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REVIEW

Molecular Markers for Predicting Response to Tamoxifen in Breast Cancer Patients

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Tamoxifen is one of the most effective treatments for breast cancer. Standard practice is to select patients who are likely to respond to this therapy through the evaluation of estrogen receptor (ER) and progesterone receptor (PR) in the primary tumor tissue. Over the past 25 yr that physicians have been using ER determination to guide tamoxifen use, numerous studies have demonstrated that this molecular marker is useful in predicting benefit from tamoxifen. ER has been analyzed for many years using ligand-binding assays. However, current practice involves the use of immunohistochemical-based assays to detect ERα. Immunohistochemistry (IHC) has several advantages. For example, IHC evaluates tumor cell heterogeneity, can be used to study small samples, is less expensive, and allows direct correlation with multiple histopathological tumor features and other molecular markers. PR, an estrogen-responsive protein, can also be useful in predicting response to tamoxifen in specific clinical situations. In recent years, several other markers of tamoxifen response have been examined, including: pS2 (another estrogen-regulated protein), heat-shock proteins 27 and 70, bcl-2 protein, c-erbB-2 (HER-2/neu) oncoprotein, and mutated p53 tumor suppressor protein. In this article, we present an analysis of the data on these new molecular markers. Overall, from numerous studies, the data indicate that in addition to ERα, bcl-2 is a potential candidate to help further improve our ability to predict response to tamoxifen. ER and bcl-2 are the most useful molecular markers to better identify breast cancer patients who will respond to tamoxifen and who will have prolonged survival.

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Introduction

Tamoxifen is a very effective antiestrogenic compound widely used for treating women with breast cancer. Tamoxifen and chemotherapy provide similar benefit in advanced disease, although tamoxifen has relatively little toxicity (compared with chemotherapy agents) and is easier to administer, offering a better quality of life. Most actions of tamoxifen are attributed to its capacity to interact with estrogen receptor (ER). Tamoxifen belongs to the first generation of pharmaceutical agents now collectively called selective estrogen receptor modulators (SERMs). In women, this drug has tissue-specific effects, acting as an estrogen antagonist on the breast, and as an estrogen agonist on bone, lipid metabolism (increasing high-density lipoprotein cholesterol and decreasing low-density lipoprotein cholesterol), and the endometrium *(1)*. The uterotropic effect on the endometrium is one of the most undesirable effects of tamoxifen, resulting in hyperplasia and carcinoma; however, because of the low incidence of carcinoma, the therapeutic benefits of tamoxifen usually outweigh the risks. Presently, second-generation SERMs are being developed and tested clinically, which may be safer and may not promote endometrial carcinoma.

Tamoxifen can be used as a chemopreventive agent to reduce the incidence of breast cancer in high-risk women *(2)*. Tamoxifen is also being used for treatment of patients with intraductal breast cancer to reduce the incidence of invasive cancer and second primaries *(3)*. However, its most useful application is to improve overall survival and decrease recurrence in women with invasive breast cancer. Here, the traditional predictor of response to tamoxifen has been the presence of ER in the tumor tissue. In this article, we review data on ER and other molecular markers that may be predictors of tamoxifen response in women with breast cancer.

The Challenge of Predicting Response to Therapy

The heterogeneity of the tumor cell population is one of the factors that can be of considerable importance when a predictive molecular marker is evaluated. Usually a predictive factor (e.g., presence of ER) is analyzed only once, when the complete primary tumor is removed at surgery. Although the presence and distribution of the marker may be homogeneous, frequently there is heterogeneity within the tumor in terms of marker expression. When only small portions of a tumor are sampled, such as with core or fine-needle biopsy sites, sampling error may result in inaccurate marker assessment, because of tumor heterogeneity. Immunohistochemistry (IHC) may be more accurate than biochemical sample assays to evaluate tumor samples. With IHC, the heterogeneity of the tissue can be directly observed, evaluating the marker more precisely in the invasive tumor, omitting the *in situ* carcinomas, hyperplasias, or "normal" epithelium usually present in tissue samples.

Tumor cells remaining in the patient are not static, but are continuously evolving, with a succession of molecular changes probably occurring in the tumor cells. Tamoxifen resistance arises from a complex interaction of genetic and epigenetic mechanisms. These include genetic instability, emergence of receptor-negative/defective clones, imbalance of growth/inhibitory factors, changes in the blood supply, and decrease in uptake/bioavailability of the drug *(4–7)*. Obviously, these changes cannot be evaluated in the primary tumor.

