

# Progesterone Receptor Loss Correlates with Human Epidermal Growth Factor Receptor 2 Overexpression in Estrogen Receptor – Positive Breast Cancer

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**Abstract** Response to endocrine therapy in breast cancer correlates with estrogen receptor (ER) and progesterone receptor (PR) status. It was originally hypothesized that the ability of PR to predict response to endocrine therapy was due to the fact that *PR* is an estrogen-regulated gene and that its levels represented a marker of functional ER activity. However, it is now known that loss of PR can occur via multiple mechanisms, many of which do not include ER function, e.g., hypermethylation of the PR promoter and loss of heterozygosity of the *PR* gene. We have shown that growth factor signaling pathways can directly down-regulate PR levels via the phosphatidylinositol 3' -kinase (PI3K)/Akt/mTOR pathway, and that this can occur independent of ER. For example, overexpression of myr-Akt in MCF-7 cells causes complete loss of PR protein and mRNA but does not reduce ER levels or activity, thus generating ER+/PR– MCF-7 cells. Therefore, the absence of PR may not simply reflect a lack of ER activity but rather may reflect hyperactive cross-talk between ER and growth factor signaling pathways. Consistent with this hypothesis, several recent clinical studies have found that ER+/PR– breast cancers overexpress human epidermal growth factor receptor (HER) 1 and HER2 compared with ER+/PR+ breast cancers. Although HER receptors can lower ER levels, one study showed that loss of PR correlated with high HER2 levels in a multivariate analysis. Furthermore, loss of PTEN, a negative regulator of the PI3K/Akt signaling pathway, has been shown to be associated with specific loss of PR and no change in ER levels. Given the well-recognized resistance of ER+/PR– breast cancer to antiestrogens, more studies are needed to better understand the etiology of ER+/PR– breast cancer, particularly the analysis of other growth factor receptors and their downstream signaling intermediates with respect to PR status.

Estrogen and the estrogen receptor (ER) play key roles in both normal breast development and breast cancer progression. Therapeutic strategies aimed at inhibiting the action of ER represent highly successful examples of targeted therapy for clinical breast cancer (1–3). ER status is a strong predictor of response to endocrine therapy in the adjuvant setting (4). Although ER is an accepted predictor of response to endocrine therapy, the role of progesterone receptor (PR) has been more controversial. For instance, the Oxford overview of all trials of tamoxifen therapy in early breast cancer found that PR status did not predict benefit (4). However, we have recently published the largest retrospective analysis of early breast cancer treated with tamoxifen and found that patients with ER+/PR+ tumors benefited much more from adjuvant

tamoxifen therapy than those with ER+/PR– tumors (5). Importantly, multivariate analyses, including lymph node involvement, tumor size, and age, show that PR status was independently associated with disease-free and overall survival. Consistent with the predictive power of PR in the adjuvant setting, several studies have shown that elevated PR levels significantly and independently correlate with increased probability of response to tamoxifen in metastatic disease (6, 7). Similarly, two recent neoadjuvant studies showed a better response (clinical outcome and proliferation rate) to endocrine therapy in PR+ tumors than in PR– tumors (8, 9).

Supporting the studies indicating a role for PR in predicting response to adjuvant antihormone therapy are recent data from the Arimidex, Tamoxifen, Alone or in Combination trial, which randomized postmenopausal women with early breast cancer to 5 years of treatment with anastrozole, tamoxifen, or the combination (10). A recent preliminary analysis of this trial with respect to hormone receptor status showed that when all three endocrine therapy arms are combined together, 7.6% of patients with ER+/PR+ tumors had a recurrence (breast cancer event) over a 47-month follow-up, whereas 14.8% of patients with ER+/PR– tumors recurred (11). Importantly, however, preliminary hormone receptor data suggests that, compared with anastrozole, the efficacy of tamoxifen was markedly reduced, specifically in the patients with ER+/PR– tumors.

