A genome-wide association study identifies locus at 10q22 associated with clinical outcomes of adjuvant tamoxifen therapy for breast cancer patients in Japanese

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Although many association studies of polymorphisms in candidate genes with the clinical outcomes of breast cancer patients receiving adjuvant tamoxifen therapy have been reported, genetic factors determining individual response to tamoxifen are not fully understood. To identify genetic polymorphisms associated with clinical outcomes of patients with tamoxifen treatment, we conducted a genome-wide association study (GWAS). We studied 462 Japanese patients with hormone receptor-positive, invasive breast cancer receiving adjuvant tamoxifen therapy. Of them, 240 patients were analyzed by genome-wide genotyping using the Illumina Human610-Quad BeadChips, and two independent sets of 105 and 117 cases were used for replication studies. In the GWAS, we detected significant associations with recurrence-free survival at 15 single-nucleotide polymorphisms (SNPs) on nine chromosomal loci (1p31, 1q41, 5q33, 7p11, 10q22, 12q13, 13q22, 18q12 and 19p13) that satisfied a genome-wide significant threshold (log-rank $P = 2.87 \times 10^{-9}$–9.41×10^{-8}). Among them, rs10509373 in *C10orf11* gene on 10q22 was significantly associated with recurrence-free survival in the replication study (log-rank $P = 2.02 \times 10^{-4}$) and a combined analysis indicated a strong association of this SNP with recurrence-free survival in breast cancer patients treated with tamoxifen (log-rank $P = 1.26 \times 10^{-10}$). Hazard ratio per C allele of rs10509373 was 4.51 [95% confidence interval (CI), 2.72–7.51; $P = 6.29 \times 10^{-9}$]. In a combined analysis of rs10509373 genotype with previously identified genetic makers, *CYP2D6* and *ABCC2*, the number of risk alleles of these three genes had cumulative effects on recurrence-free survival among 345 patients receiving tamoxifen monotherapy (log-rank $P = 2.28 \times 10^{-12}$).

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In conclusion, we identified a novel locus associated with recurrence-free survival in Japanese breast cancer patients receiving adjuvant tamoxifen therapy.

INTRODUCTION

Tamoxifen has been the gold standard for endocrine treatment of patients with estrogen receptor (ER)-positive breast cancers. However, 30–50% of patients with adjuvant tamoxifen therapy experience relapse and subsequently die of the disease (1,2), indicating individual differences in responsiveness to tamoxifen.

Tamoxifen is metabolized to the highly active metabolites, 4-hydroxytamoxifen and 4-hydroxy-*N*-desmethyltamoxifen (endoxifen). It is reported that these metabolites are the active therapeutic moieties, having 100-fold greater affinity to ER and 30–100-fold greater potency in suppressing estrogen-dependent cell proliferation than those of tamoxifen (3–5). Inter-individual differences in the formation and elimination of these active metabolites could be one of the important factors affecting variability in the response to tamoxifen. Most previous reports focused on the genes involved in the pharmacokinetics of tamoxifen and its metabolites seek genetic variations which determine the individual response to tamoxifen. Genetic polymorphisms of cytochrome P450 2D6 (CYP2D6), which is the key enzyme responsible for the generation of endoxifen, is thought to be the most promising predictor of plasma concentration of endoxifen and clinical efficacy of tamoxifen in breast cancer patients (6–14). Schroth *et al.* (15) recently reported outstanding results in 1325 breast cancer patients, providing sufficiently powered evidence for an association between *CYP2D6* genotype and clinical outcomes in patients treated with tamoxifen in the adjuvant setting. Besides *CYP2D6*, several genes, such as *CYP2C19*, *CYP3A5*, sulfotransferase 1A1 (*SULT1A1*), UDP-glucuronosyltransferase 2B15 (*UGT2B15*) and ATP-binding cassette sub-family C member 2 (*ABCC2*), were reported as possible candidates associated with the clinical outcomes of tamoxifen therapy (7,10,14,16); however, associations of these candidate genes have not yet been sufficiently validated. Therefore, individual differences in responsiveness to tamoxifen still remain, even if the effects of genetic polymorphisms of *CYP2D6* were considered, suggesting the existence of other genetic factor(s).

