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Mechanisms of endocrine resistance in breast cancer

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The term "therapy resistance", in many ways, is not a uniform term. First, it is important to distinguish therapy failure from prognostication[1]. While, on the one hand, the fact that a tumor recurrence means that some tumor cells must have survived despite the therapy applied, the fact that example lymph node negative tumors have a lower risk of relapse as compared with node positive ones relate to tumor biology, or metastatic propensity, a factor independent of those biological parameters that may cause drug resistance. Second, there are several definitions of drug resistance. Considering pre-surgical therapy or treatment for metastatic disease, therapy failure, in general, would mean lack of tumor shrinkage in response to treatment. As for adjuvant therapy, any tumor relapsing subsequent to treatment should be considered a failure. Finally, therapy failures are grouped into primary failures (or primary drug resistance) and secondary failures (secondary drug resistance), the latter occurring subsequent to an initial response.

While we are starting to learn about predictive factors (parameters associated with either drug resistance or sensitivity), even for the few predictive parameters identified, our understanding of their biological function remains incomplete. On the one hand, it is conventional wisdom that breast cancers completely devoid of the estrogen receptor (ER) do not benefit from endocrine therapy in the adjuvant or the advanced treatment setting. On the other hand, it has been shown that some breast cancers may benefit from endocrine therapy despite expressing ER immunostaining in a few percent of their tumor cells only[2]. However, the likelihood of benefiting from therapy, in the adjuvant or in the advanced setting, increases with a higher percentage of tumor cells staining positively as well as a higher receptor level as determined by conventional binding assays[3-4]. While these facts are generally accepted and provide the background for therapy guidelines, we do not understand the mechanisms beyond either tumor shrinkage, or improved survival in the primary setting, for individual tumors harboring a small fraction of ER positive cells only. In such cases, are those cells devoid of ER expression actually hormone insensitive but destroyed by other factors, which are provided by paracrine excretion from ER positive cells who respond to antihormonal manipulation? The answers to such questions may not directly impinge on therapy,

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but they are of great relevance to our understanding of the endocrine biology and potential development of future therapeutic modalities as well. While common practice in translational research is to correlate biomarkers with outcome, recent studies using second-generation DNA sequencing has further underlined the intra-tumor tissue heterogeneity with respect to genetic disturbances at the cellular level[5].

1 Estrogen receptor expression

Since the first detection of the estrogen receptor, later termed ERα, in breast cancer tissue by Elwood Jensen nearly 50 years ago[6] and the seminal studies by McGuire's group revealing its prognostic and predictive role in breast cancer[7-8], multiple studies have correlated expression of the ER, as well as the progesterone receptor (PgR) with breast cancer outcome. In 1995, the groups of Mosselman[9] and Kuiper et al[10] independent of each other cloned a second estrogen receptor, to be coined ERβ. While the biological role of ERβ in general has been affiliated with other tissue compartments, including bone metabolism[11], the potential role of this receptor, including its splice variants modulating endocrine response in breast and breast cancer tissue, has not been fully elucidated[12].

The ER expression, even at low frequency, is necessary to achieve an endocrine response; however not all ER positive tumors respond to antihormonal manipulation. While mutations affecting the gene coding for ERα are found to be a rare event[13], ER may be subject to downregulation by mechanisms like miRNA[14] and subject to acetylation as well as ubiquitination by different factors[15-16], including BRCA1. Further, ERα (as well as β) is subject to different phosphorylations, and ERα phosphorylations have been related to prognosis in breast cancer patients[17-18]. While the role of ER protein status (phosphorylations, acetylations) to its functional role *in vivo* may provide important information in the future, it should be emphasized that, at this stage, there is no routine test available by which we may discriminate functional from non-functional ER protein *in vivo*.

2 Gene expression signatures

The findings that breast cancers may be divided into distinct classes based on gene expression signatures[19] and these classes had prognostic outcome[20] revealed a novel conceptual understanding of breast cancer biology. In this classification, ER positive breast cancers in general belong to one out of two classes, the so-called luminal A or the luminal B class, each characterized by a distinct gene expression profile (Figure 1). While tumors of both classes express ER, the tumors of the luminal A class in general express ER at higher levels as compared to the luminal B tumors[21].