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Collectively, these data indicate that ER+/PR- breast tumors are less responsive to endocrine therapy compared with ER+/PR+ tumors. In the 1970s, it was hypothesized that PR might provide additional information to more accurately predict which patients will respond to hormonal therapy (12). This theory was based on the rationale that PR is induced by estrogen in ER+ breast cancer cell lines and, therefore, that PR would serve as an indicator of a functionally intact ER pathway. However, recent evidence suggests that this explanation does not account for all ER+/PR- breast cancers.

### Biological and Clinical Characteristics of ER+/PR- Tumors

Approximately 75% of primary breast cancers express ER, and over half of these also express PR (13). PR is an estrogen-regulated gene and its synthesis in normal and cancer cells requires estrogen and ER. Therefore, it is not surprising that ER+/PR+ tumors are more common than ER+/PR- tumors. However, when ER and PR are measured using quantitative assays, the levels vary over several orders of magnitude. Figure 1 shows ligand-binding values for ER and PR (fmol/mg protein) from 1,356 breast tumors from the Breast Tumor Bank at Baylor College of Medicine. Despite the well-accepted fact that ER is a major regulator of PR levels, correlation between ER and PR is fairly weak ( $r = 0.47$ ). Indeed, part of

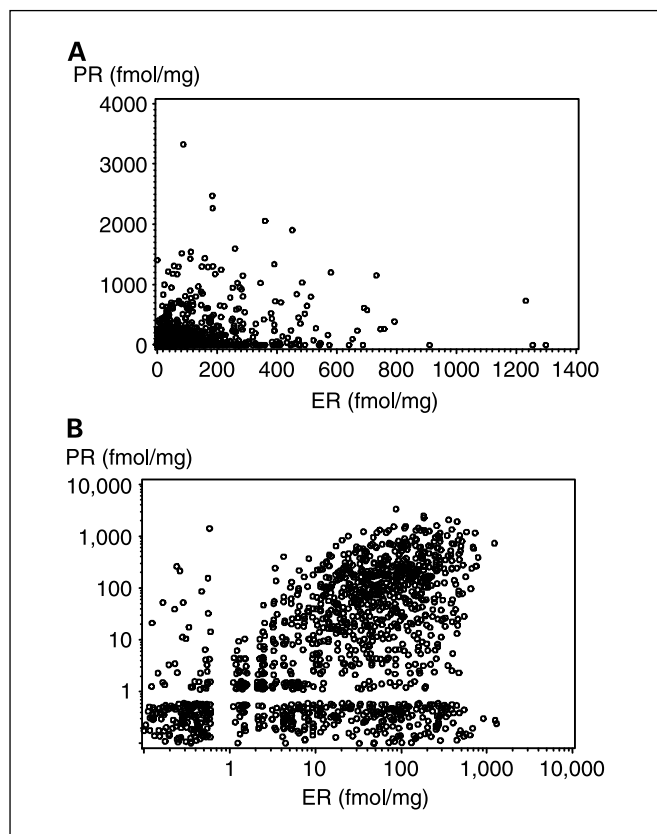
this correlation is driven by the number of tumors that are ER-/PR-, such that if only receptor-positive tumors (ER >3 and PR >10 fmol/mg) are considered, the correlation is only  $r = 0.35$ . Furthermore, the correlation when only ER+ tumors are considered (>3 fmol/mg) is the weakest ( $r = 0.31$ ). This clearly indicates that there are other regulators of PR levels distinct from ER. As Fig. 1 shows, many tumors (up to 30%) exhibit high levels of ER but a complete lack of PR. The fact that ER and PR are continuous variables was recently discussed by Konecny et al. (14) and again highlights the fact that information maybe lost by analyzing them as dichotomous variables. Consistent with this, response to tamoxifen is associated with increasing concentrations of ER (15, 16) and PR (7), not only with positive or negative status.

The etiology of ER+/PR- tumors is currently unclear. Several studies have suggested that ER+/PR- tumors seem after menopause due to low circulating levels of estrogen that are unable to stimulate the ER to synthesize PR. Consistent with this hypothesis, ER+/PR- tumors are more common in women >50 years of age (17-20). Some studies have shown that ER and PR status can change over the natural history of the disease (17) or during treatment (21).

