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Role of molecular markers in breast cancer therapy

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Translational research, in general, means to take knowledge from one area into a second. Traditional thought to represent transfer of knowledge from basic research into the clinic, translational research today covers a much broader term. Thus, most investigators will agree translational research covers a two-way process that also includes taking lessons from the clinic back to the laboratory, by which biological observations *in vivo* would create novel hypotheses to be further explored in laboratory experiments.

Such translational research may have different goals. In this paper, I like to focus on four potential aims in breast cancer, (1) to explore tumor biology in general, (2) to improve early diagnosis, (3) to enhance cancer prognostication and, most importantly, (4) to improve prediction of response to therapy, the main research topic of our own team.

1 Exploring tumor biology

Breast cancer is generally classified into the so-called luminal A and B, basal-like, HER-2 and normal-like classes[1]. More recently, a sixth class, the "lobular cell class", accounting for about 50% of all lobular cancers[2], was identified (the residual 50% of lobular carcinomas spread through the other molecular classes). While the existence of the "normal cell-like" class as a distinct group has been challenged[3], there is evidence at least a subgroup of these tumors do express a distinct gene profile characterized by low expression of several Claudins[4].

The distinction between estrogen receptor (ER) positive and negative tumors has been known for decades, also the fact that responsiveness to

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endocrine therapy depends not only on expression of the ER but, actually, on its expression value[5] and whether the progesterone receptor (PgR), transcriptional induced by estrogen stimulation[6], is co-expressed[7]. Luminal A and B class tumors, which both express ER, were found to have different gene expression profiling, and that the luminal B class, in general, harbored lower expression of ER compared to luminal A. Regarding patient prognostication, luminal A class patients did far better than the luminal B ones, but this was in particular related to patients receiving tamoxifen with a smaller prognostic impact when compared to those not exposed to adjuvant endocrine therapy[8]. The discrimination between the two groups seems to provide prognostic, but probably also predictive, information with respect to tamoxifen efficacy.

It has been known for more than two decades that 15%—25% of breast cancers have amplifications and over-express the HER-2 proto-oncogene[9]. The study by Perou *et al*[1] revealed that this was associated with over-expression of a number of genes, many of which are located on the same amplicon.

Importantly, while the luminal A and B classes in general contain ER(+) tumors, and most tumors of the HER-2 class reveal HER-2 amplification, this is not a uniform picture. Classification is based on a number of genes; thus, ER- luminal A class tumors exist, and not all tumors belonging to the HER-2 class overexpressing HER-2[3]. Notably, while most ER(+) tumors do not overexpress HER-2, using a cut-off value of 1% for ER positivity, investigators of the large BIG1-98 study reported about 7% of their ER(+) tumors to be amplified for HER-2[10]. On the contrary, about 50% of all HER-2 amplified tumors are ER(+)[11]; however, they on average express ER at lower levels as compared to HER-2 negative tumors[12]. Different subclasses are associated with a different spectrum of gene mutations.

Probably, the greatest novelty of the "Perou / Sørlie" classification[1,13] was the discovery of the so-called "basal-class" or "triple-negative" tumor type, lacking ER and PgR as well as HER-2. The two terms should not be

used synonymously; while most basal cell-like breast cancers are triple-negative, pending on additional biomarkers, including p63, EGF-R, HER-3, HER-4 and ER-beta[14], between 60% and 80% of triple negative breast cancers may be classified as basal-like. From a therapeutic prospective, it is important to recognize that about 80% of all breast cancers arising in *BRCA*1 mutation carriers belong to the basal class[13], and 10% of all basal-like tumors are diagnosed in *BRCA*1 mutation carriers[15].

A number of different groups have reported different prognostic profiles like the "stem-cell signature" by Glinsky et al[16], as well as the general prognostic signatures derived through supervised analysis by the Amsterdam and Rotterdam groups[17-18]. Most interestingly, certain genes seem to be associated with metastatic propensity toward particular organs like bones and lungs[19]. This fits well with conventional wisdom that ER(+) and ER- tumors have different metastatic locations.

