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Estimation of tamoxifen metabolite concentrations in the blood of breast cancer patients through CYP2D6 genotype activity score

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Abstract

Tamoxifen, a prodrug used for adjuvant breast cancer therapy, requires conversion to the active metabolite endoxifen through CYP 2D6. We aimed to construct an algorithm to predict endoxifen concentrations based on a patient's CYP 2D6 genotype, demographic factors, and comedication use. Eighty-eight women enrolled in the UCSF TamGen II study and 81 women enrolled in a prospective study at Dana-Farber Cancer Institute were included in this analysis. All the women had been on tamoxifen for at least 3 months before blood collection. Demographic information included the patient's age, race/ethnicity, body mass index (where available), and self-reported and measured medications and herbals that affect 2D6 activity. DNA was extracted and genotyped for 2D6 (Amplichip, Roche Diagnostics). An activity score was calculated based on genotypes and adjusted for use of medications known to inhibit 2D6. Serum was tested for tamoxifen and metabolite concentrations and for the presence of drugs by liquid chromatography/mass spectrometry. Univariate and multivariate regression analysis were computed for age, body mass index, ethnicity, and adjusted activity score to predict tamoxifen metabolite concentrations in the training data-set of UCSF patients, and the resulting algorithm was validated in the Dana-Farber patients. For the training set, the correlation coefficient (r^2) for log endoxifen and *N*-desmethyl-tamoxifen: endoxifen ratio to activity score, age, and race, were 0.520 and 0.659, respectively; 0.324 and 0.567 for the validation; and 0.396 and 0.615 for both the datasets combined. An algorithm that incorporates genotype and demographic variables can be used to predict endoxifen concentrations for women on tamoxifen therapy. If endoxifen levels are confirmed to be predictive of tamoxifen benefit, then this algorithm may be helpful to determine which women warrant endoxifen testing.

Keywords

Breast cancer; Tamoxifen; Endoxifen; Genotype; Activity score; CYP2D6

Introduction

As a selective estrogen receptor modulator, tamoxifen is widely used to treat premenopausal women with hormone receptor positive breast cancer. In vitro, tamoxifen has a relatively modest growth-inhibitory effect in comparison to its metabolites, 4-hydroxy tamoxifen and endoxifen; however, only endoxifen, which has some 100-fold higher potency in blocking estrogen receptors, has significant bioavailability [1]. Hepatic cytochrome P450 is an essential enzyme in this conversion. Several retrospective clinical studies have shown that in the adjuvant setting, breast cancer patients who carry two null alleles for 2D6 (e.g., CYP 2D6 *4/*4, termed "poor metabolizers") have lower disease-free survival and time to breast cancer recurrence than patients who are homozygous wild type (e.g., 2D6 *1/*1, termed "extensive metabolizers") when treated with tamoxifen [2, 3]. However, other investigators have failed to show an association between 2D6 genotypes and outcomes. One complexity in 2D6 testing may be the need to distinguish the importance of intermediate metabolizers. Studies have varied regarding analysis of outcomes data from heterozygotes and those with reduced function alleles (e.g., *41 and *10); most studies classify some of these as a third "intermediate metabolizer" group. Those who carry two hyper-functioning alleles are termed

“ultra-rapid metabolizers,” and are usually grouped with extensive metabolizers. Lash et al. summarized the results of 10 trials; six demonstrated a positive association between genotype and outcome and four, a negative association [4]. In addition, two large trials: “Arimidex, Tamoxifen, Alone or in Combination Trial” and “BIG 1-98” (Breast International Group) trials both recently analyzed the association between CYP2D6 and breast cancer recurrence among tamoxifen users and found no effect [5, 6]. Part of this heterogeneity may be because CYP2D6 has numerous alleles, and how to group these alleles remains controversial.

As with other pharmacogenomic tests such as the estimation of warfarin dosing to achieve stable anticoagulation, non-genetic factors, such as age, body mass index (BMI), gender, and ethnicity in addition to CYP 2C9 and vitamin K epoxide reductase genotypes, are likely to be important to produce the highest degree of dosage correlation [7]. In the case of tamoxifen, Borges et al. found that combining the genotype activity score with the presence or absence of comedications known to inhibit CYP 2D6 activity improved the regression equation to endoxifen concentrations and the ratio of endoxifen to *N*-desmethyldoxifen, a less-active intermediate metabolite [8].