The host is also evolving, with patients becoming postmenopausal, aging, experiencing dietary changes, and changes in the hormonal milieu, and alterations in the immune system. Thus, the interplay between tumor and host is constantly changing.

The metabolism of the drug might also change. In the serum there is a maintenance of steady-state tamoxifen and metabolite levels in patients after prolonged treatment *(8)*, but there is a broad range of normal tamoxifen concentration, and the serum levels of the drug have not correlated with clinical benefit *(9,10)*. There may be changes in drug metabolism over time, in normal or tumor tissue. The clinical pharmacology of tamoxifen is complicated. The drug has several metabolites, some with potent antiestrogenic actions (4-hydroxytamoxifen, *trans* isomer) and others with full estrogen agonist actions (4-hydroxytamoxifen, *cis* isomer). In tumors, these two isomers have been found at different ratios, suggesting that the accumulation of the *cis* isomer could contribute to drug resistance *(11)*. These potential changes in metabolism are quite difficult to evaluate in breast cancer patients. Finally, the drug may not only act through ER-mediated mechanisms; several actions are independent of ER, such as enhancing natural killer cell activity, blocking protein kinase C activation, binding to calmodulin, and disrupting calcium current across cell membranes *(12)*. Thus, with this degree of complexity, it is easy to understand that it is not a simple task to choose a molecular marker(s) to predict the response to tamoxifen.

Estrogen Receptor

Discussion in this section is restricted to ERα. ERβ was more recently discovered *(13)*, and there is no significant clinical data on this receptor. However, in the future it will be of interest to know whether ERβ is a useful marker for tamoxifen responsiveness. For example, through the use of mutant and chimeric receptor assays, research has found that 4-hydroxytamoxifen shows agonist activity through the ability of ERβ to drive transcription from the hRARα-1 promoter, activity that is antagonized by estrogen, suggesting that tamoxifen can specifically interact with ERβ *(14)*.

General consensus indicates that ERα is an excellent marker to predict response to tamoxifen in breast cancer patients. Tumors that are ER negative, using stringently low cut points, rarely respond to tamoxifen. Determination of this receptor was introduced in the clinic in the 1970s. It has been analyzed by different methodologies, by different antibodies, and by different laboratories worldwide, and consistently has good correlation with the clinical response to tamoxifen. This demonstrates that tamoxifen is acting on human breast tumors mainly by ERα-mediated mechanisms. Jensen and Jacobson *(15)* described the specific binding/retention of ^{3}H-estradiol in estrogen target tissues, and McGuire *(16)* introduced ER analysis as a standard practice in the management of breast cancer patients.

In a recent metaanalysis on the use of tamoxifen in 37,000 women with early breast cancer *(17)*, it was found that ER-positive patients showed significantly greater reduction in recurrence and mortality than ER-poor/negative patients. In addition, the metaanalysis suggests that adjuvant tamoxifen treatment remains a matter of research in early breast cancer patients with ER-negative tumors, because there was no statistically significant benefit in these patients.

Most data on ER in the clinic have been collected using biochemical cytosol-based ligand-binding assays. The cutoff value to consider tumor samples as ER positive is generally 10 fmol of receptor/mg of total cytosol protein, but patients whose tumors show even lower femtomoles ("ER poor") may experience better disease-free survival after tamoxifen treatment than women whose tumors completely lack ER *(18)*. To identify more effectively tamoxifen-responsive patients, other alternatives have been tested or proposed. These include determination of ERα protein and mRNA contents *(19)* and ERα variants *(20)*, a displacement assay with tamoxifen competing with estradiol for ER protein *(21)*, in vivo tumor uptake of fluorine-18 fluorodeoxyglucose, and 16α [18F] fluoro-17 β-estradiol detected with positron emission tomography *(22)*, and quantitation of the ratio of coactivator and corepressor proteins that determines the final direction of transcription by

Table 1
Recommendations for Performing IHC of ERα

1.	Do not delay tissue fixation—If radiography of the surgical specimen is performed, it should be done quickly.
2.	Use a representative paraffin block—If possible, use two. Check on the fixation quality of hematoxylin and eosin–stained slides and ensure there are an adequate number of tumor cells.
3.	Apply an antigen retrieval protocol—Different fixation and embedding protocols are used in different hospitals and institutions. The antigen retrieval protocol helps unmask ERα.
4.	Choose a good Mab—Some produce better staining than others. Select those whose predictive value has been tested clinically.
5.	Evaluation of immunostaining by using a proportion score—Choose a score that has been validated in clinical outcome studies.
6.	Perform the analysis in an experienced reference laboratory—The laboratory should run positive and negative controls, have well-trained technicians, use sensitive detection methodologies, and so on.

