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Author: Dezentjé, Vincent

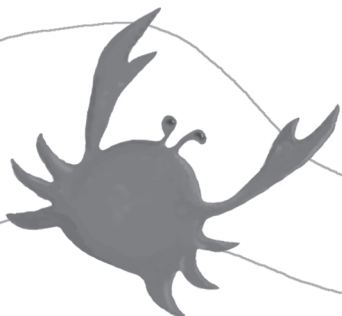
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CYP2D6 genotype in relation to tamoxifen efficacy in a Dutch cohort of the tamoxifen exemestane adjuvant multinational (TEAM) trial

Vincent O. Dezentjé, Ron H.N. van Schaik, Judith M. Vletter – Bogaartz, Tahar van der Straaten, Judith A.M. Wessels, Elma Meershoek – Klein Kranenborg, Els M. Berns, Caroline Seynaeve, Hein Putter, Cornelis J.H. van de Velde, Johan W.R. Nortier, Hans Gelderblom, Henk-Jan Guchelaar



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ABSTRACT

The clinical importance of CYP2D6 genotype as predictor of tamoxifen efficacy is still unclear. Recent genotyping studies on CYP2D6 using DNA derived from tumor blocks have been criticized because loss of heterozygosity (LOH) in tumors may lead to false genotype assignment. Postmenopausal early breast cancer patients who were randomized to receive tamoxifen, followed by exemestane in a large randomized controlled trial were genotyped for five CYP2D6 alleles. CYP2D6 genotypes and phenotypes were related to disease-free survival during tamoxifen use (DFS-t) in 731 patients. By analyzing microsatellites flanking the CYP2D6 gene, patients whose genotyping results were potentially affected by LOH were excluded. In addition, exploratory analyses on 24 genetic variants of other metabolic enzymes and the estrogen receptor were performed. For the CYP2D6 analysis, only 2.3% of the samples were excluded, because influence of LOH could not be ruled out. No association was found between the CYP2D6 genotype or predicted phenotype and DFS-t (poor vs. extensive metabolizers: unadjusted hazard ratio 1.33, 95% CI 0.52–3.43; $P=0.55$). DFS-t was associated with UGT2B15*2 (Vt/Vt + Wt/Vt vs. Wt/Wt: adjusted hazard ratio 0.47, 95% CI 0.25–0.89; $P=0.019$) and the estrogen receptor-1 polymorphism ESR1 PvuII (gene–dose effect: adjusted hazard ratio 1.63, 95% CI 1.04–2.54; $P=0.033$). In postmenopausal early breast cancer patients treated with adjuvant tamoxifen followed by exemestane neither CYP2D6 genotype nor phenotype did affect DFS-t. This is in accordance with two recent studies in the BIG1-98 and ATAC trials. Our study is the first CYP2D6 association study using DNA from paraffin-embedded tumor tissue in which potentially false interpretation of genotyping results because of LOH was excluded. Polymorphisms in the estrogen receptor-1 and UGT2B15 may be associated with tamoxifen efficacy, but these findings need replication.

INTRODUCTION

Adjuvant treatment with tamoxifen effectively decreases breast cancer recurrence. Responsiveness to tamoxifen may be partially based on a patient's ability to metabolize tamoxifen to its active metabolites 4-OH tamoxifen and the more abundant endoxifen. Biotransformation to endoxifen is mainly mediated by cytochrome P450 2D6 (CYP2D6), but several other enzymes are also involved in the formation of both active metabolites.¹ Supported by *in vitro* experiments, several cohort studies raised expectations that CYP2D6 genotype would become a clinically useful predictive marker for tamoxifen therapy.²⁻⁷ Still, clinical data on both the influence of the CYP2D6 genotype and CYP2D6 inhibitors on tamoxifen response are conflicting and some important issues remain unresolved.⁸⁻¹⁷ First, endoxifen's activity *in vivo* is uncertain, while its activity *in vitro* is apparent.¹⁰ Second, if endoxifen is the effective component in humans, the critical concentration needed for activity is uncertain, although a threshold of 5.9 ng/ml has recently been suggested.¹⁸ Finally, large inter-individual variance in endoxifen plasma concentration is only partially explained by CYP2D6 activity.^{3,7,19} While the tamoxifen metabolism is complex, variants of genes encoding other metabolic enzymes may also influence blood levels of 4-OH tamoxifen and/or endoxifen (Figure 5.1).^{1,20,21} Although it has been suggested that publications showing no association between the CYP2D6 genotype and tamoxifen efficacy are confounded, positive studies often also lack the ability to adjust for possible confounders.¹⁰ The recently performed large clinical trials that have compared the efficacy of tamoxifen with an aromatase inhibitor contain conscientiously collected patient data and are therefore well suited for pharmacogenetic analyses (e.g. ATAC, BIG1-98, IES, TEAM). Recently, two studies on patient cohorts of the BIG1-98 and ATAC trials did not result in an association between CYP2D6 genotype and tamoxifen efficacy.^{16,17} However, the CYP2D6 genotype analysis within the BIG1-98 patient cohort has been heavily criticized for using DNA from tumor instead of normal tissue.²²⁻²⁵ In the BIG1-98 study the apparent deviation from the Hardy Weinberg equilibrium may be explained by the loss of heterozygosity (LOH) of the CYP2D6 locus on chromosome 22q13 in tumor tissue.^{22,26} To justify the use of DNA retrieved from FFPE tumor blocks instead of DNA from normal tissue, a false homozygous genotype call because of LOH of chromosome 22q13 should be ruled out when CYP2D6 germline genetic variations are investigated.