In this study, to identify responsible loci for the clinical outcomes of tamoxifen therapy, we performed a genome-wide association study (GWAS) by genotyping over 610 000 single-nucleotide polymorphisms (SNPs) and identified the novel locus containing chromosome 10 open-reading frame 11 (*C10orf11*) gene associated with recurrence-free survival in the breast cancer patients treated with tamoxifen.

RESULTS

Patient characteristics

We recruited 462 Japanese patients with breast cancer receiving adjuvant tamoxifen therapy. Table 1 summarizes the characteristics of all of these patients who were pathologically diagnosed to have a hormone receptor-positive, invasive breast cancer. Their median age at the time of surgery was 51 years old (range, 27–84 years), the median follow-up period was 6.8 years (range, 0.6–23.5 years) and the median tamoxifen treatment period was 4.8 years (range, 0.6–6.3 years). Among the characteristics listed in Table 1, tumor size and nodal status showed significant associations with the recurrence-free survival [$P = 0.000215$; hazard ratio (HR), 1.71; 95% confidence interval (CI), 1.29–2.27; and $P = 0.0138$; HR, 1.83; 95% CI, 1.14–3.09, respectively] in the Cox proportional hazards analysis, whereas the other factors were not associated with recurrence-free survival (Supplementary Material, Table S1).

Genome-wide association and replication studies

We conducted a GWAS of recurrence-free survival of 240 Japanese patients with breast cancer who received tamoxifen monotherapy using Illumina Human610-Quad BeadChips. After the standard quality control, association analysis was performed for 470 796 SNPs by the trend log-rank test. We generated a quantile–quantile plot (Supplementary Material, Fig. S1) and obtained the genomic control inflation factor (λ_{GC}) of 1.023, indicating a low possibility of false-positive associations resulting from population stratification. We detected significant associations with recurrence-free survival at 15 SNPs in nine genetic regions (1p31, 1q41, 5q33, 7p11, 10q22, 12q13, 13q22, 18q12 and 19p13) that satisfied a genome-wide significant threshold of $P < 1.06 \times 10^{-7}$ (Fig. 1). To further validate the results of GWAS, we carried out a replication study using an independent 105 breast cancer patients. We genotyped 9 of the 15 SNPs because 6 of them were highly linked to another SNP ($r^2 > 0.80$; Supplementary Material, Table S2). We found that rs10509373 in *C10orf11* gene on 10q22 was significantly associated with recurrence-free survival in the replication stage (log-rank $P = 4.18 \times 10^{-4}$; Table 2 and Fig. 2). The associations of the other SNPs were not replicated (Supplementary Material, Table S2). Furthermore, a combined result of the GWAS and first replication study strongly suggested an association of this locus with recurrence-free survival in breast cancer patients treated with tamoxifen (log-rank $P = 2.19 \times 10^{-10}$). We also genotyped rs10509373 using additional 117 samples, which include the patients receiving tamoxifen after chemotherapy and observed a significant association (log-rank $P = 1.86 \times 10^{-2}$). A combined P-value of all samples was 2.19×10^{-10}, suggesting the significant association with recurrence-free survival in breast cancer patients treated with tamoxifen (Fig. 2 and Supplementary Material, Table S3). In Cox proportional hazards analysis, *C10orf11* genotype (rs10509373) was an independent indicator of the recurrence-free survival after adjustment for tumor size and nodal status ($P = 6.28 \times 10^{-8}$; Table 2). The adjusted HRs of rs10509373

