Letrozole Inhibits Tumor Proliferation More Effectively than Tamoxifen Independent of HER1/2 Expression Status

Matthew J. Ellis, Andrew Coop, Baljit Singh, Yu Tao, Antonio Llombart-Cussac, Fritz Jänicke, Louis Mauriac, Erhard Quebe-Fehling, Hilary A. Chaudi-Ross, Dean B. Evans, and William R. Miller

Duke University Comprehensive Cancer Center, Durham, North Carolina 27710 [M. J. E., Y. T.]; Lombardi Cancer Center [A. C.] and Department of Pathology [B. S.]; Washington, D. C. 20007; Instituto Valenciano de Oncologia, C/P Beltran Baguena 8 y 19, E-46009 Valencia, Spain [A. L-C.]; Universiteits Frauen-und Polikliniek UKE, Martinistrasse 25, D-20246, Hamburg, Germany [F. J.]; Institut Bergonie, 180 rue de Saint-Germain F-33076, Bordeaux Cedex, France [L. M.]; Novartis Pharma AG [E. Q-F.] and Novartis Institute for Biomedical Research, Oncology Research [H. A. C.-R., D. B. E.], CH-4002 Basel, Switzerland; and Breast Research Unit, Pudererski Building, Western General Hospital, GB-Edinburgh EH4 2UX, Scotland [W. R. M.]

ABSTRACT

Background: The biological basis for the superior efficacy of neoadjuvant letrozole versus tamoxifen for postmenopausal women with estrogen receptor (ER)-positive locally advanced breast cancer was investigated by analyzing tumor proliferation and expression of estrogen-regulated genes before and after the initiation of therapy.

Methods: Tumor samples were obtained at baseline and at the end of treatment from 185 patients participating in a double blind randomized Phase III study of neoadjuvant endocrine therapy. These paired specimens were simultaneously analyzed for Ki67, ER, progesterone receptor (PgR), trefoil factor 1 (PS2), HER1 (epidermal growth factor receptor), and HER2 (ErbB2 or neu) by semiquantitative immunohistochemistry.

Results: The treatment-induced reduction in geometric mean Ki67 was significantly greater with letrozole (87%) than tamoxifen (75%; analysis of covariance P = 0.0009). Differences in the average Ki67 reduction were particularly marked for ER-positive tumors that overexpressed HER1 and/or HER2 (88 versus 45%, respectively; P = 0.0018). Twenty-three of 92 tumors (25%) on tamoxifen and 14 of 93 on letrozole (15%) showed a paradoxical increase in Ki67 with treatment, and the majority of these cases was HER1/2 negative. Letrozole, but not tamoxifen, significantly reduced expression of the estrogen-regulated proteins PgR and trefoil factor 1, regardless of HER1/2 status (P < 0.0001). ER down-regulation occurred with both agents, although levels decreased more with tamoxifen (P < 0.0001).

Conclusion: Letrozole inhibited tumor proliferation to a greater extent than tamoxifen. The molecular basis for this advantage appears complex but includes possible tamoxifen agonist effects on the cell cycle in both HER1/2+ and HER1/2− tumors. A pattern of continued proliferation despite appropriate down-regulation of PgR expression with estrogen deprivation or tamoxifen was also documented. This observation suggests the estrogenic regulation of proliferation and PgR expression may be dissociated in endocrine therapy resistant cells.

INTRODUCTION

The nonsteroidal aromatase inhibitors (letrozole and anastrozole) are more effective endocrine therapy than tamoxifen for postmenopausal women with hormone receptor-positive metastatic (1, 2), locally advanced (3), and early stage disease (4). The molecular basis for this therapeutic advance remains uncertain, but presumably differences in the pharmacological action of aromatase inhibitors and tamoxifen are responsible (5). Unlike tamoxifen, the nonsteroidal aromatase inhibitors do not exhibit intrinsic hormonal properties but affect ER1 function indirectly by blocking the conversion of adrenal androgens to estrogen in the peripheral (i.e., nonovarian) tissues of postmenopausal women, including the breast itself (6). When deprived of estrogens, the ER cannot bind to DNA and is therefore incapable of direct involvement in transcription. In contrast, tamoxifen has an intrinsic endocrine action by binding to the ER with high affinity and activating ER dimerization and DNA binding. However, tamoxifen-bound ER has altered gene regulatory properties referred to as SERM. In several normal tissues, tamoxifen-bound ER is active and promotes, e.g., bone mineralization and endometrial proliferation. In these instances, tamoxifen is acting as an “estrogen mimic” or receptor agonist. In other tissues, most notably in breast cancer cells, tamoxifen operates as an antagonist so that the function of ER is inhibited and growth is suppressed. Unfortunately, tamoxifen does not uniformly function as an antagonist in all breast cancers. It has long been held that tamoxifen is ineffective for a subgroup of estrogen-dependent breast cancers because the drug is operating as an agonist (7). Clinically, these tumors are tamoxifen resistant but may still be aromatase inhibitor sensitive. Data from the treatment of patients with advanced breast cancer suggest that around one-third of patients with tamoxifen-resistant disease are sensitive to subsequent estrogen deprivation (8–10). These observations argue for a tamoxifen-selective resistance mechanism that can be overcome by effective estrogen deprivation treatment.

