

## Pharmacogenetics of Tamoxifen Biotransformation Is Associated With Clinical Outcomes of Efficacy and Hot Flashes

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### A B S T R A C T

#### Purpose

Polymorphisms in tamoxifen metabolizing genes affect the plasma concentration of tamoxifen metabolites, but their effect on clinical outcome is unknown.

#### Methods

We determined cytochrome P450 (CYP)2D6 (\*4 and \*6) and CYP3A5 (\*3) genotype from paraffin-embedded tumor samples and buccal cells (living patients) in tamoxifen-treated women enrolled onto a North Central Cancer Treatment Group adjuvant breast cancer trial. The relationship between genotype and disease outcome was determined using the log-rank test and Cox proportional hazards modeling.

#### Results

Paraffin blocks were obtained from 223 of 256 eligible patients, and buccal cells were obtained from 17 living women. CYP2D6 (\*4 and \*6) and CYP3A5 (\*3) genotypes were determined from 190, 194, and 205 patient samples and in 17 living women. The concordance rate between buccal and tumor genotype was 100%. Women with the CYP2D6 \*4/\*4 genotype had worse relapse-free time (RF-time;  $P = .023$ ) and disease-free survival (DFS;  $P = .012$ ), but not overall survival ( $P = .169$ ) and did not experience moderate to severe hot flashes relative to women heterozygous or homozygous for the wild-type allele. In the multivariate analysis, women with the CYP2D6 \*4/\*4 genotype still tended to have worse RFS (hazard ratio [HR], 1.85;  $P = .176$ ) and DFS (HR, 1.86;  $P = .089$ ). The CYP3A5\*3 variant was not associated with any of these clinical outcomes.

#### Conclusion

In tamoxifen-treated patients, women with the CYP2D6 \*4/\*4 genotype tend to have a higher risk of disease relapse and a lower incidence of hot flashes, which is consistent with our previous observation that CYP2D6 is responsible for the metabolic activation of tamoxifen to endoxifen.

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### INTRODUCTION

Tamoxifen is one of the most widely used drugs in the world for the treatment and prevention of estrogen receptor (ER)-positive breast cancer. When given to women with ER-positive breast cancer for 5 years after surgery, tamoxifen almost reduces by half the annual recurrence rate and reduces the breast cancer mortality rate by one third.<sup>1</sup>

Tamoxifen undergoes extensive primary and secondary metabolism, and the concentrations of tamoxifen and its metabolites vary widely.<sup>2-5</sup> Although 4-hydroxytamoxifen (4-OH tamoxifen) represents less than 10% of tamoxifen primary oxidation,<sup>2,6</sup> it has been considered to play an important role in tamoxifen's anticancer effect<sup>7</sup> given its 100-fold greater affinity for the ER and its 30- to 100-fold greater potency in suppressing

estrogen-dependent cell proliferation when compared with the parent drug.<sup>8-10</sup>

Recent studies by Flockhart et al strongly suggest that another tamoxifen metabolite, 4-hydroxy-*N*-desmethyl tamoxifen (endoxifen), previously characterized by Lien et al,<sup>11-13</sup> is more important than 4-OH tamoxifen in terms of the relative contribution to the overall anticancer effect of tamoxifen and thus to inter-individual variability in response to the drug. Endoxifen has identical properties and potency compared with 4-OH tamoxifen in terms of its binding affinity to ERs,<sup>14</sup> suppression of estradiol-stimulated cell proliferation,<sup>4</sup> and gene expression.<sup>14</sup> Furthermore, steady-state plasma endoxifen concentrations are 5- to 10-fold higher than 4-OH tamoxifen.<sup>4,5</sup> Although the metabolism of tamoxifen to 4-OH tamoxifen is catalyzed by multiple enzymes, endoxifen is formed predominantly by the CYP2D6 mediated oxidation of *N*-desmethyl tamoxifen, the most abundant tamoxifen metabolite<sup>6</sup> (Fig 1). Recent clinical studies have demonstrated that women receiving tamoxifen who either carry genetic variants associated with low or absent CYP2D6 activity or who receive concomitant medications known to inhibit CYP2D6 activity have significantly lower levels of endoxifen.<sup>4,5</sup>