tamoxifen-occupied steroid receptors *(23)*. These alternatives and the more traditional biochemical assays for ERα determination are, in general, complicated, sometimes requiring preserved frozen tissue, specialized equipment, and radioactive reagents, making them relatively difficult and expensive. With the advent of the monoclonal antibodies (Mabs), many laboratories began to evaluate ERα by enzyme immunoassay on cytosols and/or by immunochemical methods on cells and tissues (immunocytochemistry and IHC, respectively). The immunochemical assays have shown excellent correlation with the biochemical radioligand-binding assays *(24,25)*. Allred et al. *(25)* pointed out that in preliminary studies immunochemical assay on cells and tissues seemed superior than the radioligand-binding assay because it addresses the heterogeneity of ERα distribution in tumors. Presently, ERα can be reliably detected on routine formalin-fixed paraffin sections using newer Mabs with antigen retrieval procedures.

We have evaluated a number of molecular markers in tamoxifen-treated patients. Among them was a study of ERα by IHC in which paraffin blocks retrospectively collected from patients who entered the Southwest Oncology Group trial 8228 were evaluated. This study is referred to herein as the SWOG-TAM study *(26)*. These patients (n = 205) formed a very homogeneous group with the following characteristics: all had metastatic breast cancer (objectively evaluable), were ERα positive by ligand-binding assay (>3 fmol/mg of cytosol protein in either primary tumors or metastasis), and received only tamoxifen (prior adjuvant chemotherapy allowed if completed more than 3 mo prior to relapse). The study began in 1982, and median follow-up of patients is 9 yr. The SWOG-TAM study examined the predictive value of ERα using ligand-binding assay and IHC and is the largest study of its kind, to our knowledge. The controlled and prospective design of the original clinical trial strengthens the validity of the findings. The study showed that IHC scores for ERα (proportion of positive staining tumor cells: 0, no staining; 1, <1/100; 2, 1/100 to 1/10; 3, 1/10 to 1/3; 4, 1/3 to 2/3; and 5, >2/3) were significantly associated with levels determined by ligand binding. There was a significant direct relationship between higher immunohistochemical ERα score and increasing response to tamoxifen.

Important points should be kept in mind when using IHC to detect ERα (*see* Table 1). These recommendations in Table 1 apply for biopsies processed for routine formalin fixation and paraffin embedding. It is important to use an antigen retrieval protocol that includes heating with a buffer in order to achieve a high degree of unmasking of epitopes *(27)*. Moreover, there are many different Mabs against ERα, and previous studies have demonstrated considerable immunostaining variation according to the antibody selected *(28,29)*. Another important consideration is the evaluation of the immunostaining. Although it is outside the scope of this article to review all the different techniques and scoring systems that apply to the detection of ERα, we recommend the use of a score representing the estimated proportion of positive staining tumor cells (already mentioned) and evaluation of the entire slide. Tumors with scores ≥2 (≥1/100 of positive cells) should be considered positive based on previous studies calibrating IHC scores to clinical outcome *(30,31)*. Only nuclear staining should be evaluated. Cytoplasmic and membrane staining have not been validated as correlating with clinical outcome. In summary, the variations in techniques used and scoring systems applied could explain discrepant results when evaluating ERα and other molecular markers in IHC studies. Thus, there is a need to use more standardized techniques and to evaluate the immunostaining with a uniform scoring system.

ERα-positive tumors are more frequently found in postmenopausal than in premenopausal women with breast cancer. This is attributed to higher estrogen levels in younger women causing downregulation of ERα, and to the presence of more aggressive, undifferentiated tumors. In premenopausal women with breast cancer, no significant differences in ER-positive tumors have been found according to the timing of operation in relation to menstrual phase *(32)*. Immunohistochemical detection of ERα in breast cancers from pregnant women may be superior to detection by ligand binding *(33)*. The reason could be competitive blocking of receptor sites by high amounts of estrogen and

progestins present during pregnancy, which interferes with ligand-binding assays, and also to downregulation of ERα. In these cases, detection of estrogen-regulated proteins may serve as surrogate markers for ERα-dependent tumors. In a study by Encarnación et al. *(34)*, when ERα was measured in breast cancer biopsies from patients receiving tamoxifen treatment, again IHC was superior to ligand-binding assay, with ERα being falsely negative owing to receptor occupancy by tamoxifen. That study also showed that many patients having tumor progression on tamoxifen had ERα- and/or PR-positive tumors, indicating that receptor loss cannot always explain tamoxifen resistance. Moreover, certain treatments (neoadjuvant chemotherapy with mitoxantrone, methotrexate, +/– mitomycin C, and tamoxifen) may decrease ERα expression *(35)*, and this should be taken into account when the receptor is assayed in chemotherapy-treated biopsies. In general, ERα values are relatively stable and concordant in the primary tumors, compared with regional lymph node and distant metastases *(36)*.

