unlikely to increase the risk of brain metastases (Table 1) [53-61].

Besides the side effects another important problem that tends to reduce the duration of therapy is its high cost. For example in some countries like New Zealand, the adjuvant therapy is being accepted for one year due to the high public costs. Trials are in progress comparing short vs. long duration of therapy. Before any decision concerning the use of trastuzumab it is important that all invasive breast cancer patients are tested for the tumor's HER2 status [60,61].

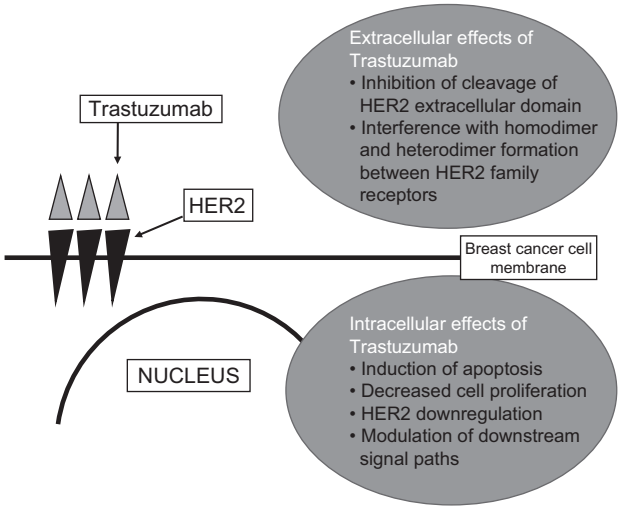

Figure 2. Trastuzumab mechanism of action.

Anti-VEGF treatment

Angiogenesis represents an important mechanism in breast cancer progression and metastasis, making anti-VEGF therapies a promising option. Overexpression of VEGF is associated with poor outcomes in patients with breast cancer. Many studies focused on the possible relationship between VEGF expression and clinical outcome in breast cancer. According to the existing knowledge VEGF leads to worse relapse-free and overall survival rates in patients with early-stage breast cancer [62]. The largest of those trials showed that VEGF was an independent prognostic marker in both node-positive and node-negative breast cancers [63]. In patients with metastatic breast cancer, VEGF overexpression led to larger tumors, negative steroid-receptor status, p53 mutations, and poor tumor differentiation. High tumor levels of VEGF led to breast tumors resisting chemotherapy with FAC (fluorouracil/doxorubicin/cyclophosphamide) or CMF (cyclophosphamide/methotrexate/fluorouracil) and hormonal therapy with tamoxifen [61]. Bevacizumab (Avastin®) (Figure 3), a monoclonal antibody, provides an effective therapy in metastatic breast cancer when combined with paclitaxel in the first-line setting. On the contrary, bevacizumab could be less effective due to the different angiogenetic mechanisms which occur beyond the

Table 1. Summary of adjuvant trastuzumab trials

Trial [Ref. number]	*Patients n*	*Recurrence HR (95% CI)*	*Mortality HR (95% CI)*
HERA [55,60]	3387	0.63 (0.53-0.75)	0.66 (0.47-0.91)
Joint Analysis N9831-31 [58]	3351	0.48 (0.39-0.59)	0.67 (0.48-0.93)
BCIRG [57]	2147	0.61 (0.048-0.76)	0.59 (0.42-0.85)
PACS 04 [56]	528	0.86 (0.61-1.22)	1.27 (0.68-2.38)
FinHer [60]	232	0.42 (0.21-0.83)	0.41 (0.16-1.08)

CI: confidence interval, HR: hazard ratio

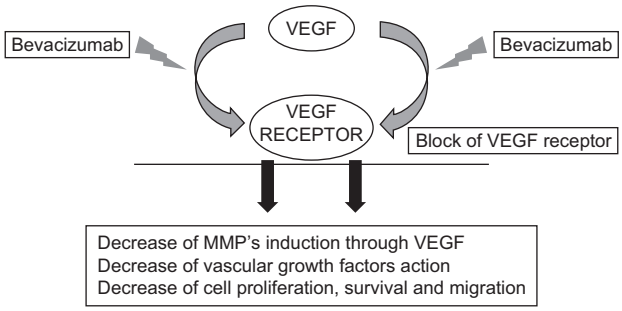

Figure 3. Bevacizumab mechanism of action.

first-line setting. Bevacizumab is a well tolerated molecule but blood pressure and urine protein levels have to be controlled during therapy. In addition, it has been postulated that the combination of multi-targeted agents could have better results. In view of this observed interaction between the different target agents it may be logical to combine anti-HER2 and anti-VEGF treatment approaches for the treatment of HER2-overexpressing breast cancers. Ongoing trials are investigating the efficacy and safety of bevacizumab in various stages of breast cancer [64,65].

Conclusions

New targeting methods have been developed during the last years in order to achieve better therapeutic results concerning breast cancer treatment. These methods include agents with different mechanisms of action such as TSG therapy or antiangiogenic factors, which lastly are believed to play an important role in cancer growth.

Yet, the majority of the trials report a low clinical response as well as low toxicity. Adenoviral vectors are reported to have a high transfection ability but tumor regression is unsatisfactory. In addition, transfer of genetic information might be blocked with antisense oligodeoxynucleotides, which are short ssDNA molecules. Lastly, the "suicide genes" are another method of molecular chemotherapy of breast cancer. Induction of antitumor immunity and transfer of phosphorylated ganciclovir in tumor cells might explain the observed "bystander effect", which is characterized by enzyme activation not exclusively of the transduced cancer cells. More efficient might be the transfection of cancer cells with two different suicide genes or the combination of suicide genes and cytokine genes (IL-2 gene).

Another mechanism which has been thought to play a role in the tumor growth is angiogenesis. Antiangiogenetic proteins, like endostatin and angiostatin, have been developed in order to block this process. The blockade of angiogenetic mechanisms in addition to the current endocrine and cytotoxic treatments is believed to have a better result. VEGF overexpression in patients with breast cancer leads to worse relapse-free and overall survival, compared with nonoverexpressing cancers. The high degree of expression, negative prognostic significance of VEGF in breast cancer, and the central role that VEGF plays in tumor angiogenesis have made this growth factor a key target for anticancer therapy. Bevacizumab has shown promising efficacy and tolerance as single agent in phase II clinical trials in metastatic breast cancer. A phase III study showed a lack of benefit of bevacizumab with capecitabine, compared with capecitabine alone, in progression-free survival. This may reflect the late disease stage and poor prognostic factors of the patient population, as well as the choice of the chemotherapeutic regimen. The contribution of other proangiogenic factors to breast cancer progression and the heterogeneity of VEGF expression in breast cancer indicate that a more focused approach to the use of anti-VEGF therapy may be beneficial. It was suggested recently that angiogenesis occurs at the very earliest stages of tumor development, when perhaps only 100-300 tumor cells are present. This suggests that antiangiogenic treatments may be most effective in micrometastatic disease, before visible disease is identified. For example, co-injection of a soluble VEGF receptor with tumor cells can inhibit subsequent angiogenesis and tumor growth in rodent models.

Combinations of bevacizumab with biologic agents, including trastuzumab and erlotinib, an inhibitor of the HER1 (epidermal growth factor receptor 1) tyrosine kinase, are also being evaluated. Further studies are warranted to determine the potential of bevacizumab in breast cancer, particularly in earlier disease stages, while methods of selecting patients who would benefit optimally from anti-VEGF therapy are urgently needed.

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