CYP3A catalyzes a number of primary and secondary metabolic routes of tamoxifen<sup>6</sup> and is the principal enzyme catalyzing the main route of elimination of tamoxifen. The activity of this enzyme system exhibits wide inter-individual variability, in part because of a polymorphism within intron 3 of the *CYP3A5* gene.<sup>15,16</sup>

We sought to determine whether common polymorphisms in the enzymes responsible for tamoxifen biotransformation affect the clinical outcomes of women receiving

adjuvant tamoxifen. In addition, we sought to determine the concordance between genotype derived from paraffin tumor with germline DNA derived from a subset of living women.

## METHODS

### Patients

The North Central Cancer Treatment Group (NCCTG) conducted a randomized phase III clinical trial in postmenopausal women with resected ER-positive breast cancer to assess the value of adding 1 year of fluoxymesterone to 5 years of tamoxifen adjuvant therapy (NCCTG 89-30-52).<sup>17</sup> No women received adjuvant chemotherapy. The details of the clinical trial including the eligibility requirements are listed in a supplementary attachment.

Within 30 days of registration, a paraffin-embedded tumor block was submitted to the NCCTG Operations Office for future research purposes. The current study evaluating the pharmacogenetics of tamoxifen biotransformation in patients randomly assigned to the tamoxifen-only arm was approved by the institutional review board of the Mayo Clinic (Rochester, MN) and the individual NCCTG sites that enrolled patients onto the clinical trial. The need for additional informed consent was waived by the institutional review boards.

Approval was also obtained from the institutional review board of Mayo Clinic Rochester and individual NCCTG sites and informed consent was obtained from living patients to obtain buccal samples and genotype for polymorphisms within tamoxifen metabolizing genes.

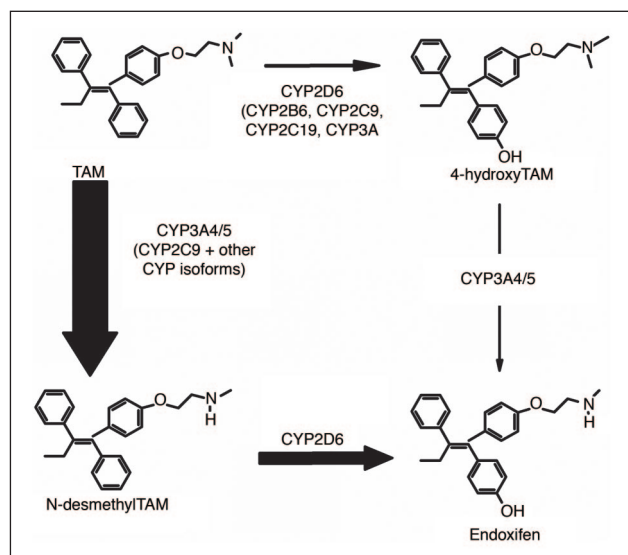
### Sample Preparation

Using paraffin-embedded tissue blocks from the women enrolled onto the tamoxifen only arm, three sections (10- $\mu$ m thick) and one hematoxylin and eosin slide were prepared from each block and a 1 cm area of high tumor cellularity was identified, microdissected, deparaffinized, and DNA extracted using the method described previously.<sup>18</sup> In living women who supplied a buccal sample, DNA was extracted using a QIAamp DNA Mini kit (Qiagen, Valencia, CA) per instructions.