How accurate is ERα in predicting the response to tamoxifen? In advanced breast cancer the cytostatic/cytotoxic effects of tamoxifen are temporary, and unfortunately the disease will advance in most patients. However, the disease may regress or be stable for many years, enough to significantly prolong the disease-free survival and overall survival of patients. In the SWOG-TAM study *(26)*, patients with intermediate scores by IHC (2 and 3) showed a 46% response, and those with high ERα scores (4 and 5) showed a 66% response. In patients with early invasive breast cancer, distant microscopic tumor foci may or may not be present, and one cannot be sure whether microscopic foci of tumor cells are completely eliminated and controlled by tamoxifen. Long-term benefit is evident, however, with the metaanalysis of tamoxifen in women with early breast cancer *(17)*, demonstrating that the proportional reduction in recurrence rates in the trials of about 5 yr of tamoxifen treatment was 50% in patients with ER-positive tumors and 60% in patients with ER-positive tumors with at least 100 fmol/mg of cytosol protein. The proportional reduction in mortality was 26% for ER-positive women and 36% for women with ER-positive tumors with at least 100 fmol/mg of cytosol protein (all of them treated for about 5 yr with tamoxifen). Together, these data illustrate that there is a direct correlation between the amount of ER and the benefit of tamoxifen in early and late stage disease.

Finally, the study of ERα has been utilized also as a prognostic factor to help determine whether a particular breast cancer patient with early stage disease will have a low or high risk of developing gross distant metastases. ERα is more frequently present in well-differentiated and less aggressive tumors. The prognostic power of ERα is evident, but not strong. It may be better to combine this molecular prognostic factor with histopathological tumor features and expression of other molecular markers when assessing the prognosis in a breast cancer patient *(37)*.

Progesterone Receptor

The study of PR was introduced as a standard practice in the management of breast cancer patients to improve the detection of endocrine-responsive tumors. The rationale is that in estrogen target tissues, estrogen treatment induced PR expression *(38)*, and this was also found in human breast cancer cells *(39)*. Therefore, it was hypothesized that PR is a surrogate marker of intact, functional molecular machinery within ERα-positive tumor cells (intact pathway: estrogen-ERα-DNA with transcription of PR mRNA leading to PR protein expression). Early clinical data supported the concept that evaluation of PR in addition to ER improved the prediction of response to endocrine therapy *(40)*. There are tumors with an ER^-/PR^+ phenotype, in which apparently PR is synthesized independent of ER pathways (e.g., meningiomas) *(41)*. However, when ER^-/PR^+ breast tumors identified by ligand-binding assay were reexamined by monoclonal anti-ERα antibodies, it was found that these tumors were in fact ERα positive *(42)*. Nevertheless, when immunochemical methods to detect ER and PR are performed, ER^-/PR^+ tumors are still infrequently found (about 5% of cases). Existing data suggest that ER^-/ PR^+ tumors will respond to tamoxifenal, although the prognosis may be somewhat worse than for the ER^+/ PR^+ phenotype.

To make more functional use of PR analysis, induction of PR by tamoxifen has been explored. Tamoxifen showed estrogen agonistic activity on cultured cancer cells, inducing PR within 1 or 2 d after treatment, reaching maximum induction levels at 4–6 d *(43)*. In vivo studies examining tamoxifen induction of PR have been performed. Howell et al. *(44)* reported that in 40% of cases PR levels were higher in a second biopsy taken at a median of 13 d after tamoxifen treatment, and that 90% of these patients ($n = 19$) responded to continued endocrine therapy. In this study, PR levels were lower in the second biopsy in 37% of the tumors, and these patients ($n = 19$) did not have a longer time to progression. By contrast, another study has shown that short-term tamoxifen treatment did not modify PR levels in breast carcinomas (using lower tamoxifen doses and a shorter time for the second biopsy) *(45)*. In a study of ER and PR in breast cancer biopsies from patients on tamoxifen *(34)*, six patients with ER-negative tumors by ligand binding and IHC expressed PR, suggesting that ER could still be functional or that PR was constitutively expressed. Moreover, in a subset of patients with receptor assays prior to tamoxifen and at the time of progression while taking the drug it was shown that PR remained unchanged *(34)*. A more recent study examined the effects on PR levels of four, three times weekly cycles of mitoxantrone, methotrexate, +/– mitomycin C, and tamoxifen; the researchers found that at 10 and 20 d posttreatment PR did not change significantly compared with the pretreatment biopsy *(35)*. Therefore, at present there is insufficient evidence to support the use of PR as a "functional PR induction test" in the

clinic. In a research setting, it could be performed on patients with relatively large primary tumors, by taking an initial biopsy before starting treatment, then a second biopsy after administration of high doses of tamoxifen.