Although there are many studies on CYP2D6 genotype and breast cancer outcomes, there are fewer studies on endoxifen levels and outcomes. Recently however, in the Women’s Healthy Eating and Living (WHEL) study, Madlensky et al. showed that women in the lowest quintile of endoxifen concentration had worse clinical outcomes than women whose endoxifen concentration were in the upper four quintiles combined [9]. There was no association with tamoxifen levels or with levels of any of the other metabolites or ratios of metabolites. This initial study suggests that endoxifen concentrations may be important in the success of tamoxifen therapy.

We performed a detailed investigation of CYP2D6 genotype and endoxifen levels in two cohorts: a University of California, San Francisco (UCSF) cohort, and a Harvard-based cohort. We sought to build different correlation models for tamoxifen and metabolite concentrations including a traditional genotype score (i.e., extensive, ultra, intermediate, and poor metabolizer), an activity score (i.e., intermediate scores between genotypes), and a regression model that included age, gender, and BMI.

Materials and methods

Patients

The UCSF and Dana-Farber’s tamoxifen trials involved CYP 2D6 genotyping for consenting patients on or considering tamoxifen for breast cancer adjuvant therapy. The UCSF TamGEN II cohort has been previously described [10]. These studies were approved by human subjects committees at UCSF and Dana-Farber, and all the patients provided written consent. Both the CYP 2D6 and endoxifen results were reported to attending oncologists in the Dana Farber’s study. Only the CYP 2D6 results were reported to providers in the UCSF study. Of the 245 women enrolled in the UCSF study, 88 patients qualified for the substudy as they were on tamoxifen for at least 4 months and had given permission for tamoxifen testing analysis. BMI was available on a subset of 56 patients. A written questionnaire

regarding co-medications including a list of generic and brand names was given to each subject in the UCSF cohort. Eligibility criteria for the Dana-Farber study included tamoxifen use for early stage breast cancer for at least 3 months. The validation set consisted of 81 patients from the Dana Farber study, with BMI available on all. All patients provided a self-reported co-medications history at the time of enrollment. Patients were only enrolled who reported at baseline that they “always” or “often” reported tamoxifen drug use according to the Morisky scale [11]. Patients were not included in the study if they self-reported less than “often” adherence.

Laboratory testing

Blood was collected via venipuncture into plasma tubes containing EDTA for CYP 2D6 testing, and no anticoagulants for serum tamoxifen metabolites. Serum samples were centrifuged within 1 h of collection and the serum stored frozen at -70°C until analysis. The DNA was extracted using QIAamp DNA blood Mini Kit (Qiagen, Valencia, CA, USA), amplified by PCR, and tested using the Amplichip CYP450 test (Roche Molecular Systems Inc., Pleasanton, CA, USA). Patients with a rare 2D6 genotype as determined by the Amplichip interpretative software were omitted. For all patients, genotyping and tamoxifen metabolite assays were conducted in the Department of Laboratory Medicine at UCSF. Serum for all the samples was tested for tamoxifen and metabolites using liquid chromatography tandem mass spectrometry (Model 3200, Applied Bioscience Inc., Foster City, CA, USA). A description of the tamoxifen/metabolite assay has been published previously [12]. The qualitative analysis for the presence of drug inhibitors was conducted in serum using LC-time-of-flight mass spectrometry (Model 6230, Agilent Technologies, Santa Clara, CA, USA) [13]. In this assay, we have targeted 32 drugs and 7 herbal medications known to inhibit CYP2D6. A listing of these drugs and the limit of sensitivity is available in the Supplementary Table 1. Only drugs found in therapeutic concentrations were reported. This drug assay was targeted toward common medications known to inhibit CYP 2D6 and commonly used to treat the side effects of tamoxifen. Serum samples were available for drug inhibitors in 83 of 88 (94%) and 56/81 (69%) of the UCSF and Dana Farber cohorts, respectively.