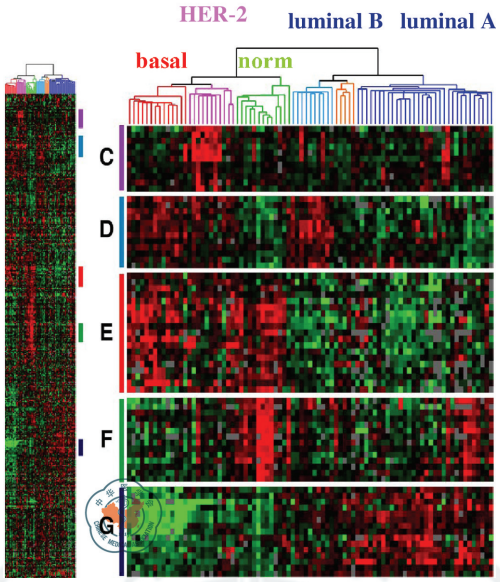


Figure to the left: full cluster diagram. The C-G letters refer to the HER-2 amplicon cluster (C), cluster of unknown significance (D), basal epithelial cell-enriched cluster (E), normal breast-like cluster (F) and luminal epithelial gene cluster containing the estrogen receptor-associated genes (G).

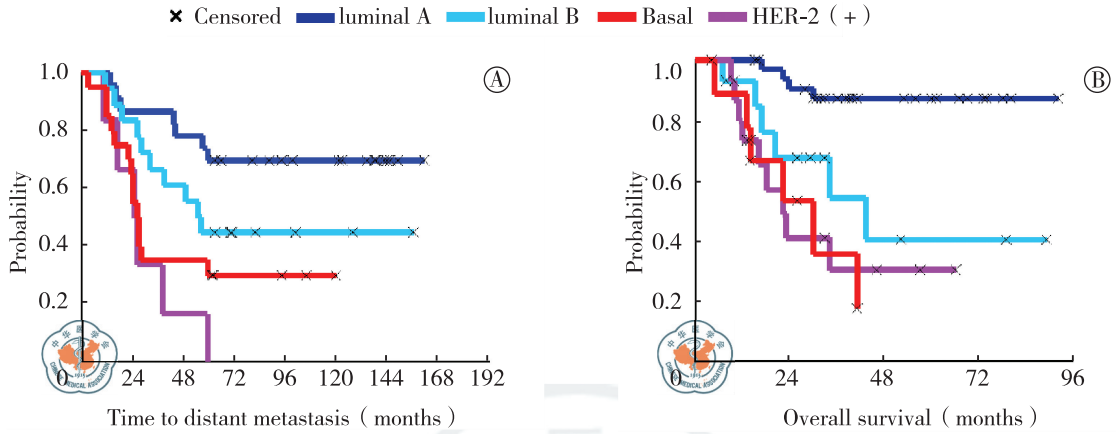
Figure 1 Gene expression patterns of 85 experimental samples from 78 breast cancers, three benign tumors and four benign tissue samples[20].

In addition to the classification by Perou and colleagues, several gene expression profiles have been developed. These profiles, in contrast to the hierarchical signature by Perou et al, in general have been developed by supervised analysis. Thus, the Oncotype DX 21-gene signature[22] as well as the 2-gene signature by Ma et al[23] both revealed prognosis among tamoxifen-treated patients. However, as untreated patients were not included as controls, it is impossible to address whether this effect relates to tumor biology per se (meaning the signature acts as a "pure" prognostic factor) or, to some extent, predicted response to therapy, meaning that its association with outcome could be influenced by therapy.

Notably, while tumors belonging to the luminal B class as well as tumors expressing a high Oncotype DX score in general seem to benefit more from adjuvant chemotherapy as compared to tumors of the luminal A class and those having a low Oncotype DX score, provided that endocrine therapy is applied in concert, long-term prognosis in general is more favorable to the luminal A tumors and those tumors having a low Oncotype DX score[24-25].

Taken together, these findings indicate a prognostic role of gene expression signatures in ER positive breast cancers. The difference in long-term outcome between luminal A and luminal B tumors in individuals receiving adjuvant tamoxifen as compared to individuals not receiving adjuvant endocrine treatment (Figure 2) is consistent with a predictive role of these classes to endocrine sensitivity[26]. However, the fact that luminal A class tumors in general reveal a higher ER concentration as compared to tumors of the luminal B class suggests that

this effect could be due to the difference in ER level only.



A: van't Veer data set; No adjuvant endocrine therapy (P<0.01, compared with each other); B: Norway/Stanford data set; adjuvant TAM (P<0.01, compared with each other); Notice that part A depicts relapse-free survival while B relates to overall survival. The figures depict a larger difference in outcome in-between tumors of the luminal A and luminal B class provided that adjuvant endocrine therapy is applied.