In the Tamoxifen Exemestane Adjuvant Multinational (TEAM) trial, the efficacy of tamoxifen followed by exemestane (after 2.5 to 3 years) was compared to that of 5 years of exemestane in postmenopausal hormone receptor positive early breast cancer patients.²⁷ In the current study, we investigated possible associations between the CYP2D6 genotype / phenotype and disease

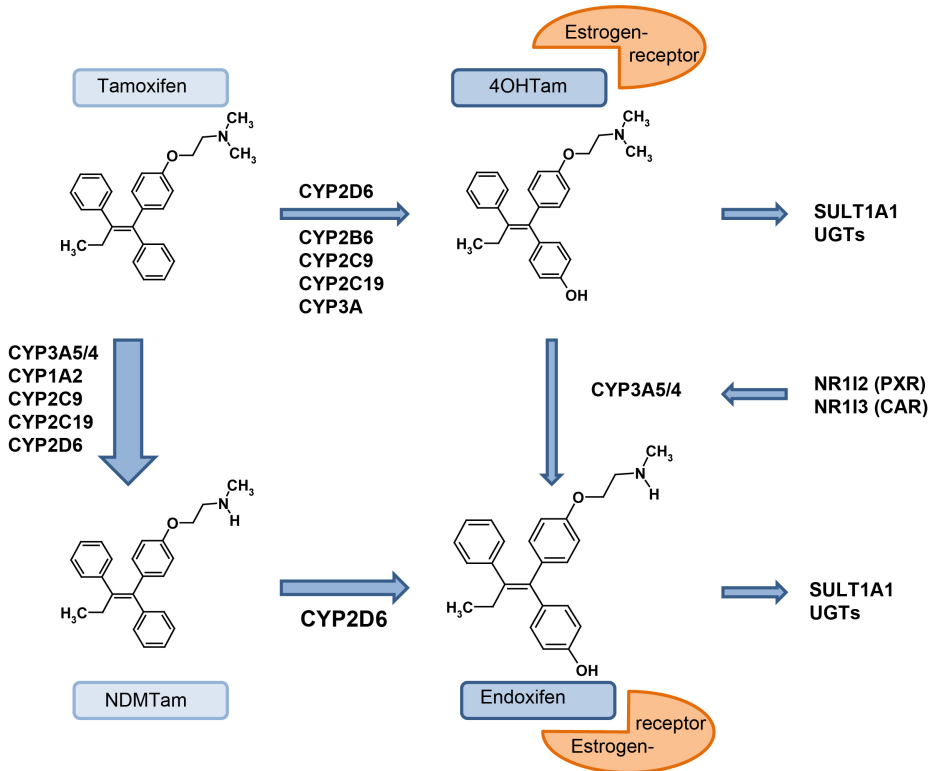


Figure 5.1 Tamoxifen metabolism. Abbreviations: 4OHTam, 4-hydroxytamoxifen; CYP, cytochrome P450 isoenzyme; SULT, sulfotransferase; UGT, UDP-glucuronosyltransferase; NDMTam, N-desmethyltamoxifen; NR1, nuclear receptor subfamily 1; PXR, pregnane X receptor; CAR, constitutive androstane receptor.

free survival in a Dutch cohort of the TEAM trial. Patients were excluded from our analysis when the possibility of a false CYP2D6 genotype because of LOH in the tumor could not be ruled out. In addition, associations with other genetic variants of metabolic enzymes and the estrogen receptor were explored.

METHODS

Patients

Of in total 9,779 postmenopausal early stage breast cancer patients enrolled in the TEAM trial, 2,753 were included in The Netherlands and 1,379 were randomized to tamoxifen (20 mg once

daily) with a planned switch to exemestane after 2.5–3 years. Tumor blocks of 746 of these 1,379 patients (54.1%) were available for genotyping. Information on tumor and patient characteristics including concomitant medication use, the tamoxifen start date as well as planned and unplanned stop dates were locally registered on case record forms and centrally collected at the datacenter in Leiden, The Netherlands. The current pharmacogenetic study was separately approved by the central medical ethics review board of the Erasmus University Medical Center in Rotterdam, The Netherlands.

Endpoint

The endpoint of the core TEAM trial was disease free survival (DFS-c), defined as the time from randomization to locoregional or distant recurrence, second breast cancer, or death without recurrence.²⁷ To avoid effect modification by subsequent aromatase inhibitor use, a new endpoint was created for the purpose of this pharmacogenetic study, being disease free survival defined as the time from the tamoxifen start date to the tamoxifen discontinuation date (DFS-t). Patients were censored at the time of tamoxifen discontinuation for reasons other than an event (e.g. planned or unplanned switch to exemestane or another aromatase inhibitor or intolerable side effects) or loss to follow-up. The original endpoint of complete disease free survival (DFS-c), as used in the core TEAM trial analysis, was used in a sensitivity analysis.²⁷

Genotyping

Germline genetic variants in candidate genes of enzymes involved in the tamoxifen metabolism and of the estrogen receptor (Figure 5.1) were selected based on assumed clinical relevance, high allelic frequency or the assumption that nonsynonymous amino acid change leads to altered protein functionality. The polymorphisms included in our analyses are listed in Table 5.1. Genotyping was performed on formalin fixed paraffin embedded tumor (FFPE) tissue as described previously.²⁸ In brief, from three slides of 20 µm, DNA was extracted with the Maxwell forensic DNA isolation kit (Promega, Leiden, The Netherlands). Before genotyping a pre-amplification step was used to increase the percentage of successfully genotyped samples without loss of reliability and with minimal use of DNA mass.²⁸ For genotyping Taqman assays (Applied Biosystems, Foster City, CA, USA) were used on the Biomark (Fluidigm, San Francisco, USA). In case of failure of genotyping using the Taqman based method, pyrosequencing was performed (Qiagen, Chatsworth, CA, USA) on a Pyrosequencer 96 MA (Biotage, Uppsala, Sweden). CYP2D6 genotypes were translated to predicted phenotypes (extensive, intermediate or poor metabolizer) as described in the supplementary methods section.