A study of premenopausal women with breast cancer, found that the phase of the menstrual cycle at the time of operation did not influence PR status *(32)*. Therefore, breast cancer tissue is not particularly sensitive to the cyclical estrogen/progesterone changes that occur during the menstrual cycle. In the normal endometrium, there is more PR during the follicular than in the luteal phase. Moreover, even higher hormonal levels such as those found during pregnancy had little influence on PR values (determined by ligand-binding assay) in women with breast cancer. We found that the incidence of PR-positive tumors from pregnant women was not significantly different from age-matched control women *(33)*. However, by IHC we found in five of eight ER-negative tumors, evidence of "functioning" ER as determined by positive staining for PR.

An especially important question is, is PR analysis useful to the prediction of the response to tamoxifen? Table 2 shows the response to tamoxifen according to PR status. In advanced breast cancer patients, the SWOG-TAM study *(26)* revealed that PR determinations by ligand binding were significantly associated with PR scores by IHC. Moreover, in this study, response increased with increasing PR. ER^+/PR^+ tumors were modestly more likely to respond than ER^+/PR^- tumors, and ER^+, PR^+ patients had a longer median survival, but these differences were not significant. Other studies evaluating PR by IHC in advanced breast cancer patients have shown inconsistent results *(26,35,46)*. In early breast cancer patients, the metaanalysis *(17)* showed that in women with ER-positive tumors, the PR status was of little additional value in predicting the response to tamoxifen. However, in a subset of patients who were ER^-/PR^+, reduction in recurrence and mortality was similar to that of ER^+ patients, thus demonstrating the utility of PR determination. A recent article *(18)* evaluating PR as a continuous variable showed that PR did not predict the response to tamoxifen. Overall, PR determination in the management of breast cancer patients to predict response to tamoxifen is of modest utility. Better, more powerful markers are needed, perhaps to replace or complement PR.

pS2

The pS2 protein was first found in the human breast cancer cell line MCF-7 after estrogen stimulation. It is expressed in approx 50% of human breast tumors but not in normal breast tissue *(47)*. The function of pS2 protein is still unclear. In normal tissues, it is found in male and female stomach epithelial cells lacking ER and in salivary glands at low levels. In abnormal tissues, pS2 is found in ulcerative lesions of the gastrointestinal tract and in certain malignant tissues (i.e., ovarian cancer, colon, and stomach tumors). The pS2 gene contains transcriptional enhancer elements responsive to estrogen, epidermal growth factor (EGF), and *c-jun*, among others *(47)*. Because pS2 is under estrogen regulation, it is of interest to know whether this protein can be a useful predictive marker for hormonal responsiveness of breast cancers. Foekens et al. *(48)* found pS2 positivity almost exclusively in ER-positive tumors (96%). In another retrospective study of advanced breast cancer patients ($n = 72$), those with pS2-positive tumors by IHC had a 76% response (stable disease, partial response, or complete response) to hormone therapy, but no correlation was found between pS2 expression and duration of response *(49)*. In the same study, ER-positive patients (by ligand binding) showed a 54% response. Moreover, in this study no association between pS2 and PR was found, and the correlation of pS2 and ER reached 83%.

In the SWOG-TAM study *(26)*, the response rate to tamoxifen was 52, 48, and 72% for pS2-negative, pS2-intermediate, and pS2-high levels, respectively (determined by IHC). In multivariate analysis, pS2 was marginally predictive for time to treatment failure (TTF) and was not significant for overall survival. That study also showed that likelihood of response, TTF, or overall survival was not significantly improved by adding pS2 data to ER data. Overall, the data indicate that pS2 is predictive of response to tamoxifen; however, it is not additionally useful to ER and PR and thus has not been routinely incorporated into clinical practice.