Figure 2 Prognostic impact of the different tumor classes among patients not receiving any systemic adjuvant treatment (A) and patients with stage Ⅲ breast cancers treated in our clinic with neoadjuvant chemotherapy and tamoxifen in cases of ER(+) tumors(B)[20].

3 Proliferation rate

While the gene expression profile of the Oncotype DX includes genes associated with proliferation rate, a more direct and easy assessment ofthe cell cycle is the immunostaining of the proliferation marker Ki67. Thus, a high Ki67 staining index indicates a high proliferation rate. Among ER(+) breast cancers, a high Ki67 staining, similar to low ER expression levels, in general is seen among tumors of the luminal B class[27]. Interestingly, Dowsett and colleagues have observed a low Ki67 expression, in particular when measured in tumor samples collected 2 weeks after commencing endocrine therapy[28-29], to be associated with improved long-term outcome during treatment with novel third-generation aromatase inhibitors as well as treatment with tamoxifen (Table 1). In the IMPACT study[30], neoadjuvant treatment with anastrozole caused more profound suppression of Ki67 compared with tamoxifen as well as the combination (anastrozole + tamoxifen) treatment arm. While this challenging observation indicates Ki67 to be a most potent surrogate marker assessing endocrine treatment efficacy, further confirmatory studies are warranted.

While a drop in Ki67 expression indicates slowing of the cell cycle and, thus, a reduced growth rate, endocrine treatment is associated with tumor shrinkage. While the potential contribution of permanent growth arrest, or senescence, to the anti-tumor efficacy of chemotherapy in animal models remains a conflicting issue[31-32], little is known regarding its potential contribution to anti-tumor effects *in vivo*.

Table 1 Key studies reporting different parameters predicting benefits of endocrine adjuvant/neoadjuvant therapy in ER(+) breast cancers

Regimen	Setting	Predictive factor	Result	Reference
tamoxifen	adjuvant	ER level by IHC	correlation ER(+) level / effect therapy	4
tamoxifen	adjuvant	ER level by IHC	correlation ER(+) level / effect therapy	61
anastrozole	adjuvant	ER level by IHC	correlation ER(+) level / effect therapy	61
tamoxifen	adjuvant	HER-2(+)/(−) by IHC	HER-2 overexpression reduced benefit of therapy	61
anastrozole	adjuvant	HER-2(+)/(−) by IHC	HER-2 overexpression reduced benefit of therapy	61
tamoxifen	adjuvant	HER-2(+)/(−) by IHC	HER-2 overexpression reduced benefit of therapy	62
letrozole	adjuvant	HER-2(+)/(−) by IHC	HER-2 overexpression reduced benefit of therapy	62
tamoxifen	neo-adjuvant	Ki67drop at 2 weeks	Ki67 reduction predicted outcome	28
letrozole	neo-adjuvant	Ki67drop at 2 weeks	Ki67 reduction predicted outcome	28

4 Adaption to estrogen deprivation?

Potential explanations why treatment with aromatase inhibitors fails in some patients include lack of effective intratumor estrogen suppression, a mutant-derived aromatase protein, poor drug uptake, *etc*. Noteworthy, studies assessing intratumor estrogen levels before and during the treatment with third-generation aromatase inhibitors have shown consistent suppression of intratumor estradiol[33-34], lending no support to such a hypothesis. *In vitro*, several groups[35-37] have shown MCF-7 cells gradually exposed to estradiol at low concentration over months (so-called Long Term Estrogen Deprivation, LTED) to develop a state of "estrogen hypersensitivity" get maximum growth stimulation by estradiol at a concentration of 1/1000 to 1/10 000, the concentration required or optimal growth stimulation of the wild-type cells. Notably, while LTED is an *in vitro* phenomenon, the hypothesis that tumor cells may "adapt" to an external environment with respect to estradiol concentration, may explain certain clinical observations. Strikingly, pre- and postmenopausal patients harboring ER(+) tumors both respond to estrogen suppression (ovarian ablation in premenopausal women, aromatase inhibition among the postmenopausals) despite the fact that plasma as well as tissue estradiol concentrations are 10-fold higher in premenopausal women as compared to postmenopausal women[38-39]. Furthermore, premenopausal patients developing acquired resistance toward ovarian suppression may subsequently respond to aromatase inhibition, and anecdotal evidence indicates response to aromatase inhibition among patients with very low plasma estrogen levels due to previous adrenalectomy[40].