Table 1. Characteristics of patients

Characteristic	No. of patients (%) GWAS	First replication	Second replication	Total
No.	240	105	117	462
Age at surgery (years)				
Median	51	50	48	51
Range	31–83	35–84	27–71	27–84
Follow-up (years)				
Median	7.2	5.2	6.2	6.8
Range	1.1–23.5	0.6–19.3	1.0–15.5	0.6–23.5
Tamoxifen treatment (years)				
Median	4.9	4.0	4.7	4.8
Range	1.0–6.1	0.6–6.0	0.7–6.3	0.6–6.3
Menopausal status				
Pre-menopause	101 (42.1)	40 (38.1)	75 (64.1)	216 (46.8)
Post-menopause	131 (54.6)	40 (38.1)	35 (29.9)	206 (44.6)
Unknown	8 (3.3)	25 (23.8)	7 (6.0)	40 (8.7)
Tumor size (cm)				
≤2	138 (57.5)	57 (54.3)	48 (41.0)	243 (52.6)
2.1–5	92 (38.3)	34 (32.4)	56 (47.9)	182 (39.4)
>5	1 (0.4)	2 (1.9)	12 (10.3)	15 (3.2)
Unknown	9 (3.8)	12 (11.4)	1 (0.9)	22 (4.8)
Nodal status				
Negative	193 (80.4)	88 (83.8)	74 (63.2)	355 (76.8)
Positive	44 (18.3)	13 (12.4)	41 (35.0)	98 (21.2)
Unknown	3 (1.3)	4 (3.8)	2 (1.7)	9 (1.9)
ER status				
Positive	173 (72.1)	87 (82.9)	98 (83.8)	358 (77.5)
Negative	24 (10.0)	2 (1.9)	12 (10.3)	38 (8.2)
Unknown	43 (17.9)	16 (15.2)	7 (6.0)	66 (14.3)
PR status				
Positive	167 (69.6)	77 (73.3)	87 (74.4)	331 (71.6)
Negative	28 (11.7)	11 (10.5)	22 (18.8)	61 (13.2)
Unknown	45 (18.8)	17 (16.2)	8 (6.8)	70 (15.2)
Her-2				
Positive[a]	3 (1.3)	5 (4.8)	6 (5.1)	14 (3.0)
Negative	82 (34.2)	28 (26.7)	60 (51.3)	170 (36.8)
Unknown	155 (64.6)	72 (68.6)	51 (43.6)	278 (60.2)
Treatment				
Tamoxifen alone	240 (100.0)	105 (100.0)	0 (0.0)	345 (76.7)
Tamoxifen + AC or EC	0 (0.0)	0 (0.0)	41 (35.0)	41 (8.9)
Tamoxifen + CMF	0 (0.0)	0 (0.0)	32 (27.4)	32 (6.9)
Tamoxifen + other chemotherapies	0 (0.0)	0 (0.0)	44 (37.6)	44 (9.5)
Events				
No event	210 (87.5)	89 (84.8)	98 (55.4)	397 (85.9)
Locoregional events	9 (3.8)	0 (0.0)	0 (0.0)	9 (1.9)
Distant metastasis events	12 (5.0)	15 (14.3)	17 (9.6)	44 (9.5)
Contralateral breast events	9 (3.8)	1 (1.0)	2 (1.1)	12 (2.6)

AC, adriamycin + cyclophosphamide; EC, epirubicin + cyclophosphamide; CMF, cyclophosphamide + methotrexate + 5-fluorouracil.
[a]Score of 3+ in immunohistochemistry.

C allele was 4.51 (95% CI, 2.72–7.51), suggesting that C allele was a risk allele for breast cancer recurrence.

To further identify SNPs associated with recurrence-free survival in patients receiving tamoxifen therapy, we genotyped 130 tag SNPs for fine mapping on chromosome 10q22 (Chr. 10: 77.35–78.70 Mb) where the most significant association with the recurrence-free survival was observed (Fig. 3 and Supplementary Material, Table S4). Although no SNPs showed a stronger association than the landmark SNP, rs10509373, fine mapping of this region indicated that a 172-kb linkage disequilibrium (LD) block (77.67–77.84 Mb) including *C10orf11* was likely to contain the genetic variant(s) associated with recurrence-free survival in patients receiving tamoxifen therapy.

Combination analysis with previously identified gene loci

As we previously identified significant associations of *CYP2D6* and *ABCC2* rs3740065 genotypes with recurrence-free survival in patients treated with tamoxifen among the patients receiving tamoxifen monotherapy (Supplementary Material, Table S5) (7), we investigated a combined effect of *C10orf11* genotype in addition to *CYP2D6* and *ABCC2* genotypes on the recurrence-free survival by classifying the 345