Statistics

The traditional genotype classification scheme produces whole numbers for poor (0), intermediate (1), extensive (2), and ultra-metabolizers (3). Individuals heterozygous for poor (e.g., 2D6 *4) and extensive (e.g., 2D6 *1) are given an intermediate classification [14]. Those heterozygous for intermediate (e.g., 2D6 *10) and extensive are given an extensive classification. In addition, a genotype “activity score” was determined using a previously reported scheme [8] where points are summed for each 2D6 allele. A score of 0 was given to alleles associated with poor metabolism (*3, *4, *5, *6, and *7), 0.5 for intermediate metabolism (*9, *10, *17, *29, and *41), 1.0 for extensive metabolism (*1, *2, and *35), and 2.0 for ultrametabolism (*1XN, *2XN, *35XN, and *41XN, gene duplications). For the modified activity score, the sum was lowered by 0.5 points if the subject reported the presence of a weak 2D6 inhibitor (citalopram, trazodone, sertraline, paroxetine, quinine, or methylene dioxymethamphetamine) and lowered by 0.75 if the subjected patients reported a

strong inhibitor (bupropion, paroxetine, or diphenhydramine). No adjustment was made for patients on venlafaxine, as this drug has no effect on 2D6 activity [15].

Univariate and multivariate regression analyses were computed for age, BMI, ethnicity, and adjusted activity score to predict tamoxifen metabolite concentrations, measured in ng/ml. For the UCSF cohort, BMI data were available on 56 of 88 subjects. The distribution of serum tamoxifen and metabolite results across the study sites was non-parametric. Therefore, tamoxifen and metabolite results and ratio of endoxifen-*N*-desmethyltamoxifen ratio were log (base 10) transformed, resulting in parametrically distributed data. All the calculations were performed using MedCalc ver 11.5.1.0. (Mariakerke, Belgium).

Results

A summary of the patients enrolled into the training and validation sets is shown in Table 1. Racial/ethnic categories differed between the two sites, with more Asians in the UCSF cohort and more Caucasians in the Dana-Farber cohort. There was no significant difference in the usage of individual drugs known to inhibit 2D6 (Table 1). Citalopram and venlafaxine, used to control hot flashes and vaginal dryness associated with tamoxifen use, were the drugs most frequently cited. In the UCSF cohort, there were 8 women who did not report citalopram use, but for whom citalopram was found in serum. In this cohort, nine women who self-reported usage did not have detectable drug levels in their serum. In the Dana-Farber cohort, undisclosed use of citalopram was discovered in five women, venlafaxine in seven, diphenhydramine in one, quinidine in two, and the herbal Kavaine in two. Eleven women self-reported usage did not have detectable drug levels in their serum. In the Dana-Farber cohort, undisclosed use of citalopram was discovered in five women, venlafaxine in seven, diphenhydramine in one, quinine in two, and the herbal Kavine in two. Drugs were found in 10 women who did not self-report usage, while seven women self-reported drugs did not have detectable drug levels in their serum. Regarding unadjusted activity scores, there were no differences in the incidence between the two cohorts.

Table 2 lists the univariate and multivariate proportions of variance explained (r^2) by the linear regression models for genotype scores and demographic variables to predict tamoxifen and metabolites for the training, validation, and combined cohorts. Across the various genotyping schemes, the associations between genotypes and tamoxifen, 4-hydroxytamoxifen, and *N*-desmethyltamoxifen were poor. While CYP 2D6 is the principal enzyme for conversion of tamoxifen to the 4-hydroxy metabolite, this intermediate is present in low concentrations and has a shorter half-life.

By contrast, the association between genotypes and endoxifen as well as between genotypes and the ratio of endoxifen to *N*-desmethyltamoxifen were considerably higher [$r^2 = 0.342$ and 0.465 , respectively]. Results progressively improved when the activity score scheme was used ($r^2 = 0.415$ and 0.532) and when data on CYP 2D6 drug inhibitors were incorporated ($r^2 = 0.469$ and 0.656 , respectively). There was no significant difference in the correlation between drug inhibitor usage determined as measured by mass spectrometry or self-reported (data not shown). Therefore, it was considered positive if either was present.