The growth stimulation curve for MCF-7 cells, whether wild-type or exposed to LTED, is "bell-shaped", meaning that estradiol at concentrations above which is needed for optimal stimulation actually suppresses cellular growth. During LTED, this "bell-shaped" curve moves to the left; thus, estradiol at a concentration stimulating the growth of wild-type MCF-7 cells now becomes growth inhibitory.

Actually, estradiol induces apoptosis in cells becoming resistant to estrogen deprivation[41]. High doses of estrogens were used as additive treatment for breast cancer prior to introduction of contemporary treatment[42]. Hypothesizing that estrogen deprivation may sensitize breast cancer cells *in vivo* toward estrogen treatment, we treated patients with advanced breast cancer developing acquired resistance to aromatase inhibitors with diethylstilbestrol 15 mg daily[43]. In this study, 10 out of 32 patients achieved an objective response; in addition, two patients obtained stable disease beyond six-month duration. Similar results were obtained in a confirmatory study by Ellis et al[44]. Interestingly, in this study, they obtained a similar response rate at a medium dose of estradiol (6 mg daily) as to high-dose (30 mg daily) treatment.

Several explanations to the phenomenon of LTED have been proposed, including activation of the ERK MAP kinase and the PI3kinase pathways, non-genomic effects of estradiol, or activation of growth factor receptors or proto-oncogenes as IGF-IR, EGFR or HER-2; the readers are referred to several excellent reviews on this topic[45-47]. Based on the "proof of principle", the clinical observations that estrogen therapy works subsequent to estrogen deprivation, the mechanism of "estrogen adaption"[48] is likely to be a mechanism of relevance to endocrine resistance *in vivo*.

5 Activation of growth signal pathways

Following the seminal papers by Slamon and colleagues revealing overexpression of the HER-2 proto-oncogene as a prognostic factor in breast cancer[49-50], much work has concentrated on the biological role of HER-2 in breast cancer biology as well as therapy resistance. Notably, while only about 10% of ER(+) breast cancers reveal HER-2 overexpression[51], among the subgroup (15%–20%) of breast cancers revealing HER-2 amplification and / or overexpression, about half are ER positive[52-53]. Thus, endocrine treatment remains an option for about 50% of all patients with HER-2 amplified breast cancers. Similar to tumors of the luminal B class, tumors in the so-called "HER-2" class, when ER is positive, generally express ER at a lower concentration as compared to tumors of the luminal A class[54].

Studies conducted more than a decade ago revealed HER-2 overexpression to reduce the response to endocrine therapy with tamoxifen as well as to other endocrine options, including droloxifen, the second-generation aromatase inhibitor fadrozole and megestrol acetate in metastatic breast cancer[55-58]. Interestingly, data from two neoadjuvant studies indicated the benefit of letrozole[59] as well as anastrozole[60] over tamoxifen to be particularly high in HER-2 overexpressed tumors. However, more recent data from the TransAtac study[61] and the BIG 1-98 study[62] both reveal that HER-2 overexpression can reduce long-term relapse-free

survival among patients treated with an aromatase inhibitor as well as those treated with tamoxifen. Moreover, both studies indicate that the aromatase inhibitor may reduce the hazard ratio (HR) for a relapse compared with tamoxifen to about the same extent among HER-2 positive and HER-2 negative tumors.

Two randomized studies addressed the impact of adding either trastuzumab to anastrozole[63] or lapatinib to letrozole[64] in patients with ER-positive, HER-2-positive metastatic breast cancer. The studies both revealed a significant benefit from combining an anti-HER-2 agent with an aromatase inhibitor. This hypothesis gained further support from two small clinical studies[65-66]. Interestingly, in the study by Johnston and colleagues, they also enrolled patients with ER-positive but HER-2 negative tumors. While no benefit from adding lapatinib was observed for the whole group with HER-2 negative tumors, a pre-defined sub-analysis revealed a non-significant benefit from having lapatinib in the group of patients who experienced a relapse less than 6 months after terminating adjuvant tamoxifen. The hypothesis behind enrolling such patients in the study were that HER-2 upregulation may also confer resistance to endocrine therapy in a subgroup of ER-positive tumors not overexpressing HER-2. Furthermore, trastuzumab has been shown to counteract letrozole resistance in cells and xenografts not overexpressing HER-2[67]. Finally, for the patients treated with aromatase inhibitors in the neoadjuvant setting, we found treatment with aromatase inhibitors to up-regulate HER-2 mRNA expression levels in tumors not amplified for HER-2[68].