Although ER+/PR- primary untreated tumors may simply evolve by loss of PR from ER+/PR+ tumors, the differences in the biology and outcome of ER+/PR- tumors suggest that some of these may initially evolve separately as ER+/PR- tumors, representing their own individual, stable phenotype from the outset. Indeed, recent prospective studies have shown that ER+/PR- tumors have their own unique epidemiologic risk factors (22). For instance, the incidence of each of the four receptor tumor subtypes (ER+/PR+, ER+/PR-, ER-/PR+, and ER-/PR-) differs with age (23), pregnancy history, postmenopausal hormone use, and body mass index after menopause (24). Importantly, many of these associations remain for PR by itself, even when corrected for quantitative ER levels, highlighting the significance of both receptors not only in breast cancer treatment but also in its etiology.

### Growth Factor Down-Regulation of PR in Breast Cancer Cell Lines

There are many mechanisms that may explain the generation of ER+/PR- breast tumors (Table 1). The simplest is that the ER is nonfunctional and unable to stimulate PR production and that the tumor is, therefore, no longer dependent upon estrogen for growth and survival. In contrast to the loss of ER function, recent studies by our group and others suggest novel mechanisms for loss of PR expression in breast cancer (25). Previous studies of breast cancer cell lines have implicated growth factor signaling in repression of PR expression (26). In a recent report, Konecny et al. (14) compared ER and PR levels in breast cancer cell lines transfected with human epidermal growth factor receptor (HER) 2. In ZR-75 breast cancer cells, overexpression of HER2 caused PR levels to decrease by 500-fold to levels normally observed in ER- cell lines, whereas ER levels only dropped by half. These data suggest that growth factors down-regulate PR levels independent of ER levels or activity in breast cancer cells, a mechanism further substantiated by results from clinical studies (see next section).



**Fig. 1.** Quantitative measurement of ER and PR in human breast tumors. ER and PR were measured by ligand-binding assay (fmol/mg) and are plotted on a linear (A) and a log (B) scale. A small amount of uniformly distributed noise was added to both ER and PR in (B) to separate points at the low end.

**Table 1.** Molecular mechanisms for the loss of PR in breast tumors and generation of the ER+/PR- phenotype

1. Nonfunctional ER (42, 43)
2. Low circulating levels of estrogen (44)
3. Hypermethylation of PR promoter (45)
4. Loss of heterozygosity at *PR* gene locus (46, 47)
5. Growth factor down-regulation of *PR* (25)
6. Selective ER modulator or growth factor-induced membrane ER activity (48)
7. Altered ER coregulator activity (29)

These unresolved phenomena raise the question of how growth factors modulate PR expression. In exploring this issue, we found that short-term treatment (hours) with insulin-like growth factor-I (IGF-I), as well as epidermal growth factor and heregulin, all sharply lowered PR levels and progestin-induced PR activity in breast cancer cells (25). This is in contrast to other estrogen-regulated genes, such as *pS2*, whose expression is increased by IGF-I (27). The phosphatidylinositol 3'-kinase (PI3K)/Akt pathway was specifically involved in this growth factor down-regulation of PR levels because inhibitors of this pathway could reverse the PR down-regulation. We originally showed that overexpression of myr-Akt1 in MCF-7 cells resulted in loss of PR, although the ER was functional and active (25). To support this, we have also found that overexpression of dominant-active Akt3 (myr-Akt3) can repress PR and cause cells to become ER+/PR- (Fig. 2). In this experiment, we used anti-HA immunoblot to isolate MCF-7 stable transfectants that overexpressed HA-myr-Akt3 at different levels (clone 1—low to undetectable; clone 2—medium; and clone 3—high expression). Stimulation of clone 1 with IGF-I resulted in the detection of p-Akt. In clone 2, basal levels of p-Akt were increased due to the expression of the constitutively active myr-Akt; however, IGF-I still increased levels of p-Akt to those seen in clone 1. In clone 3, constitutive basal levels of p-Akt were detectable to the levels of the IGF-I stimulation in clone 1. Furthermore, stimulation of clone 3 with IGF-I did not increase p-Akt levels further. PR levels were unaffected by the short 15-minute stimulation with IGF-I (down-regulation is first evident after 2 hours of IGF-I). However, the constitutive phosphorylation of Akt in clone 2 resulted in a reduction of PR levels that was even more evident in clone 3. The decrease in PR occurred in the absence of changes in ER levels. The loss of PR protein was associated with reduced *PR* mRNA (Fig. 2B) consistent with our previous data (25). A recent report suggests that activator protein 1 may be involved in repression of the *PR* promoter (28).