The molecular mechanisms that determine responsiveness to tamoxifen and aromatase inhibitors are under intense investigation. These may include second messenger pathways, modulation of ER α function by ER β, and alterations in the composition of the complex that forms between ER and a host of transcription coactivator and corepressor proteins (11, 12). From the standpoint of clinical investigation, a number of studies have focused on the effect of HER1 (epidermal growth factor receptor) and HER2 (ErbB2, neu) on the efficacy of endocrine therapy (13). These closely related oncogenic plasma membrane tyrosine kinases predict a poor outcome for patients with advanced breast cancer receiving a spectrum of endocrine treatments (14). One possible model to explain these clinical observations is that the requirement for estrogen in HER1 and/or HER2-positive breast cancers has been bypassed, i.e., ER positive, HER1/2 positive tumors are functionally ER negative. Another possibility for tamoxifen-treated patients is that active HER1 and HER2 signaling promotes tamoxifen-dependent tumor growth (15, 16). This latter mechanism is specific to agents with SERM activity, i.e., tamoxifen and raloxifene. Aromatase inhibitors do not interact with ER, and so this resistance mechanism is not pertinent to this class of agents.

Recently, we conducted a prospective study of the relationship between HER1 and HER2 expression and the clinical activity of tamoxifen and letrozole in the context of a neoadjuvant endocrine therapy trial that compared the effectiveness of these two endocrine agents for patients with hormone receptor positive locally advanced primary breast cancer (17). This double blind randomized study
(Letrozole 024) demonstrated that letrozole was a more effective neoadjuvant therapy than tamoxifen with a superior response rate and a higher incidence of breast conserving surgery (3). HER1 and HER2 are heterodimerization partners, share downstream signal transduction pathways, and both linked to the development of endocrine resistance (14, 18). On this basis HER1 and HER2 were examined as a combined category in which trial outcomes were compared between ER+ tumors that were negative for both receptors versus a group that were positive for either HER1 or HER2 (or both). Within the subset of HER1+ and/or HER2+ (HER1/2+) tumors, letrozole proved much more effective than tamoxifen (clinical response rate of 88 versus 21%). These data therefore suggest that ER+, HER1/2+ primary breast cancers are usually estrogen dependent, and potent estrogen deprivation therapy is an effective treatment. However, the efficacy of tamoxifen is compromised.

This study further investigates these clinical observations by examining several indices of ER function at baseline and after treatment at the time of surgery. These biomarkers were chosen prospectively and included the proliferation marker Ki67. Ki67 is a simple way to gauge the effectiveness with which estrogen-dependent cell cycling is inhibited by endocrine treatment in clinical samples (19), and a recent preliminary report from investigators in Edinburgh suggests a correlation between suppression of Ki67 during neoadjuvant endocrine therapy and the subsequent efficacy of adjuvant endocrine therapy (20). The Royal Marsden group reported a randomized neoadjuvant trial that compared the aromatase inhibitor vorozole with tamoxifen. Ki67 levels fell within 2 weeks of treatment and remained suppressed at surgery 3 months later (21). The same group collected enough ER+, HER2+ cases from three different neoadjuvant or short-term perioperative endocrine therapy studies to have enough cases to examine the relationship between HER2 expression and the combined antiproliferative effects of either vorozole, tamoxifen, idoxifene, or anastrozole. They concluded that ER+, HER2+ breast cancers showed an impeded response to endocrine therapy, although the numbers were too small to allow a comparison between the antiproliferative effects of a SERM versus an aromatase inhibitor (22). We also studied changes in PgR and trefoil factor 1 (PS2 or TFF1) expression because these genes are directly regulated by ER through estrogen responsive promoter elements (23–26). Measurements of treatment-induced changes in expression from these genes reflect the transcriptional activity of ER at least with respect to the promoter activity of these two commonly used indicator genes.

