Endocrine resistance in breast cancer: gene expression profiling and modifications of the estrogen receptor
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Chapter 9
Cytochrome P450 2C19 genotype predicts outcome on tamoxifen treatment in advanced breast cancer
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Abstract

Background
The anti-estrogen tamoxifen is metabolized by cytochrome-P450 (CYP) enzymes, with endoxifen as most active metabolite. Recently, CYP2D6 and CYP2C19 genotypes were reported associated with therapy outcome of adjuvant tamoxifen for breast cancer. However, contradictory results are published, and their value for advanced disease is yet unknown. We present association of functional polymorphisms in CYP2C19 and CYP2D6 with outcome of tamoxifen for advanced breast cancer.

Methods
This retrospective multicenter study was performed on genomic DNA of 499 estrogen receptor (ER)-positive primary breast tumor specimens of patients treated with tamoxifen as first-line therapy for advanced disease. Primary endpoint was the time to treatment failure (TTF). Genomic DNA was analyzed using Taqman allele-discrimination assays.

Results
Pooled analysis of three independent patient cohorts showed a longer TTF for CYP2C19*2 carriers, encoding lower enzymatic activity, compared to non-carriers (HR (95% CI): 0.72 (0.57-0.90), p=0.004). Neither the ultra-active CYP2C19*17 nor the inactive CYP2D6*4 allele were associated with TTF. Also in a pooled multivariable analysis, stratified for center and time between primary diagnosis and occurrence of metastases, corrected for menopausal status at start of treatment, adjuvant chemotherapy and CYP2D6*4 status, CYP2C19*2 was independently associated with TTF (HR (95% CI): 0.73 (0.58-0.91), p=0.007). CYP2D6*4 and CYP2C19*2 were not prognostic for breast cancer outcome.

Conclusion
CYP2C19*2 is associated with a longer TTF in hormone-sensitive breast cancer patients treated with tamoxifen for advanced disease. This finding sheds new light on the involvement of CYP2C19 in tamoxifen metabolism, offering possibilities for further treatment individualization and novel management strategies.
Introduction

The anti-estrogen tamoxifen has been used for the treatment of ER-positive breast cancer for more than 30 years both in the adjuvant and advanced setting\(^1,2\). However, despite tamoxifen’s successes, only half of the advanced breast cancer patients who have ER-positive tumors respond to tamoxifen therapy\(^3\) underlining the need to get better insight into factors associated with outcome.

*In vitro* and *in vivo* studies have demonstrated that tamoxifen is subjected to extensive oxidation, predominantly by cytochrome P450s (CYP450). Activity of CYP450 enzymes may vary considerably between patients due to the presence of variant alleles, causing variation in exposure to tamoxifen itself and/or its metabolites\(^4,5\). Such variations in exposure may contribute to the observed differences in clinical response to this drug. The major pathway (~90%) in tamoxifen metabolism involves the conversion by cytochrome P450 3A4 (CYP3A4) to N-desmethyl tamoxifen, which is subsequently converted by CYP2D6 to the highly active metabolite endoxifen (*Figure 9.1*). A minor pathway (~10%) involves the 4-hydroxylation by CYP2D6 to 4-hydroxytamoxifen (4-OHTAM) (*Figure 9.1*)\(^6,7\). Both 4-OH-TAM\(^8-10\) and endoxifen have a much higher binding affinity for the ER than tamoxifen\(^5,11\). On average, plasma concentrations of endoxifen are approximately 8-10 times higher than those of 4-OH-TAM, making endoxifen the most potent metabolite of tamoxifen\(^12\) to contribute to the anti-estrogenic effects of tamoxifen. This was confirmed in *in vitro* studies\(^13\).

Recently, CYP2D6 genotype was shown to correlate with plasma concentrations of endoxifen\(^5,12\) and with outcome to adjuvant tamoxifen therapy. Compared to wild type patients, individuals with a CYP2D6 variant allele (*CYP2D6*\(^*4\)) poorly metabolized tamoxifen, and did worse after tamoxifen therapy\(^14,15\). However, also opposite results have been published reporting increased survival of CYP2D6 poor metabolizers on adjuvant tamoxifen\(^16\). In addition to *CYP2D6*, also *CYP2C19* variants have been described to impact outcome to tamoxifen. *CYP2C19*\(^*17\), an allele encoding increased enzymatic activity, was favorably associated with prolonged survival on tamoxifen in the adjuvant setting for postmenopausal women\(^17\), suggesting a clinical role for this enzyme in tamoxifen therapy outcome as well.