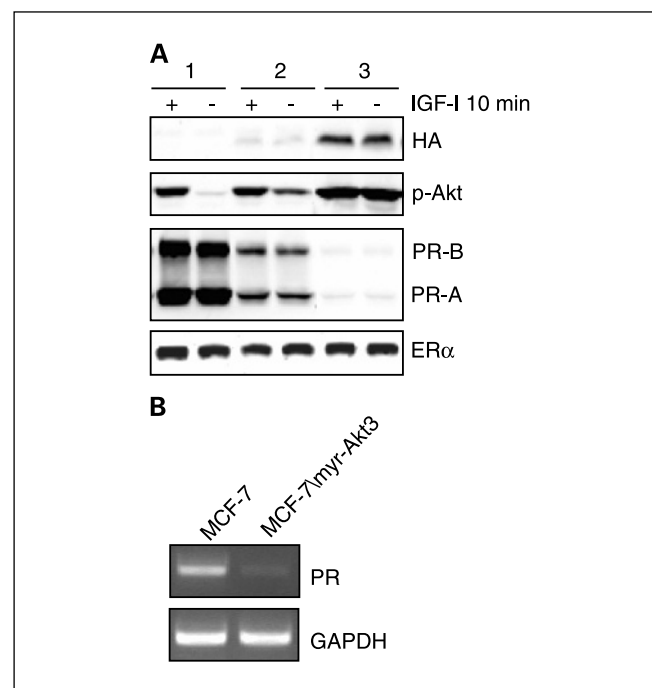
Importantly, the lowering of PR levels occurred in the absence of a change in ER levels, thus creating MCF-7 cells that are ER+/PR-. Furthermore, the activity of ER was also unaltered as assessed by ERE-tk-luciferase reporter assay (Fig. 3). In this experiment, MCF-7 cells with or without myr-Akt expression were transfected with an estrogen activity reporter. Irrespective of the myr-Akt, all clones showed estrogen induction and tamoxifen antagonism of ER activity. This is an important point to consider as studies using growth factor receptors, such as HER2, are complicated by the fact that when

these signaling pathways are overexpressed at high levels, ER is ultimately eliminated, thus causing a loss of PR attributable to the "nonfunctional ER" theory. However, in the MCF-7 cells expressing myr-Akt3, ER levels are unchanged and the ER is active and able to respond to estrogen in a transfection reporter assay, confirming that loss of PR in these cells is not explicable by the "nonfunctional ER" theory.

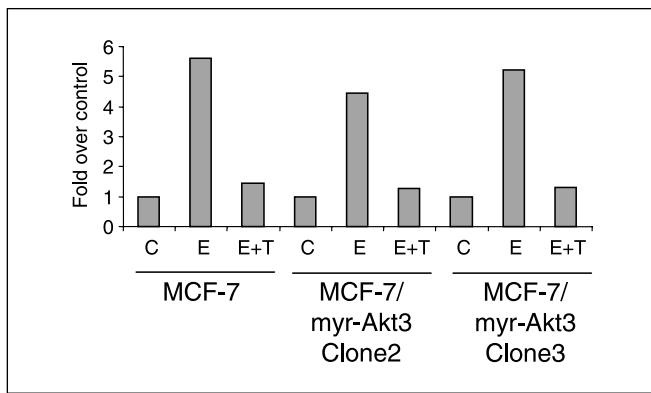
Consistent with our cell culture data showing PI3K/Akt-mediated loss of PR are data from transgenic mice that overexpress the ER coregulator AIB1 in the mammary gland (29). In these transgenic mice, AIB overexpression results in mammary tumorigenesis that is associated with increased IGF-I activation of the PI3K/Akt/mTOR pathway. Importantly, mammary tumors from AIB1 transgenic mice show persistently elevated ER but loss of PR. This loss of PR may be due to the elevated IGF-I signaling, which again highlights the possibility of ER-independent regulation of PR levels.