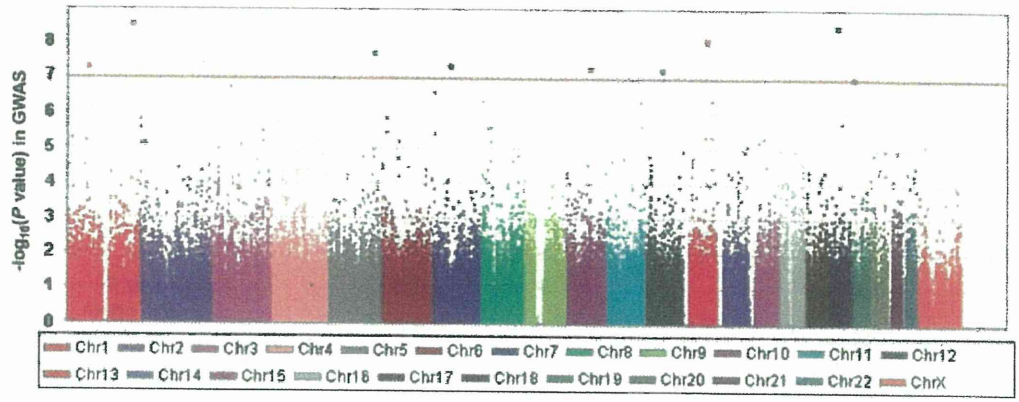


Figure 1. Results of the GWAS. Manhattan plot showing the $-\log_{10}$-transformed *P*-value of SNPs in the GWAS for 240 Japanese patients with breast cancer receiving tamoxifen monotherapy. The red line indicates the genome-wide significance level ($P = 1.06 \times 10^{-7}$).

Table 2. Association analysis of rs10509373 in C10orf11 with recurrence-free survival in breast cancer patients receiving tamoxifen therapy

SNP	Chr	Chr location[a]	Allele (risk)	Study set	Risk allele frequency		Log-rank *P*	Univariate		Multivariate[b]	
					Event	No event		HR (95% CI)[c]	*P*-value	HR (95% CI)[c]	*P* value
rs10509373	10	77827578	T/C (C)	GWAS	0.117	0.021	5.19×10^{-8}	7.70 (3.25–18.22)	3.41×10^{-6}	9.64 (3.85–24.12)	1.29×10^{-6}
				First replication	0.094	0.017	4.18×10^{-4}	7.93 (2.06–30.58)	2.65×10^{-3}	5.96 (1.49–23.86)	1.17×10^{-2}
				GWAS + first replication	0.109	0.020	2.19×10^{-10}	7.34 (3.58–14.98)	4.90×10^{-8}	9.18 (1.39–22.62)	6.07×10^{-9}
				Second replication	0.132	0.036	1.86×10^{-2}	2.72 (1.13–6.53)	2.53×10^{-2}	2.92 (1.14–7.49)	2.55×10^{-2}
				Combined replications	0.114	0.027	2.02×10^{-4}	3.21 (1.65–6.22)	5.67×10^{-4}	3.20 (1.53–6.69)	1.97×10^{-3}
				Combined all	0.115	0.024	1.26×10^{-10}	4.51 (2.72–7.51)	6.29×10^{-9}	4.53 (2.62–7.83)	6.28×10^{-8}

Chr, chromosome; CI, confidence interval; GWAS, genome-wide association study.
[a]Based on NCBI 36 genome assembly.
[b]Adjusted for tumor size and nodal status.
[c]HR per one allele.

patients into 6 groups (0, 1, 2, 3, 4 and 5 risk allele groups) according to the number of risk alleles of the three genes. Kaplan–Meier analysis revealed the number of risk alleles of these three genes to have cumulative effects on recurrence-free survival (log-rank $P = 2.24 \times 10^{-12}$; Fig. 4). In the Cox proportional hazards analysis of 345 patients, the *CYP2D6* and *ABCC2* genotypes showed similar associations with recurrence-free survival to those in previous analysis of 282 patients ($P = 1.99 \times 10^{-4}$ and 8.51×10^{-4}, respectively; Supplementary Material, Table S6) (7). In the multivariate analysis, rs10509373 in *C10orf11* still showed a significant association even after adjustment of *CYP2D6* and *ABCC2* genotypes in addition to tumor size and nodal status ($P = 4.74 \times 10^{-7}$; Supplementary Material, Table S6), indicating that rs10509373 is an independent risk factor of breast cancer recurrence. Furthermore, combined analysis of *C10orf11*, *CYP2D6* and *ABCC2* revealed that genotypes of the three genes have cumulative effects on recurrence-free survival ($P = 2.28 \times 10^{-12}$), and adjusted HR for risk of recurrence computed for patients carrying three or more risk alleles increased from 6.51-fold (three risk alleles) to 119.51-fold (five risk alleles) compared with those carrying one risk allele (Supplementary Material, Table S6). In the subgroup analysis of menopausal status, we identified the significant associations in both subgroups of pre- and postmenopausal patients, although the stronger association was observed in postmenopausal group than in the premenopausal patients (Supplementary Material, Table S7).