MATERIALS AND METHODS

Patient and Tumor Bank Description. Between March 1998 and August 1999, patients were enrolled from 55 centers in 16 countries. The baseline characteristics of both treatment groups were well balanced for stage, age, surgical intent, and histological grade. A detailed description of patient characteristics and clinical outcomes has been published elsewhere (3). All patients provided written informed consent, and institutional review board approvals for 4 months unless the patient was withdrawn for PD, an adverse event, or on request by the patient or investigator. Surgery was scheduled 4 months from the date the patient received her first treatment to ensure there was no interval between the last study treatment day and the patient’s operation. A 3-year follow-up program to examine recurrence and survival is ongoing.

Study Assessment. Initial evaluation included clinical measurement of the primary breast lesion and regional lymph nodes, pathological diagnosis by core needle biopsy, and ER and PgR analysis by immunohistochemistry. Mammogram and ultrasound-based tumor measurements were also obtained, as well as two core biopsies for correlative science analysis. After initiating treatment, patients were assessed monthly for clinical response, adverse events, and concomitant medications/therapies, as well as months two and three by breast ultrasound. An ultrasound measurement was conducted after 1 month if there was a suspicion of progression. At 4 months, a surgical assessment was conducted, a final ultrasound and mammogram were obtained, and subsequently surgical outcomes were recorded. The primary efficacy end point was overall objective response, determined by breast palpation (clinical response), expressed as the percentage of patients in each treatment arm with a CR or a PR. Responses categories were defined according to WHO criteria as CR, PR, no change, PD, and not evaluable. Palpable ipsilateral axillary lymph node involvement downgraded a clinical CR. Clinical CR, PR, or no change was downgraded to “not evaluable” in cases of treatment for <4 months. Secondary efficacy endpoints were the percentage of patients who underwent breast-conserving surgery; the response rate (CR + PR) determined by mammography at 4 months; and the response rate (CR + PR) determined by ultrasound at 4 months.

Predictive Marker Analysis. Samples were shipped at ambient temperature to the Lombardi Cancer Center in 10% (volume for volume) buffered formalin and were paraffin embedded on receipt. All study analyses were conducted blind with respect to clinical outcomes, patient identity, and drug assignment using an anonymous sample coding system. Specimens containing invasive breast cancer in both the baseline specimen and post-treatment specimen were simultaneously immunostained using an automated immunostainer (Biogenex; San Ramon, CA) and the multistep Biotin–Avidin Complex amplification colormetric method (27). Sections were mounted on slides, incubated at 60°C for 1 h, and then deparaffinized and rehydrated. To improve antigenicity, slides were subjected to antigen retrieval by boiling sections in 10 mM citrate buffer (pH 6.0) for 10 min, and the incubation was continued for an additional 20 min as the specimen cooled. Slides were then incubated with 3% H2O2 for 5 min, washed with PBS, and blocked with normal goat serum in PBS (Biogenex) at 37°C for 5 min. After washing, samples were incubated with the appropriate antibody in diluent (Biogenex) at 37°C for 45 min. The following antibodies were used: for ER, PgR, and HER2 Clones ER1D5, PR1A6, and 3B5, respectively, (Immunotech/Coulter, Marseilles, France), HER1 Clone 31G7 (Zymed; San Francisco, CA) all at dilutions reported previously (17), Ki67 antibody at a dilution of 1:50 (Zymed) and Trefoil factor 1 antibody at a dilution of 1:50 (Zymed) and Trefoil factor 1 antibody at 47°C for 20 min with biotinylated multilink complex (Biogenex), washed, and finally with 3, 3′-diaminobenzidine tetrahydrochloride dihydrate (Biogenex) for 10 min for color development. Sections were lightly counterstained with 6% Mayers hematoxylin solution, washed, dehydrated, and cover-slipped.