An important issue remains: to what degree is mRNA over-expression related to DNA amplifications? Pollack *et al*[20] found that more than 60% of amplified genes over-express mRNA. On the other hand, only 12% of mRNA variations could be explained by an increased DNA copy number, suggesting other mechanisms to play a more important role.

2 Early diagnosis

There is much ongoing work evaluating use of DNA as well as protein markers for early cancer detection. This, in particular, concentrates on blood markers[21]. In addition to problems related to method sensitivity and specificity, few cancers have a "single marker", and parameters like epithelial DNA in plasma, although a potential sensitive marker, may lack specificity for tumor organ location. Thus, detection of a positive but non-specific screening marker in an asymptomatic patient pose medical as well as ethical dilemma; to look for a potential early cancer through multiple organ systems may be as looking for a needle in a haystack. Obviously, such testing is ethically justified only in case the likelihood of identifying the primary tumor is high.

3 Improving prognostication

While traditional prognostic factors like lymph-node (LN) status have been used to select patients for therapy, the huge number of prognostic factors identified over the years has added moderately to our understanding of the biology of breast cancer. Notably, while there have been expectations to novel prognostic factors as bone marrow micrometastases, disappointingly, a recent meta-analysis revealed this parameter to lack significant prognostic information among LN(−) breast cancers[22]. The key issue however regarding use of prognostic factors selecting patients for therapy is the need for knowledge about their potential predictive value. The fact that LN(+) as well as LN(−) patients respond well to endocrine therapy as well as chemotherapy does not preclude the fact that a novel prognostic factor may be predictive for response to therapy. Thus, mutations in the TP53 gene is a general marker for a poor prognosis but also predict a poor response to anthracycline therapy[23]. As such, while novel gene expression signatures have been defined that they could potentially be applied to prognostication, there is a mandatory need to learn their potential predictive value, to avoid selecting high-risk patients for therapeutic strategies to which they may be resistant. Thus, to be suitable for therapy selection, no prognostic marker should be considered independent of its potential predictive value.

Much interest has been directed toward developing prognostic gene signatures. Thus, the "Perou/Sørlie" classification has revealed prognostic information in patient groups treated with anthracycline-based chemotherapy as well as patients not exposed to systemic adjuvant therapy[8,13]. Furthermore, the poor prognosis of basal-like tumors has been confirmed in several large patient series identifying these tumors through immunostaining (IHC) of protein markers[24]. Interestingly, separating triple-negative tumors into those harboring a basal-like profile versus those that do not express a basal-like one (i. e., normal-like tumors) revealed an inferior prognosis for those of the basal cell-like class[14].

The 21-gene OncotypeDX and 2-gene Theros signature were both derived as prognostic indexes in patients treated with tamoxifen. The

OncotypeDX signature has been further validated[25] and claimed superior to conventional individual parameters. The 70-gene Mammoprint signature revealed prognostic information among patients treated with adjuvant chemotherapy as well as untreated patients[17, 26]. Also, it has been shown that "high-risk" patients as identified through the different signatures, including the Rotterdam 76-gene profile[18] and PAM50[3] to a large extent overlap, despite the fact that the individual genes composing each signature overlap to a limited extent[27].

4 Improving prediction of response to therapy

4.1 Endocrine treatment

Expression of ER is a requirement for sensitivity to hormonal manipulation. However, the mechanism of endocrine resistance is far from completely understood. Thus, we do not know why many tumors do not respond to endocrine therapy despite expressing high ER levels. While different mechanisms, including receptor mutations, pharmacokinetic alterations and growth factor expression has been proposed[28-29], so far none of these mechanisms has explained endocrine resistance in ER(+) tumors. On the other hand, beneficial effects of tamoxifen have been recorded among tumor expressing ER positivity among 1% of cells only[30]. If this involves paracrine loops, identification of these mechanisms may provide new targets for therapeutic manipulation.

