

Metabolic Pathway Analysis and Effectiveness of Tamoxifen in Danish Breast Cancer Patients



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ABSTRACT

Background: Tamoxifen and its metabolites compete with estrogen to occupy the estrogen receptor. The conventional dose of adjuvant tamoxifen overwhelms estrogen in this competition, reducing breast cancer recurrence risk by nearly half. Phase I metabolism generates active tamoxifen metabolites, and phase II metabolism deactivates them. No earlier pharmacogenetic study has comprehensively evaluated the metabolism and transport pathways, and no earlier study has included a large population of premenopausal women.

Methods: We completed a cohort study of 5,959 Danish nonmetastatic premenopausal breast cancer patients, in whom 938 recurrences occurred, and a case-control study of 541 recurrent cases in a cohort of Danish predominantly postmenopausal breast cancer patients, all followed for 10 years. We collected formalin-fixed paraffin-embedded tumor blocks and genotyped 32 variants in 15 genes involved in tamoxifen metab-

olism or transport. We estimated conventional associations for each variant and used prior information about the tamoxifen metabolic path to evaluate the importance of metabolic and transporter pathways.

Results: No individual variant was notably associated with risk of recurrence in either study population. Both studies showed weak evidence of the importance of phase I metabolism in the clinical response to adjuvant tamoxifen therapy.

Conclusions: Consistent with prior knowledge, our results support the role of phase I metabolic capacity in clinical response to tamoxifen. Nonetheless, no individual variant substantially explained the modest phase I effect on tamoxifen response.

Impact: These results are consistent with guidelines recommending against genotype-guided prescribing of tamoxifen, and for the first time provide evidence supporting these guidelines in premenopausal women.

Introduction

Tamoxifen and its metabolites compete with estrogen for estrogen receptor (ER) binding (1–3). The conventional dose overwhelms estrogen in this competition (3), inhibiting estrogen-dependent

growth of tumor cells and reducing the risk of recurrence among early-stage breast cancer patients by nearly half (4). Tamoxifen remains guideline endocrine therapy for ER α^+ premenopausal breast cancer patients and an important alternative or sequential therapy in ER α^+ postmenopausal patients (5, 6). Although tamoxifen is sometimes labeled a prodrug, optimal metabolism is probably not necessary to achieve steady-state concentrations that overwhelm estrogen in competition for ER binding (1–3). Nonetheless, the potential for women with reduced metabolic capacity to accrue less than the full benefit of tamoxifen adjuvant therapy has been an ongoing controversy (3, 7–14).

Tamoxifen is metabolized by phase I reactions to active compounds primarily by hydroxylation and demethylation (Fig. 1; refs. 15, 16). The 4-hydroxylated metabolites bind the ER a hundred-fold more readily than the parent compound and its demethylated metabolite (17), but have less than 10% the steady-state concentration (1, 2). Glucuronidation (18, 19) and sulfonation (20), which are phase II reactions, yield inactive water-soluble metabolites that are excreted. Hydroxylation of the demethylated compound to endoxifen (4-hydroxy *n*-desmethyl tamoxifen) is catalyzed almost entirely by cytochrome P450 2D6 (CYP2D6; ref. 21). Women who carry two knockout or reduced function CYP2D6 alleles have reduced endoxifen concentrations (22–28). CYP2D6 variants have therefore received the most attention in clinical epidemiology studies of gene–drug interactions that might reduce tamoxifen’s effectiveness (3, 8, 10, 14, 29, 30). Multiple enzymes catalyze most steps in the metabolic pathway, and multiple pathways yield the same metabolites (15, 18–21), so focusing on a single enzyme is unlikely to capture the full importance of metabolism in achieving an optimal tamoxifen response. No earlier

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Note: Supplementary data for this article are available at Cancer Epidemiology, Biomarkers & Prevention Online (<http://cebp.aacrjournals.org/>).