Up to now, the impact of *CYP2D6* and *CYP2C19* genotypes on outcome to tamoxifen therapy has been assessed only in studies with tamoxifen administrated as adjuvant treatment. However, in the adjuvant setting, outcome is determined by both tumors’ response to tamoxifen as well as by the intrinsic aggressiveness of the tumor cells. In this study, we investigated the role of two *CYP2C19* variants, one yielding an enzyme with low activity (*CYP2C19*\(^*2\)) and one encoding an enzyme with high activity (*CYP2C19*\(^*17\)), and one *CYP2D6* variant resulting in a low activity enzyme (*CYP2D6*\(^*4\)) in three separate Dutch cohorts of ER-positive patients treated with tamoxifen for metastatic disease.
Figure 9.1 Tamoxifen metabolic pathway. Major (bold) and minor contributors (mostly based on in vitro experiments with recombinant enzymes) are indicated. Translation into in vivo situations may be affected by protein expression, drug concentrations and site of action. CYP2C19 involvement is indicated in red. The role in trans- to cis-endoxifen conversion is at this moment speculative.

Methods

Patient cohorts

Genomic DNA was isolated from primary breast tumor specimens of women who entered three Dutch clinics between 1978 till 1997, who had been given tamoxifen for advanced disease and from whom detailed clinical follow-up information was available. In brief: patients were selected according to the following criteria: 1) invasive ER-positive breast carcinoma, 2) no (neo)adjuvant endocrine treatment, 3) advanced disease deemed not curable by surgery and/or radiotherapy for which first-line tamoxifen mono-therapy had been given, 4) at least 4 weeks of tamoxifen treatment 5) frozen or paraffin embedded tumor material available. For this study the Erasmus MC included 294 patients (median age at diagnosis: 59.5, range 32-83 years; at start tamoxifen: 63, range 33-85 years), the NKI-AVL (126 patients; median age at diagnosis: 60.3, range 36-83 years; at start tamoxifen 66, range 37-92 years) and the Radboud Nijmegen (79 patients; median age at diagnosis: 62.6, range 30-86 years; at start tamoxifen: 67, range 33-90 years). Thirty-five patients presented with metastatic disease (including supraclavicular lymph node metastasis) at diagnosis or within 1 month after primary surgery. Fifty-eight patients previously received adjuvant chemotherapy: 13/58 patients
anthracyclin-based (FAC/FEC) and 45/58 patients non-anthracyclin-based (CMF) (Table 9.1). Detailed patient characteristics have been described previously\textsuperscript{18-20}. To assess the prognostic value of these genotypes, genomic DNA from a distinct Rotterdam series of breast tumor specimens was available: i.e. untreated, node-negative and ER-positive patients (n=293). In brief, patients underwent surgery for primary breast cancer at the median age of 58 year (range, 22-88 yr). Analyses of disease free survival were performed in those patients of which CYP genotypes and complete follow-up was available. ER protein status of the tumors was determined by routine ligand-binding assays, enzyme immunoassays or immunohistochemistry.

These retrospective studies were approved by the Medical Ethics Committee of the Erasmus MC (MEC 02.953), Rotterdam, by the Institutional Review Board of the Netherlands Cancer Institute, Amsterdam, and by the Institutional Review Board of the Radboud Ziekenhuis Nijmegen, The Netherlands. The present study, in which coded DNAs were used, was conducted in accordance with the Code of Conduct of the Federation of Medical Scientific Societies in the Netherlands (http://www.fmww.nl). When possible we adhered to the Reporting Recommendations for Tumor Marker Prognostic Studies (REMARK)\textsuperscript{21}.

CYP2C19 and CYP2D6 genotyping
Genotyping for CYP2C19*2 (681G>A, rs4244285), CYP2C19*17 (-806C>T, rs12248560) and CYP2D6*4 (1846G>A, rs3892097) was performed on 5 ng genomic DNA, using Taqman allelic discrimination assays (Applied Biosystems, Nieuwerkerk ad IJssel, The Netherlands) on an ABI Prism 7000 Sequence Detection system (Applied Biosystems). Each assay consisted of two allele-specific minor groove binding (MGB) probes, labeled with fluorescent dyes VIC and FAM. The assays IDs are C_25986767_70(CYP2C19*2), C___469857_10 (CYP2C19*17) and C_27102431_D0 (CYP2D6*4). Thermal profile consisted of 95oC for 15 minutes, followed by 50 cycles of 15 seconds at 92°C and 90 seconds at 60°C. Genotypes were scored by allele-specific fluorescence using SDS 2.2.2 software (Applied Biosystems). Assay performance was validated via direct sequencing of wildtype, heterozygous and homozygous variant samples.