An interesting phenomenon relates to Long Term Estrogen Deprivation (LTED). Briefly, estrogen-stimulated MCF-7 cells grown for long-term periods (months) in culture exposed to estrogens at decreasing concentrations develop estrogen "hypersensitivity", meaning that they may be growth stimulated by estradiol at a concentration of 1/1000 to 1/10 000, the concentration needed to stimulate wtMCF-7 cells. The growth stimulation curves (with respect to wt as well as to LTED cells) express a bell-shaped profile, meaning that estradiol at high concentrations inhibits cell growth; actually, others[31] have shown estradiol to cause apoptosis in sensitized cells. The fact that patients developing resistance towards

aromatase inhibitors may subsequently respond to estrogen therapy[32] suggest that LTED may be a mechanism of resistance to estrogen deprivation *in vivo*.

The potential contribution of HER-2 overexpression to endocrine resistance is incompletely understood. Zhu *et al*[33] found HER-2 amplification to predict response to antiaromatase therapy in the neoadjuvant setting. Similarly, Ellis *et al*[34] reported a selective benefit for letrozole compared to tamoxifen among tumors overexpressing either HER-1 or HER-2. A particular benefit for aromatase inhibition among HER-2 overexpressing tumors was further supported by the results from the IMPACT study, although the numbers here were too small to reach statistical significance[35]. While adjuvant studies have revealed a better outcome among HER-2 negative as compared to HER-2 positive tumors when treated with tamoxifen as well as an aromatase inhibitor[10], this discrepancy between results observed in the adjuvant and neoadjuvant setting could reflect an effect on primary tumor cells not related to micrometastases.

Recently, we observed estrogen deprivation with aromatase inhibitors to be associated with HER-2 upregulation. Thus, Johnston *et al*[36] reported a benefit from adding lapatinib in concert with letrozole among HER-2 non-amplified tumors with an early relapse on adjuvant tamoxifen. The interesting observations warrants further studies exploring the potential role of HER-2 conferring endocrine resistance in HER-2 non-amplified tumors.

4.2 Anti-HER-2 therapies

Causing a modest anti-tumor effect when applied as monotherapy and when administered concurrently with chemotherapy in advanced breast cancer (ABC), trastuzumab has been shown to dramatically reduce relapse rate in the adjuvant setting[37]. Lapatinib, a tyrosine kinase inhibitor attacking HER-1 as well as HER-2 revealed significant anti-tumor effects in metastatic disease [36, 38], while gefitinib, a HER-1 inhibitor, was found ineffective among unselected patients[39].

While the mechanism(s) of acquired resistance toward anti-HER-2

therapies *in vivo* remains poorly understood, certain clinical observations of interest have been recorded. Patients failing on trastuzumab in ABC may subsequently benefit from treatment with either lapatinib[38] or neratinib[40], the second compound inhibiting the HER-4 tyrosine kinase in addition. Pertuzumab is a second HER-2 antibody blocking the HER-2 heterodimerization domain. Interestingly, preliminary findings suggest a benefit from administering trastuzumab and pertuzumab to patients resistant to either drug applied as monotherapy[41]. Also, lapatinib plus trastuzumab was found superior to lapatinib monotherapy in patients with ABC progressing on trastuzumab[42].

HER-2 activation leads to activation of different downstream signaling pathways including the MAP kinase system as well as PI3K-Akt[43]. There is evidence suggesting activating PI3K mutations may predict resistance toward trastuzumab but not lapatinib[44-45]. PI3K mutations are detected in 16% — 40% of breast cancers[46-47]. While some breast cancers also harbor Akt mutations[48] or reveal lack of PTEN staining[49], so far these alterations have not been linked to trastuzumab resistance. In contrast, overexpression of AXL has been associated with lapatinib resistance in experimental systems[50].