### Assay Methods

Patient samples (both tumor and buccal) were genotyped for CYP2D6\*4 (1846G > A), and CYP2D6\*6 (1707T > del) polymorphisms as described previously<sup>5</sup> using the Applied Biosystems' Taqman Allelic Discrimination Assay (Foster City, CA) according to the manufacturer's instructions. Briefly, 10 ng of DNA was added to a 5- $\mu$ L reaction containing forward and reverse primers along with two allele-specific labeled probes (one wild-type and one variant allele specific). The polymerase chain reaction and fluorescence measurements were performed using the ABI Prism 770 sequence detection system. Additional CYP2D6 alleles less common in the white population (\*3 and \*5) were not analyzed because inadequate amounts of DNA were available for testing.

Additionally, patient samples (both tumor and buccal) were genotyped for the *CYP3A5*\*3 polymorphism (6986 G > A) using the method described by Hustert et al<sup>16</sup> with minor modifications. Briefly, a 280-base pair product was amplified using an initial 10-minute incubation at 94°C, followed by 45 cycles of 1 minute at 94°C, 1 minute at 67°C, and 1 minute at 72°C, with a final 5-minute extension at 72°C. Amplification was confirmed on a 2% agarose gel, and the samples were sequenced by the dye terminator method in both the forward and reverse directions. Sequencing



**Fig 1.** Selected transformation pathways of tamoxifen and the main CYP enzymes involved. The relative contribution of each pathway to the overall oxidation of tamoxifen is shown by the thickness of the arrow, and the principal P450 isoforms responsible are highlighted in larger fonts.

results were manually evaluated at the single nucleotide polymorphism location.

### Study Design and End Points

The primary objectives in this study were to determine the relationship between genotype and relapse-free time, disease-free survival, and overall survival. A secondary objective was to determine whether the incidence of hot flashes differed with respect to genotype.

Relapse-free time (RF-time) was defined as the time from randomization to documentation of a breast event, where a breast event is any recurrence (local, regional, or distant) of breast cancer or the documentation of contralateral breast cancer (including ductal carcinoma-in-situ). When estimating the distribution of RF-time, patients who developed a nonbreast second primary cancer (other than squamous or basal cell carcinoma of the skin, carcinoma-in-situ of the cervix, or lobular carcinoma-in-situ of the breast) before the diagnosis of a breast event were censored on the day their second primary was diagnosed. Patients (alive or dead) without a breast recurrence, contralateral breast cancer, or a second nonbreast primary cancer were censored at the date of their last disease evaluation.

Disease-free survival (DFS) was defined as the time from randomization to documentation of the first of the following events: any recurrence (local, regional, or distant) of breast cancer, the documentation of contralateral breast cancer, or death from any cause. Patients who were alive without a breast recurrence, contralateral breast cancer, or a second nonbreast primary cancer were censored at the date of their last disease evaluation. Patients who developed a second primary (before breast recurrence or a contralateral breast cancer) were censored on the date the second primary was diagnosed. Overall survival (OS) was estimated as the time from registration to death from any cause.

The distributions of RF-time, DFS, and OS were estimated overall using the Kaplan-Meier method. For each patient and pathologic factor and each clinical outcome, the following approach was used to assess the strength of the relationship between the factor and the outcome and to assess the proportional hazards assumption. A log-rank test was used to assess the association between the factor and the outcome of interest. To examine whether the proportional hazards assumption was appropriate, a plot of the log of the stratum-specific cumulative hazard functions of that factor (determined using Kaplan and Meier estimates) against time was constructed and examined for lack of parallelism. Also, a Cox model with the factor of interest and the interaction term composed of the factor with log (time) was fit to the data, and then the significance of the interaction term was assessed to evaluate whether the hazard depended on time.

Cox multivariate modeling was then used to determine which subset of patient and pathologic characteristics from among age (< 65 years  $\nu$  > 65 years), extent of surgery (mastectomy  $\nu$  breast conservation therapy), primary tumor size (< 3 cm  $\nu$  > 3 cm), axillary lymph node status (positive  $\nu$  negative), and ER status (10 to 49 fmol  $\nu$  50 fmol or greater  $\nu$  positive by immunohistochemical assay) was significantly associated with each clinical end point. Model assumptions and adequacy of fit were examined using residual plots, such as dfbeta statistic (a transform of the score residual) versus patient rank order of study entry, Martingale residuals versus linear predictor scores, and deviance residual versus linear predictor scores.