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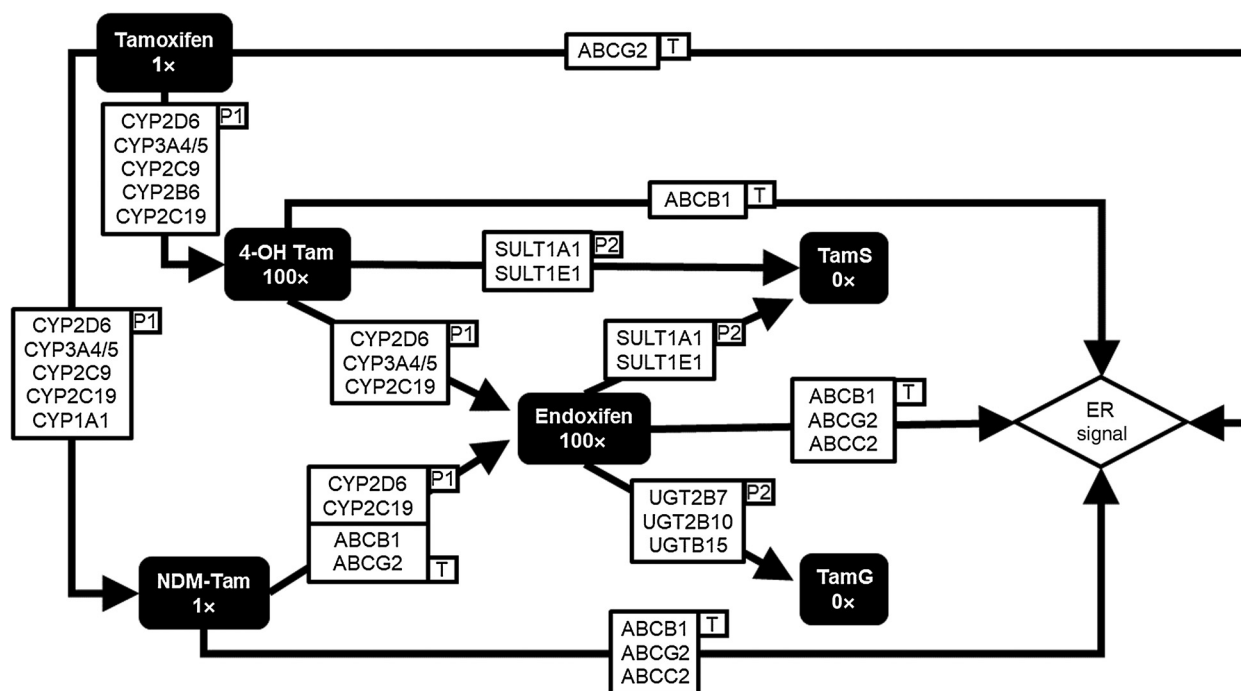


Figure 1.

Tamoxifen metabolic pathway. Tamoxifen and its metabolites appear as black boxes, which include relative ER binding affinities. Arrows denote transitions between compounds and interactions with the estrogen receptor (ER signal). White boxes overlaying the arrows show the polymorphic genes encoding the enzymes involved in metabolism or transport of tamoxifen at each transition. Flags on these boxes indicate the pathway concepts to which the boxes of genes belong (P1, phase I metabolism; P2, phase II metabolism; T, transporter; refs. 44, 45). 4-OH Tam, 4-hydroxy tamoxifen; NDM-Tam, N-desmethyl tamoxifen; TamG, tamoxifen glucuronide; TamS, tamoxifen sulfate.

study of tamoxifen adjuvant therapy has taken a comprehensive analytic approach to determine the importance of the metabolic pathway. Furthermore, no earlier study has been restricted to premenopausal women, for whom tamoxifen remains guideline-recommended adjuvant endocrine therapy and in whom estrogen concentrations are much higher, possibly heightening the impact of reduced metabolic capacity.

To address limitations of previous studies, we used Bayesian pathway analyses (31, 32), which incorporate prior knowledge of the metabolic pathway biology and of the functional consequences of genetic variation within the complete tamoxifen pathway. This novel strategy allowed for analysis of multiple genetic variants and the complex interactions between them to assess the impact of specific variants, pathway proteins, transporters, and key summary concepts in the tamoxifen pathway on tamoxifen effectiveness in a cohort study restricted to premenopausal women and a case-control study nested in primarily postmenopausal women. The use of these two independent studies allowed comparison of results to examine the replicability of pathway effects.

Materials and Methods

This project was approved by the Institutional Review Board of Emory University, the ethical committee of Aarhus University, and review committees at other participating institutions. Danish law does not require informed consent for registry-based studies but does require approval from the Danish Data Protection Board. The Danish ethical committee must approve studies based on biological samples, but can grant, as in this case, exceptions from informed consent.

Additional details of the methods appear in the Online Data Supplement.

Populations

The cohort included premenopausal women, identified from the Danish Breast Cancer Group (DBCG) clinical registry, diagnosed with nonmetastatic first invasive primary breast cancer 2002–2011 (33). Participants were restricted to ER α^+ , tamoxifen-treated (ER α^+ /TAM $^+$), and ER α^- /TAM $^-$ patients, who did not receive neoadjuvant chemotherapy or neoadjuvant endocrine therapy, and who were followed from diagnosis to recurrence, death, 10 years of follow-up, loss to follow-up due to emigration, diagnosis with another primary malignancy, or September 25, 2017, whichever occurred first. The study's hypothesis is that reduced metabolic function reduces the effectiveness of tamoxifen, so genetic markers of reduced metabolic function should only be associated with recurrence in the ER α^+ /TAM $^+$ patients. We included ER α^- /TAM $^-$ patients in the premenopausal cohort as a negative control stratum to ensure that the genetic markers of reduced metabolic function had a null association with recurrence in women not treated with tamoxifen.