Heat-Shock Proteins

We have examined the value of two heat-shock proteins, hsp70 and hsp27, in predicting response to tamoxifen in the SWOG-TAM study *(50)*. Heat-shock proteins participate in numerous molecular/cellular events, and, in general, they behave as molecular chaperones, being involved in cytoprotection, and reestablishing protein homeostasis. Different heat-shock proteins interact with defined substrates. For example, hsp70 has been implicated in the assembly and trafficking of steroid receptors *(51)*. *Hsp27* is also of interest because this gene is under dual control, both by estrogens and by heat-shock transcription factors. Hsp27 is expressed in estrogen target tissues *(52)*. Moreover, in human breast and endometrial cancers, hsp27 expression correlates with the presence of ER. In advanced breast cancer patients, hsp70 and hsp27 have been associated with resistance to chemotherapeutic agents *(53)*. Thus, biologically, testing of these two heat-shock proteins was attractive, and they were analyzed by IHC in the SWOG-TAM study. There was good correlation between the expression of the two heat-shock proteins in the tumors. However, increased hsp70 and hsp27 expression was not significantly associated with response to tamoxifen, TTF, or survival. Perhaps the complexity of biological functions exerted by

Table 2
Response to Tamoxifen According to PR Status

	Advanced breast cancer *(26)*	
PR by IHC	Response (%)	Overall survival
Negative	46	—
Intermediate (score 2 to 3)	55	—
High (score 4 to 5)	70 ($p = 0.03$)	—
ER and PR combined		
$ER\alpha^+/PR^+$	62	37 mo
$ER\alpha^+/PR^-$	55	28 mo
	Early breast cancer (metaanalysis) *(17)*	
ER and PR combined	Recurrence reduction (%)	Mortality reduction (%)
ER^+/PR^+	37	16
ER^+/PR poor	32	18
ER^-/PR^+	23	9

the heat-shock proteins and the dynamics of heat-shock proteins content changes in the tumor restrain their utility as markers of response to tamoxifen.

bcl-2

The bcl-2 oncogene was discovered in human follicular B-cell lymphomas at the chromosomal translocation t(14;18). This translocation placed bcl-2 in the same transcriptional orientation as the immunoglobulin heavy chain locus, causing elevated expression levels of the bcl-2 (25-kDa) protein. This protein, present also in normal cells, interacts with other bcl-2 family members (i.e., bax), regulating the susceptibility of cells to apoptosis *(54)*. Because bcl-2 protein is mainly involved in the control of cell death as an antiapoptotic molecule, it could be hypothesized that high bcl-2 levels would be associated with inhibition of apoptosis and hence with drug resistance (many drugs including tamoxifen act on cancer cells by activating apoptosis). In fact, there are reports associating high bcl-2 expression levels with resistance to a number of oncologically active drugs *(55)*. However, the area of apoptosis is under active investigation, and we are still learning many of the complex mechanisms involving bcl-2 family members in the regulation of cell death and survival. For example, in a recent review it has been stressed that bcl-2 is not a panacea for mammalian cell death, that there are cell lines in which bcl-2 is ineffectual against the signal induced by "death receptors," and that additional mammalian death pathways remain to be discovered *(56)*. For this reason, it is not surprising that translational research reports have associated high bcl-2 expression levels with drug sensitivity rather than with drug resistance *(57–59)*. These particular studies were performed on biopsies from breast cancer patients, and all of them indicated that higher bcl-2 expression was associated with response to endocrine therapy. For example, Gasparini et al. *(58)* investigated bcl-2 by IHC in lymph node–positive breast cancer patients ($n = 81$) receiving adjuvant tamoxifen therapy. They found in multivariate analyses that only the interaction between ERα and bcl-2 was statistically significant. Among the patients with ERα-negative tumors, the odds of relapse of bcl-2-positive patients were significantly lower than those of the bcl-2-negative ones. These findings also suggested that bcl-2 added significant prognostic information in ERα-negative tumor-bearing patients.

bcl-2 protein expression was also examined by IHC in the SWOG-TAM study *(60)*. There was a significantly better response to tamoxifen for patients whose tumors had higher bcl-2 levels (62% for high expressers vs 49% for low expressers). TTF was also better for bcl-2-positive patients. The significance of adding bcl-2 after considering ER and PR levels was not strong, but patients with ER-positive tumors with high bcl-2 expression were more likely to respond to tamoxifen. Moreover, in multivariate analyses, a high bcl-2 level was independently related to a better rate of survival (median survival of 40 mo for high expressers vs 25 mo for low expressers). In a recent study on elderly breast cancer patients ($n = 145$) with lymph node–positive cancer treated with surgery and radiotherapy followed by adjuvant tamoxifen, low/negative bcl-2 levels in the tumor were associated with a higher probability of relapse *(61)*.