The potential prognostic value of each genotype in terms of RF-time, DFS, and OS was expressed using three categories: no variant alleles, one variant allele, and two variant alleles. The

log-rank test and the generalized Wilcoxon test were then used to assess whether RF-time, DFS, or OS differed with respect to genotype. For each end point and each genotype, the Cox model that was previously found to provide the best fit from among those containing a subset of patient and pathologic characteristics was expanded to include the genotype represented in terms of two indicator variables. The likelihood ratio tests were then used to ascertain whether one or both indicator variables made a significant contribution to the model. For all CYP2D6\*4 analyses, we refer to the wild-type allele as the absence of the \*4 allele.

Hot flashes were graded using the NCCTG supplement to the National Cancer Institute Common Toxicity Criteria (version 1) as follows: 0, none or no change; 1, mild; 2, moderate; or 3, severe. The Wilcoxon rank sum test was used to assess whether the severity of hot flashes (0 to 3) differed with respect to genotype, and the one-sided Fisher's exact test was used to assess whether the proportion of women with moderate or severe hot flashes was smaller for those with the CYP2D6\*4/\*4 genotype than those without the CYP2D6\*4/\*4 genotype.

## RESULTS

### Patient Characteristics

Of the 256 women enrolled onto the tamoxifen-only arm, 213 paraffin-embedded tumor blocks and 10 normal tissue blocks were available for DNA extraction. Table 1 lists the preregistration characteristics of the 256 eligible patients randomly assigned to the tamoxifen-only arm who did and did not have a specimen from their primary surgery available for genotyping. The overall patient characteristics were similar, although a higher percentage of patients with available tissue had a tumor size greater than 3 cm (22%) compared with the group without tissue available (15%).

For the group of 223 patients whose paraffin sample was available, the first documented event was as follows: local, regional, or distant breast recurrence (43 patients), contralateral breast cancer (12 patients), a second nonbreast primary cancer (16 patients), and death without a breast recurrence or second primary cancer (40 patients). At last follow-up, 112 women are alive without evidence of a breast event or second primary, 25 women are alive after a breast event or second primary cancer, 33 women died with disease recurrence, 13 women died having developed a second primary cancer, 32 women died of other causes, and eight women died of unknown causes. The Kaplan-Meier estimates for the 10-year RF-time, DFS, and OS were as follows: 75.0% (95% CI, 69.1% to 81.4%), 61.0% (95% CI, 54.7% to 68.0%), and 68.4% (95% CI, 62.5% to 74.9%), respectively. The median length of follow-up among the 137 patients still alive was 11.4 years (range, 5.7 to 14.1 years).

For the clinical end points of RF-time, DFS, and OS, Cox modeling demonstrated that positive nodes and tumor size greater than 3 cm were significantly associated with decreased RF-time, DFS, and OS.

**Table 1.** Preregistration Characteristics of the Patients on the Tamoxifen Arm Who Did and Did Not Have Paraffin-Embedded Tumor Tissue Available From Their Primary Breast Surgery and Patients in the CYP2D6\*4/\*4 Group

	% of Patients		
	Women With Paraffin Tissue (n = 223)	Women Without Paraffin Tissue (n = 33)	CYP2D6*4/*4 Genotype (n = 13)
Operative procedure			
Mastectomy	83	73	92
Breast conservation	17	27	8
Prior hysterectomy	42	45	40
Prior BSO	25	21	23
Exogenous estrogens	16	15	0
No. of positive nodes			
0	62	64	31
1-3	26	15	54
4-9	7	15	8
10+	4	6	8
Tumor size $\geq$ 3 cm	22	15	38
ER status			
10-49 fmols	21	15	15
$\geq$ 50 fmols	67	61	62
Positive	12	24	23
Age, years			
Median	68	69	73
Range	42-87	48-83	56-87
White	92	91	100

Abbreviations: BSO, bilateral salpingo-oophorectomy; ER, estrogen receptor.