Data analysis and statistics
Associations between clinicopathological characteristics and genotypes were tested using Pearson chi-squared statistics for categorized or Kruskal-Wallis test for continuous when appropriate. Deviations from the genotype frequencies from those expected were tested with the Hardy-Weinberg equilibrium equation. Survival curves were generated using the method of Kaplan and Meier and a log-rank test was used to test for differences. Cox proportional hazard regression analysis was applied to compute the HR. Due to limited numbers at risk all analyses were censored at 36 months. The endpoint was time to treatment failure (TTF). Time to treatment failure was defined as the time between start of tamoxifen and documentation of progression of the lesion or the appearance of new lesions according to standard criteria (IUAC or WHO), or death due to breast cancer. Patients who passed away from a different cause within one month after stopping tamoxifen treatment and who had not started a second line of treatment were censored for TTF on the stopping date of tamoxifen. The validity of the proportional hazard assumptions was checked using a test based on Schoenfeld residuals. The proportional hazards were violated for the length of the disease free interval (DFI), defined as the time elapsing between primary diagnosis and the development of metastatic disease, as a dichotomised variable, divided at 24 months. Therefore all multivariable analyses were stratified
for DFI. In multivariable analyses, HRs was adjusted for the traditional predictive factors, menopausal status at start therapy and prior adjuvant therapy. For pooled analyses, center was added as an additional stratification factor. A pooled analysis was performed using a random-effect model using the Der Simonian and Laird method. The results of the pooled analyses were compared with the results from the pooled Cox proportional analyses stratified for center and DFI. Computations were performed with the STATA statistical package, release 10.1 (STATA Corp., College Station, TX). All P-values were two-sided and P<0.05 was considered statistically significant.

**Table 8.1. Patient characteristics**

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<td>&lt;0.00</td>
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* At start tamoxifen

**Results**

**Study population and genotypes**

Patient and tumor characteristics of the three cohorts are summarized in **Table 9.1**. Genotyping of all patients (n=499) resulted in minor allele frequencies (MAF) of 16% for CYP2C19*2, 19% for CYP2C19*17 and 22% for CYP2D6*4, with 13, 21 and 31 homozygote variant patients, respectively. For each series, except for CYP2C19*17 in cohort 2 (p=0.03), variants were in Hardy Weinberg equilibrium. No significant differences between allele frequencies in the three cohorts were observed (**Table 9.1**). Clinical variables significantly differed between cohorts: menopausal status, disease-free interval, and adjuvant chemotherapy (**Table 9.1**).
Figure 9.2 Forest plot of pooled analysis including all patients. Random effects model, using the Der Simonian and Laird method. Squares proportional to size of cohort. Vertical line, width = 95% confidence interval. Diamond middle, effect estimate.

Figure 9.3. Kaplan-Meier analysis. Time to treatment failure after start of tamoxifen for recurrent disease as function of CYP2C19*2 (left) and CYP2D6*4 (right) genotype in three cohorts of ER-positive breast tumor patients. At X-axis the number of patients at risk are indicated. Heterozygotes and homozygotes are analyzed as one group (red) versus wild type patients (blue).
Association of genotypes with time to treatment failure (TTF)