### Increased Levels of HER1 and HER2 in ER+/PR- Breast Tumors

Clinical studies have addressed the correlation between steroid hormone receptors and HER2 amplification or overexpression in breast cancer (30). Konecny et al. recently reported a quantitative analysis of ER, PR, and HER2 in



**Fig. 2.** Overexpression of myr-Akt3 in MCF-7 cells causes complete loss of PR protein and mRNA and results in ER+/PR- cells. MCF-7 cells were transfected with hemagglutinin (HA)-tagged myr-Akt3. Stable clones were isolated and characterized. *A*, low (1), medium (2), and high (3) MCF-7/myr-Akt3-overexpressing clones were incubated overnight in serum-free medium and then stimulated with or without IGF-I (5 nmol/L) for 15 minutes. Cells were lysed, proteins were separated by SDS-PAGE, and then immunoblotted. *B*, MCF-7 wild-type cells or MCF-7Akt3 clone 3 cells were lysed and RNA isolated. Reverse transcription-PCR was done for PR and for glyceraldehyde-3-phosphate dehydrogenase (*GAPDH*) as a loading control. Reverse transcription-PCR products were separated on 1% agarose gel and stained with ethidium bromide.



**Fig. 3.** myr-Akt3 overexpression in MCF-7 cells does not affect ER activity. MCF-7 cells or stable transfectants overexpressing myr-Akt3 were transiently transfected with ERE-tk-luciferase reporter and a Renilla reporter as a control. Cells were incubated overnight in serum-free medium and then stimulated for 16 hours with estradiol (E, 1 nmol/L) or estradiol and 4-OH-tamoxifen (E+T, 100 nmol/L). Cells were lysed and activity measured using a Dual Luciferase Reporter kit (Promega, Madison, WI) according to the instructions of the manufacturer. Columns, fold over control.

1,595 tumors (14) and found negative correlations between ER or PR and HER2. Intriguingly, their study revealed that relatively low levels of HER2 amplification/overexpression were associated with marked decreases of PR, but not of ER, thus causing tumors to be ER+/PR-. High amplification of HER2 ultimately led to the loss of ER and resulted in ER-/PR- tumors.

Relatively few studies have assessed the effect of HER2 on PR levels in the subset of ER+ tumors (Table 2). Three small studies showed an increase in HER2 positivity in ER+/PR- breast cancer. Dowsett et al. (9) showed a 2.2-fold increase in HER2 positivity in ER+/PR- compared with ER+/PR+ breast tumors. Additionally, they showed that the ER+/PR- tumors showed a reduced response (lowering of Ki-67) to neoadjuvant endocrine therapy. Dixon et al. (31) reported a 1.7-fold increase in HER2 positivity in ER+/PR- breast cancers. Bamberger et al. (32) measured the two isoforms of PR (PR-A and PR-B) and correlated levels with HER2 positivity. They found a 3.5-fold increase in HER2 positivity in ER+/PR-B- compared with ER+/PR-B+ breast tumors, although no significant change was seen with PR-A.

Two subsequent larger studies also reported an increase in HER2 positivity in ER+/PR- breast cancer. Taucher et al. (33) measured ER, PR, and HER2 in 923 tumors and found a 2-fold increase in HER2 positivity in ER+/PR- breast tumors. Similarly, Huang et al. (34) measured the same variables in 1,362 tumors and also found a 2.2-fold increase. Importantly, although previous studies had shown a correlation between ER and PR loss and high HER2 levels in univariate analyses, Huang et al. found that this also held true in a multivariate analysis, indicating that the loss of PR is not simply accounted for by the lowering of ER levels. We have recently performed the largest and most comprehensive quantitative analysis of ER, PR, and HER2 in breast tumors. We found that ER+/PR- tumors had a 3-fold elevation in HER1 levels and a 1.4-fold increase in HER2 (20).