bcl-2 is an estrogen-regulated protein, and estrogen administration increases bcl-2 mRNA and protein and produces downregulation of bcl-x(L), an effect that is blocked by tamoxifen *(62)*. Moreover, tamoxifen increases apoptosis and decreases bcl-2 in breast cancer cells *(63)*. These findings help elucidate the endocrine control of bcl-2 family members and of apoptosis. More clinical studies are needed to explore the usefulness of bcl-2 family members. Overall, the data indicate that bcl-2 is promising

for better identification of patients who will respond to tamoxifen and who will have prolonged survival.

c-erbB-2 (HER-2/neu)

The c-erbB-2 protooncogene was first identified in rat neuroglioblastomas carrying a point mutation induced by carcinogen administration. It belongs to a tyrosine kinase family of membrane receptors (EGFR/c-erbB-1, HER-2/c-erbB-2, HER-3/c-erbB-3, and HER-4/c-erbB-4), and in human tumor tissues the c-erbB-2 protooncogene can be amplified, causing inappropriately high expression of the 185-kDa receptor protein. Activation of this orphan receptor results from an interactive network of receptor–receptor interactions following binding of cooperative receptors with a multiplicity of growth factors, all of which serve to amplify signal transductions *(64)*. In breast cancer patients, HER-2/neu protein expression has been correlated with more aggressive tumor behavior and hence with worse prognosis *(37)*, with resistance to chemotherapy *(65,66)*, and with response to humanized anti-HER-2/neu Mab administration *(67)*.

Of interest is the regulation of HER-2/neu by estrogens and tamoxifen. In human breast cancer cell lines, estrogen administration caused a decrease in HER-2/neu mRNA/protein levels, and this downregulation could be partly prevented by tamoxifen *(68,69)*. Less clear are the actions in vivo on MCF-7 cells grown as solid tumors in nude mice. Here, estrogen caused an increase in HER-2/neu levels, whereas tamoxifen showed a modest reduction in HER-2/neu immunostaining *(70)*. Another report showed increased cytoplasmic HER-2/neu expression following tamoxifen administration *(71)*. Immunostaining of frozen sections (more sensitive than paraffin sections) from human normal breast tissues revealed weak/moderate HER-2/neu positivity in both ductal and lobular epithelia, and the staining did not vary significantly during the menstrual cycle (the same occurred in endometrial samples) *(72)*. Moreover, menopausal status did not influence the percentage of breast tumors with amplified c-erbB-2 *(73)*. Thus, HER-2/neu expression is not significantly altered by the physiological changes in steroid hormone levels that occur in vivo, except possibly during pregnancy. In a very small study, breast cancers from pregnant women contained relatively elevated HER-2/neu levels *(33)*. Whether these findings were owing to a physiological effect or is to chance alone awaits validation by further studies.

Clinically, HER-2/neu expression is inversely correlated with the expression of ER. In a large study of more than 1000 breast cancers, only 15% of ER-positive patients showed HER-2/neu overexpression compared with 26% of ER-negative patients *(74)*. A more important question is, is this subset of ER^+/HER-2/neu^+ breast cancer patients more resistant to endocrine therapy? In vitro studies seem to support this, because some (but not all) ER-positive MCF-7 cell clones transfected with plasmids carrying HER-2/neu showed tamoxifen resistance *(75,76)*. Another study showed that overexpression of HER-2/neu in MCF-7 cells caused upregulation of bcl-2 and suppression of tamoxifen-induced apoptosis *(77)*. Clinical studies following these initial observations have been conflicting *(78–87)*. Several reasons may explain the contradictory results, e.g., analysis of HER-2/neu in ER-positive and ER-negative patients without discriminating the true ER^+/HER-2/neu^+ subset; low number of patients with ER^+/HER-2/neu^+ tumors; measurement of HER-2/neu by different methodologies and evaluating the response to second-line endocrine therapies rather than to tamoxifen. In our analysis of HER-2/neu in the SWOG-TAM study *(86)*, we found HER-2/neu positivity associated with lower ER values and low bcl-2 but not significantly with response rate to tamoxifen, TTF, or survival. When different cut points for HER-2/neu were explored, no significant evidence of a progressive relationship between an increasing proportion of HER-2/neu-positive cells and shorter TTF or survival was found. In a more recent study, tamoxifen significantly improved relapse-free survival and overall survival in breast cancer patients (n = 331) irrespective of HER-2/neu status, concluding that all HER-2/neu-positive, ER-positive patients should receive tamoxifen as part of their adjuvant treatment *(88)*.