The source population of the nested case-control study of primarily postmenopausal breast cancer patients consisted of female residents of the Jutland Peninsula in Denmark ages 35 to 69 years when diagnosed with a nonmetastatic first invasive primary breast cancer between 1985 and 2001, and who were registered in the DBCG (34). The population was restricted to 1,826 ER α^+ /TAM $^+$ patients who did not receive neoadjuvant chemotherapy or neoadjuvant endocrine therapy, were intended to be treated with tamoxifen for at least 1 year, and were followed until

breast cancer recurrence, death from any cause, loss to follow-up (e.g., emigration), 10 years of follow-up, or September 1, 2006, whichever occurred first. For each case, we selected without replacement one control from the source population who was alive and had no recurrence after the same amount of follow-up. Controls were matched to cases on menopausal status at diagnosis (premenopausal or postmenopausal), date of breast cancer surgery (caliper matched ± 12 months), county of residence at time of diagnosis, and cancer stage at diagnosis. Almost all $ER\alpha^+/TAM^+$ patients in this case-control study were postmenopausal because tamoxifen was not DBCG guideline therapy for premenopausal women until 1999 (35).

Data collection

We used the Danish Civil Personal Registration number to link data sets (36). We used the DBCG registry for data on demographics (age, menopausal status, and hospital of diagnosis), tumor characteristics (UICC stage, histologic grade, and ER expression), and therapy characteristics (primary surgical tumor management, receipt of radiotherapy, receipt of adjuvant chemotherapy, receipt of tamoxifen therapy, and completion of tamoxifen therapy in the premenopausal cohort). We used the Danish National Patient Registry (37) for data on the conditions in the Charlson comorbidity index (38) that were present at breast cancer diagnosis.

DNA isolation and genotyping

Laboratory personnel were blinded to clinical information. We used the Danish National Pathology Registry and Data Bank (39) to identify and collect formalin-fixed, paraffin-embedded (FFPE) primary tumor tissue blocks from pathology department archives of treating hospitals. FFPE sections were cut from each sample, and DNA was extracted using the Omega Mag-Bind FFPE DNA 96 Kit (M6958-01) with RNase treatment and heat deparaffinization on the Thermo Fisher KingFisher Flex with the Omega KF script (Omega_M6958_WaterDip_100uLElution_KF). We have earlier compared genotypes for three variants obtained from DNA extracted from 106 tumor-affected tissues with the genotypes obtained from DNA extracted from paired nonneoplastic tissues and found near-perfect concordance (40).

A comprehensive review of tamoxifen pharmacogenetic and pharmacokinetics literature yielded a set of 126 genetic variants in 17 genes known to be involved in tamoxifen metabolism or transport. We restricted genotyping to single-nucleotide polymorphisms (SNP) with minor allele frequencies $\geq 5\%$ in populations of European ancestry and with known functional importance, yielding a set of 32 variants in 15 genes (Supplementary Table S1). TaqMan allelic discrimination was conducted using the dry down method as directed by the manufacturer. Post-read fluorescent data were collected, and initial automatic genotyping calls (auto-calls) were made using Thermo Fisher SDS v2.4 and TaqMan Genotyper v1.3 software suites, respectively. Genotype custom calls were defined by manual adjustment of genotype regions or point-by-point reclassification on TaqMan allelic discrimination plots by consensus between five investigators. Missing genotypes were multiply imputed using observed genotypes from the same gene family as that of the missing value, in addition to patient and tumor characteristics. In this manner, we created 50 complete data sets using the “MICE” package for R (41), which we aggregated into a single data set for analysis. We tested whether the genotypes for each variant, for each calling protocol, were in Hardy-Weinberg equilibrium (42). Complete genotyping results for each variant are presented in Supplementary Fig. S3, pages S22–S85.

Definitions of variables

For each variant, patients were classified as having two, one, or no functional allele. We used the DBCG definitions of breast cancer recurrence (any type of breast cancer or distant metastases diagnosed subsequent to the initial course of therapy; ref. 43) and menopausal status (35). We included the following covariates: calendar time of breast cancer diagnosis, age at diagnosis, Charlson comorbidity index at diagnosis (38), county of residence at diagnosis (matched in the postmenopausal case-control study), UICC stage at diagnosis, histologic grade, surgery type, receipt of radiotherapy, receipt of adjuvant trastuzumab, and receipt of adjuvant chemotherapy.