In a pooled analysis of three Dutch breast cancer cohorts, using univariate Cox regression analysis, patients carrying the inactive CYP2C19*2 genotype had a significant longer TTF (HR (95% CI): 0.72 (0.57-0.90), p=0.004; n=484) (Figure 9.2). In multivariable analysis, corrected for menopausal status and adjuvant chemotherapy, stratified for center and DFI, CYP2C19*2 genotype status was significantly associated with TTF (HR (95% CI): 0.71 (0.58-0.90), p=0.004), showing that this finding is independent from the traditional predictive factors. When CYP2D6 genotype status was included in the model, the CYP2C19*2 coefficient did not change (HR (95% CI): 0.73 (0.58-0.91), p=0.007). Investigating the separate cohorts, patients with at least one CYP2C19*2 allele showed a significant longer TTF (HR (95%CI): 0.74 (0.55-0.98), p=0.037; n=283) compared to non-carriers in cohort 1 (Table 9.2, Figure 9.3). Similar HRs, although not statistical significant due to the lower numbers, were observed for cohorts 2 and 3, showing HRs of 0.70 (95% CI 0.44-1.11, n.s.; n=122) and 0.65 (95% CI 0.35-1.21; n.s.; n=79), respectively (Table 9.2, Figure 9.3). For the CYP2C19*17 allele, no significant association with TTF was observed in any of the cohorts investigated.

For CYP2D6*4, analysis of the three separate cohorts yielded different patterns (Figure 9.3): no effect (HR (95%CI): 0.97 (0.75-1.25), n.s.), a shorter TTF (HR (95%CI): 1.5 (0.98-2.30), n.s.) and a longer TTF (HR (95%CI): 0.58, (0.32-1.02), n.s.). Pooled analysis, including all three cohorts, yielded overall no significant association of CYP2D6*4 with TTF (Figure 9.2).

Association of genotypes with disease-free survival (DFS)

To explore the prognostic value of the CYP2C19 and CYP2D6 variant alleles, the association between genotype and tumor recurrence/aggressiveness, was studied retrospectively in a separate cohort of untreated patients with node-negative disease and ER-positive tumors. Patients carrying the ultra-active CYP2C19*17 genotype had a significant longer DFS (HR (95% CI): 0.66 (0.46-0.95), p=0.025; n=243). By contrast, carriers of the inactive CYP2C19*2 allele or the inactive CYP2D6*4 allele showed no significant association with DFS (HR (95% CI): 0.81 0.58-1.15 n.s.; n=259 and 1.10 (0.78-1.53), n.s.; n=279), respectively.

Table 8.2. Univariate Cox regression analysis of the risk of treatment failure after tamoxifen according to CYP450 genotypes

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<th>Cohort 2 HR (95% CI)</th>
<th>p</th>
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<td>CYP2C19*2</td>
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<td>0.70 (0.44-1.11)</td>
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<td>0.65 (0.35-1.21)</td>
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<td>CYP2C19*17</td>
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<td>CYP2D6*4</td>
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<td>0.063</td>
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Discussion

We demonstrate, for the first time, that a significant association exists between the CYP2C19*2 allele and outcome in terms of TTF for ER-positive breast cancer patients treated with first-line tamoxifen for advanced disease in a large group of breast cancer patients, in a multi-centric study. Additionally, it was shown that this variant associates with response to tamoxifen, and not with tumor aggressiveness since no prognostic value for this genotype was revealed in the untreated ER-positive patient cohort. The finding of CYP2C19*2 being associated with outcome was reflected in the separate cohorts by comparable hazard ratio’s (HRs 0.74, 0.70, 0.65). Due to limited cohort sizes, however, this finding was only statistically significant in the largest (n=294) cohort, and not in the two smaller cohorts (sample sizes n=126 and n=79). In spite of significant differences in patient characteristics between the three cohorts, the trend and HRs confirmed the observation made in the first cohort for CYP2C19*2. Although prospective confirmation is needed, these results imply that a decreased CYP2C19 activity may result in a better outcome after tamoxifen treatment.

Our data regarding CYP2C19 genotyping seemingly contradict a recent report on patients treated with tamoxifen in the adjuvant setting. In that study, increased CYP2C19 activity, as predicted by carriership of the ultra-rapid CYP2C19*17 variant, was associated with an increased DFS (i.e. HR=0.45, p=0.03) \(^7\). The mechanism suggested by the authors is an increased transformation of tamoxifen into endoxifen by CYP2C19*17. However, a direct correlation between CYP2C19*17 genotype and endoxifen concentrations has to our knowledge as yet not been shown. In our study, investigating tamoxifen use for advanced disease, we did not find a significant association of CYP2C19*17 allele and clinical outcome, neither in any of the three cohorts nor in the pooled series. A possible explanation for the differences found for CYP2C19 genotype between our study and the study by Schroth et al \(^7\) could be that the effect of genotypes studied in an adjuvant setting is different from the use of tamoxifen in advanced disease. Since outcome in the adjuvant setting is not only dependent on the response to tamoxifen of potentially present micro-metastases but is also greatly affected by a tumor’s aggressiveness, the CYP2C19*17 allele could represent a prognostic factor, unrelated to tamoxifen therapy. Accordingly, in an independent cohort of ER-positive node-negative primary breast cancer patients who did not receive any adjuvant systemic treatment, we observed that CYP2C19*17 is indeed significantly associated with a longer disease-free interval. Recently, also another study indicated that CYP2C19*17 is a protective genotype\(^22\). As a consequence, the reported favorable association of CYP2C19*17 with outcome in the adjuvant setting is likely to represent attenuated tumor aggressiveness rather than increased response to tamoxifen.

