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Locally advanced breast cancer as a model for biomarkers research

ABSTRACT

The presence of the tumor in locally advanced breast cancer as an in vivo model offers the possibility of studying the effect of primary anticancer therapy on biological parameters. The hypothesis is that changes in certain molecular biomarkers, particular determinants of tumor growth such as proliferation or apoptosis, or molecular biomarkers of angiogenesis modulation, may predict clinical outcome. In this review, numerous molecular biomarkers of proliferation (ERα and ERβ, PR, EGF-R family) and apoptosis (p53, Bcl-2 family), as well as molecular biomarkers of angiogenesis (FGF, VEGF) are discussed for their possible role in locally advanced breast cancer growth.

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INTRODUCTION

A balance between proliferation, differentiation, and cell loss by apoptosis in the stem-cell population and throughout the cells of the mammary gland is critical for normal development. Perturbations in this balance can contribute to cancer development. Conditions that up-regulate cell proliferation or down-regulate apoptosis can allow the accumulation of mutations that contribute to the subsequent development of breast cancer.

Tumor development depends not only on the nature of the tumor cells themselves, the specific oncogenic or tumor suppressor alterations occurring within the malignant cell itself, but also on the modifying effects of normal host cells. Briefly, fibroblast and endothelial cells are believed to favor tumor development, as well as invasion and metastasis of malignant cells. On the contrary, normal myoepithelial cells and epithelial cells have been shown to exert a tumor suppressor effect, inhibited growth and induced apoptosis of breast cancer cells.

The most prominent characteristics of locally advanced breast cancer is the marked increase in the number of malignant cells, resulting from an imbalance between cell proliferation and apoptosis, with absence of the clinically verified distant metastatic site.

The use of neoadjuvant anticancer therapy presents a major change in the management of locally advanced breast cancer as a systemic disease. In general, there have been three main objectives for the use of neo-adjuvant anticancer therapy:
- To reduce tumor and lymph node bulk;
- To improve overall survival;
- To preserve, by leaving the tumor *in vivo*, an important indicator of tumor response to the therapy and a possible predictor of overall survival.

It would be very important and useful to compare pre-therapy with post-therapy molecular biomarkers values because in this way we could distinguish the endogenous molecular biomarkers values from molecular biomarkers values changed by the therapy. In that context, determination of pre- and post-therapy molecular biomarkers in locally advanced breast cancer, which are predictive of the tumor biology, may be helpful for the prognosis and therapeutic stratification of individual patients.

MOLECULAR BIOMARKERS OF PROLIFERATION

Steroid hormone receptors-related proliferation

Normal development of mammary glad is critically dependent on the ovarian steroid hormones, estrogen and progesterone. Changes in steroid hormone action, in particular estrogen action, occur during the development of breast cancer as well as during breast cancer progression.

The effects of steroid hormones are mediated by their respective receptors, which function as transcriptional factors. Estrogen receptor-α (ERα) and the ER-regulated progesterone receptor (PR) are of special interest, because their protein levels are elevated in breast carcinomas as opposite to normal tissue. However, the majority of normal human breast epithelial cells express ER-β only. The function of ER-β, that is independent of ER-α expression, is unknown, although when both receptors are coexpressed it has been speculated that ER-β isoforms may negatively modulate ER-α activity (1), resulting in inhibition of ER-α-positive breast cancer cells proliferation. The ratio of ER-α and ER-β may be of particular importance for proliferation of breast carcinoma cells. Therefore, increasing of this ratio may be responsible for increasing of estrogen-regulated proliferation of breast carcinoma cells resulting in increasing tumor mass and spreading of tumor mass to surrounding tumor stroma in locally advanced breast cancer.

Receptors for estrogen belong to the steroid/thyroid hormone superfamily of nuclear receptors. They are composed of three independent but interacting functional domains:
- The NH$_2$-terminal or A/B domain;
- The C or DNA binding domain;

- The D/E/F or ligand-binding domain.

Binding of a ligand to ER triggers conformational changes in the receptor and this leads, via a number of events, to changes in the rate of transcription of estrogen-regulated genes. These events include receptor dimerization, receptor-DNA interaction, interaction with coactivator and other transcriptional factors, and formation of preinitation complex (2).

The receptor contains two transcriptional activation functions by which are able to regulate the expression of target genes; AF-1, which is located in the N-terminal or A/B domain, and AF-2 in ligand binding domain. AF-1 and AF-2 can function independently or synergistically depending on gene promoter and/or the cell type (3). Among the members of the family of steroid hormone receptors, the N-terminal region has the highest degree of amino acids sequence variability whereas the DNA binding domain has the most shared homology. AF-1 domain of ER-α is very active in stimulation of receptor-gene expression, but activity of the AF-1 domain of ER-β is negligible. Another striking difference between ER-α and ER-β is their distinctive response to the tamoxifen; Tamoxifen is partial estrogen agonist with ER-α but is pure estrogen antagonist with ER-β. Further, the DNA binding domain of ER-α and ER-β are highly homologous. Thus ER-α and ER-β can be expected to bind to various estrogen response elements with similar specificity and affinity.