Statistical analysis

Analyses were conducted within strata of $ER\alpha^+/TAM^+$ and $ER\alpha^-/TAM^-$. We computed the frequency and proportion of patients within categories of genotypes, of assigned protocols of tamoxifen duration, and of the covariates. We estimated adjusted associations and 95% confidence intervals between individual SNPs and breast cancer recurrence using Cox proportional hazards regression models in the premenopausal cohort and using unconditional logistic regression in the postmenopausal case-control study. We allowed for nonlinearity of associations across the number of minor alleles by modeling genotypes as factor variables. We then repeated the analyses with enforced linearity to examine a possible dose response of increasing numbers of minor alleles. Each analysis was performed using the genetic data generated from the auto-call and custom-call genotyping data. All genetic associations were calculated with reference to homozygote wild-type patients.

We estimated the impact of genetic variation in the tamoxifen metabolism/transport pathway on breast cancer recurrence using ALPS pathway analysis. ALPS is a Bayesian method for deriving the effect of a set of interacting nodes on an outcome, incorporating prior information about the pathway (31). To implement ALPS, we codified the well-established tamoxifen metabolic pathway (Fig. 1) as a prior on the tree structures considered. This structure represents the relationships between metabolites, genes, and gene variants on the routes between tamoxifen administration and breast cancer recurrence. We ran one million Markov Chain Monte Carlo (MCMC) iterations of ALPS from a random starting point in the prior forest using imputed, aggregated data from the studies. We also ran one million iterations without observed data to obtain prior probabilities for pathway features encoded in the prior. From these MCMC results, we calculated posterior probabilities and Bayes factors (BF) for individual variants, for pathway interaction structures derived by ALPS, and for key summary concepts in the tamoxifen pathway. These concepts were defined according to (i) gene function (phase I metabolism, phase II metabolism, or transporter; refs. 44, 45); (ii) tamoxifen metabolite formation (4-OH tamoxifen, N-desmethyl tamoxifen, endoxifen, tamoxifen sulfate, and tamoxifen glucuronide); and (iii) variants in the gene encoding CYP2D6. We computed BFs by dividing the posterior odds for each gene variant or pathway concept in the three study groups by the corresponding prior odds.

Conventional analyses were carried out with SAS v.9.4 (SAS Institute, Cary, NC, USA). Multiple imputation and ALPS analyses were carried out with R v.3.5.1 (R Foundation for Statistical Computing, Vienna, Austria).

Results

The premenopausal cohort included 4,600 $ER\alpha^+/TAM^+$ and 1,359 $ER\alpha^-/TAM^-$ patients (Table 1), in whom 656 and 282 recurrences

Table 1. Distribution of clinical and tumor characteristics by study population, ER α status, and receipt of tamoxifen among participants in population-based postmenopausal case-control and premenopausal cohort studies of Danish women diagnosed with a first primary breast cancer.

ER α status and receipt of tamoxifen	Postmenopausal case-control study	Premenopausal cohort	
	N cases (%)/ N controls (%) ER α ⁺ /TAM ⁺	ER α ⁺ /TAM ⁺	ER α ⁻ /TAM ⁻
Menopausal status at diagnosis			
Premenopausal	34 (6.3)/34 (6.3)	4,600 (100)	1,359 (100)
Postmenopausal	507 (94)/507 (94)	0 (0)	0 (0)
Age at diagnosis			
<35	0 (0)/0 (0)	222 (4.8)	182 (13.4)
35-44	16 (3.0)/13 (2.4)	1,610 (35.0)	550 (40.5)
45-54	116 (21)/111 (21)	2,701 (58.7)	597 (43.9)
\geq 55	409 (76)/417 (77)	67 (1.5)	30 (2.2)
Diagnosis year			
1985-1993	235 (43)/234 (43)	0 (0)	0 (0)
1994-1996	113 (21)/112 (21)	0 (0)	0 (0)
1997-2001	193 (36)/195 (36)	0 (0)	0 (0)
2002-2010		4,600 (100)	1,359 (100)
UICC stage at diagnosis			
Stage I	9 (1.7)/9 (1.7)	1,184 (26)	402 (30)
Stage II	250 (46)/250 (46)	2,476 (54)	702 (52)
Stage III	282 (52)/282 (52)	917 (20)	246 (18)
Unknown		23 (0.5)	9 (0.7)
Histologic grade			
Unsuitable	108 (25)/144 (35)	10 (0.2)	13 (1.0)
I	234 (54)/215 (52)	955 (21)	21 (1.5)
II	92 (21)/57 (14)	2,391 (52)	216 (16)
III	107 (20)/125 (23)	950 (21)	884 (65)
Unknown		294 (6.4)	225 (17)
Type of primary surgery			
Mastectomy	483 (89)/470 (87)	2,033 (44)	627 (46)
Breast-conserving surgery	58 (11)/71 (13)	2,567 (56)	732 (54)
Progesterone receptor expression			
PR ⁻	0 (0)/0 (0)	383 (8.3)	1,121 (83)
PR ⁺	0 (0)/0 (0)	2,680 (58)	19 (1.4)
Unknown	541 (100)/541 (100)	1,537 (33)	219 (16)
HER2 expression			
HER2 ⁻	0 (0)/0 (0)	2,887 (63)	692 (51)
HER2 ⁺	0 (0)/0 (0)	619 (14)	354 (26)
Unknown	541 (100)/541 (100)	1,094 (24)	313 (23)
Duration of intended tamoxifen therapy			
None	0 (0)/0 (0)	0 (0)	1,359 (100)
1 year	247 (46)/249 (46)	0 (0)	0 (0)
2 years	98 (18)/92 (17)	0 (0)	0 (0)
5 years	196 (36)/200 (37)	4,600 (100)	0 (0)
Receipt of systemic adjuvant chemotherapy			
No	471 (87)/476 (88)	437 (9.5)	109 (8.0)
Yes	70 (13)/65 (12)	4,163 (91)	1,250 (92)
Receipt of radiotherapy			
No	358 (66)/350 (65)	655 (14)	267 (20)
Yes	183 (34)/191 (35)	3,945 (86)	1,092 (80)
Receipt of anti-HER2 therapy			
No	541 (100)/541 (100)	2,887 (63)	692 (51)
Yes	0 (0)/0 (0)	619 (13)	354 (26)
Unknown	0 (0)/0 (0)	1,094 (24)	313 (23)
Charlson comorbidity index score			
0	450 (83)/444 (82)	4,587 (99)	1,344 (99)
1 or 2	81 (15)/86 (16)	6 (0.13)	7 (0.52)
\geq 3	10 (1.8)/11 (2.0)	7 (0.15)	8 (0.59)