4.3 Chemotherapy

While the molecular mechanisms causing resistance to chemotherapy is poorly understood, a number of empirical observations have linked tumor "phenotype" to adjuvant chemotherapy efficacy. Considering contemporary adjuvant chemotherapy, it is impossible to directly compare the benefit of adding say a taxane to anthracycline-containing chemotherapy among ER (+) versus ER(−) patients because ER(+) patients will also receive endocrine therapy generally; thus, comparisons basically evaluate the benefit of adding endocrine treatment to chemotherapy for patients harboring ER(+) tumors. While these studies reveal an additional benefit for chemotherapy also among patients harboring ER(+) tumors[51], the benefit of chemotherapy is smaller as compared to patients harboring ER(−) tumors. A possible explanation could be that ER negativity

correlates to low tumor grade and high growth rate which have been associated with response to chemotherapy[52-53]. High expression of Ki67 seems to identify a subgroup of patients that may benefit from adding a taxane to anthracycline-containing adjuvant chemotherapy[54].

While amplification of topoisomerase-II (Topo-II) has been suggested to predict sensitivity to treatment with anthracyclines, recent evidence has shown the effect to be better correlated to amplification of the centromere of chromosome 17 on which Topo-II as well as other genes critical to breast cancer growth as HER-2, *TP*53 and *BRCA*-1 are located[55].

Gene signatures have revealed prognostic information in breast cancer, but their application in selecting patients for chemotherapy has been limited. Predictive signatures derived by supervised analysis in general have revealed statistical correlations to efficacy of anthracycline- as well as taxane-containing regimens and their combinations[56-57]. However, with a few exceptions most of them have not been confirmed in independent studies. Interestingly, Hanneman *et al*[58] revealed alterations in gene expression during treatment to predict response to anthracycline-containing primary chemotherapy. While Potti *et al*[59] took an interesting approach developing drug sensitivity signatures across a panel of cell lines, the practical implications of their finding predicting drug resistance *in vivo* remains to be confirmed.

Particular interest relates to the Mammoprint and OncotypeDx signatures, as they are increasingly taken into clinical use. The main idea beyond the Mammoprint test is to identify patients at low risk of relapse not in need of chemotherapy. On the contrary, even low risk patients may be suitable candidate for chemotherapy provided the benefit is high; ideally, selection of patients for cytotoxic therapy should be based not on a risk assessment but on the potential benefit derived. Regarding the OncotypeDx signature, Paik *et al*[60] reported benefit for CMF-based chemotherapy among ER(+) patients on tamoxifen with a high risk score for relapse, while patients with a low-risk score experienced no benefit. A similar finding has recently been reported with respect to anthracycline-containing

chemotherapy[61]. Regarding Mammoprint, Straver *et al*[62] found pathologic complete response (pCR) to primary anthracycline-containing chemotherapy to be correlated to a "poor" prognostic signature.

Analyzing tumors of the original dataset from which the "Perou / Sørlie" hierarchical classification was developed[13], we found tumors belonging to the luminal B class in particular to reveal resistance against weekly doxorubicin treatment[63]. Applying gene expression analysis or IHC, other groups have shown tumors belonging to the basal cell-like class or triple negativity in general to predict a pCR[64]. However, despite being more likely to achieve a pCR a general poor prognosis for triple-negative tumors as compared to other breast cancers was observed. Hugh *et al*[65] reported TAC to improve relapse-free and overall survival compared to FAC among patients harboring tumors in luminal B class, HER-2 class, and triple negative tumors in general (covering the basal cell-like and normal cell-like subgroups), but not for tumors in luminal A class. Although the luminal B tumors in general are ER(+), they express receptors at a lower level, reveal a higher Ki67 index, and respond more poorly to tamoxifen as compared to luminal A class tumors[8], suggesting many of these tumors actually to be endocrine insensitive. The data accordingly fits to the general observation that ER- tumors may benefit more from dose-dense treatment as well as the addition of taxanes to anthracycline-containing regimens as compared to ER(+) tumors[51].