Table 5.1 Genetic variants of enzymes involved in the tamoxifen metabolism and estrogen receptor-1 included in the survival analysis

Gene	Allele name	Polymorphism	RS number	χ^2 test for Hardy Weinberg equilibrium	P-value
CYP2D6	*3	2549 A/del	rs4986774	0.07	0.80
	*4	1846 G/A	rs3892097	11.17 ^a	0.001
	*6	1707 T/del	rs5030655	20.97 ^a	4.7x10 ⁻⁶
	*14	1758 G/A	rs5030865	0.00	0.98
	*41	2988G>A	rs28371725	32.30 ^a	1.3x10 ⁻⁸
CYP2C9	*2	3608 C/T	rs1799853	0.16	0.69
	*3	42614 A/C	rs1057910	15.00 ^a	1.1x10 ⁻⁴
CYP2C19	*2	19154 G/A	rs4244285	4.23 ^a	0.04
	*17	-806 C/T	rs12248560	27.91 ^a	1.3x10 ⁻⁷
CYP2B6	*6	516 G/T	rs3745274	4.79 ^a	0.03
	*8	415 A>G	rs12721655	0.04	0.85
CYP3A5	*3	6986 A>G	rs776746	4.21 ^a	0.04
UGT1A4	*2	70 C/A	rs6755571	1.87	0.17
		-163 G/A	rs3732218	0.28	0.60
		-219 T/C	rs3732219	1.18	0.28
UGT1A8	*2	518 C/G	rs1042597	1.27	0.26
UGT2B7		-840 G/A	rs7438135	0.32	0.57
UGT2B15	*2	253 G/T	rs1902023	21.04 ^a	4.5x10 ⁻⁶
NR1I2 (=PXR)		8055 C/T	rs2276707	0.92	0.34
		7635 A/G	rs6785049	0.02	0.88
		-24113 C/T	rs2276706	0.64	0.42
		-25385 C/T	rs3814055	0.00	1.00
		b	10620 C/T	rs1054190	0.06
NR1I3 (=CAR)		10799 G/A	rs1054191	0.10	0.76
	b	47636 T/G	rs4073054	0.39	0.53
		45518 C/T	rs2307424	0.81	0.37
ESR1		47537 A/C	rs2307418	1.30	0.25
	Pvull	453-397 T/C	rs2234693	0.00	0.96
	Xbal	453-351 A/G	rs9340799	8.96 ^a	0.003

^a not in Hardy Weinberg equilibrium but genotype frequencies in accordance with previous literature/ NCBI

^b analyzed as haplotype

CYP, cytochrome P450 isoenzyme; UGT, UDP-glucuronosyltransferase; SULT, sulfotransferase; NR1, nuclear receptor subfamily 1; PXR, pregnane X receptor; CAR, constitutive androstane receptor; ESR1, estrogen receptor 1

Loss of heterozygosity

The ratio between tumor and germline DNA in a sample derived from FFPE tumor tissue, differs between samples. A high percentage of tumor DNA may result in falsely called genotypes because of LOH in the tumor. If a certain germline homozygous CYP2D6 genotype was assumed while in

fact one of the alleles has been lost in tumor but not in normal tissue a false test is the result.²² To avoid such incorrect interpretation of CYP2D6 genotyping results, three microsatellite markers D22S276, D22S2284 and D22S423 near the CYP2D6 gene on chromosome 22q13 with a high frequency of heterozygosity (>80%) were additionally determined (Table S5.4 supplementary files). The chance that a patient is homozygous for all three markers would be less than $0.20^3=0.8\%$. Thus, in nearly all patients including those with a homozygous germline CYP2D6 genotype, heterozygosity should be demonstrated for ≥ 1 microsatellite markers. We hypothesized that LOH of the CYP2D6 gene would also lead to LOH of the microsatellites given the proximity of the markers to the 22q13 locus. Heterozygosity for one of these microsatellite markers then validates a true homozygous germline CYP2D6 genotype tested in the same tumor block. Patients with a homozygous CYP2D6 genotype were excluded from our CYP2D6 analysis if influence of LOH on the CYP2D6 genotype in the tumor block could not be ruled out (i.e. in case of "homozygosity" of all microsatellite markers). Further details can be found in the supplementary methods section.

Statistical analysis

For comparison of proportions and means, χ^2 statistics and the Student's t-test were used. Cox regression analysis was used to assess whether DFS differed with respect to age at diagnosis, surgical procedure, tumor size, grade, nodal status, adjuvant chemotherapy or radiotherapy and the CYP2D6 genotypes or phenotypes. In an additional exploratory analysis, 24 genetic variants of other metabolic enzymes and the estrogen receptor were associated with DFS. Statistical analyses were performed using SPSS 20.0 (SPSS Inc., Chicago, IL). Covariates were included in the multivariable model if they were of clinical significance (tumor size, nodal status, grade and chemotherapy) or had a univariable P-value<0.1. Genetic variants were initially tested in a general model (2 degrees of freedom). If this test resulted in a P-value<0.1, the genetic variant was fitted and the most appropriate model (gene-dose, dominant or recessive) was selected. The distributions of DFS were estimated overall using the Kaplan-Meier method. A log-rank test was used to assess the association between the genetic variant and the outcome of interest. All results from the multivariable Cox regression analysis with a P-value<0.05 were considered significant. No correction for multiple testing for the 24 genetic variants other than CYP2D6 was applied, since this was an exploratory analysis.