occurred, respectively, during the follow-up period. The postmenopausal case-control study included 541 ER α ⁺/TAM⁺ recurrent cases and 541 matched controls, of whom 94% were postmenopausal. ER α ⁺/TAM⁺ patients in the premenopausal cohort were all assigned to a protocol of 5 years of tamoxifen therapy, whereas some of the postmenopausal case-control patients were originally assigned to receive 1 or 2 years of tamoxifen. Most patients assigned to 1- or 2-year protocols actually received tamoxifen treatment for a longer duration (34), probably because of accumulating contemporaneous evidence that longer treatment duration was more effective. Among 8 women with intended duration of 1 year of tamoxifen, the minimum received duration was 0.02 years, the maximum was 5.0 years, and the median was 1.0 year. Among 10 women with intended duration of 2 to 5 years of tamoxifen, the minimum received duration was 1.8 years, the maximum was 5.0 years, and the median was 2.8 years. In the premenopausal cohort, only 21% of women failed to complete the intended 5 years of tamoxifen therapy, and this proportion varied little with categories of the genetic variants (Supplementary Table S10). The

Online Data Supplement also presents a more complete description of the study populations' demographics and trends in treatment. All comparisons of tamoxifen metabolic capacity reported here are within the postmenopausal case-control and premenopausal cohort studies, so are unaffected by these patterns of breast cancer care.

Of the 32 gene variants assayed, 6 were excluded from the analysis due to poor amplification of the minor allele or substantial departure from genotype frequencies expected under Hardy-Weinberg equilibrium. Estimates of the associations between the genetic variants and breast cancer recurrence from the conventional analyses are presented in Supplementary Figs. S1 and S2 for the premenopausal cohort and postmenopausal case-control studies, respectively. Estimates of associations within studies and strata of ER α /TAM status are tabulated in Supplementary Tables S2-S9. There was no genetic variant that had non-null associations with recurrence in the ER α ⁺/TAM⁺ strata of both the premenopausal cohort and the postmenopausal case-control study, and a null association with recurrence in the ER α ⁻/TAM⁻ stratum of the premenopausal cohort. This pattern would be expected

Table 2. ALPS marginal BFs for tamoxifen pathway gene variants.