The best correlations were observed with inclusion of age, gender, activity score, and drug inhibitors ($r^2 = 0.520$ and 0.659 , respectively). The inclusion of BMI did not further improve the regression. The best correlation was observed between the ratio of endoxifen to *N*-desmethyltamoxifen.

Table 2 also shows the results of the regression coefficients for the validation cohort. The correlation coefficients were consistently lower in the validation cohort than the development cohort. Table 3 shows the log-linear regression equation for endoxifen and the endoxifen/*N*-desmethyltamoxifen ratio from the multiethnic UCSF cohort. From this equation, a prediction of these metabolite concentrations can be rendered within the stated variability. This cohort was selected because it produced the highest degree of correlation. A prediction of endoxifen concentrations may be possible if there is good adherence to tamoxifen dosing and reliable information on demographics and the use of CYP2D6 drug inhibitors. Figure 1a, b shows the calculated versus observed endoxifen and endoxifen/*N*-desmethyltamoxifen ratios for the entire cohort using the regression equations shown in Table 3.

Discussion

The aim for pharmacogenomic testing in clinical practice is to link genotype variances directly into clinical management decisions that can improve on therapeutic outcomes [16]. Because of the metabolism of tamoxifen into active metabolites, it has been an important test case for clinical pharmacogenomics. Should low endoxifen be confirmed to heighten risk of recurrence of early stage breast cancer, outcomes might be improved by developing an understanding of which patients are likely to have low endoxifen, and therefore which might benefit from a different hormonal therapy regimen or dose. Irvin et al. recently showed that doubling the tamoxifen dose from 20 to 40 mg among intermediate and poor CYP2D6 metabolizers results in increased endoxifen concentrations at steady state [17]. Rae et al. showed that patients who were wild type for CYP2D6 had high genotype activity scores with lower adherence to tamoxifen [18]. The authors suggested that these individuals had a higher rate of undesirable side effects such as hot flashes. We do not have direct data on adherence, which is a limitation of this study. However, in the Dana-Farber cohort, a qualitative assessment of adherence using the Morisky scale was attempted. We have developed a detailed model that includes both genotypic and clinical and demographic data to predict serum endoxifen levels among women on tamoxifen. Our analysis demonstrates that activity score is far more predictive than the traditional classification of “normal,” “intermediate,” and “poor metabolizer.” These results are consistent with the analysis of Borges et al. [8].

The results of this study demonstrate a very high explanatory power of CYP2D6 activity score on endoxifen levels and on endoxifen/*n*-desmethyl ratios. We were able to explain ~50–60% of the variance in the training dataset and ~50% of the variance with the modified activity score that accounts for the presence of drug inhibitors. Considering that activity score results may still group some alleles with slightly different levels of activity together, this is a very high level of the variance explained by a single gene. These data demonstrate that CYP2D6 is the key enzyme for determining endoxifen levels.

A unique aspect of this study was the direct measurement of drug and herbals known to inhibit CYP 2D6. Our results showed that there were some patients where we found drugs but were not declared. Many breast cancer patients are on a variety of medications and may not be able to recite which drugs they are taking. This is true even if a list of potential drugs is given that includes brand and generic names, as was the case in the UCSF cohort. For patients who self-reported 2D6 drug inhibitor but no drug was found in the serum, we concluded that there may have been abstinence at the time of blood collection. Despite this, there still may have had an effect on tamoxifen metabolism on a long-term basis, assuming that they were truthful in taking the drug. There was little difference in the degree of correlation between the self-reported and the found drug usage, and therefore, we counted them as present if either of the two criteria was met.

One might have expected an inverse correlation of 2D6 genotype with tamoxifen, i.e., higher blood concentrations in poor metabolizers, but no correlation was observed. The rate of tamoxifen metabolism to *N*-desmethyltamoxifen is dependent on CYP3A4/5 enzyme activity, which likely accounts for the weakness of the association with CYP 2D6 genotype. The levels of the other metabolites may also be influenced by other enzymes. However, since none of these are known to have strong biological activity against cancer except 4-hydroxytam, and since the latter is not bioavailable at significant levels, genotypes at other genes are less likely to be relevant for breast cancer.