Immunohistochemical Scoring. All samples were scored by both A.C. and B.S. who were blinded to drug assignment, treatment outcome, and timing. To qualify the specimen for this analysis, a simple cutoff method was used that considered an ER tumor positive if ≥10% of the nuclei in the invasive component of the tumor stained positive. To further assess the level of ER and PgR expression in both the baseline and surgical specimen, a
BIOMARKER MODULATION BY LETROZOLE VERSUS TAMOXIFEN

RESULTS

Clinical and Biomarker Characteristics of the Study Population. Only samples that were ER positive were included in this analysis. These “study biopsy proven” ER-positive cases were then reviewed for information on baseline HER1 and HER2 status and baseline and post-treatment Ki67. Only cases with complete information on all four of these biomarkers were included. This selection process generated a set of 185 cases for further study; 92 were treated with tamoxifen and 93 with letrozole. Paired information on ER was also available in 184 of these cases, paired PgR data in 183 cases and paired TFF1 information in 182 cases. The loss of a few data points was attributable to depletion of malignant tissue on deeper sectioning of a specimen.

Clinical trial outcomes were examined in the new “paired” biomarker subset to ensure that clinical findings and baseline biomarker relationships described in our initial analyses were reproduced despite the loss of cases attributable to the unfeasibility of biomarker analysis (Table 1). The difference in clinical response rates, 61 and 37% for letrozole versus tamoxifen, respectively (P = 0.0007), in the paired biomarker subset was similar in magnitude to the intent to treat analysis (55 versus 36%; Ref. 3) and to the “on study biopsy-confirmed” hormone receptor-positive subset (60 versus 41%; Ref. 17). Furthermore, the advantage for letrozole in terms of responses recorded by mammography was replicated (39 versus 23%; P = 0.0266). Letrozole was superior to tamoxifen for ER positive, HER1, and/or HER2 positive cases in the new biomarker subset with 87% of these tumors responding to letrozole versus 17% with tamoxifen (P = 0.0002). As with the study biopsy-confirmed hormone receptor positive subset, the advantages of letrozole over tamoxifen for ER-positive, HER1/2-negative cases were less striking, but nonetheless, an advantage was evident at both the level of clinical response (56 versus 41%; P = 0.0534) and mammography response (40 versus 23%; P = 0.0116). These similarities in clinical outcome measures between the intent to treat analysis, the confirmed hormone receptor positive subset, and the new paired biomarker subset suggested a random selection of cases from the intent to treat population without systematic bias in favor of one or the other treatment arm.

Table 2 provides information on the distribution of ER, HER1, and HER2 status on the letrozole arm versus the tamoxifen arm. The distribution of ER Allred scores was similar between the two arms and most cases fell within the strongly positive categories of 6, 7, and 8, and 9 (95% for letrozole and 97% for tamoxifen). A similar number of cases on the two arms were HER1 and/or HER2 positive (16% for letrozole and 18% for tamoxifen). The HER1/2-positive cases had a comparable ER Allred distribution to the rest of the biomarker subset (94% within the Allred score range of 6, 7, and 8), i.e., there was no tendency for the HER1/2-positive subset to have a lower ER score than the HER1/2-negative subset. This finding is in contrast to other investigators (32) but probably reflects the fact that this analysis specifically excluded any case in which the ER IHC was below the cutoff value of 10%. We concluded from these data that our analyses of biomarker changes with treatment were focused on unequivocally ER-positive cases without an imbalance between the two treatment arms in terms of the baseline expression of ER, HER1, or HER2.

Table 1 Clinical outcomes for cases with a complete paired biomarker analysis. P values were based on a stratified Mantel-Haenszel chi-squared test adjusted for tumor size and clinical nodal status

<table>
<thead>
<tr>
<th></th>
<th>Letrozole</th>
<th>Tamoxifen</th>
<th>P</th>
</tr>
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<tbody>
<tr>
<td>Overall response CR plus PR</td>
<td>93</td>
<td>92</td>
<td>0.0007</td>
</tr>
<tr>
<td>Clinical</td>
<td>57 (61%)</td>
<td>34 (37%)</td>
<td>0.0266</td>
</tr>
<tr>
<td>Mammmogram</td>
<td>36 (39%)</td>
<td>21 (23%)</td>
<td>0.0759</td>
</tr>
<tr>
<td>HER1/2-positive subset</td>
<td>15</td>
<td>17</td>
<td>0.0002</td>
</tr>
<tr>
<td>Overall response CR plus PR</td>
<td>13 (87%)</td>
<td>3 (17%)</td>
<td>0.0002</td>
</tr>
<tr>
<td>Clinical</td>
<td>5 (33%)</td>
<td>1 (6%)</td>
<td>0.0597</td>
</tr>
<tr>
<td>Mammmogram</td>
<td>78</td>
<td>75</td>
<td>0.0534</td>
</tr>
<tr>
<td>HER1 and HER2-negative subset</td>
<td>44 (56%)</td>
<td>31 (41%)</td>
<td>0.0316</td>
</tr>
<tr>
<td>Overall response CR plus PR</td>
<td>31 (40%)</td>
<td>21 (23%)</td>
<td>0.0316</td>
</tr>
<tr>
<td>Mammmogram</td>
<td>6525</td>
<td></td>
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To validate the Ki67 analysis, the significance of these changes was initially examined by ANCOVA according to methodology recommended by Vickers and Altman (31). To use this parametric test, the data were log transformed to simulate a normal distribution. Within the whole study, letrozole had a statistically significant greater effect on proliferation than tamoxifen (87% inhibition of geometric mean Ki67 versus 75%; \( P = 0.0009 \)). This advantage for letrozole was observed in both the HER1/2-negative subset (86 versus 79%; \( P = 0.0149 \)) and the HER1/2-positive subset (88 versus 45%; \( P = 0.0018 \)). These data are consistent with the conclusion that letrozole was a more effective antiproliferative treatment than tamoxifen, regardless of HER1/2 expression status.