Pathway concept	Gene	Variant	MAF	ALPS marginal BFs		
				Postmenopausal ER α ⁺	Premenopausal ER α ⁻	Premenopausal ER α ⁺
Phase I metabolism						
	CYP1A1	rs1048943	0.08	n/a	n/a	n/a
	CYP2B6	rs3745274	0.24	0.43	0.41	0.46
	CYP2B6	rs8192709	0.06	1.55	3.16	0.43
	CYP2C19	rs12248560	0.17	4.32	2.12	1.78
	CYP2C19	rs4244285	0.14	0.71	0.46	2.99
	CYP2C9	rs1057910	0.10	2.25	0.75	0.56
	CYP2C9	rs1799853	0.17	0.66	0.70	0.81
	CYP2D6	rs1065852	0.21	0.44	1.42	1.06
	CYP2D6	rs16947	0.34	0.47	1.15	3.80
	CYP2D6	rs3892097	0.19	2.29	0.44	1.41
	CYP2D6	rs28371706	0.06	n/a	n/a	n/a
	CYP2D6	rs28371725	0.11	0.53	0.44	1.30
	CYP3A	rs10273424	0.11	0.68	2.29	0.74
	CYP3A5	rs776746	0.38	3.14	0.80	0.90
Phase II metabolism						
	SULT1A1	rs1042157	0.30	n/a	n/a	n/a
	SULT1A1	rs1801030	0.11	n/a	n/a	n/a
	SULT1A1	rs9282861	0.23	n/a	1.02	0.55
	SULT1E1	rs3775775	0.14	1.48	0.45	0.53
	SULT1E1	rs3775778	0.27	9.48	1.06	0.46
	UGT2B7	rs7434332	0.33	1.86	6.16	0.45
	UGT2B10	rs294769	0.48	1.04	0.71	2.40
	UGT2B15	rs1902023	0.47	2.89	0.82	2.57
Transporters						
	ABCB1	rs10248420	0.35	n/a	n/a	n/a
	ABCB1	rs1045642	0.41	0.61	0.68	0.79
	ABCB1	rs1128503	0.43	0.44	0.61	1.82
	ABCB1	rs2032582	0.33	0.56	0.43	0.46
	ABCC2	rs3740065	0.2	0.97	1.03	0.68
	ABCC2	rs717620	0.2	0.60	2.18	0.65
	ABCC2	rs8187710	0.08	0.45	0.78	1.53
	ABCG2	rs1564481	0.35	n/a	1.01	3.06
	ABCG2	rs2231164	0.43	n/a	n/a	n/a
	ABCG2	rs2622604	0.23	n/a	4.77	0.66

Note: BFs >1 indicate the data more strongly support an effect on recurrence than no effect, and support increases with the magnitude of the BF. Abbreviations: BF, Bayes factor; MAF, minor allele frequency; n/a, not available.

for a genetic variant truly predictive of optimal tamoxifen therapy response (46). *CYP2D6**4 (rs3892097) is a knockout allele that eliminates enzyme function and has minor allele frequency near 20% in populations of European descent (47). The predictive utility of this variant has been emphasized in clinical epidemiology studies of tamoxifen pharmacogenetics (13). In our custom-call genotyping data, homozygote carriers of this variant did not have a notably increased hazard of recurrence, compared with homozygote wild-type carriers, in the ER α ⁺/TAM⁺ stratum of the premenopausal cohort (HR = 1.33; 95% CI, 0.83–2.12) or the postmenopausal case–control study (OR = 1.58; 95% CI, 0.87–2.88). Heterozygotes, compared with homozygote wild-type carriers, also had no notable association in either study (HR = 0.83; 95% CI, 0.62–1.11 and OR = 1.12; 95% CI, 0.82–1.53, respectively).

Results of the ALPS pathway analyses are reported as BFs in Tables 2 and 3. BFs >1 indicate the data more strongly support an effect on recurrence than no effect, and support increases with the magnitude of the BF (48). BFs for the premenopausal cohort should be compared between ER α /TAM strata, because variants and pathway concepts that predict tamoxifen effectiveness are expected to show effects only in the ER α ⁺/TAM⁺ stratum. BFs for the postmenopausal case–control study should be compared with the ER α ⁺/TAM⁺ from the premenopausal cohort, because variants and pathway concepts that predict tamoxifen effectiveness should have similar BFs in both studies.

Table 2 shows BFs for main effects of individual gene variants, grouped according to enzyme function. In the premenopausal cohort, we observed some evidence for recurrence effects in the ER α ⁺/TAM⁺ stratum for variants in *CYP2C19* (rs4244285; BF = 2.99), *CYP2D6* (rs16947; BF = 3.80), *UGT2B10* (rs294769; BF = 2.40), *UGT2B15* (rs1902023; BF = 2.57), and *ABCG2* (rs1564481; BF = 3.06). In the postmenopausal case–control study, we observed some evidence for recurrence effects of variants in *CYP2C19* (rs12248560; BF = 4.32), *CYP2C9* (rs1057910; BF = 2.25), *CYP2D6* (rs3892097; BF = 2.29), *CYP3A5* (rs776746; BF = 3.14), *SULT1E1* (rs3775778; BF = 9.48), and *UGT2B15* (rs1902023; BF = 2.89). Thus, only *CYP2C19*, *CYP2D6*, and *UGT2B15* had notable BFs in the ER α ⁺/TAM⁺ stratum of both studies.

