A Single-Nucleotide Polymorphism in the Aromatase Gene Is Associated with the Efficacy of the Aromatase Inhibitor Letrozole in Advanced Breast Carcinoma

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Abstract

Purpose: To evaluate the efficacy of treatment with the aromatase inhibitor letrozole in breast cancer patients segregated with respect to DNA polymorphisms of the aromatase gene CYP19.

Patients and Methods: Postmenopausal patients (n = 67) with hormone receptor–positive metastatic breast cancer were treated with the aromatase inhibitor letrozole. PCR allelic discrimination was used to examine three single-nucleotide polymorphisms (SNP) in DNA obtained from breast carcinoma tissue. Two SNPs analyzed (rs10046 and rs4646) were located in the 3’ untranslated region and one (rs727479) was in the intron of the aromatase CYP19 gene. The primary end point of treatment efficacy was time to progression (TTP).

Results: Median age was 62 years and median number of metastatic sites was 2. Observed allelic SNP frequencies were rs10046, 71%; rs4646, 46%; and rs727479, 63%. Of the 67 patients, 65 were evaluable for efficacy. Median TTP was 12.1 months. We observed no relationship between TTP and the rs10046 or rs727479 variants. In contrast, we found that TTP was significantly improved in patients with the rs4646 variant, compared with the wild-type gene (17.2 versus 6.4 months; P = 0.02).

Conclusion: In patients with hormone receptor–positive metastatic breast cancer treated with the aromatase inhibitor letrozole, the presence of a SNP in the 3’ untranslated region of the CYP19 aromatase gene is associated with improved treatment efficacy. Testing for the CYP19 rs4646 SNP as a predictive tool for breast cancer patients on antiaromatase therapy deserves prospective evaluation.

Aromatase inhibitors, when administered to postmenopausal women, prevent the conversion of androgens to estrogens via inhibition of the aromatase enzyme. The antiaromatase compounds have emerged as a family of potent target-directed agents in the hormonal treatment of breast cancer. Third-generation aromatase inhibitors are used in the treatment of metastatic breast carcinoma (1, 2) and in the adjuvant setting (3–5).

Currently, the clinical indication for the use of aromatase inhibitors in breast cancer patients is guided by two criteria: a postmenopausal status and a positive hormone receptor status. Menopause is the critical criterion because functioning ovaries synthesize estrogen in an amount that would preclude aromatase inhibitors from being active. Positivity of estrogen or progesterone receptors in breast carcinomas has been related to the efficacy of tamoxifen as well as aromatase inhibitors. A recent review concluded that, when using anastrozole or letrozole as first-line treatment of patients with metastatic breast cancer, positive hormone receptor status is of prime importance in improving the time to disease progression (6). However, the clinical relevance of hormone receptors when using aromatase inhibitors is moderate because only 30% of the patients exhibit an objective clinical response (7–9) and, therefore, the power to discriminate potentially responding from nonresponding patients is low. Additional biomarkers that could help in predicting the efficacy of aromatase inhibitors in the clinical setting are being avidly sought as guides in the use of these target-directed drugs (10).

Approximately two thirds of human breast carcinomas express aromatase protein or show aromatase enzyme activity (11–14). However, to date, the levels of aromatase protein or biochemical activity measured in breast carcinomas have failed to show a clear clinical benefit following the administration of aromatase inhibitors (15–17).
Polymorphisms in the aromatase CYP19 gene have been shown to alter aromatase activity in postmenopausal women (18, 19). Although CYP19 polymorphisms have been examined in relation to breast cancer risk in healthy women, and several CYP19 gene variants have been associated with a lower cancer risk (20–24), no study of CYP19 polymorphisms has addressed the possible relationships between these variants and the prognosis of breast cancer patients, or with the efficacy of antiaromatase agents.

We undertook the present investigation to assess whether the presence of CYP19 gene polymorphisms can have an effect on the clinical activity of the aromatase inhibitor letrozole in patients with hormone receptor–positive advanced breast carcinoma.