Two model of ligand-dependent activation of ER exist to date: the so called "classical" activation induced by agonist which results in direct interaction of the ER with DNA and subsequent transcriptional activation, and nonclassical activation induced by agonist which cause the interaction of ER with other proteins (AP-1, SP1 or NF-κB).

Ligand-independent regulation of ER activity occurs via phosphorylation of various serine and tyrosine residues in the AF-1 and AF-2 domains controlled by signaling pathways of growth factor receptors, such as EGF-R, IGF-R and HER2/neu-R. Estrogen receptor, in return, regulates the transcription of genes required for growth factor activity, which results in a complex interaction between ER and growth factor-receptor pathways (4).

Consequently, ligand-dependent activation of ER as well as ligand-independent regulation of ER activity may result in increasing tumor mass and spreading of tumor mass to surrounding tumor stroma in locally advanced breast cancer.

Growth factors-related proliferation

Epidermal growth factor-receptor (EGF-R) family members are activated by a large group of EGF-related growth factors. Common to all these growth factors is the EGF domain with six conserved cytosine residues characteristically spaced to form three intramolecular disulphide bridges. In general, EGF-like ligands are synthesized as glycosylated transmembrane precursors, which are proteolytically cleaved from the cell surface to yield the mature growth factor (5). All EGF-R family members are characterized by a modular structure consisting of an extracellular ligand binding, and the intracellular part harboring the highly conserved tyrosine domain.

Legend binding induces the formation of homo- or heterodimers, which subsequently trigger the autophosphotilation of cytoplasmic tyrosine residues (6). These phosphorilated amino acids represent docking sites for a variety of signal transducers which regulate membrane-proximal steps of a complex signaling network ultimately defining the biological response to a given signal. Deregulation of this tightly controlled system by overexpression, amplification or constitutive activation of mutant receptors and/or autocrine stimulation through aberrant growth factor loops is frequently linked to hyperproliferative disease such as breast cancer (7).

MOLECULAR BIOMARKERS OF APOPTOSIS

Tumor suppressor gene p53 - related apoptosis

The p53 tumor suppressor gene encodes a 393 amino acid nuclear phosphoprotein. The p53 wild-type is composed of three independent but interacting functional domains (8):
- The N-terminal part is involved in transcriptional control;
- The middle portion is responsible for the DNA binding;
- The carboxyl-terminal part is involved in its function..

The p53 gene appears to act as a real tumor suppressor gene since its function is not required for normal development but lack of its function confers an enormously elevated risk of developing cancer. The current and most powerful model of wild-type p53 function is one in which p53 monitors the genome for DNA damage, participating in the maintenance of genomic stability (9). Consequently, mutations in p53 may lead to genomic instability (10).

Intrinsic or environmental DNA damage change the transcriptional level of several cell cycle check points related genes, including mdm2, GADD45, and p21 (WAF1/CIP1), through the increased synthesis of p53 wild-type protein. The mdm2 gene product interacting with p53 wild-type protein can inhibit p53-mediated transcription, and it has been suggested that p53 and mdm2 reciprocally regulate each other (11). Overexpression of mdm2 protein therefore might be one of the mechanisms of inactivation of p53 function. Gene product of GADD45 (Growth Arrest DNA Damage) prolongs G1 phase, and thereby permits the cell to repair the DNA damage before entering S phase (12). p21 as product of p21(WAF1/CIP1) inhibits the activity of cyclin-dependent kinase complexes, the progression of the cell cycle, the replication DNA, and represent the primary mediator of the effect of p53 on the cell cycle (13). Further, it is known that the proliferating cell nuclear antigen, PCNA, interacts with both GADD45 and p21, and could serve as a "switching mechanism" blocking PCNA interaction with DNA polimerase δ. Deregulation of PCNA expression occurs in a breast cancer and serves as a marker of marked tumor growth, such a common feature of locally advanced breast cancer.

The normal p53 function can be inactivated by somatic and germ line mutations, binding to the mdm2 and to different viral oncoproteins. The main mutations sites are the zinc binding domains L2 and L3 and the evolutionarily conserved region II and V (14). p53 mutations change the conformation of the protein and lead to stabilization of p53 and its accumulation in the nuclei of cancer cells (15). Cells lacking normal p53 function have a selective growth advantage and are more resistant to ionizing radiation and some widely used anti-cancer drugs than cells with wild-type p53 protein.

In the presence of wild-type p53, oncogene-expressing cells can form tumors, but cells survival is limited by their increased susceptibility to apoptosis (16). As a consequence, selection against p53 often occurs late in tumor progression. Anticancer agents may simply activate the apoptotic program intrinsic to these sensitized cells. Thus, genetic alterations that accompany malignant transformation can increase the therapeutic index by radiation or chemo/hormone therapy. Other mutations may have the opposite effect, leading to tumor resistance.

Mutations in p53 may lead to genetic instability such as amplification of the HER-2/neu locus sited in the same chromosome or activation of the apoptotic program of cell death that is regulated by anti-apoptotic bcl-2 (inhibited by p53) and pro-apoptotic bax (promoted by p53) genes.