To try and gain insights into the nature of endocrine therapy resistance, an exploratory analysis was conducted in which treatment-induced changes in the Ki67 score were examined on a case-by-case basis. Fig. 1 presents raw data on changes in the percentage of Ki67-positive cells in HER1/2+ tumors treated with either tamoxifen (Fig. 1A) or letrozole (Fig. 1B). On treatment with letrozole, 11 HER1/2+ cases showed a decrease in Ki67, 1 exhibited no change, and 3 showed an increase of which only one was >2-fold (0.1–0.3%). At such low levels of staining, classifying this particular tumor as showing an “increase” is probably spurious. Of the 17 HER1/2+ cases treated with tamoxifen, 10 showed a decrease and 7 an increase in Ki67, of which 3 were relatively dramatic (9.5–22.7%, 20.9–40.7%, and 0.1–17.3%). Thus, a more complete interpretation of the data are to state that tamoxifen often reduced proliferation in HER1/2+ cases, but this effect was balanced by a similar number of cases that showed an increase in proliferation with treatment. These data therefore demonstrate that HER1/2 analysis is not a rigorous test for whether tamoxifen will be an effective antiproliferative agent in any given tumor. Nonetheless, the HER1/2+, ER+ subgroup was “enriched” for cases in which tamoxifen therapy was associated with an increase in Ki67 (41% showing an increase in Ki67 with treatment in the ER+, HER1/2+ subset versus 21% in ER+, HER1/2− subset). This was not the case for letrozole, where only 13% of the ER+, HER1/2+ cases showed an increase in Ki67 versus 15% in the ER+, HER1/2− subset. An additional point to make is that the majority of cases in which an increase in Ki67 was observed were HER1 and HER2 negative. If cases in which the Ki67 estimates were <1% in both the baseline and surgical specimen were excluded because of measurement uncertainty, 23 cases treated with tamoxifen exhibited an increase (25%), but of these, only 7 were HER1/2 positive (30%). On the letrozole arm, 14 cases showed an increase, 2 of which were HER1/2 positive (14%).