The linkage of pharmacogenomics data to phenotypic characterization has been previously accomplished for other widely used drugs, such as warfarin. Using pharmacogenomics data for CYP 2C9 and vitamin K epoxide reductase complex 1, algorithms have been established that predict the optimum warfarin doses that predict stable anticoagulation (as measured by the international normalized ratio) [7]. These algorithms can predict approximately 37–55% of the variability in dosing [19]. We found that a similar degree of statistical correlation was obtained between CYP 2D6 genotyping and tamoxifen metabolite concentrations as was seen previously for CYP 2C9 and warfarin. Consistent with data reported by Borges et al. [8], the highest correlation was observed with the ratio of endoxifen to *N*-desmethyltamoxifen.

To date, the significance of endoxifen levels for breast cancer outcomes remains unclear. In longitudinal tamoxifen breast cancer outcome studies, the endoxifen/*N*-desmethyltamoxifen ratio was not predictive of adverse outcomes in the WHEL study [9]. Therefore, the stronger correlation observed between this ratio and 2D6 genotypes is of uncertain clinical relevance. It may be harder to predict the absolute level of endoxifen because this may be more affected by adherence to tamoxifen and gastrointestinal absorption.

The difference in the degree of correlation between the UCSF and Dana-Farber cohorts may be caused by clinical variation in patient populations or the effect of sample shipping and testing, and variations in use of concurrent medicines or medical supplements (Table 1) [20]. Although some herbal medications inhibit CYP 2D6 [21] and as we tested for the presence of eight of them, we did not specifically ask patients for their herbal medication usage. In addition, the presence of a larger number of Asian patients who were in the UCSF dataset may have also improved the information content of the model since a large number of

Asians carry the *10 allele which is usually classified as an “intermediate activity.” We found some differences in the number of women who self-reported CYP 2D6 drug inhibitors versus those found by mass spectrometry. Despite these differences, the correlation coefficients from the validation cohort, at least for the endoxifen/*N*-desmethyldoxifen ratio at 0.591, were as high as the best of those reported by Borges et al. [8].

One limitation of this study is that it measured total tamoxifen and metabolite concentrations based on what was known more than a year ago, when the research began. Recent reports however have shown that the metabolism tamoxifen to endoxifen is considerably more complex than previously thought. Murdter et al. showed that tamoxifen is further broken down into E, Z, and Z' isomers [22]. Analytic methods based on LC/MS–MS have been developed for the measurement of these isomers [23]. The clinical significance of these isomers has yet to be determined, though in vitro models have suggested that the E isomers have weaker affinity for estrogen receptors. In this article, we did not speciate the isomers of the major tamoxifen metabolites. If the Z and Z' isomers are more clinically significant, then it is likely that there is a closer link between the active isomers of tamoxifen metabolites and genotype activity scores than we found here with total metabolite concentrations.

More refined studies of factors that impact levels of active isomers of tamoxifen metabolites may further clarify when low levels should be suspected. Ongoing research focusing on the clinical relevance of tamoxifen metabolites will be needed to determine the true impact of pharmacogenomics in breast oncology.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Abbreviations

CYP	Cytochrome
UCSF	University of California, San Francisco
ATAC	Arimidex, Tamoxifen, Alone or in Combination
BIG	Breast International Group
WHEL	Women's Healthy Eating and Living
TamGEN	Tamoxifen genetics
PCR	Polymerase chain reaction
BMI	Body mass index

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Fig. 1.

a Regression of calculated endoxifen concentrations for all subjects using the modified activity score using the equation shown in Table 1. **b** Regression of calculated endoxifen:*N*-desmethylenoxifen ratios for all subjects using the modified activity score using the regression equation shown in Table 1

Table 1

Demographic characteristics and co-medication use for UCSF and Dana Farber Studies