RESULTS

Tumor blocks were collected from 746 patients enrolled in the TEAM trial and randomized to tamoxifen followed by exemestane from 59 of the 69 Dutch hospitals. Fifteen patients were ineligible because of distant metastasis at diagnosis (n=1), an ER/PgR negative primary tumor (n=4), a history of previous breast cancer (n=8) and because the patient never started tamoxifen therapy (n=2). The primary analysis therefore was performed on the 731 eligible patients. Twenty-nine genetic variants were successfully genotyped using Taqman assays except for CYP2D6*3 which was genotyped with pyrosequencing (Table 5.1).²⁸ Genotype frequencies of 10 selected genetic variants showed deviation from Hardy Weinberg equilibrium, but were still considered appropriate to analyze, because they did not differ from the frequencies previously reported in literature or on the NCBI website (Table 5.1).

In Table 5.2 the 731 eligible patients are described. These patients were similar to the whole group of Dutch patients randomized to the sequential arm of tamoxifen followed by exemestane (n=1,379) with regard to age, surgery, tumor size, grade, nodal status, adjuvant chemotherapy and radiotherapy (P>0.05, data not shown). The total number of DFS-t events in the 731 patients was 60 with a median follow-up time of 2.5 years until tamoxifen discontinuation. 25.3 and 59.1% of patients received adjuvant chemo- and radiotherapy respectively (Table 5.2). We translated the data from the 5 CYP2D6 alleles (*3, *4, *6, *14, *41) and concomitant CYP2D6 inhibitor use to a predicted poor, intermediate or extensive metabolizer phenotype. Of note, ultrarapid metabolizers could not be defined since it was not possible to detect gene duplication on this source of DNA.

Analysis of the CYP2D6 alleles and the three microsatellite markers D22S276, D22S2284 and D22S423 flanking the CYP2D6 gene demonstrated heterozygosity for at least one of the CYP2D6 alleles or microsatellite markers in 97.7% of patients with a specified CYP2D6 phenotype. The 14 patients (2.3%) with a homozygous CYP2D6 genotype in which no heterozygosity could be demonstrated for the microsatellite markers (because of homozygosity or test failure) were excluded from the analysis. The separate CYP2D6 alleles (most commonly *4 and *41) and the CYP2D6 phenotypes were not associated with DFS-t (Figure 5.2A-C and Table 5.3). Including the 14 patients in the analysis did not alter these results (Figure 3 supplementary files).

In the exploratory multivariable analysis UGT2B15*2 (Vt/Vt + Wt/Vt vs. Wt/Wt: HR 0.47, 95% CI 0.25-0.89; P=0.019) and ESR1-PvuII (gene-dose effect Wt/Wt>Wt/Vt>Vt/Vt: HR 1.63, 95% CI 1.04-2.54; P=0.033) seemed associated with DFS (Table 5.3). In the sensitivity analysis using the complete disease free survival (DFS-c) as was used in the core TEAM trial analysis, CYP2D6

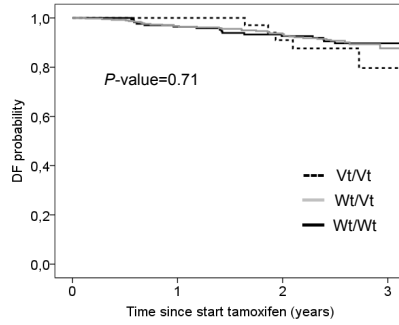
genotypes and phenotypes were also not associated (data not shown). In the exploratory analyses, only CYP2C19*2 (Vt/Vt vs Wt/Vt + Wt/Wt: HR 2.39, 95% CI 1.03-5.54; P=0.043) was associated with DFS-c in multivariable analysis. In contrast, the more frequent CYP2C19*17 ultrarapid metabolizer allele was neither associated with DFS-t nor DFS-c.

Table 5.2 Characteristics of 731 Dutch patients in the TEAM trial available for genotyping

	n	%
Age (years)		
Mean		66.1
Range		44.8-90.7
SD		9.2
Type of surgery		
Mastectomy	405	55.4
Breast Conserving	326	44.6
Tumor stage		
T1	310	42.4
T2	374	51.2
T3/ T4	44	6.0
T0/ Tis	1	0.1
Unknown	2	0.3
Nodal stage		
N0	241	33.0
N1	437	59.8
N2/3	53	7.3
Tumor grade		
1	111	15.2
2	310	42.4
3	269	36.8
Unknown	41	5.6
Adjuvant chemotherapy		
Yes	188	25.7
No	542	74.1
Unknown	1	0.1
Adjuvant radiotherapy		
Yes	432	59.1
No	298	40.8
Unknown	1	0.1
Concomitant CYP2D6 inhibitor use		
Paroxetine	9	
Fluoxetine	3	
Amiodarone	2	
Total	14	1.9

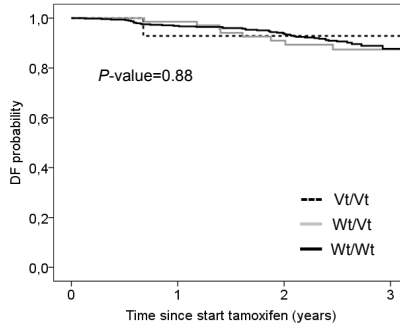
n, number of patients; SD, standard deviation