Triple negative and basal cell-like tumors remain with a poor prognosis, despite initial responsiveness to therapy[64]. Similarly, breast cancers arising in *BRCA*1 mutation carriers seem to respond adequately to anthracycline-containing chemotherapy[66], but so far we lack evidence of a significantly improved long-term outcome. Thus, many efforts are spent on improving treatment for this group of tumors and, in particular, the subgroup of tumors harboring *BRCA*1 mutations.

*BRCA*1 mutations cause a defect in DNA double-strand repair, and experimental evidence suggest these tumors may be particularly sensitive to drugs like platinum compounds generating inter-strand cross-links[67].

While evidence so far is limited, the results from two small trials suggests a high rate of pCR among *BRCA*1 mutation carriers[68] as well as in triple negative tumors[69] to cis-platinum treatment. Tumors arising in *BRCA*1 and *BRCA*2 mutation carriers express a different biology, with *BRCA*2 mutated tumors in general being ER(+)[70]. Despite this, tumors harboring *BRCA*2 mutations seems to carry a similar defect with respect to DNA double break repair as *BRCA*1 mutated ones. While we lack data confirming efficacy of cis-platinum in *BRCA*2 mutated tumors, experimental evidence suggests a beneficial effect. Interestingly, secondary deletions removing the gene fragment carrying a *BRCA*2 mutation have been shown to restore wt *BRCA*2 function and confer resistance to platinum compounds[71]. However, more data are needed evaluating efficacy of platinum compounds in this group of patients before making therapy recommendations.

A second most interesting option relates to targeted therapy with respect to tumors in *BRCA*1 but also *BRCA*2 mutation carriers as well as to basal cell-like tumors in general. The fact that *BRCA*2 mutated tumors, similar to those carrying a *BRCA*1 mutation, are defective in double-strand DNA repair, means both tumor types may depend on single strand repair to survive DNA damage. Poly (adenosine diphosphate ribose) polymerase (PARPs) plays a critical role with respect to single-strand repair. Now, studies applying the PARP inhibitor olaparib as monotherapy have revealed anti-tumor effects in breast cancer occurring in *BRCA*1/2 mutation carriers[72]. Furthermore, the PARP inhibitor BSI-201 has been shown to enhance antitumor effects of gemcitabine and carboplatin chemotherapy in ABC[73]. Notably, in that study, inclusion criteria were not based on *BRCA*1/2 testing; the inclusion criterion was triple negativity as evaluated by IHC. This raises the question whether many triple negative / basal cell-like tumors actually harbor defects in other genes apart from *BRCA*1/2 involved in double-strand repair. If so, identification of such defects as predictive markers could significantly improve therapy for this group of patients.

5 Way forward

While the whole human genome contains approximately 30 000 genes,

notably, each individual gene may activated multiple down-stream genes. Considering the merging evidence of alternative transcripts[74], the number of mRNA transcripts in a cell is probably in the range of ten-fold the number of coding genes. Considering alternative splices, for some genes, as example p73, splices have been identified which express antagonistic activities toward the main protein translation. Thus, to detect gene expression profiling at the mRNA level for a gene expressing several transcripts, multiple probes will be required for each gene. Adding the fact that proteins undergo post-translational modifications with acetylations, phosphorylations and ubiquinations, there exist several millions of alternative protein transcripts within an individual cell.

Now, with high through-put sequencing becoming available, this reveals the potential of sequencing the full genome in individual tumors. Improvement with respect to proteomics as well as development of general protein arrays allows protein expression exploration at a large scale. However, such techniques heavily depends on informatics; in addition, to fully define the role of different functional pathways, we probably need a large amount of tumor samples for which gene alterations may be correlated to clinical outcome.