The HER family of receptors are upstream of the PI3K/Akt/mTOR signaling cascade (35). PTEN is a negative regulator of PI3K signaling, and loss of PTEN, which is associated with

activation of this survival pathway, has recently been correlated with loss of PR in clinical breast cancer specimens (36). In another smaller study, loss of PTEN correlated with loss of both ER and PR; however, this study did not specifically examine ER+/PR- tumors (37). Importantly, it has been reported that loss of heterozygosity at chromosome 10q23, which harbors the *PTEN* gene, occurs in ~30% to 40% of sporadic breast cancers, and that this loss of heterozygosity is associated with higher histologic grade and specific loss of PR, but not ER, expression (38). In contrast to data with *PTEN*, mutation of *PI3K* has been positively associated with increased ER and PR levels (39), although this was not noted in two smaller studies (40, 41). This last observation highlights the complicated interplay between ER, PR, and the *PI3K* pathway, and differences in studies may reflect the interaction of this pathway both with the etiology of ER+/PR- breast cancers and also with changes in receptor levels during disease progression.

## Summary

The late Bill McGuire was seminal in the development and measurement of ER and PR as predictive markers for endocrine therapy of breast cancer. Together with Kate Horwitz, he hypothesized in the 1970s that loss of PR reflected a loss of ER activity and that this was a marker of poor response to antiestrogens. Since that time, a relatively small amount of attention has been paid to the etiology and biology of ER+/PR- breast cancer. Few clinical studies dichotomize these subgroups, with most normally including response to ER and/or PR. We have recently proposed that generation of ER+/PR- breast cancers is more complicated than simple loss of ER function and may actually represent growth factor activation of ER and independent down-regulation of PR. This theory seems to hold up in clinical specimens and provides a novel hypothesis for the antiestrogen resistance of these tumors. This hypothesis predicts that PR is a marker of active growth factor signaling and that these tumors should be treated with a combination of endocrine therapy and growth factor signaling inhibitors. Further studies on other growth factor signaling cascades that down-regulate PR in clinical specimens are warranted.

**Table 2.** Increase in HER2 positivity in ER+/PR- tumors

Investigators	ER+/PR+	ER+/PR-	Fold increase
Dowsett et al. (9)	9.3% (7/75)*	20.5% (8/31)	2.2
Bamberger et al. † (32)	7.7% (2/26)	26.9% (7/26)	3.5
Taucher et al. (33)	7.6% (33/436)	15.2% (32/211)	2.0
Dixon et al. (31)	23.5% (4/17)	40% (2/5)	1.7
Huang et al. (19)	5.4% (48/806)	11.5% (29/223)	2.1
Arpino et al. (20)	14% (158/972)	21% (119/448)	1.5

\*Data are percentage of tumors showing HER2 positivity (number of tumors are in parentheses). Several different assays and cutoff points for defining HER2 positivity were used.

† Bamberger et al. (32) found increased HER2 levels to be associated with loss of PR-B but not PR-A or total PR.

## Open Discussion

**Dr. Mitch Dowsett:** If you look at the most recent Overview Analysis (*Lancet* 2005;365:1687–717), the benefit of tamoxifen versus nothing is 41% and 40% for the PR+ and PR– groups; thus, it is identical. Tamoxifen seems to be as beneficial, irrespective of PR status, in the Oxford Overview, the NSABP B-14 trial, and the NATO/CRC data. PR is a prognostic factor but is not a predictive factor for tamoxifen benefit. It is a predictive factor for even more benefit from aromatase inhibitors.

**Dr. Lee:** It may come down to the issue of how the steroid receptors were measured. Dr. Osborne has argued against the Overview Analysis on that basis. It took a long time for the Overview to show an ER predictive effect because of poor measurement of the receptors. In our hands, measured in-house at a single institution, there is strong predictive value of PR (*J Clin Oncol* 2003;21:1973–9).

**Dr. Kathleen Pritchard:** There was a quite a large number of patients in the Overview who didn't have PR status determined.