### Genotype and Allele Frequency

The CYP2D6 (\*4 and \*6) and CYP3A5 (\*3) alleles were successfully amplified in 190, 194, and 205 patients, respectively, and their allelic frequencies are listed in Table 2. No CYP2D6 (\*6) variants were detected. The genotype and allelic frequencies for each variant were similar to published reports in a predominantly white population.

### Clinical Outcome by Genotype

**CYP2D6\*4.** Patient characteristics for women with the CYP2D6\*4/\*4 genotype are listed in Table 1. A greater proportion of women with the CYP2D6\*4/\*4 genotype had node-positive disease relative to that of the entire group. Women with the CYP2D6 \*4/\*4 genotype (n = 13) had

significantly worse RF-time and DFS but not OS compared with either the \*4/wt (n = 40) or the wt/wt genotype (n = 137; log-rank  $P = .030$ ,  $P = .020$ , and  $P = .360$ , respectively; Fig 2). Cox proportional hazards modeling demonstrated that nodal status and tumor size were significantly associated with RF-time, DFS, and OS. Once nodal status and tumor size were accounted for, women with the CYP2D6 \*4/\*4 genotype still tended to have worse RF-time and DFS but not OS, compared with patients with one or no variant alleles. Table 3 lists the unadjusted and adjusted hazard ratios comparing patients with the CYP2D6\*4/\*4 genotype with the wt/wt or \*4/wt genotypes.

**CYP3A5 (\*3).** Neither RF-time, DFS, nor OS was found to differ in terms of CYP3A5 \*3 genotype (A/A  $\nu$  A/G  $\nu$  G/G; log-rank  $P = .854$ , .937, and .950 respectively).

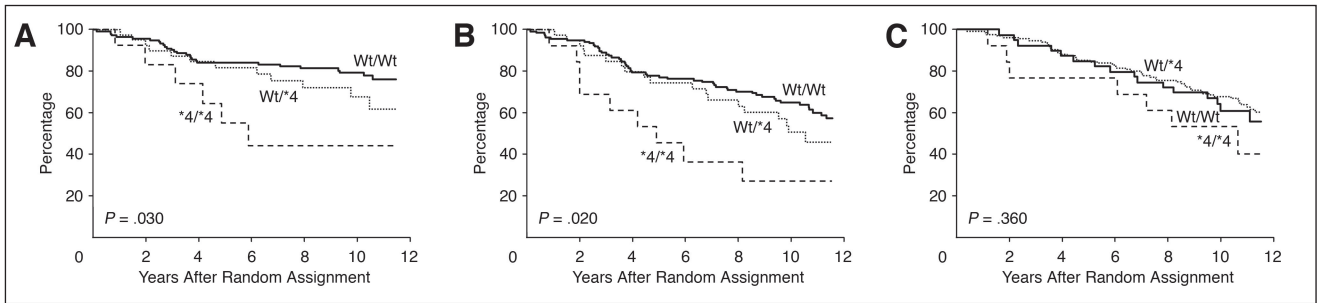
### Hot Flashes

Among the 223 patients, a total of 61% (n = 136) reported having hot flashes, with 40% (n = 90) reporting mild (grade 1), 15% (n = 34) reporting moderate (grade 2), and 5% (n = 12) reporting severe (grade 3) hot flashes. There were no differences in hot flash severity with respect to CYP3A5. However, for CYP2D6\*4, none (0 of 13) of the women with the CYP2D6 \*4/\*4 genotype had moderate or severe hot flashes compared with 20% (36 of 177) for patients with either the \*4/wt or wt/wt genotypes (one-sided  $P = .06$ ; Table 4).