Table 3 shows BFs for tamoxifen pathway concepts. For enzyme function concepts, we saw weak evidence for an effect of phase I

(activating) metabolism on recurrence in the ER α ⁺/TAM⁺ stratum of both studies (BFs = 2.12 and 2.26), but not in the ER α [−]/TAM[−] stratum of the premenopausal cohort (BF = 0.95). There was no such consistent pattern for the phase II (deactivating) metabolism concept (BFs = 3.00 and 0.77) or transporter concept (BFs = 0.24 and 1.05) on recurrence in the ER α ⁺/TAM⁺ strata. For tamoxifen metabolite concepts, variants affecting 4-hydroxy tamoxifen and N-desmethyl tamoxifen were weakly associated with recurrence in both studies, but variants affecting 4-hydroxy-N-desmethyl tamoxifen were weakly associated with recurrence in only the premenopausal cohort (BF = 1.65 in the ER α ⁺/TAM⁺ stratum). *CYP2D6* activity was weakly associated with recurrence in premenopausal women (BF = 1.75) and not associated with recurrence in postmenopausal women (BF = 0.66).

Discussion

In this large study applying comprehensive genotype analysis across multiple tamoxifen pathway enzymes and transporters, we found a weak association between phase I activating metabolism and risk of recurrence among tamoxifen-treated women, regardless of menopausal status, consistent with the pattern expected of a predictive biomarker (46). Patterns for phase II deactivating metabolism and transporter function were inconsistent with this pattern. The BF for the 4-hydroxy-N-desmethyl tamoxifen (endoxifen) concept was only weakly associated with recurrence risk and only for premenopausal women. This result is consistent with a recent trial that showed no association between endoxifen concentrations and recurrence in early-stage breast cancer patients receiving adjuvant tamoxifen (49). Formation of 4-OH tamoxifen and N-desmethyl tamoxifen was weakly associated with recurrence, and both of these metabolites are upstream of endoxifen.

The availability of systematically archived FFPE tumor blocks as a source of DNA facilitated our large studies of unselected patient populations with long follow-up, which is a strength, but also resulted in some limitations. First, both the premenopausal cohort and the postmenopausal case–control studies were stratified by ER α expression at diagnosis. Assay methods and criteria for ER α positivity have evolved over time (50). Recall that the breast cancer patients in the postmenopausal case–control study were diagnosed between 1985 and 2001. DBCG guidelines recommended

Table 3. ALPS BFs for tamoxifen pathway concepts.

Pathway concept	ALPS BFs		
	Postmenopausal ER α ⁺	Premenopausal ER α [−]	Premenopausal ER α ⁺
Enzyme function			
Phase I metabolism	2.26	0.95	2.12
Phase II metabolism	3.00	1.12	0.77
Transporter	0.24	1.01	1.05
Tamoxifen metabolites			
4-Hydroxy tamoxifen	2.58	1.08	2.42
N-Desmethyl tamoxifen	1.74	0.76	2.25
4-Hydroxy-N-desmethyl tamoxifen	0.80	0.77	1.65
Tamoxifen sulfate	2.09	0.47	0.28
Tamoxifen glucuronide	2.06	2.54	1.93
<i>CYP2D6</i> activity	0.66	0.49	1.75

Note: BFs >1 indicate the data more strongly support an effect on recurrence than no effect, and support increases with the magnitude of the BF. Abbreviation: BF, Bayes factor.

biochemical assays from 1982 to 1989 and predominantly biochemical assays from 1990 to 1998, after which IHC assay was recommended (51). We therefore compared ER α expression at diagnosis with centralized reassay by IHC in the postmenopausal case-control study (51). Positive ER α expression at diagnosis was confirmed by centralized reassay for 94% of patients, whereas negative ER α expression at diagnosis was confirmed by centralized reassay for only 75% of patients. Concordance improved with calendar time among women who had positive ER α expression at diagnosis, possibly reflecting improvements in assay quality. The patients included in the premenopausal cohort were diagnosed between 2002 and 2011, so all would have had estrogen expression assayed by IHC, which is more accurate and a better predictive and prognostic marker (52, 53). Given the uniform use of the more accurate assay during the diagnostic era of the premenopausal cohort, we did not reassay ER α expression in that study population. Few of the women categorized as ER α^+ were likely to be truly ER α^- in either study population. A higher proportion of ER α^- women were likely to be truly ER α^+ , both because of the higher potential for assay errors described above and because current guidelines recommend classifying tumors as ER α^+ when $\geq 1\%$ of cells express the receptor (52), whereas almost all of our patients were diagnosed in an era when $\geq 10\%$ of cells or its equivalent in the biochemical assay (52) was the threshold for positivity (54). We confirmed that all women in the ER α^- strata did not receive tamoxifen, so even these false-negative women can validly serve as negative controls for this predictive study.