Oncogene Bcl-2 - related apoptosis

The Bcl-2 family proteins are key regulators of the apoptotic pathway. Members of the Bcl-2 family can either suppress or promote the cell death signal. These proteins share at least one of four homologous regions termed Bcl homology (BH) domains (BH1 to BH4). Based on their homology function and sequence homology, Bcl-2 family members can be classified into three main categories:
- Antiapoptotic proteins such as Bcl-2 and Bcl-x that inhibit cell death;
- Pro-apoptotic proteins such as Bax and Bak that promote cell death:
- "BH3-only" proteins such as Bad and Bid that are pro-apoptotic and share sequence homology only in the BH3 domain.

While opposing biological functions and wide differences in amino acid sequences, experimentally determined structures of Bcl-2, Bcl-x, Bax and Bad

are surprisingly similar.

Homo- and heterotypic dimmers are observed among members of the Bcl-2 family. Through heterodimerization anti-apoptotic and pro-apoptotic proteins neutralize the biological activity of opposing partners and thus the fate of a cell is determined by the ratio of these proteins and the different combinations of their complexes (17).

Other mechanisms by which Bcl-2 family proteins regulate apoptosis independently are suggested to be involved with their direct interactions with the voltage-dependent anion channel (18). It is suggested that cytochrome c is released from the mitochondria through volt-dependent anion channel and that antiapoptotic and pro-apoptotic Bcl-2 family members exert different effects of closing and opening the channel, respectively (19).

The role of Bcl-2 in development and progression of breast cancer is still unclear. Based on the hypothesis that Bcl-2 facilitates and extends survival of transformed cells, thereby providing an opportunity to accumulate further genetic aberrations and promoting malignant progression, a relationship between Bcl-2 overexpression and unfavorable clinical outcome is to be expected. However, most studies performed until now have found a strongly positive correlation between Bcl-2 expression and parameters of good prognosis and/or prediction (20). A strongly positive correlation between Bcl-2 expression with increasing levels of both estrogen and progesterone receptor expression was observed. Tumors with lower histological grading were usually Bcl-2 positive, whereas marked Bcl-2 expression was rare in case with the highest histological grading. High proliferating activity and overexpressions of c-cerbB-2 were inversely correlated with Bcl-2 positivity.

To explain the complex relation of Bcl-2 with the biological behavior of the tumor there is one possible mechanism to consider. The function of Bcl-2, as an antioxidant metabolic factor, is to protect macromolecules, including DNA, from damage caused by the generation of oxygen free radical species (21). Interestingly, oxygen free radical species have been demonstrated not only to damage cells but also, within a certain concentration range, to induce proliferation (22). It may be that Bcl-2 down-regulates proliferative activity by its counteraction against high reactive oxygen free radicals. Such suggestion is in accordance with the finding that proliferation of solid tumor cell lines in vitro was suppressed after introduction of Bcl-2 expression vectors (23).

It may be speculated that the essential biological impact of Bcl-2 in breast cancer is based not so much on the prevention of programmed cell death as on the down regulation of proliferation through neutralization of the mitotic effect of high reactive oxygen free radical levels. Thus, the selection advantage of prolonged survival would be counteracted by low proliferation rates, and clonal expansion would be delayed.

ANGIOGENESIS MODULATION

Angiogenesis, the formation of new blood vessels from the existing vascular network, is essential for continued tumor development, growth, invasion and metastasis (24). There is now good evidence that tumors produce a variety of positive and negative angiogenic factors that influence the vascularization process (25). Some of the positive factors encourage the formation of new blood vessels include members of the fibroblast growth factor (FGF) and vascular endotothelial growth factor (VEGF) families, while the angiogenic inhibitors include angiostatin and endostatin. Proangiogenetic factors, in particular FGF and VEGF, activate endothelial cells, which leads to secretion and activation matrix metalloproteinases (MMPs), plasminogen activators (e.g. urokinase plasmin activator complex (uPA-PAI) and cathepsins (26). This results in degradation of basement membrane, which allows the endothelial cells to invade the surrounding matrix. Subsequently, endothelial cells migrate, proliferate, and eventually differentiate to form a new, lumen-containing vessel. Finally, the endothelial cells deposit a new basement membrane and secrete growth factors that attract supporting cells to stabilize the new vessels. It is, therefore, essential for tumors to establish their own vasculature in order to be able to survive and grow.

CONCLUSION

Neoadjuvant/presurgical medical therapy of breast cancer provides a unique opportunity to derive biological information related to tumor response. There are very few studies that have set out to study the biology of neo-adjuvant anti-tumor therapy in breast cancer. However, a numbers of studies have tried to identify molecular biomarkers or changes in molecular markers that are associated with response or resistance to treatment, with the goal of introducing them as predictive markers. Areas of key importance are (27):
- What changes occur to underpin the regression of tumors?
- What biological processes are required to facilitate the changes and which, if deficient, may lead to resistance?
- Are there identifiable features in the cells that remain at the end of anti-tumor therapy, which allow their survival?

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