**Table 2.** Genotype and Allele Frequencies (q) for CYP2D6 (\*4) and CYP3A5\*3

	No.	%	
CYP2D6 (*4), n = 190			q = 0.17
wt/wt	137	72.1	
wt/*4	40	21.1	
*4/*4	13	6.8	
CYP3A5 (*3), n = 205			q = 0.088
A/A	6	2.9	
A/G	24	11.7	
G/G	175	85.4	

NOTE. No CYP2D6 (\*6) variants were observed.



**Fig 2.** Kaplan-Meier estimates of (A) relapse-free time, (B) disease-free survival, and (C) overall survival for patients with the CYP2D6\*4 genotype.

### Tumor and Germline Genotype Concordance

A total of 17 living women submitted a buccal specimen. For CYP2D6\*4, there were 15 patients with concomitant tumor and buccal genotype, and the concordance between tumor and buccal genotype was 100% (15 of 15). Similarly, for CYP3A5\*3, there were 13 patients with concomitant tumor and buccal genotype, and the concordance rate was 100% (13 of 13).

## DISCUSSION

Using a prospective adjuvant tamoxifen study for the treatment of estrogen-dependent breast cancer, we have examined the effect of variation within tamoxifen metabolizing genes on disease outcome and toxicity in the form of hot flashes.

Women homozygous for the most common allele associated with the CYP2D6 poor metabolizer (PM) phenotype, CYP2D6\*4, tend to have worse RF-time (hazard ratio [HR], 1.85;  $P = .176$ ) and DFS (HR, 1.86;  $P = .089$ ), once nodal status and tumor size were accounted for. The biologic importance of CYP2D6 genotype was further supported by the finding that none of the women with the \*4/\*4 genotype experienced moderate or severe hot flashes, compared with 20% of the women with either the \*4/wt or wt/wt genotypes. By obtaining germline CYP2D6\*4 genotype from living patients, we demonstrated 100% concordance between tumor and buccal genotype, suggesting that tumor

CYP2D6\*4 genotype is an accurate means by which to obtain CYP2D6\*4 germline status.

Approximately 7% to 10% of white patients lack functional CYP2D6, and the genetic basis of the PM phenotype has been well defined. In this study, we examined the association between clinical outcome and the CYP2D6\*4 allele, which accounts for 75% of CYP2D6 PMs in white patients,<sup>19</sup> as well as for the CYP2D6 (\*6) allele that accounts for a minority of CYP2D6 PMs. Because of the small amount of DNA obtained from the paraffin-derived tissue, not all alleles associated with CYP2D6 PM phenotype could be examined; furthermore, alleles associated with CYP2D6 intermediate metabolizer phenotype (eg, CYP2D6\*41)<sup>20</sup> and the ultrarapid metabolizer phenotype, although likely rare in this population, were not evaluated. Nevertheless, these data suggest that CYP2D6 genetic variation is an important determinant of tamoxifen effect and that lower or absent CYP2D6 activity may increase the risk of tamoxifen treatment failure. It will be important that our findings be corroborated in a larger prospective tamoxifen study in which a greater number of alleles corresponding to the CYP2D6 metabolizer status are evaluated.

Because we were unable to ascertain the effect of the CYP2D6\*4 allele in a group of women who did not receive tamoxifen, we cannot rule out the possibility that the CYP2D6\*4 allele is a prognostic marker. However, a recent study by Nowell et al<sup>21</sup> demonstrated no statistically significant effect of the CYP2D6\*4 allele on either progression-free

**Table 3.** Unadjusted and Adjusted Hazard Ratios and Corresponding 95% CI and  $P$  Values Comparing Patients With the CYP2D6\*4/\*4 Genotype With the wt/wt or \*4/wt Genotypes

	Unadjusted			Adjusted*		
	Hazard Ratio	95% CI	$P$	Hazard Ratio	95% CI	$P$
Relapse-free time	2.71	1.15 to 6.41	.023	1.85	0.76 to 4.52	.176
Disease-free survival	2.44	1.22 to 4.90	.012	1.86	0.91 to 3.82	.089
Overall survival	1.73	0.79 to 3.76	.169	1.12	0.50 to 2.50	.780

NOTE. Hazard ratios for CYP2D6\*4 \*4/\*4 relative to \*4/wt and wt/wt are shown.