**Letrozole versus Tamoxifen: Effects on the ER-regulated Genes PgR and TFF1.** To examine the effects of letrozole and tamoxifen treatment on estrogen-regulated gene expression, statistical summaries were prepared for the changes in PgR and TFF1 Allred scores on each treatment arm. The data presented in Fig. 2, A and B illustrate the effect of treatment on PgR expression by plotting the proportion of cases in each Allred category at baseline and after treatment at surgery. Average tumor PgR expression decreased dramatically on letrozole treatment (\( P = 0.0001 \); one sample Wilcoxon signed rank test).
test) with a corresponding marked increase in the proportion of PgR-negative cases (Allred scores of 0 and 2) from 22 to 78% (Fig. 2A). The effect of letrozole was so profound that only 4.4% of surgical specimens exhibited an Allred score of between 6 and 8. In contrast, changes in PgR expression with tamoxifen therapy were not consistent with both increases and decreases in expression frequently observed. Consequently, there was no significant overall change in the average PgR Allred score, although there was a trend for the proportion of PgR-negative cases to decrease rather than increase in the post-treatment specimens (from 29.6 to 20.9%; Fig. 2B). The relationships between the degree of PgR change and likelihood of a response were explored using the Mann-Whitney test. There was no significant difference in the degree of reduction in ER expression (Fig. 3, A and B). In 14.3% of the cases treated with tamoxifen, the ER level fell into the negative range versus 4.3% with letrozole, and overall ER Allred scores decreased more in the tamoxifen arm than in the letrozole arm (P = 0.0001, two-sample Wilcoxon signed rank test). In vitro data have demonstrated a role for overactive growth factor signaling in the down-regulation of ER expression (34). We therefore examined ER levels in the HER1/2-positive subset to determine whether HER1/2 expression affected the degree of change in ER expression. (Fig. 3, C and D). As with the entire population, reductions in ER expression occurred. Four of 17 HER1/2+ cases (23%) become ER negative (Allred score of 0 or 2) on the tamoxifen arm compared with only 1 of 15 cases (6%) treated with letrozole. These proportions were similar to the unselected cases, and the two sample signed rank test indicated no statistical differences in the change in ER status according to subsets defined by HER1/2 status. We therefore concluded that the effects of endocrine treatment on ER expression were variable but marked down-regulation frequently occurred by the time surgery took place particularly for tumors treated with tamoxifen. The highly significant difference in the degree of reduction in ER expression between letrozole and tamoxifen suggests that the down-regulation mechanisms activated by these two endocrine agents may be distinct. HER1/2 status did not obviously impact on the down-regulation process, although the total number of HER1/2-positive cases was small, and a modest effect could have been missed.

A Comparison of Treatment-induced Changes in Ki67 and PgR Expression. Theoretically, if an increase in Ki67 with treatment was the result of agonist effects of tamoxifen, then there should be a concordant increase in both Ki67 and PgR. In instances where Ki67 increased on the tamoxifen arm (Fig. 4A), the treatment effect was variable (PgR increased in 9, decreased in 8, and was unchanged in 1). In five cases, PgR was not expressed in either sample, and so these cases could not be categorized. Increases and decreases were seen in both HER1/2-positive cases (indicated by the dashed lines), as well as HER1/2-negative cases. For the 14 letrozole-treated tumors exhibiting an increase in Ki67, PgR expression did not change in one, and in two, there was no expression, leaving 11 cases with a definitive change with treatment (Fig. 4B). Of these, PgR decreased in 8 and increased in 3. These data illustrate that changes in PgR expression and proliferation with treatment are not coordinated processes, with down-regulation of PgR expression still occurring in response to estrogen deprivation despite lack of cell cycle arrest.

DISCUSSION

The biological basis for the efficacy of breast cancer endocrine treatment is complex but includes the induction of G1-S phase cell cycle arrest, apoptosis, and changes in gene expression related to inactivation of ER-dependent transcription. In addition, endocrine agents may impact on angiogenesis, differentiation, invasion, and
metastasis to produce a therapeutic effect, although exactly how estrogen regulates these biological processes is far from clear. Given these wide-ranging effects of endocrine therapy, the surrogate marker analysis described in this study is admittedly narrowly focused on only two classes of biomarkers, a measure of proliferation (Ki67), and two surrogates for the transcriptional activity of the ER (PgR and TFF1). Despite this simple approach, our analysis does provide insight into the biological basis for differences in the clinical efficacy between aromatase inhibitors and tamoxifen.

Inhibition of estrogen-dependent proliferation is a major component of the clinical activity of effective endocrine therapy. Inhibition of cell growth by antiestrogens was believed to occur largely through modulation of the transcriptional functions of ER (12). However, an intricate interplay between ER, other transcription factors from the AP1 family (35), together with coactivators and corepressors (26), G1 cyclins, cyclin-dependent kinases, and kinase inhibitors (36), is now thought to promote cell cycle progression on estrogen exposure. Recent experimental results suggest that endocrine treatment must appropriately modulate all these components of the G1-S transition mechanism to avoid the development of resistance (36). In this study, we used the Ki67 biomarker to gain an insight into the effect of endocrine therapy on tumor proliferation. This simple but well-validated measure of active cell cycling was used in three types of analysis: (a) to compare the effectiveness each endocrine therapy had on proliferation; (b) as a means to compare antiproliferative effects of each drug in subgroups defined by HER1 and HER2 status; and (c) to classify tumors as resistant or sensitive to the cell cycle effects of endocrine therapy to assist in the examination of other biomarkers that might provide insights into why a tumor would fail to undergo growth arrest in response to an endocrine intervention.