Materials and Methods

Patients. Patients included in this open prospective study were required to have the following inclusion criteria: (a) ambulatory and postmenopausal with advanced breast cancer previously treated with antiestrogens (tamoxifen) and proven objective tumor progression; (b) performance status of 0 to 2 on the Eastern Cooperative Oncology Group scale; (c) estrogen receptor positive and/or progesterone receptor positive; (d) age > 18 years; (e) life expectancy > 3 months; (f) written informed consent to participation in the study; and (g) serum sample available. The exclusion criteria were (a) life-threatening or rapidly progressing metastases (central nervous system, pulmonary lymphangitis, inflammatory breast cancer), evidence of liver metastases involving > 30% of the organ detected by ultrasound and/or computed tomography scan; (b) other concurrent or previous malignancies except for contralateral breast carcinoma, cone biopsy in situ carcinoma of the cervix, or adequately treated basal or squamous cell carcinoma of skin; (c) patients who have lymphedema, ascitis, central nervous system metastases, or medullary suppression as the unique manifestation of the disease; (d) concomitant antineoplastic treatments; (e) other concomitant endocrine therapy except topical, inhaled, or intra-articular corticosteroids; (f) known hypersensitivity to letrozole or to any other component of the drug; (g) any other investigational medications without a washout period of 7 days for a topical drug and 30 days for a systemic drug; (h) involvement in another clinical trial within the previous 4 weeks; and (i) legal and/or other circumstances indicating that patient is not able to understand the design of the study and its consequences.

The study included 67 postmenopausal patients recruited between April 1999 and November 2000. All had advanced hormone receptor–positive breast cancer. All patients provided signed informed consent, and the study received approval from the ethics committees of the participating institutions.

Pretreatment evaluation included the following clinical variables: patient characteristics, menopausal status, disease history and diagnosis, hormone receptor status, prior antineoplastic treatments, Eastern Cooperative Oncology Group performance status, tumor assessment, and serum CA 15-3, a surrogate marker of metastatic tumor burden (25).

The treatment used was letrozole (2.5 mg, oral, daily) until disease progression or unacceptable toxicity occurred. A complete tumor assessment was done at baseline, and areas found positive for metastatic target lesions were monitored every 3 months for response assessment. Patients were assessed until there was evidence of disease progression, as defined by WHO criteria. All patients were monitored for survival at 6 months after conclusion of treatment. No local treatments of the target lesions were allowed within the study protocol. Complete response required the complete disappearance of all disease for at least 4 weeks, and partial response was defined as a reduction of ≥ 50% in tumor volume. Time to progression (TTP) was the principal end point for treatment efficacy. TTP was calculated from the date of entry into the study up to the date of progression, or death. Overall survival was calculated from the date of entry into the study up to the time of death. Patients who were alive and were not known to have disease progression were censored at the last date they were known to be alive. Presentation of the data conforms to the REMARK criteria (reporting recommendations for tumor marker prognostic studies; ref. 26).

DNA isolation. DNA was obtained from paraffin-embedded tissue obtained from biopsy of breast carcinomas. Samples were de waxed with two washes of xylene, followed by the addition of 1 mL of 100% ethanol to remove residual xylene. After de waxing, DNA was obtained using the commercial DNeasy tissue kit (Qiagen) according to the manufacturer’s protocol. The DNA obtained was incubated at 95°C for 10 min to inactivate the proteinase K used in the extraction procedure.

Single-nucleotide polymorphism selection. We searched the National Center for Biotechnology Information single-nucleotide polymorphism (SNP) database16 for polymorphisms of the CYP19A1 (aromatase) gene. At the time of the search (January 2004), there were 275 SNPs in the database, of which 41 had a rate of heterozygosity > 20%. Of these, 30 were found in the 3’ untranslated region (UTR), 10 were in the intronic region, and 1 was exonic. We selected three sequences of the aromatase CYP19 gene, two located in the 3’-UTR and one in the intronic region. At the time of the study,

<table>
<thead>
<tr>
<th>dbSNP</th>
<th>% Allele frequency</th>
<th>Genotype frequency (literature values)</th>
<th>Nucleotide position</th>
<th>Gene location</th>
<th>Primers and probes: supplier’s codes</th>
</tr>
</thead>
<tbody>
<tr>
<td>rs10046</td>
<td>C 54.7 T 55.3</td>
<td>CC 0.39 CT 0.41 TT 0.19</td>
<td>1,642</td>
<td>3’-UTR</td>
<td>C_8234731_1</td>
</tr>
<tr>
<td>rs4646</td>
<td>G 67.9 T 32.1</td>
<td>GG 0.45 GT 0.43 TT 0.12</td>
<td>1,814</td>
<td>3’-UTR</td>
<td>C_8234730_1</td>
</tr>
<tr>
<td>rs727479</td>
<td>T 75.6 G 24.4</td>
<td>TT 0.559 TG 0.354 GG 0.087</td>
<td>96,248</td>
<td>Intronic</td>
<td>0007139326</td>
</tr>
</tbody>
</table>

Abbreviation: dbSNP, data base single-nucleotide polymorphism.
there were no available data on functional correlates. Due to the explanatory nature of the study, the number of SNPs analyzed was limited to three. Cases were categorized as either normal homozygous [wild-type (WT)] or polymorphic variant (heterozygous or variant homozygous).