Several questions remain to be settled. Notably, we do not know whether adding either trastuzumab or lapatinib improves outcome by reversing endocrine resistance or the two treatment options, anti-HER-2 treatment and endocrine manipulation, are acting independent of each other or not. Clearly, further studies are warranted, including adding more extensive anti-HER-2 treatment to endocrine manipulation. Notably, treatment with trastuzumab and lapatinib in concert was revealed superior to lapatinib monotherapy among patients progressing on trastuzumab[69], and the combination of trastuzumab and pertuzumab, with or without concomitant chemotherapy, looks promising[70-71]. Finally, the potential role of other members of the HER-family, like HER-3 and HER-4, remains to be addressed.

Another potential mechanism of aberrant growth stimulation that, potentially, may cause resistance to endocrine treatment, is through deregulation of the phosphatidylinositol 3-kinase (PI3K)-Akt/mTOR pathway[72-73]. mTOR interacts with the estrogen receptor by phosphorylation[74]. Everolimus is an mTOR inhibitor[75] presenting synergistic effects with aromatase inhibitors on proliferation and also apoptosis in experimental systems[76]. Thus, in a randomized phase Ⅱ trial adding everolimus improved efficacy of letrozole in the neoadjuvant setting[77], and in a large phase Ⅲ study, everolimus added to exemestane improved median

progression-free survival for patients on exemestane treatment from 4.1 to 10.6 months, respectively[78]. Ongoing studies are addressing the benefit of adding everolimus and related compounds to different types of endocrine therapy.

6 Clinical observations; lack of cross resistance to endocrine regimens

Lack of cross-resistance to different endocrine treatment optionshas been known for decades. Patients who acquired resistance to tamoxifen subsequently responded to progestins administered as high-dose regimens or the first-generation aromatase inhibitor aminoglutethimide[79], and the selected patients were known to respond to several different endocrine regimens administered in sequence[80]. While patients over time develop resistance to all forms of endocrine manipulation, strikingly, in some cases there even is a lack of cross-resistance between non-steroidal and steroidal aromatase inhibitors[81]. While steroidal and non-steroidal aromatase inhibitors bind differently, with steroidal aromatase inhibitors causing irreversible protein binding[82], data from *in vivo* studies do not suggest any major difference with respect to total body estrogen synthesis or plasma estrogen suppression[83-85]. Interestingly, exemestane reveals a slight androgen-agonistic effect *in vivo*[86]; whether this may contribute to its anti-tumor effect remains unknown.

7 Conclusion

Despite decades of intensive experimental research, our knowledge regarding the mechanisms of endocrine resistance in ER-positive breast cancers remains poor. However, certain findings, like effect of estrogen therapy subsequent to aromatase inhibition, and the findings that anti-HER-2 therapy and everolimus improve outcome in concert with endocrine therapy, reveal future directions. As for the next generation of studies, we need to not only collect observations, but also perform translational research aiming at understanding the mechanisms regulating sensitivity to therapy. Importantly, novel laboratory techniques, like second-generation sequencing allowing for full genome or exome sequencing, holds promises for the future[87]; indeed, the first paper reporting full genome sequencing[88] in tumors revealing differential responses to aromatase inhibition has recently been published.

A major challenge is the design of optimal translational studies taking the full advantage of these new techniques. To explore the mechanisms of resistance, we need to characterize individual tumors with respect to genetic as well as epigenetic disturbances and to correlate these molecular findings with the clinical outcome. Of similar importance, perhaps, we should explore tumor tissue alterations in response to therapy (Figure 3). While the findings that endocrine therapy suppresses Ki67 expression in responding tumors are of significant interest, we need to learn what

alterations may occur in other parameters during therapy as well.

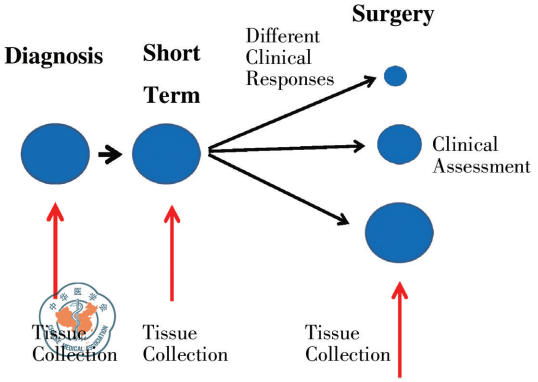


Figure 3 Schematic presentation for translational trials exploring primary biomarkers as well as short-time (days, 1–2 weeks) and long-term (more than 3 months) alterations with correlation to clinical response in the primary medical (neoadjuvant) setting.

【**Key words**】 endocrine therapy; therapy resistance; breast neoplasms

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