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Review

Tamoxifen resistance: From cell culture experiments towards novel biomarkers

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ABSTRACT

Tamoxifen is still the most frequently used antiestrogen for the treatment of patients with premenopausal, estrogen receptor positive breast cancer. However, in 20–30% of these cases, tamoxifen therapy fails due to an existing or developing resistance. The prediction of tamoxifen resistance by appropriate biomarker analysis and the development of novel therapies for tamoxifen resistance in premenopausal breast cancer is, therefore, an important goal of ongoing research.

Tamoxifen resistance is associated with altered estrogen receptor expression especially on the plasma membrane, including the alternative G-protein coupled receptor GPR-30 (GPER) and estrogen receptor splice products, such as ERα36. Tamoxifen resistant cells often use alternative pathways to promote proliferation in the absence of genomic estrogen signaling. These pathways involve the epidermal growth factor EGF, the inflammation associated transcription factor NF-κB- and the IGF-1 pathway.

Tamoxifen resistant mamma carcinoma cell lines are useful models to understand tamoxifen resistance *in-vitro* and to search for prognostic or predictive biomarkers. Furthermore, such cell lines can be used to identify potential targets for therapy.

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Introduction

Breast cancer is the most frequent neoplasm in women, and about 70% of these carcinomas express the α-estrogen receptor (ESR1, ERα), which is associated with a good prognosis. Today, about 85% of ERα-positive breast cancer patients have an overall survival of more than five years after diagnosis [1], mainly due to endocrine therapy [2]. However, the ERα-positive group is also the most diverse class of mamma carcinomas and it is likely that patients with certain subclasses need different treatment.

For endocrine therapy, which is currently the standard treatment for patients with ER-positive cancers, estrogen action can either be blocked by selective estrogen receptor mediators

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(SERMs), such as tamoxifen or by down-regulating estrogen receptor expression by compounds, such as fulvestrant or by inhibiting estrogen synthesis by aromatase inhibitors. In premenopausal women with ER positive breast cancer, tamoxifen is still the most frequently used SERM, whereas in postmenopausal patients, aromatase inhibitor therapy or a combination of both is more favorable [3]. The positive effect of a five year treatment with tamoxifen on disease recurrence remained evident for 5–10 years after diagnosis, and sustained even in the following five years, although recurrence rates became similar after year 10 in the two groups [4]. Although, this remarkable success, about 20–30% of cases the tumors are resistant to tamoxifen therapy [5,6], which was either present before treatment, called *de-novo* resistance, or develop resistance under therapy, which is entitled acquired tamoxifen resistance [7]. To prevent failure of antiestrogen therapy with tamoxifen, it is desirable to predict the resistance phenotype by biomarkers and to develop alternative treatment options.

Cell culture models

As early as 1981 [8] and 1984 [9], the first cell culture models for tamoxifen resistance became available. Surprisingly, commonly used ER-positive breast cancer cell lines such as MCF-7 become tamoxifen resistant by a simple incubation for about three months with the drug, usually in its active form 4-OH-tamoxifen. Nevertheless, several protocols of tamoxifen treatment, differing in concentration, time and serum supplementation have been developed. In some cases, resistant cells were cloned, in other laboratories resistant cells were cultivated together, so that the best adapted and fastest proliferating cells have been selected. It would, therefore, not be surprising to find several different mechanisms of tamoxifen resistance, as it seems also evident for individual breast cancer patients. However, to our knowledge, a comprehensive comparison of tamoxifen resistant cell lines has not been published yet.

There are several hypothetical possibilities how an ER-expressing cell can become resistant to tamoxifen or that tamoxifen itself serves as a growth stimulus. Firstly, there can be mutations in the ESR1-gene or the associated effectors, or secondly, there can be an adaptive response using other pathways to bypass the blocked estrogen signal. Current research favors the idea that mutations might not be necessary, especially for the development of acquired tamoxifen resistance. Another option could be an altered metabolism of tamoxifen, resulting in decreased concentrations of the active 4-OH tamoxifen, however, as this is not a cell autonomous feature it is not topic of this review.