**Primers and probes.** Primers and probes were obtained from Applied Biosystems Assays-on-Demand SNP Genotyping product. Three primers and probes for CYP19A1 were used; two in 3'-UTR location and one in intronic location. The primers and probes were designed in the following reverse DNA sequences to detect the SNPs: CYP19A1 #1 (rs10046), TCTGTTGTAACAGGAGCAGATGCA/[A/C]AATGCCTGTACGGTGACAGCCA, where [A/C] is the SNP region and A is labeled with VIC fluorochrome and C with FAM fluorochrome; CYP19A1 #2 (rs4646), CATCTGATGGAGAATGCTCCAGT[TA/G]G/GTTACTGCCGCCCTTTCTCTGACT, where [TA/G] is the SNP region and A is labeled with VIC fluorochrome and G with FAM fluorochrome; and CYP19A1 #3 (rs727479), TCTCTCCCTTGACCACCTTTCTCTAA/[A/C]GAGAGATTTTGCGTTTGCCAGGATTT, where [A/C] is the SNP region and A is labeled with VIC fluorochrome and C with FAM fluorochrome. The detailed characteristics of the SNPs analyzed are summarized in Table 1.

**Genotyping.** SNP analysis was done using a real-time PCR allelic discrimination TaqMan assay according to the manufacturer’s (Applied Biosystems) instructions with minor modifications. All PCR reactions were run in duplicate and contained 100 ng of patient DNA, 12.5 μL of TaqMan Universal Master Mix (Applied Biosystems), 1.2 μL of primers and probes, and water up to a final volume of 25 μL. Appropriate negative controls were also run. Real-time PCR was done on an ABI Prism 7500 Sequence Detection System (Applied Biosystems) using the following conditions: 50°C for 2 min, 95°C for 15 s and 62°C for 1 min. For each cycle, the software measures the fluorescent signal from the VIC- or FAM-labeled probe.

**Marker analyses.** Human epidermal growth factor receptor 2 (HER2) in primary tumor tissue was assessed with the anti-HER2 antibody CB11 (Biogenex) at a dilution of 1:80 as well as with a streptavidin-biotin detection system (kit LSAB2, DAKO). Color development was with diaminobenzidine using Harris’ hematoxylin counterstain. Membrane HER2 staining was quantified in percentages (0-100%). Cases were considered positive when ≥10% of tumor cells had intense membrane staining (HER2 3+ score). CA 15-3 was determined in the serum before the start of letrozole therapy. Values ≤30 units/mL were considered negative whereas values >30 units/mL were considered positive.

**Statistical methods.** Discrete variables were described by absolute and relative frequencies. Continuous variables were described by the mean, median, SD, and range. The association of aromatase SNPs with clinical parameters was evaluated with the χ² test. Although this was an exploratory study, we adjusted the P value of the response evaluation using the Bonferroni correction. Kaplan-Meier survival curves were used to compare time-to-event variables such as TTP for the groups of patients. The long-rank test was used to compare the survival curves. Multivariate analysis were done using a Cox regression test. All statistical analyses were done using SPSS (version 11.5) for Windows.

### Results

Median age of the patients was 62 years, and median Eastern Cooperative Oncology Group performance status was 0 (range, 0-2). Median number of metastatic sites was 2 (range, 1-6), and the number of patients with elevated serum CA 15-3 was 27, whereas 19 patients had normal values. Forty tumors were estrogen receptor+/progesterone receptor+, 16 were estrogen receptor+/progesterone receptor-, and 8 were estrogen receptor-/progesterone receptor-. HER2 status was negative in 49 cases and positive in 15 cases.