The alternative is to search for genetic alterations based on a functional hypothesis. Most genetic alterations discovered in a tumor may actually be co-called "passengers" and not drivers, meaning they have a limited impact on tumor cell-behavior. As such, these alterations will just disturb the picture when we try to elucidate the mechanism of drug resistance. We now have much information considering key biological events like regulation of the normal cell cycle and, at least to a certain extent, growth-arrest and apoptosis, although the integrated regulation of these processes in response to cellular damage is not yet fully understood. Understanding the biology behind chemoresistance, important clues are provided by identification of germ-line genetic alterations associated with particular cancer risk syndromes. Genes involved in apoptosis and growth-arrest like p53 as well as genes involved in DNA repair to be associated with distinct inherited

cancer syndromes have all been found to be associated with lack of responsiveness to certain types of chemotherapy in different cancer forms. Taking into account the fact that much of the DNA damage created by carcinogenic agents resembles the damage caused by chemotherapy, it is reasonable to postulate that gene defects associated with an impaired ability to handle DNA damage in response to carcinogenic agents may also interact with responsiveness to certain types of chemotherapy. Based on our own experience[36], we found *TP*53 mutations to predict lack of responsiveness to anthracycline and mitomycin in breast cancer patients (Table 1a[75] and b[76]). However, *TP*53 mutations were not fully predictive for drug resistance; some tumors expressed therapy resistance despite harboring wild-type *TP*53, while other tumors respond despite harboring *TP*53 mutations. It is suggested that other genes involved in the p53 cascade or redundant pathways could be involved as well. The scenario is depicted and explained in Fig 1. Most importantly, this figure illustrates how tumors harboring mutations located in different genes in the same functional pathway may reveal a different gene expression profile[77]. Thus, our hypothesis is that genes associated with germ-line cancer syndromes could provide "beacons" for identification of functional pathways associated with chemotherapy resistance.

Table 1a Relationship TP53 mutations and chemoresistance doxorubicin monotherapy[75]

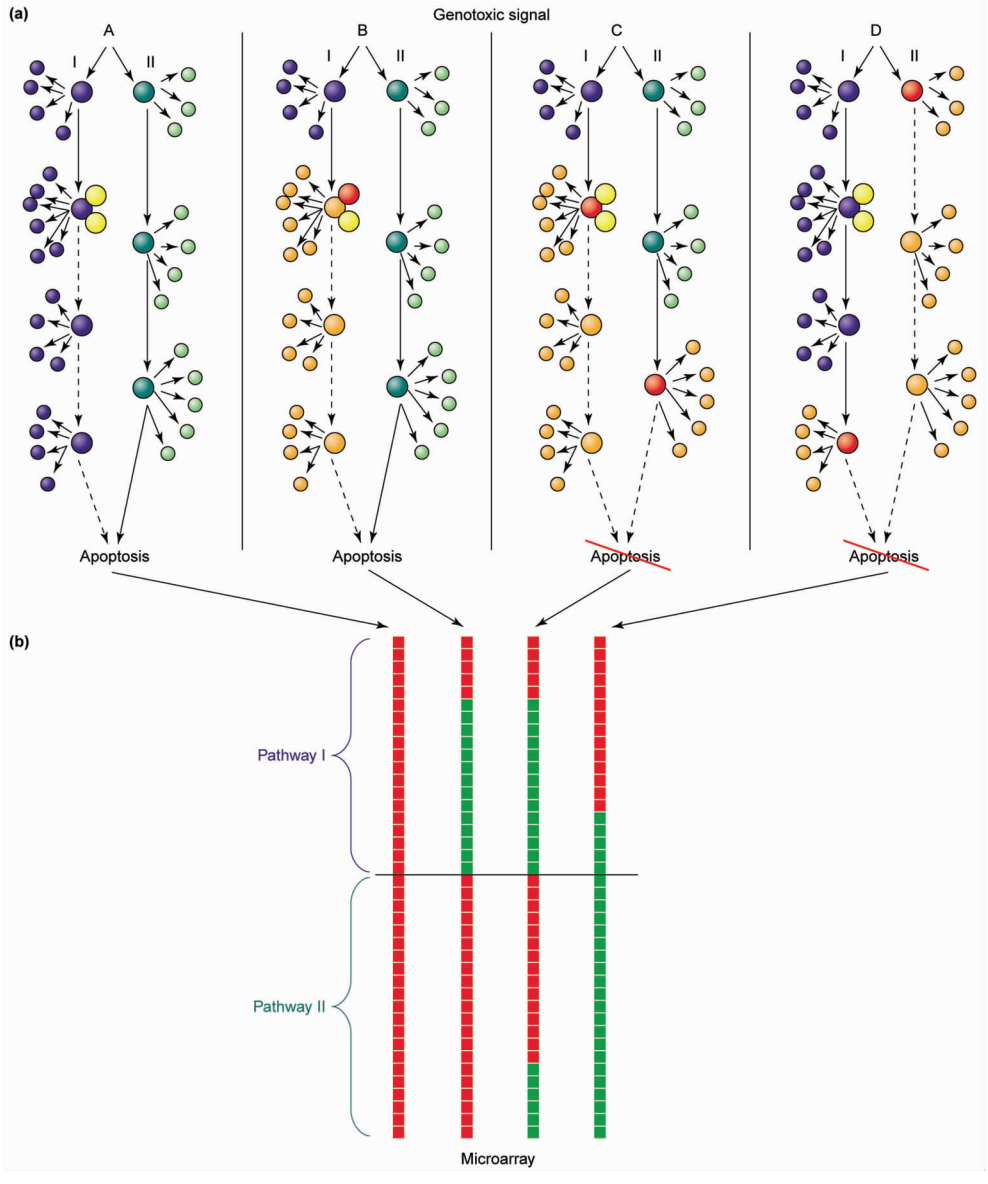
	PR/SD	PD
TP53 WT	67	4
TP53 mutated L2/L3	14	5