A. CYP2D6*4 possible LOH excluded



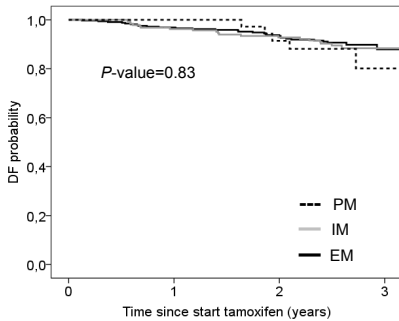
n at risk					n events	
Vt/Vt	42	34	29	5	5	
Wt/Vt	180	158	139	18	16	
Wt/Wt	399	340	294	36	36	

B. CYP2D6*41 possible LOH excluded



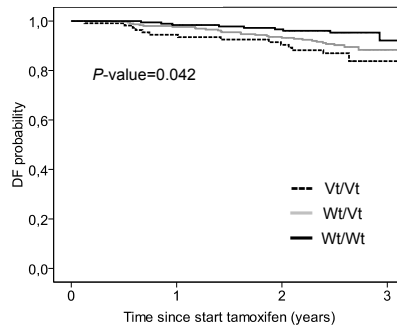
n at risk					n events	
Vt/Vt	16	13	11	2	1	
Wt/Vt	76	69	56	8	8	
Wt/Wt	525	446	394	50	47	

C. CYP2D6 phenotype possible LOH excluded



n at risk					n events	
PM	47	36	31	5	5	
IM	202	175	154	21	19	
EM	345	296	256	32	31	

D. ESR1 PvuII



n at risk					n events	
Vt/Vt	120	97	83	7	14	
Wt/Vt	314	275	240	29	29	
Wt/Wt	207	181	163	20	10	

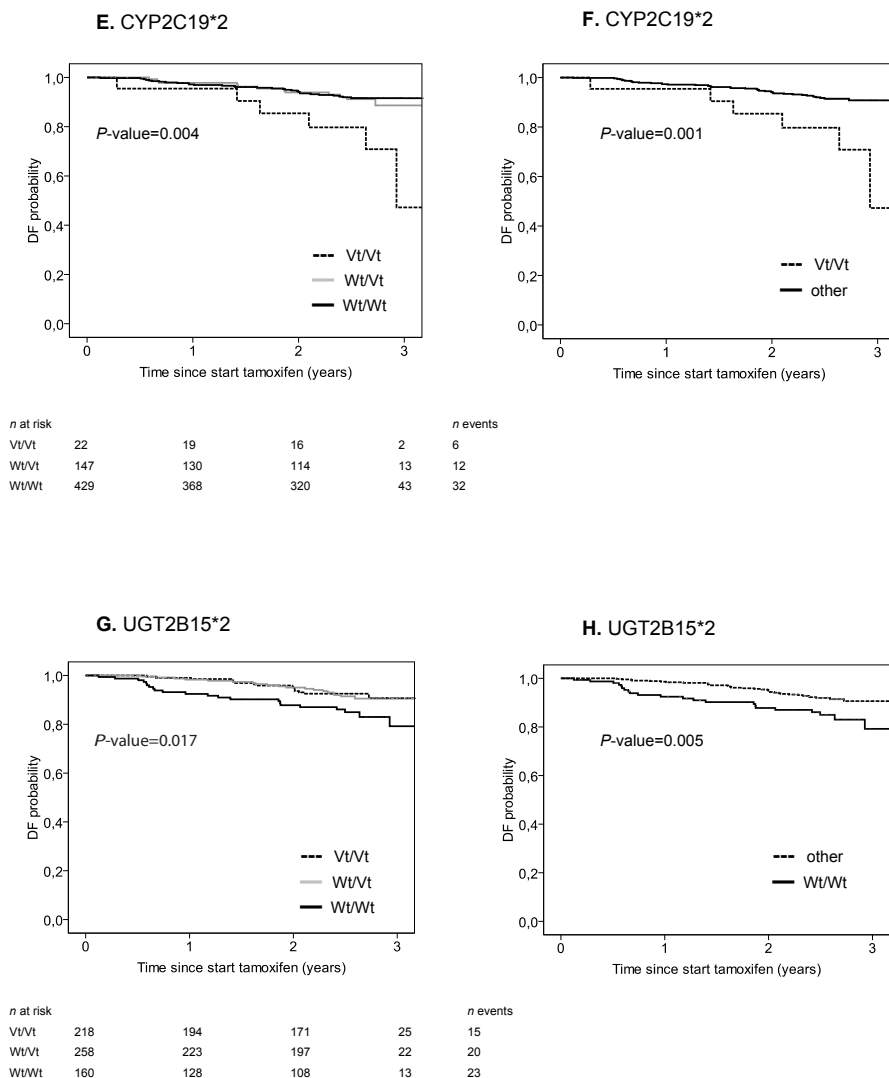


Figure 5.2 Kaplan Meier probabilities for Disease Free Survival during tamoxifen use (DFS-t) of: (A) CYP2D6 *4 genotypes (patients excluded if influence of LOH on assigned genotype cannot be ruled out); (B) CYP2D6 *41 genotypes (patients excluded if influence of LOH on assigned genotype cannot be ruled out); (C) predicted CYP2D6 phenotypes based on detection of *3, *4, *6, *14, *41 alleles and concomitant CYP2D6 inhibitor use (patients excluded if influence of LOH on assigned genotype cannot be ruled out); (D) ESR1 PvuII genotypes; (E) CYP2C19*2 genotypes; (F) CYP2C19*2 genotypes grouped according to a dominant model; (G) UGT2B15*2 genotypes; (H) UGT2B15*2 genotypes according to a recessive model. LOH, loss of heterozygosity; DF, disease free; Vt, variant type allele; Wt, wild type allele; PM, poor metabolizer; IM, intermediate metabolizer; EM, extensive metabolizer.