The results from this Ki67 analysis demonstrate that letrozole more effectively inhibits the proliferation of ER-positive breast cancer than tamoxifen. This result is in accordance with clinical efficacy data that indicate letrozole is more effective therapy for locally advanced and metastatic ER+ breast cancer. As well as providing an explanation for these clinical observations, this biomarker/clinical outcome concordance further validates the choice of Ki67 as an effective surrogate end point biomarker for the efficacy of endocrine agents in breast cancer treatment. Another conclusion that can be drawn from these data are that the efficacy of breast cancer endocrine therapy is dependent on the successful induction of the arrest of cell proliferation, although further analysis would be required to see if any therapeutic activity of
endocrine treatment could be identified in the absence of an effect on tumor proliferation.

A comparison of the antiproliferative effects of letrozole and tamoxifen according to HER1/2 status (Table 3) underscores the conclusion that letrozole, at least in the neoadjuvant treatment setting, is more effective than tamoxifen for the treatment of ER+ primary breast cancers. To explain this observation, we had earlier proposed that HER1/2 signaling promoted the agonist properties of tamoxifen. Agonist effects are not possible with letrozole, and so estrogen deprivation proved to be an effective treatment strategy in this tumor subtype (17). The Ki67 proliferation analysis presented in this study provides some support for this hypothesis because the letrozole maintained a significant inhibitory effect in the presence of HER1/2 expression, whereas tamoxifen did not. However, if arguments regarding agonism rest on documenting the number of cases exhibiting a paradoxical increase in Ki67, a more complicated picture emerges. Although there were more increases with tamoxifen (23 of 92) than letrozole (14 of 93), this difference only trended toward significance ($P = 0.09$ Mantel Haenszel $\chi^2$ test). However, this exploratory analysis based on categorical assignment of Ki67 outcomes is a weaker statistical approach than ANCOVA analysis, and the number of “resistant” tumors in the study is too small to generate a definitive statistical conclusion using contingency tables. The fact that increases were seen with both agents demonstrates that tamoxifen agonism should be viewed as only one potential explanation for an increase in Ki67. An additional hypothesis is that clonal selection over 4 months of treatment can uncover a subclone of cells that grow in an estrogen-independent and endocrine therapy-resistant manner. Assay imprecision could also account for some instances in which Ki67 increased. Random variability in Ki67 measurement is difficult to quantify in the absence of data from a placebo control arm, which would be unethical in the context of a neoadjuvant study. However, the question of variability can be addressed from studies of brief endocrine therapy exposures conducted in the window between diagnosis and definitive surgery. These investigations indicate that some degree of fluctuation in paired biomarker measurements can be expected in the absence of treatment, although the median percentage change in Ki67 on the placebo arm of a recently reported perioperative study was low (7% median difference post-treatment versus baseline; Ref. 37). In light of these data, the large changes in Ki67 we observed with treatment are unlikely to be consistently caused by assay imprecision.
These data contribute to ongoing concerns regarding the reliability of HER1/2 analysis as a biomarker for tamoxifen resistance. Examination of the tamoxifen-treated ER+, HER1/2+–treated cases as a group indicates that tamoxifen was not an efficient antiproliferative agent overall; however, the antiestrogen was not completely inactive with several instances of quite marked falls in Ki67 in post-treatment samples. In addition, the majority of the cases exhibiting an increase in Ki67 were HER1/2 negative, indicating other explanations for the failure of endocrine therapy to induce growth arrest must be explored.

A more complete view of the role of HER1/2 in ER function needs to evolve, and the additional factors that determine whether tamoxifen will be effective in the presence of HER1/2 must be identified. A role for the estrogen regulated transcription factor AIB1 has recently been proposed because patients with HER2+ and AIB1+ tumors fair particularly poorly on adjuvant tamoxifen therapy (38).