$P=0.008$ comparing mutation status among patients with progressive disease (PD) to those having a partial response (PR) or stable disease (SD).

Table 1b Relationship TP53 mutations and chemoresistance mitomycin/5FU[76]

	PR/SD	PD
TP53 WT	22	3
TP53 mutated L2/L3	3	6

$P=0.006$ comparing mutation status among patients with progressive disease (PD) to those having a partial response (PR) or stable disease (SD).



(a): Large blue and green circles connected by unbroken arrows represent the critical signal cascades. Yellow circles represent potential co-factors for a gene product, and small blue and green circles represent other activated genes that do not belong to the critical signal cascade but might be involved in other biological functions. Large red circles represent genes inactivated by mutations or other mechanisms, and orange circles represent genes whose expression is affected by these events. Each of the two critical signal pathways (I and II) is able to induce apoptosis. A: The normal situation in which apoptosis is induced in response to genotoxic stress. B: Apoptosis might occur despite one of the two pathways being damaged. C and D: Loss of apoptotic function caused by damage to both pathways. (b): Hypothetical mRNA gene expression profiles of A-D (by microarray). Each square on the array represents the mRNA expression of a single gene in a single tumour. Each column represents one tumour. Red squares represent high mRNA levels (i. e. normal gene expression), and green squares represent downregulation. Gene expression from pathway I is at the top of the column, from pathway II at the bottom. Note that each situation (A-D) would provide a different gene expression profile as evaluated by microarray. In this example, B and C might produce global gene expression profiles with a higher degree of similarity compared with A and B, or C and D. (Reproduced with permission from reference 77).

Fig **1** Schematic representation of the functional pathway redundancy hypothesis

In conclusion, we believe we now have the laboratory tools for identifying functional defects controlling tumor behavior, including abilities like metastatic propensity as well as therapy resistance. However, to take full advantage of these opportunities, we need to base our research on functional hypotheses regarding tumor biology and to develop appropriate clinical trials to test our hypotheses *in vivo*.

【**Key words**】 Breast neoplasms; Therapy; Prognostication; Prediction

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(Received: June 22, 2010)

(Edited by:Zhao Bin)

Lønning PE. Role of molecular markers in breast cancer therapy[J/CD]. 中华乳腺病杂志:电子版,2010,4(5):479-495.