Median follow-up was 19.7 months. Median TTP was 12.1 months. Median survival from the time of diagnosis of metastasis was not reached at the time of the present analysis. Best response evaluation indicated that 8 cases had achieved complete response, 10 had partial response, 28 had stable

### Table 2. Genotype frequency observed in the population studied

<table>
<thead>
<tr>
<th>n</th>
<th>Homozygous WT, n (%)</th>
<th>Variant forms</th>
<th>Heterozygous variant, n (%)</th>
<th>Homozygous variant, n (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>rs10046</td>
<td>65</td>
<td>CC 19 (29)</td>
<td>CT 29 (45)</td>
<td>TT 17 (26)</td>
</tr>
<tr>
<td>rs4646</td>
<td>67</td>
<td>GG 36 (54)</td>
<td>GT 26 (39)</td>
<td>TT 5 (7)</td>
</tr>
<tr>
<td>rs727479</td>
<td>64</td>
<td>TT 23 (37)</td>
<td>TG 27 (41)</td>
<td>GG 14 (22)</td>
</tr>
</tbody>
</table>

**Table 3. Correlations of CYP19 and clinical variables**

<table>
<thead>
<tr>
<th>Variable</th>
<th>CYP19 gene rs4646</th>
<th>n</th>
<th>WT, n (%)</th>
<th>Variant, n (%)</th>
<th>P</th>
</tr>
</thead>
<tbody>
<tr>
<td>ECOG performance status</td>
<td>0</td>
<td>36</td>
<td>20 (56)</td>
<td>16 (44)</td>
<td>NS</td>
</tr>
<tr>
<td>1-2</td>
<td>29</td>
<td>16 (55)</td>
<td>12 (45)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>No. metastatic locations</td>
<td>1</td>
<td>26</td>
<td>11 (42)</td>
<td>15 (58)</td>
<td>NS</td>
</tr>
<tr>
<td>2</td>
<td>18</td>
<td>12 (67)</td>
<td>6 (33)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>&gt;2</td>
<td>22</td>
<td>13 (59)</td>
<td>9 (41)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>CA 15-3</td>
<td>Negative</td>
<td>19</td>
<td>11 (58)</td>
<td>8 (42)</td>
<td>NS</td>
</tr>
<tr>
<td>Positive</td>
<td>27</td>
<td>14 (52)</td>
<td>13 (48)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>ER status</td>
<td>ER+/PR+</td>
<td>40</td>
<td>20 (50)</td>
<td>20 (50)</td>
<td>NS</td>
</tr>
<tr>
<td>ER+/PR-</td>
<td>16</td>
<td>9 (56)</td>
<td>7 (44)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>HER2 status</td>
<td>Negative</td>
<td>49</td>
<td>28 (57)</td>
<td>21 (33)</td>
<td>NS</td>
</tr>
<tr>
<td>Positive</td>
<td>15</td>
<td>8 (53)</td>
<td>7 (47)</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

**NOTE:** WT, wild-type (GG); variant, heterozygous or homozygous for the SNP rs4646 (GT or TT).

Abbreviations: ER, estrogen receptor; PR, progesterone receptor; ECOG, Eastern Cooperative Oncology Group.
Our results describe a relationship between polymorphic variants of the aromatase gene and the efficacy of antiaromatase therapy in postmenopausal women with breast cancer. Previous attempts to link expression of aromatase mRNA, aromatase protein, or aromatase enzyme activity with therapeutic activity have been unsuccessful. In the present study, one of the three aromatase CYP19 gene variants that we investigated (rs4646) was associated with greater efficacy of letrozole administered in postmenopausal women with advanced hormone receptor–positive breast carcinoma.

The effect was observed in the TTP parameter of the disease, which was the primary efficacy end point of the study. Patients with the rs4646 SNP variant had a TTP that was three times longer than those who did not have this variant. This finding can be of considerable clinical relevance. To assess whether the rs4646 polymorphism is a predictive factor of the activity of aromatase inhibitors or a prognostic factor in advanced breast cancer, a series of patients treated with other forms of therapy such as chemotherapy or antiestrogens would need to be investigated. Recently, a prospective study of objective response to short-term preoperative course of letrozole in 86 postmenopausal women with primary breast cancer identified rs4646 as one of the aromatase gene polymorphisms that generate a predictive model of response to letrozole (27).

Our study has some limitations. The number of SNPs tested was limited to three, which was conditioned by the exploratory nature of the study. Having tested additional aromatase gene SNPs might have provided additional informative variants. In our study, there might be possible sources of false positive results, among which is sample size or imbalance between ethnic groups. In our series, the small sample size was compensated by an almost 50% distribution of the variant forms. Furthermore, all patients were from a homogeneous population (Table 1). We observed no correlation of SNPs with age, performance status, the number of metastatic sites, serum CA 153 levels, estrogen or progesterone receptor status, and HER2 status (Table 3).