The exact mechanism behind our findings that CYP2C19 genotype status is of clinical importance for outcome to tamoxifen in advanced disease is not completely clear yet. In the metabolism of tamoxifen, CYP2C19 has been implicated in the conversion of
tamoxifen itself and several metabolites, yet nowhere as main catalyst (Figure 9.1). Nonetheless, specific inhibition of CYP2C19 activity in human liver microsomes resulted in a decrease in concentrations of endoxifen, alpha-OH-TAM and didesmethyl-TAM\(^23\), indicating CYP2C19 activity is truly contributing to tamoxifen metabolism. In addition, conversion of 4-OH-TAM to 3,4 dihydroxy-TAM was decreased in the presence of CYP2C19 inhibitors whereas also inhibition in the conversion of 4-OH-TAM to endoxifen was seen\(^23\). An interesting pathway to consider is the involvement of CYP2C19 in the conversion of *trans*- to *cis*-4-OH-TAM\(^24,25\) (Figure 9.1). *Trans*-4-OH tamoxifen has a high affinity for the estrogen receptor, whereas the *cis*-isomer of 4-OH TAM is a much less potent anti- estrogen\(^26\). Williams et al\(^24\) described *cis*- to *trans*-4-OH-TAM conversion in 5 out of 12 liver microsomes, indicating this process actually occurs in vivo, but is not equal in all individuals. Data on tumor bearing mice and human breast cancer patients suggested earlier that resistance to tamoxifen was associated with lowered intracellular concentrations of both tamoxifen itself and of *trans*-4-OHTAM, the latter due to its isomerization to *cis*-4-OH-TAM\(^27,28\) Possibly,*cis*/*trans* isomerization may also occur for endoxifen, which might then explain the favorable effect of a reduced CYP2C19 activity by a decreased conversion of *trans*-endoxifen to *cis*-endoxifen. Thus far, no studies have addressed this particular mechanism and therefore further research is warranted.

We did not reveal a clear association of CYP2D6\(^*4\), the most frequent CYP2D6 null allele in Caucasians, as either a prognostic or a predictive factor for outcome to tamoxifen in advanced disease. Although none of these associations were statistically significant with respect to TTF, we found different trends in our cohorts: i.e. no association, association with a shorter and association with a longer TTF. The different effect of the CYP2D6 status may be explained by intrinsic differences between the three cohorts studied (Table 9.1). The discrepant findings, however, do reflect the contradictory results in the current literature in which, compared to wild types, for CYP2D6 poor metabolizers a worse outcome\(^12,14,15\), a better outcome\(^29\) as well as no association\(^30\) have been published. These and other reports on CYP2D6 genotype in relation to tamoxifen therapy were recently summarized\(^31\) and indicate that additional factors may modify the effect of CYP2D6 genotype on tamoxifen outcome. One potential confounder in studies examining the association between CYP450 genotypes and outcome to tamoxifen is the use of co-medication. In particular Selective Serotonin Reuptake Inhibitors (SSRIs) are thought to affect the formation of antiestrogenic metabolites via inhibition of CYP2D6. Since our study is performed retrospectively, we cannot totally exclude that our data are influenced by the use of SSRIs as co-medication. However, a dominant role for SSRIs in the inhibition of CYP2D6 activity in our study is very unlikely: over 90% of the patients included in our study received tamoxifen before the introduction of SSRIs in the Netherlands.
In conclusion, we found that CYP2C19*2 genotype status may contribute to a more appropriate selection of patients for tamoxifen therapy. Furthermore, elucidation of the underlying mechanisms accounting for better outcome to tamoxifen in patients with a CYP2C19*2 allele may yield improved therapeutic strategies in treating breast cancer patients with tamoxifen.

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