Table 5.3 Cox proportional hazards model estimates for disease free survival during tamoxifen use

	Univariable			Multivariable					
	HR	95% CI	P	Single SNPs adjusted for T, N, grade, surgery and chemotherapy			Adjusted for T, N, grade, surgery, chemotherapy and other SNPs		
				HR	95% CI	P	HR	95% CI	P
Age	1.01	0.98-1.03	0.72						
Tumor size									
≤ 2cm	1.00	Reference					1.00	Reference	
> 2 and ≤ 5cm	1.36	0.78-2.36	0.27				1.16	0.59-2.25	0.67
> 5cm	2.64	1.12-6.21	0.026				1.13	0.24-5.27	0.88
Nodal status									
N0	1.00	Reference					1.00	Reference	
N1	0.73	0.42-1.28	0.27				0.74	0.38-1.46	0.38
N2/3	2.64	1.24-5.63	0.012				1.34	0.43-4.18	0.62
Grade									
1	1.00	Reference					1.00	Reference	
2	1.12	0.45-2.81	0.80				1.12	0.36-3.51	0.85
3	2.29	0.96-5.47	0.06				2.63	0.87-7.95	0.09
Surgery									
Mastectomy	1.00	Reference					1.00	Reference	
Breast conserving	0.46	0.26-0.80	0.006				0.55	0.27-1.11	0.10
Previous chemotherapy									
No	1.00	Reference					1.00	Reference	
Yes	1.25	0.71-2.20	0.44				0.61	0.28-1.36	0.23
Previous radiotherapy									
No	1.00	Reference							
Yes	0.77	0.46-1.28	0.31						
CYP2D6 phenotype									
EM	1.00	Reference							
IM	1.01	0.57-1.78	0.99						
PM	1.33	0.52-3.43	0.55						
CYP2C19*2									
Wt/Wt + Wt/Vt	1.00	Reference		1.00	Reference		1.00	Reference	
Vt/Vt	3.78	1.61-8.89	0.002	2.83	0.99-8.08	0.052	1.56	0.37-6.53	0.55
UGT2B15*2									
Wt/Wt	1.00	Reference		1.00	Reference		1.00	Reference	
Wt/Vt + Vt/Vt	0.47	0.27-0.80	0.005	0.49	0.29-0.86	0.012	0.47	0.25-0.89	0.019
ESR1 P _{vull}									
Wt/Wt > Wt/Vt > Vt/Vt	1.64	1.11-2.41	0.013	1.64	1.10-2.45	0.015	1.63	1.04-2.54	0.033

SNP, single nucleotide polymorphism; T, tumor size; N, nodal status; HR, hazard ratio; EM, extensive metabolizer; IM, intermediate metabolizer; PM, poor metabolizer; Wt, wild type allele; Vt, variant type allele

NOTE: CYP2C19*2 genotypes are analyzed according to a recessive model; UGT2B15*2 genotypes are analyzed according to a dominant model; ESR1 P_{vull} genotypes are analyzed according to a multiplicative model indicated by (>) between the genotypes

DISCUSSION

No association between the CYP2D6 genotypes or phenotypes and tamoxifen efficacy was found in our study. Our findings are in line with the data of two studies recently published in the Journal of the National Cancer Institute in which DNA from tumor blocks was used to genotype CYP2D6.^{16,17} These studies were criticized because LOH in tumors would have led to false interpretation of CYP2D6 genotyping results. Indeed, the study of Regan reports a higher CYP2D6 *4/*4 frequency than expected in Caucasians.¹⁶ To avoid this, we performed the first CYP2D6 association study using DNA from FFPE tumor blocks in which potentially false genotypes resulting from LOH in tumor tissue were excluded. Of note, even after controlling for LOH, CYP2D6*4 and *41 still exhibited statistically significant departures from the Hardy Weinberg Equilibrium (Table 5.1). The genotype frequencies in the current study however, did not significantly differ from those in a population of Dutch early breast cancer patients, enrolled in a prospective trial in which DNA derived from whole blood was used for genotyping (CYPTAM: NTR1509, unpublished data). The reason why LOH did not affect our genotyping results in the majority of patients is probably because the slices of FFPE tumor tissue from which DNA is isolated contained substantial amounts of normal tissue. Previous studies in which 100% concordance between CYP2D6 genotype in normal and tumor tissue is demonstrated strengthen this finding.^{8,29} This however does not implicate that future genotyping studies can be validly performed using DNA from tumor blocks when the gene is known to suffer from LOH in the tumor. In tumor enriched samples LOH may still cause false interpretation of the genotyping result. Especially when genetic variants with low allele frequency are studied, a small amount of false genotyping results could notably impact the study results. Exclusion of those possibly false genotypes by using microsatellite analysis as presented here is recommended and will enable the future use of archived FFPE tumor blocks for pharmacogenetic studies, especially when genes are studied that exhibit LOH in tumor. This may be very useful because large clinical trials contain valuable clinical data but often only have FFPE tumor tissue available for genotyping.²⁵