**Dr. Lee:** Yes, there are a substantial proportion of PR unknowns in BIG 1-98 as well and this becomes a problem in analyzing the data. In the 48-month follow-up ATAC data presented by Dr. Dowsett at San Antonio [*Breast Cancer Res Treat* 2003;82 (Suppl 1):S7], patients with PR– disease were doing much better with anastrozole compared with tamoxifen. But, in the BIG 1-98 trial, which is a very similar design of letrozole versus tamoxifen for 5 years, there is essentially no difference by PR status and letrozole is favored in both the PR+ and the PR– groups. So you end up with two trials that look very similar but have very different results. Obviously, this is going to require some serious consideration of how to clean these data up. Probably, there are several reasons for this, some of them technical, how many PR unknowns were in there, and how the measurement of ER and PR was done.

**Dr. Steven Come:** Because the differences in the ATAC analysis were pretty large, is it really plausible that technical difficulties could smooth all that out? It is only 1,600 patients, but the hazard ratio is huge.

**Dr. Dowsett:** If you look at the ER–/PR– subgroup, you get a hazard ratio of 1. If you look at the tamoxifen and the combination arms, the PR+ and PR– groups are juxtaposed in the survival curves, which is what you'd expect them to do. So there is a corroborative internal consistency there which makes me believe that the data are reasonable.

**Dr. James Ingle:** Can we talk about ER? You have dichotomized ER+/ER–. Is there value to quantitating the ER, discussing what is the threshold for ER positivity? This came up in the St. Gallen's overview, where level of endocrine sensitivity was the determinant for selection of therapy.

**Dr. Dowsett:** I think some of this is historic. Unlike the ligand-binding assay, current immunohistochemical techniques have a very narrow dynamic range. We have sensitized it to a degree, so that the ER– tumors are truly ER–. Then, we

have a bimodal distribution as we get to the higher values. So the tools we are currently using are not very good for quantitating ER. Now, the reverse transcription-PCR, which Dr. Lee showed, had a much broader dynamic range. Dr. Paik's recent data from the NSABP B-14 trial suggests that there is a really important interaction between ER level and tamoxifen benefit.

**Dr. Soonmyung Paik:** The B-14 data clearly show that if you have a meaningful quantitative measure, the response is linear, where increasing levels of ER translate into an increasing degree of clinical benefit in the adjuvant setting.

**Dr. Lee:** The only problem with those data is that RNA doesn't equal protein.

**Dr. Paik:** The ligand-binding assay data versus the reverse transcription-PCR data show only a modest correlation. But the ligand-binding assay data alone show an interaction with increasing levels of response. I think the direct test for your hypothesis, with PR being essentially a surrogate for HER2 function, is looking at the trastuzumab adjuvant trial.

**Dr. Lee:** Exactly. If HER2 is a direct regulator of PR, then patients who are PR– should respond better to trastuzumab than ones that are PR+.

**Dr. Aman Buzdar:** In our small neoadjuvant study in HER2-positive patients, we looked at the ER and PR status in patients who were treated with trastuzumab (*J Clin Oncol* 2005;23:3676–85). Pathologic complete response rate was very similar in patients who were both receptor negative versus either receptor positive. So, in this small study, receptor status did not predict benefit. Roughly half the patients were receptor positive, and half were both receptor negative. Our study is very small but all the assays were done in a single lab.

**Dr. Carlos Arteaga:** Based on what Dr. Dowsett was saying, you will find very few HER2-positive tumors that are positive for both steroid receptors, correct?

**Dr. Lee:** There are some ~10%. You would have to do a meta-analysis to get enough data. When you look at the literature, most of the literature is either ER+ or PR+; people have very rarely have subtracted that ER+/PR– subset out. That's the problem.

**Dr. Arteaga:** We've talked about examples in which too much activation of growth factor pathways may negate the effect of antihormone therapies. What about the opposite? That is, in tumors with high ER/PR levels, do growth factor receptor signaling inhibitors not work as well?

**Dr. Lee:** Possibly. If so, that doesn't bode well for these signaling inhibitors because you would predict that if that's true, they won't work very well in a double receptor-positive population, where there is probably low growth factor signaling. If we can design a study in a setting where the patients have some benefit, as in a neoadjuvant setting, then we can address that question. There is no addressing that issue in the advanced setting because clinical response is so rare.

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