Second, none of the women in the postmenopausal case-control study and half of the women in the premenopausal cohort study were diagnosed after 2006, when HER2 testing became routine according to DBCG guidelines. Although this precludes categorization of the participants according to luminal subtype, there is no reason to believe that HER2 expression would be related to metabolic capacity as measured by the genotypes we assayed.

Third, DNA extracted from neoplastic cells may not have the same genotype as DNA extracted from nonneoplastic tissue (12). The latter would more often reflect the germline genotype, which is most relevant for understanding the metabolic capacity of the liver, where most tamoxifen metabolism takes place. This concern has led to recommendations that studies of gene-drug interactions that rely on DNA extracted from neoplastic tissue should be discarded from the evidence base (12, 55, 56). However, concordance between genotypes obtained from DNA extracted from neoplastic tissue compared with genotypes obtained from DNA extracted from paired nonneoplastic tissue has been very good (12, 13, 40, 57–60), possibly because DNA extracted from neoplastic tissue is mixed with DNA extracted from nonneoplastic tissue in the tumor microenvironment (58). Furthermore, this concern fundamentally applies to genotype misclassification. The strength of the bias induced by this misclassification would be driven by the specificity of genotype classification (i.e., the proportion of true wild-type homozygotes correctly classified; ref. 61). This specificity is likely to be nearly 100%, because true wild-type homozygotes would not be misclassified as heterozygotes or as variant homozygotes by the DNA rearrangements arising from tumorigenesis. Our quantitative bias analyses, which bias-adjusted earlier studies' estimates of association to account for genotyping error informed by the concordance studies, yielded bias-adjusted estimates that differed little from the conventional estimates (13), consistent with the expectation of negligible bias.

We have not genotyped all variants for all genes involved in tamoxifen metabolism. We selected a set with known functional consequences and $\geq 5\%$ minor allele frequency in populations of European descent. A more complete set of variants may have yielded stronger estimates of association, as has been reported earlier for single gene effects (59, 62). Our emphasis, however, was on pathway effects, so incorporating known and prevalent functional variants should be sufficient to identify aspects of the pathway instrumental in optimal tamoxifen response. The analysis did identify phase I metabolism as important in both studies, suggesting that bias toward the null from genotyping an incomplete set of variants was inconsequential. Earlier studies of the association between use of concomitant medications and breast cancer recurrence yielded null results in the postmenopausal case-control study (63, 64), so use of these medications was not included in our models. Nondifferential selection bias arising from missing FFPE blocks for some participants and from genotyping failures was also unlikely to have biased our results and inferences substantially.

Our study fills two important gaps in the evidence base regarding the effect of metabolic capacity on tamoxifen's effectiveness. First, our study is the first to include a large cohort restricted to premenopausal women, 79% of whom completed the intended 5 years of tamoxifen therapy. The competition between tamoxifen and its metabolites and estrogen and its metabolites may be more likely to require full metabolic capacity in premenopausal women, but our results do not suggest that any individual variant in metabolic or transport genes reduces tamoxifen's effectiveness to a clinically relevant extent in such women. Second, ours is the first study to take a comprehensive analytic approach to assessing the importance of tamoxifen's metabolic pathway, rather than analyzing individual or several variants in individual or several genes encoding the metabolic enzymes. We found some evidence supporting a small effect of variable phase I metabolism on tamoxifen's effectiveness. This result is consistent with the importance of demethylation and especially hydroxylation in generating the most active tamoxifen metabolites. Nonetheless, no individual variant substantially explained the phase I effect on tamoxifen response, and the overall prognostic utility was small, so this finding does not portend clinical utility. Our results overall, therefore, are consistent with the current guidelines that recommend against genotype-guided tamoxifen therapy (5, 65), and for the first time bolster the evidence base supporting these guidelines for premenopausal women. We have made the statistical computing code for the pathway analysis publicly available (<https://github.com/tpahern/ALPS-Bayesian-Pathway-Analysis>), and encourage others with relevant data on the pharmacogenetics of tamoxifen to apply the method to their data.

Disclosure of Potential Conflicts of Interest

J.W. Baurley is an LLC member and has ownership interest (including patents) in BioRealm. B. Ejlersen reports receiving commercial research grants from NanoString Technologies, Roche, Novartis, and Oncology Venture and is a consultant/advisory board member for PUMA. T. Tramm is listed as a co-inventor on international patent number WO2013132354A3, titled "Gene signatures associated with efficacy of postmastectomy radiotherapy in breast cancer," and is a consultant/advisory board member for Roche. T.L. Lash is a Methods Advisory Council member for Amgen. No potential conflicts of interest were disclosed by the other authors.

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