Compared to other publications on heterogeneous populations the current study was performed in a trial population, with good documentation of patient data enabling a broad multivariable analysis. Concomitant CYP2D6 inhibitor use and tamoxifen adherence may interact with the CYP2D6 genotype and may be associated with clinical outcome.^{12,13,30} Therefore, these factors may cause confounding. In this study information on concomitant medication including CYP2D6 inhibitors (e.g. paroxetine) was available, enabling a more accurate classification into CYP2D6 phenotypes. However, the low prescription frequency (1.9%) may suggest incomplete registration.¹² To our knowledge adjustments for

tamoxifen compliance have only been made in one previous report.³¹ In the current TEAM trial cohort planned and unplanned tamoxifen discontinuation dates were registered. By censoring disease free survival at the time of tamoxifen discontinuation (DFS-t), effect modification by aromatase inhibitors is prevented and the chance of confounding by compliance is reduced. This method however limits the follow-up duration and number of events, thus decreasing the study's statistical power. For the CYP2D6 phenotype our study is powered ($1-\beta=80\%$) to detect a 2.1-fold increased risk for poor and intermediate metabolizers compared to extensive metabolizers. By performing a sensitivity analysis of CYP2D6 phenotype using the complete DFS (DFS-c), not censored at the end of tamoxifen use, thus including the years on exemestane, the number of events increases from 55 to 138. In this analysis our study is powered ($1-\beta=80\%$) to detect a 1.6-fold increased risk for poor and intermediate metabolizers compared to extensive metabolizers. However, despite this increase in statistical power the CYP2D6 phenotype is still not associated with disease free survival ($P=0.42$). The sequential exemestane use may have decreased the detrimental effect of CYP2D6 genotype on tamoxifen efficacy. In current daily practice however, optimal endocrine therapy for postmenopausal patients includes an aromatase inhibitor, which generally is given after 2.5 years of tamoxifen. Our study results therefore apply to most postmenopausal early stage breast cancer patients that are currently treated with tamoxifen.

Kiyotani suggested in a subgroup analysis that chemotherapy modifies the effect of CYP2D6 genotype on clinical outcome.³² An association between CYP2D6 genotype and outcome was only found in patients treated with tamoxifen, but not receiving chemotherapy, in contrast to patients who also received adjuvant chemotherapy. In our study however, subgroup analyses of patients with and without chemotherapy still resulted in a null association. Another possible explanation for our findings is that the CYP2D6 genotype mostly affects the late breast cancer recurrences. As was shown by Schroth the differences in DFS between the various CYP2D6 phenotypes in patients treated with 5 years of adjuvant tamoxifen became more apparent after 5 years of follow-up.⁹ Finally, the possibility of a real association between CYP2D6 genotype and tamoxifen efficacy with a smaller effect size cannot be precluded.

Although the association with CYP2D6 could not be replicated, our exploratory analyses suggest that polymorphisms in UGT2B15 and the estrogen receptor-1 impact DFS-t in breast cancer patients treated with adjuvant tamoxifen. The UGT2B15*2 and ESR1-PvuII polymorphisms are common and therefore potentially clinically relevant. The UGT2B15*2 polymorphism has been linked to decreased glucuronidation and clearance and may lead to accumulation of active metabolites and thus a better response to tamoxifen.^{20,33} In contrast, previous studies failed to show an association between UGT2B15 and clinical outcome in breast cancer patients using tamoxifen.^{10,34} In our study a

decreased DFS-t was found with increasing number of variant (C) alleles of ESR1 PvuII. The ESR1 PvuII polymorphism has been associated with different side effects of tamoxifen.³⁵⁻³⁷ We hypothesized that polymorphisms in ESR1 may change tamoxifen efficacy by alterations in estrogen receptor binding or signalling. The ESR1 PvuII genotype has been associated with susceptibility to the effects of hormone therapy on mammographic density in postmenopausal women: increased mammographic density was observed in women on hormone replacement therapy harbouring the ESR1 PvuII C/T (=Wt/Vt) and T/T genotype, but not the C/C genotype.³⁸ While high mammographic density is a known risk factor for breast cancer, reduction in mammographic density is observed during tamoxifen use and therefore may be a marker for tamoxifen response in breast cancer patients.³⁹ Hypothetically, the ESR1 PvuII genotype may be associated with susceptibility to the effect of tamoxifen on mammographic density and breast cancer recurrence. Although this hypothesis has not been supported by published data, an association between ESR1 PvuII genotype and tamoxifen efficacy has been reported.⁴⁰ In the sensitivity analysis UGT2B15*2 and ESR1 PvuII were not associated with the complete DFS suggesting effect modification by exemestane. Because of the exploratory nature of these analyses no adjustments for multiple testing were made, therefore these findings may also be caused by chance. Additionally, LOH in tumor tissue may have influenced these results as we did not account for potential LOH in our exploratory analyses.

In conclusion, we could not detect an association between CYP2D6 genotype or phenotype and tamoxifen efficacy, which is in line with previous data from other large studies.¹⁴⁻¹⁷ The current study however, is the first CYP2D6 association study in which the potential influence of LOH in tumor blocks was accounted for, justifying the use of DNA retrieved from FFPE tumor blocks. Our broader exploratory pathway analysis showed that UGT2B15*2 and ESR1-PvuII may be associated with DFS, although these findings need validation.