TTP in the 65 evaluable patients was significantly prolonged in the cases with the rs4646 SNP variant of CYP19, when compared with those showing the WT form of the gene (525 versus 196 days; \( P = 0.02 \); Fig. 1). The hazard risk was 0.52 (95% confidence interval, 0.28–0.94). This relationship was not observed for the rs10046 variant (288 versus 500 days; \( P = 0.3 \)) or for the rs727479 variant (370 versus 294 days; \( P = 0.9 \)).

Table 4. Correlation between response to treatment and adverse effects segregated with respect to SNP rs4646

<table>
<thead>
<tr>
<th>Response and adverse events</th>
<th>CYP19 gene rs4646</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>( n ) WT, ( n (%) )</td>
</tr>
<tr>
<td>Best response</td>
<td></td>
</tr>
<tr>
<td>Complete response</td>
<td>8</td>
</tr>
<tr>
<td>Partial response</td>
<td>10</td>
</tr>
<tr>
<td>Stable disease</td>
<td>28</td>
</tr>
<tr>
<td>Disease progression</td>
<td>15</td>
</tr>
<tr>
<td>Clinical benefit</td>
<td></td>
</tr>
<tr>
<td>Benefit*</td>
<td>42</td>
</tr>
<tr>
<td>No benefit</td>
<td>19</td>
</tr>
<tr>
<td>Adverse effects</td>
<td></td>
</tr>
<tr>
<td>No</td>
<td>35</td>
</tr>
<tr>
<td>Yes</td>
<td>32</td>
</tr>
</tbody>
</table>

NOTE: WT, wild-type (GG); variant, heterozygous or homozygous for the SNP rs4646 (GT or TT).

*Clinical benefit = complete response + partial response + stable disease >6 mo.
ethnic group, and there was a good correlation between the genotype frequencies that we observed and the reported literature values in European populations (shown in Table 1). Nevertheless, statistical fluctuation for a P value of 0.02 in a genetic association needs a prospective validation before a definitive association is established, and our finding must be viewed as hypothesis-generating.

The favorable response to therapy that we observed in the patients carrying the rs4646 SNP could be related to a greater estrogen-reducing effect of letrozole. Baseline estradiol levels have been shown to be related to some aromatase gene polymorphisms in healthy postmenopausal women (18, 28). However, there is a paucity of data about the effect of circulating estrogen levels on response to aromatase therapy in women. Letrozole suppressed plasma estrogen by >90% in postmenopausal women with breast cancer (29), but the effect on tumor response was not evaluated.

The mechanism by which the genetic variant affected letrozole activity was not addressed in our study. However, several hypotheses can be formulated. Whereas most SNPs are “silent” and do not alter the function or the expression of a gene (30), the aromatase SNP that we have described seems to be active. This could be related to an advantage induced in the aromatase protein structure that makes it more active (31). Other mechanisms could involve mRNA stabilization, enhanced transcription, or posttranslational regulation of expression. The measurement of circulating estradiol concentrations in our patients would have added important information. Unfortunately, serum samples were not available for the determination of estradiol in the present study. It is a reasonable hypothesis that carrying the normal rs4646 allele might imply a diminished functional efficacy of aromatase inhibitors when used at the recommended dose. This would imply that estrogen synthesis can be maintained in a subset of postmenopausal patients carrying the normal aromatase gene while receiving treatment with aromatase inhibitors. This hypothetical relationship of rs4646 and circulating estradiol in antiaromatase-treated women contrasts with the published relationship of the rs10046 SNP and estradiol in untreated women. In a study of postmenopausal women not receiving hormonal therapy, the variant rs10046 polymorphism was associated with higher estradiol levels (18). Estradiol levels in women receiving antiaromatase therapy, however, have not been studied until now in relationship with aromatase gene variants.

To conclude, we have found that the presence of a SNP in the 3’-UTR of the CYP19 aromatase gene is associated with improved treatment efficacy in patients with hormone receptor–positive metastatic breast cancer treated with the aromatase inhibitor letrozole. Testing for the CYP19 rs4646 SNP as a predictive tool for breast cancer patients on antiantiromatase therapy deserves clinical prospective evaluation.

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References


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