Therefore, in light of the conflicting results, more clinical studies are needed to evaluate HER-2/neu protein in large and homogeneous groups of breast cancer patients in order to have a clearer picture on the use of this marker for predicting responsiveness to tamoxifen.

p53 and Other Molecular Markers

A multiplicity of functions are performed by p53. It is a nuclear phosphoprotein (transcription factor) involved in the transcriptional modulation of numerous genes participating in the control of the cell cycle and apoptosis. Inactivation of p53 is relatively common in breast cancer. Therefore, the biological rationale for investigating p53 in tamoxifen-treated patients is that p53-inactivated tumors should be more resistant to tamoxifen because one of the important molecular mechanisms involved in the control of the cell cycle and apoptosis is deficient. To test this hypothesis, we analyzed p53 in the SWOG-TAM study *(60)*. In this study, p53 analysis was performed by IHC as a surrogate end marker of mutated/inactivated p53. The results showed that p53 positivity (mutated/inactivated) was inversely correlated with ER (but not PR) level and to bcl-2 expression; however, there was no significant relation to the rate of response to tamoxifen. In multivariate analyses, mutated/inactivated p53 did not predict TTF but, as expected, was associated with shorter survival. Thus, in this study, p53 status, as determined by IHC, was not significantly associated with response to tamoxifen.

Table 3
p53 and Other Molecular Markers of Tamoxifen Response[a]

Factor	*n*	Patients	Result (reference)
Cathepsin D (IHC)	83	Node-negative	No correlation with recurrence *(89)*
SPF (flow cytometry)	647	PR^+, adjuvant	High PR^+/high SPF, more benefit from tamoxifen *(90)*
Cathepsin L (ELISA)	56	Postmenopausal adjuvant	High cathepsin L, higher risk of recurrence *(91)*
Angiogenesis (IHC, CD31)	178	$Node^+$, adjuvant	High angiogenesis, poor response to tamoxifen *(92)*
EGFR (IHC)	155	Progressing on tamoxifen	Presence of EGFR, poor response *(85)*
p53 (IHC)	205	Metastatic	No correlation with response with TTF; high p53 correlated with shorter survival *(60)*
CD36 (RNA, differential display)	2	Biopsies before and after tamoxifen	Downregulation of CD36 mRNA caused by tamoxifen *(93)*
PSA (ELISA, cytosol)	434	Recurrent disease	High PSA, poor response to tamoxifen *(94)*
Ki-67 (fine-needle cytology)	158	Neoadjuvant (chemotheraphy and tamoxifen)	Decreased Ki-67 at 10/20 d, good clinical response *(46)*
fos, Ki-67 (IHC)	71	Sequential biopsies during tamoxifen therapy and on disease progression	Decreased fos/Ki-67/cellularity after therapy, better response *(95)*

[a]*n*, number of patients entered into the study; EGFR, epidermal growth factor receptor; ELISA, enzyme-linked immunosorbent assay; TTF, time to treatment failure; PSA, prostate-specific antigen; SPF, S-phase fractions.

Because detection of p53 by IHC is an imperfect marker of disabled p53, these findings do not entirely exclude the possibility that specific p53 mutations/inactivations are associated with tamoxifen resistance. However, p53 mutations are found throughout the gene in breast cancer, there are no significant hot spots for p53 mutations, and all mutations appear to be inactivating. At present there are no studies on specific p53 mutations for tamoxifen resistance.

Table 3 presents other molecular markers that have been examined in relation to tamoxifen response. Some of these appear promising, but, at present, there is not enough clinical data to draw a valid conclusion about the usefulness of these markers in the clinic. Breast cancer is clinically, pathologically, and molecularly a heterogeneous disease, and more clinical studies incorporating homogeneous groups of cancer patients are needed to decrease, as much as possible, reports of conflicting results.

Conclusion

Identification of breast cancer patients who will respond to tamoxifen is extremely important because the drug is effective only in a subpopulation of cancer patients—those expressing ER in the tumor. ER can be detected by ligand-binding assays as well as by immunochemical assays. Because less tissue is needed to perform IHC assays and because multiple other molecular prognostic/predictive markers can be assessed on the same specimen, it is more advantageous to analyze ER (ERα) by IHC. Additional advantages of IHC are evaluation of tumor heterogeneity and lower cost. To more effectively identify tamoxifen-responsive patients, functional ER assays have been tested and/or proposed, but these assays are complex and costly, and there are few clinical data to support their use. In some clinical situations, in addition to ER, PR determination can be of value. In recent years, several other molecular mark-

ers have been evaluated, including pS2, hsp27, hsp70, bcl-2, HER-2/neu, and p53. At present, bcl-2 is a potential candidate to be added to ER determination to predict more accurately the response of breast cancer patients to tamoxifen.

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