\*A Cox model including nodal status and tumor size was used to estimate the adjusted hazard ratios.

**Table 4.** Incidence of Moderate (grade 2) or Severe (grade 3) Hot Flashes Within Genotype Subgroups

Genotype	Patients Who Developed Moderate or Severe Hot Flashes		
	No. of Patients	Total Patients	%
CYP3A5 (*3), n = 205			
G/G	37	175	21
G/A	6	24	25
A/A	1	6	17
CYP2D6 (*4), n = 190			
*4/*4	0	13	0
*4/wt	9	40	23
wt/wt	27	137	20

NOTE. For CYP2D6 \*4/\*4 patients, 0 (0%) of 13 patients experienced grade 2 or 3 hot flashes compared with 35 (20%) of 177 patients with the \*4/wt or wt/wt genotypes ( $P = .064$ ).

survival or overall survival in 166 breast cancer patients who did not receive tamoxifen, suggesting that the CYP2D6\*4 allele is not a prognostic marker.

The optimal dose of tamoxifen is unknown. The Oxford overview suggested no differences in either recurrence or survival when cross-trial comparisons were made between studies using higher doses (30 to 40 mg/d) with 20 mg/d.<sup>22</sup> Furthermore, lower doses of tamoxifen (1 mg/d and 5 mg/d) have similar effects on biologic markers of proliferation when compared with 20 mg/d,<sup>23</sup> but have not been tested in large randomized adjuvant trials. Although the overall effect of tamoxifen is due to the sum of multiple different tamoxifen metabolites, our findings suggest that the optimal biologically active dose of tamoxifen may differ with respect to inter-individual variation in CYP2D6.

One of the most common classes of medications coprescribed with tamoxifen for the treatment of hot flashes or depression are the newer antidepressants, which include the selective serotonin reuptake inhibitors and the serotonin and norepinephrine reuptake inhibitors.<sup>24-26</sup> Several drugs be-

longing to this class, specifically paroxetine and fluoxetine, are known potent inhibitors of CYP2D6 and significantly reduce the serum concentrations of endoxifen in women receiving adjuvant tamoxifen.<sup>5</sup> Because concomitant medication information was not recorded in this trial, the effect of CYP2D6 inhibitors on the clinical outcomes of women in this study is still unknown. Further studies are needed to determine the effect of concomitant CYP2D6 inhibitors on the treatment outcomes of women receiving adjuvant tamoxifen.

Recent studies have also attempted to look at the effect of CYP2D6 genotype on the outcomes of women receiving adjuvant tamoxifen.<sup>21,27</sup> However, because of the small sample size and the confounding inclusion of receptor-negative patients, these studies have been unable to ascertain the effect of the CYP2D6\*4 allele on treatment outcomes.

In summary, our findings suggest that inter-individual variability in the response to tamoxifen may be explained in part by genetic variation in CYP2D6. These findings have the potential to improve the ability of physicians to select the optimal hormonal therapy for the treatment of ER-positive breast cancer. Further studies are needed in women receiving tamoxifen to fully define the effect of CYP2D6 genetic polymorphisms and medications that inhibit CYP2D6 on tamoxifen response.

### Acknowledgment

We thank the women who participated in this clinical trial as well as the individual NCCTG investigators who enrolled patients.

### Appendix

The Appendix is included in the full-text version of this article, available online at [www.jco.org](http://www.jco.org). It is not included in the PDF (via Adobe® Acrobat Reader®) version.

### Authors' Disclosures of Potential Conflicts of Interest

The authors indicated no potential conflicts of interest.

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