Characteristic	UC San Francisco Number/ mean (%)	Dana Farber Number/ mean (%)	<i>P</i>
<i>n</i>	88	81	
Age, years	48.4 (±9.3)	49.7 (±8.2)	NS
Self-reported ethnicity			
Caucasian	63 (71.6%)	75 (93.8%)	<0.0005
Asian/East Asian	18 (20.4%)	2 (2.5%)	<0.001
Latina/Hispanic	5 (5.7%)	1 (1.2%)	NS
African American	1 (1.1%)	2 (2.5%)	NS
Declined/refused/do not know	1 (1.1%)	0 (0%)	NS
Self-reported or found 2D6 drug inhibitor usage ^a			
Citalopram	17 (19%)	16 (20%)	NS
Bupropion	2 (2.3%)	0 (0%)	NS
Venlafaxine	4 (4.5%)	10 (12%)	NS
Trazodone	2 (2.3%)	2 (2.4%)	NS
Sertraline	2 (2.3%)	0 (0%)	NS
MDMA	1 (1.1%)	0 (0%)	NS
Diphenhydramine	2 (2.2%)	2 (2.4%)	NS
Paroxetine	0 (0%)	1 (1.2%)	NS
Methadone	1 (1.1%)	0 (0%)	NS
Quinine	1 (1.1%)	2 (2.4%)	NS
Kavaine	0 (0%)	2 (2.4%)	NS
Total drug mentions	32 (36%)	33 (41%)	NS
Unadjusted activity score			
0.0	4 (4.5%)	2 (2.5%)	NS
0.5	5 (5.7%)	7 (8.6%)	NS
1.0	25 (28.4%)	23 (24.5%)	NS
1.5	30 (34.1%)	18 (22.2%)	NS
2.0	20 (27.2%)	31 (38.3%)	NS
2.5	2 (2.3%)	0 (0%)	NS
3.0	2 (2.3%)	1 (1.2%)	NS

^aMore than 1 drug inhibitor may have been used per patient

Univariate (activity gene scores) and multivariate (with demographics) correlation (r^2) to log transformed tamoxifen and metabolite concentrations

Table 2

Criteria	Tam	4-OH	Endox	NDM	Endox/NDM
Traditional genotype score					
Training, $n = 88$	0.006	0.073	0.342	0.033	0.465
Validation, $n = 81$	0.024	0.198	0.266	0.014	0.371
Total, $n = 169$	0.012	0.078	0.289	0.019	0.412
Activity score					
Training, $n = 88$	0.002	0.077	0.415	0.026	0.532
Validation, $n = 81$	0.010	0.076	0.341	0.044	0.547
Total, $n = 169$	0.006	0.073	0.361	0.027	0.537
Modified activity score ^a					
Training, $n = 88$	0.002	0.087	0.469	0.054	0.656
Validation, $n = 81$	0.005	0.059	0.319	0.068	0.568
Total, $n = 169$	0.000	0.074	0.382	0.046	0.613
Multivariate analysis model 1 ^b					
Training, $n = 56$	0.078	0.150	0.520	0.158	0.659
Validation, $n = 81$	0.024	0.069	0.324	0.079	0.567
Total, $n = 137$	0.029	0.098	0.396	0.066	0.615
Multivariate analysis model 2 ^c					
Training, $n = 88$	0.172	0.184	0.468	0.189	0.624
Validation, $n = 81$	0.026	0.076	0.322	0.080	0.577
Total, $n = 169$	0.035	0.086	0.352	0.082	0.56

^aWith inclusion of self-reported and measured drugs that inhibit CYP 2D6

^bModel includes modified activity score, age, and body mass index

^cModel includes modified activity score, age, and race

Table 3

Log-linear regression equation for endoxifen and endoxifen/*N*-desmethyldoxifen from the UCSF cohort

$\text{Log (endoxifen)} = 0.372 + 0.0036 (\text{age}) + 0.020 (\text{race}) + 0.272 (\text{modified activity score})$
$\text{Log (endox/N-desmethyldox)} = -1.81 + 0.00072 (\text{age}) - 0.0114 (\text{race}) + 0.332 (\text{modified activity score})$

Where, age is in years, race 0 = white, 1 = Latina, 2 = African American, 3 = Asian and 4 = other