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SUPPLEMENTARY DATA

METHODS

CYP2D6 predicted phenotypes

CYP2D6 genotypes were translated to predicted phenotypes (extensive, intermediate or poor metabolizer). By definition, the CYP2D6 intermediate metabolizer phenotype predicted by genotype consisted of patients homozygous for a decreased activity allele (e.g. *41/*41) or heterozygous for an absent activity allele (e.g. *1/*4 and *41/*4). A patient could only be classified to a certain CYP2D6 phenotype if genotyping was successfully done for the CYP2D6 alleles with a reported frequency in Caucasians of more than 5%. In case of an allele frequency of less than 5% a missing genotyping result for that allele was accepted. For that specific allele the wild type was assumed. For example, if in a patient the assay for CYP2D6*4 resulted in a heterozygous (Wt/Vt) genotype but no result was available for the less frequent *3 allele (allele frequency=3%), the patient was considered to have a *1/*4 genotype and was thus classified as an intermediate metabolizer. Additionally, concomitant use of a CYP2D6 inhibitor could reclassify the CYP2D6 phenotype predicted by genotype.⁴¹

Loss of heterozygosity

DNA samples were pre-amplified for the three microsatellite markers as described by Fletcher et al.⁴² Each PCR reaction consisted of 1 pmol of each primer, 4 µl Qiagen Hotstar PCR mastermix (Qiagen, Venlo, The Netherlands), 3 µl DNA in total volume of 8 µl. PCR conditions for pre-amplification were as follows: 15 minutes at 95°C, 20 cycles at 94°C-55°C-72°C for respectively 20, 20 and 60 seconds. PCR was finalized by 10 minutes at 72°C. Next, to the PCR products 100 µl sterile water was added and 1 µl was used for second round PCR using primers listed in Table S5.4 (supplementary files). Each microsatellite marker was separately amplified by PCR. Each reaction consisted of 2.5 pmol reverse and forward primer (of which one primer was labeled with FAM-fluorescent dye) 5 µl Qiagen Hotstar PCR mastermix (Qiagen, Venlo, The Netherlands), 1 µl diluted pre-amplified DNA in total volume of 10 µl. PCR conditions for pre-amplification were as follows: 15 minutes at 95°C, 35 cycles at 94°C-55°C-72°C for 30 seconds each step and PCR was finalized by 10 minutes at 72°C. To PCR product 100 µl sterile water was added and 1 µl was used for fragment length analysis using ABI-3130 and peakscanner software according to manufacturers prescription (Life Technologies, Nieuwerkerk aan den IJssel, The Netherlands).

Table S5.4 Microsatellite markers for loss of heterozygosity analysis

Marker	Primers for second round PCR 5'-3'	Expected size
D22S276	AAATGGGCTTGTAAGAAAAATA* AAATATGAAGTACTTCTTACCAC	165 +/- 18bp
D22S284	GAGCAAGACCTGTCTCAAGA* ACAGCAAAATGATATTAGTTTGAGC	88 +/- 16bp
D22S423	GAGTGAGTGACTGAGTAAATGTAGTG* ATCCCTGAAATACACATATATGTAC	200 +/- 26bp

* Primers are labeled with FAM fluorescent dye at 5'-end

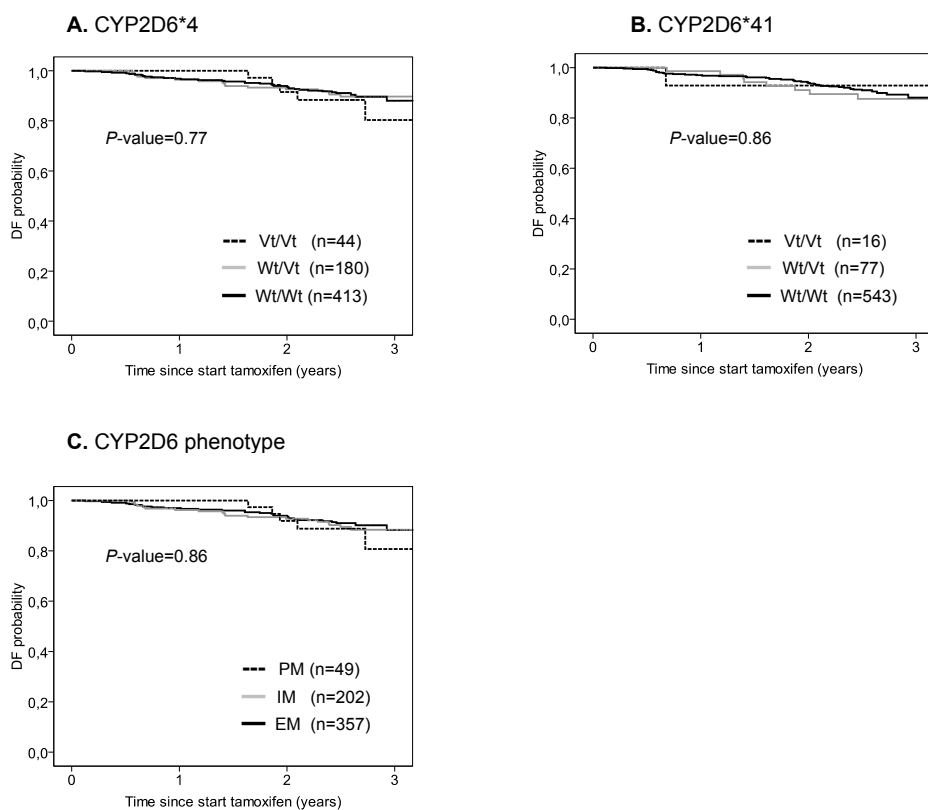


Figure S5.3 Kaplan Meier probabilities for Disease Free Survival during tamoxifen use (DFS-t) of: (A) CYP2D6 *4 genotypes (patients included with uncertain influence of LOH on assigned genotype); (B) CYP2D6*41 genotypes (patients included with uncertain influence of LOH on assigned genotype); (C) predicted CYP2D6 phenotypes based on detection of *3, *4, *6, *14, *41 alleles and concomitant CYP2D6 inhibitor use (patients included with uncertain influence of LOH on assigned genotype). LOH, loss of heterozygosity; DF, disease free; Vt, variant type allele; Wt, wild type allele; PM, poor metabolizer; IM, intermediate metabolizer; EM, extensive metabolizer.

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