Estrogen receptors

To understand the action of tamoxifen and possible ways to escape this inhibitor a detailed knowledge of the estrogen receptor "network" is necessary. Currently, there are three genes identified that encode proteins which bind estrogens and are able to transduce a proliferative signal. Two of these receptors are classical nuclear receptors and are encoded by the ESR1 and ESR2-genes, usually known as α- and β-estrogen receptors (ERα, ERβ). These two genes are rather similar and the encoded proteins exhibit a common organization in six domains (A–F) [10,11]. A/B domains are responsible for ligand independent transcriptional activation. The C-domain harbors the two DNA-binding Zn-fingers, as well as, a dimerisation domain. The following D-domain contains a nuclear localization signal and the E-domain is responsible for ligand binding but contains also another nuclear localization signal [12]. E contains also the activation domain 2 which is responsible for ligand depending transcriptional activation. The F domain modulates the transcriptional activation mediated by the regions A/B and E. Although the ERβ-protein is highly similar to the ERα protein, especially in the ligand binding and DNA binding domain, the ligand specificity is different [13,14]. Indeed, only two of the binding pocket amino acids are different between the two receptors [15]. For both receptors, more than 10 splice forms exist that might exhibit different localization, ligand binding specificity and, thus, different biological functions.

The genomic signaling for both receptors has been analyzed in great detail (for review, see i.e. [16]). Generally, after ligand binding, the receptors undergo dimerisation and are then transferred to the nucleus, where they bind to specific estrogen response elements (ERE) and initiate transcription together with cofactors, such as AP1 and SP1. But there is also a non-genomic signaling that is initiated at, or at least near the plasma membrane. These signaling results in fast events that involve G-proteins, MAP-Kinases, protein kinase B (AKT) and also adenylyl cyclase and growth factor receptors, such as EGFR [17]. As a result, a proliferative signal can be generated without genomic estrogen signaling. There is also preliminary evidence for a promoter element that is responsible for transcriptional effects of membrane estrogen signaling [18].

Additionally, estrogen receptors can be phosphorylated at the ligand-independent activation domain by, i.e. MAP-kinases and AKT. This results in cross talk between genomic and non-genomic signaling of the estrogen receptors and other signal transduction pathways [19]. Also located at the membrane is the G-protein coupled receptor GPER, formerly known as GPR-30. It has been shown that this receptor accepts estrogens as ligands and then signals through cAMP and calcium fluxes [20]. It is also clear that such signaling will cross talk with the pathways used by ESR1 and ESR2. Altogether, estrogen signaling involves a set of receptors with different isoforms that show a remarkable degree of cross talk. As these receptors also exhibit different ligand specificity, it is very reasonable that tamoxifen resistance can be mediated by a differential usage of this receptor network.

Estrogen receptor α (ESR1)

Expression of this estrogen receptor significantly determines the prognosis of breast cancer and is therefore applied in standard diagnostic procedures. Its major form is a 66 kDa protein that confers mostly genomic signaling but can also be detected at the plasma membrane [21] by biochemical means. Detection in the nucleus is most frequently seen and relevant for pathological diagnosis. A positive signal in the cytoplasm or at the membrane is only observed for a small part of cases and its significance, therefore, questioned [22]. However, cytoplasmic and membrane staining has also been reported to be associated with poor prognosis, but this cytoplasmic/membrane staining has been mainly attributed to the truncated ERα36 (see below) isoform [23].

Although, at least 13 splice forms are listed so far in the Ensembl database [24], only four proteins have been characterized in detail yet. On Western blots, α-estrogen receptors of 66, 46, 39, 35 kDa can easily be detected with a serum directed against the whole receptor. The 66 kDa represents the "full size" ERα, whereas the other isoforms are missing certain domains. The 46 kDa form is devoid the AF-1 domain and found at the plasma membrane [25]. Nevertheless, it can dimerize with the 66 kDa form, resulting in competition [26] but it can also activate genomic estrogen effects in otherwise ERα negative cells and is down-regulated in tamoxifen resistant cell lines [27]. The regulation of transcription and splicing of ESR1 gene products is not understood in detail; however, a few factors have been identified. In fact, nearly every exon of the gene can be regulated from its own promoter [28]. The homeobox transcription factor BARX2 has been identified to up-regulate the 46 kDa form in favor of the 66 kDa receptor [29]. The nuclear protein NPE3-3 binds to the estrogen receptor α as well to splicing factors and its