The finding that letrozole induced profound down-regulation of PgR and TFF1 expression is not surprising given the estrogen-dependent expression patterns of these genes and recapitulates findings in other neoadjuvant aromatase inhibitor studies (19, 33). This down-regulation is in marked contrast to the mixed agonist/antagonist effect of tamoxifen on PgR and TFF1 expression and underscores profound differences in the molecular effects of these two agents. Interestingly, the degree of PgR suppression was greater for tumors in which a mammographic response was documented. This raises the possibility that PgR expression in post-treatment samples could be a useful surrogate biomarker for the effectiveness of estrogen deprivation therapy. This possibility will have to be explored further in new studies. Another novel feature of these data were to have enough cases to compare PgR and TFF1 changes within the HER1/2–positive subset. Several studies have suggested that in the presence of active HER1/2 signaling, ER activity becomes ligand independent (39, 40). If this mechanism was in operation, treatment with an aromatase inhibitor might fail to alter ER-dependent gene expression because ER function would continue without a ligand. We found this was not generally the case, however, because PgR levels were often profoundly inhibited by letrozole in ER+, HER1/2+ cases. These data, in conjunction with the changes in Ki67, suggest that the ER remains largely ligand dependent in HER1/2+ primary breast cancer. One could reasonably argue, however, that simply examining PgR expression is an incomplete look at this question because ER could operate in a ligand-independent manner only when operating indirectly through nonclassical genomic pathways (AP-1). An expression analysis that covers a wide spectrum of estrogen sensitive genes regulated by both classical and nonclassical genomic pathways is now under way to gain a more complete insight into how tumor response relates to treatment-induced changes in gene expression.

Down-regulation of ER with endocrine treatment is a long-standing observation, and in early studies, it was recognized that initially positive breast cancers treated with adjuvant tamoxifen can become ER negative on relapse (41). More recently, it has been shown that down-regulation of ER can occur very rapidly on initiation of endocrine therapy. In a study by Harper-Wynne et al. (21), both vorozole and tamoxifen induced a decrease in ER expression within weeks of treatment. The mechanism of tamoxifen and aromatase inhibitor–induced ER down-regulation is unclear and extremely variable. However, our observation that ER down-regulation occurs to a greater extent with tamoxifen than letrozole implies that the mechanisms associated with aromatase inhibitors and tamoxifen may be different. ER down-regulation is known to occur on estrogen treatment and has been considered a homeostatic effect (42, 43). Theoretically therefore, down-regulation with letrozole is more difficult to explain because up-regulation of ER is the expected response to estrogen deprivation. Additional mechanisms may therefore come into play to alter ER expression levels in estrogen-deprived breast cancers, perhaps including clonal selection of preexisting ER-negative subclones. One practical conclusion of these findings is that ER measurements after the initiation of endocrine therapy could give false negative results, and so every effort should be made to establish the ER status of the tumor before initiating neoadjuvant endocrine treatment.

Changes in Ki67 were used to identify a subgroup of tumors that failed to exhibit endocrine therapy-induced cell growth arrest. Defining resistance as any tumor that exhibited an increase in Ki67 with treatment, 25% of tamoxifen-treated tumors and 15% of letrozole-treated tumors had a defective “cell cycle response” to endocrine therapy. Recent studies suggest that estrogen-independent growth may occur though an imbalance in the functions of the cyclins, cyclin-dependent kinases, and cyclin-dependent kinase inhibitors, e.g., experimental suppression of the cyclin-dependent kinase inhibitor p27 in MCF7 cells leads to proliferation in the presence of tamoxifen or the absence of estrogen (36, 44). Similarly, overexpression of G1-S cyclins can also overcome endocrine therapy-induced growth arrest, presumably by sequestering cyclin-dependent kinase inhibitors to inactive complexes (45–47).

Discordance between the estrogenic modulation of PgR expression and cell growth has been noted in preclinical studies (48). In MDA MB 134 cells, estrogen stimulates cell growth but does not increase PgR expression in the manner observed in MCF7 cells. Our data suggest that the opposite situation may also occur in vivo, with estrogen deprivation with letrozole failing to suppress proliferation under circumstances in which PgR expression is nonetheless inhibited. In such cases, we speculate that specific lesions prevent G1-S arrest might sever the link between estrogen exposure and the cell cycle but leave other aspects of estrogen regulation, such as modulation of PgR expression, intact. Ongoing studies are attempting to relate the expression of the G1-S checkpoint components (such as p27) with clinical and biomarker outcomes in this data set.

The examples of treatment-induced or “dynamic” biomarker findings presented in this study can be viewed as an example of how neoadjuvant therapy trials can be used to reveal the molecular fundamentals of endocrine treatment for breast cancer. This study also supports the evolving concept that endocrine therapy resistance is not attributable to a single event or pathway. With more complete insights into these mechanisms, it may be possible to diagnose hormone receptor positive yet endocrine therapy resistant breast cancer at the time of diagnosis, through presurgical exposure to estrogen deprivation therapy.

ACKNOWLEDGMENTS
We thank the physicians, research staff, and patients who participated in the Letrozole 024 clinical trial.

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BIOMARKER MODULATION BY LETROZOLE VERSUS TAMOXIFEN


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