DISCUSSION

This study represents the first GWAS which attempts to identify genetic variants associated with clinical outcomes of tamoxifen therapy and successfully revealed that a marker SNP, rs10509373, on chromosome 10q22 was significantly associated with recurrence-free survival in 462 Japanese patients with breast cancer receiving tamoxifen monotherapy. Furthermore, combined analysis of this SNP with previously identified predictors, *CYP2D6* and *ABCC2*, revealed that the number of risk alleles of the three genes have cumulative effects on recurrence-free survival in tamoxifen-treated breast cancer patients.

The most significantly associated SNP in this study, rs10509373 (combined $P = 1.26 \times 10^{-10}$), is located in a 172-kb LD block which contains the 3′ region of *C10orf11*

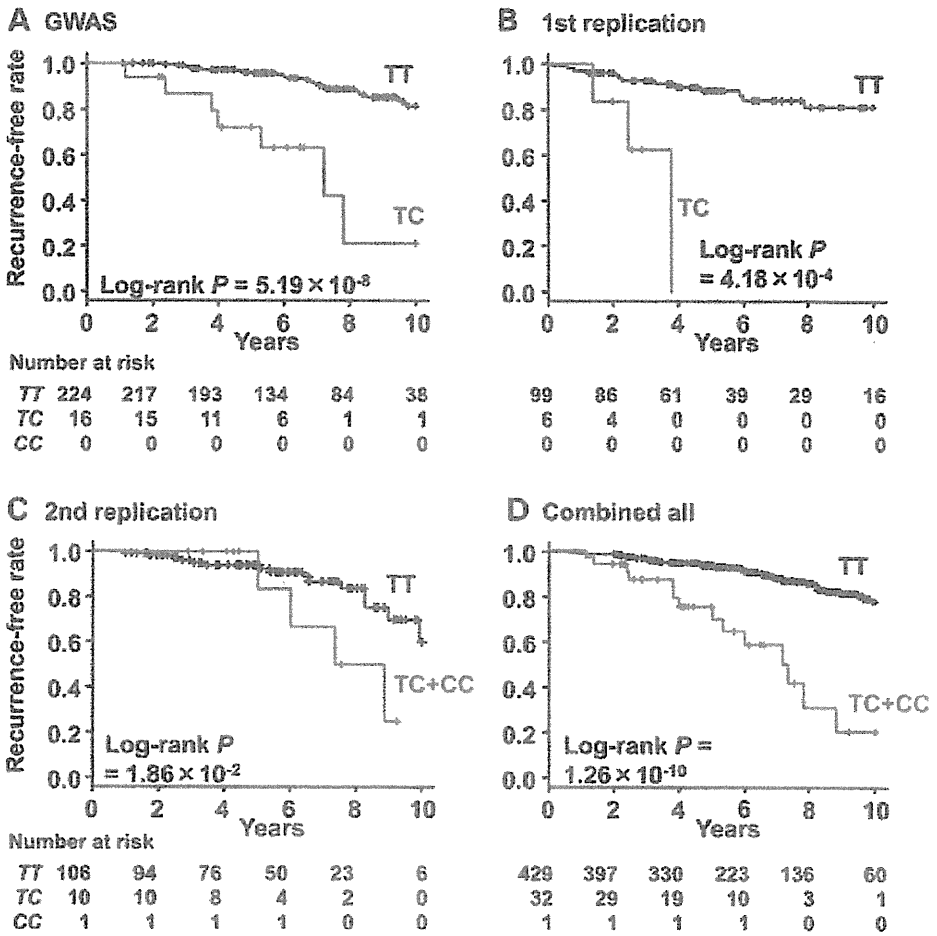


Figure 2. Kaplan–Meier estimates of recurrence-free survival for *C10orf11* rs10509373 genotype in 240 patients genotyped in the genome-wide association study (A), in 105 patients genotyped in the first replication study (B), in 117 patients genotyped in the second replication study (C) and in 462 patients in the combination analysis (D).

gene. The fine mapping of this region indicated that a peak association was located in intron 5 of *C10orf11* gene, suggesting that *C10orf11* is likely to be a causative gene to determine the clinical outcomes of breast cancer patients treated with tamoxifen. Because no associated SNPs were found in exon region by re-sequencing of *C10orf11* (Supplementary Material, Table S8), a genetic variant(s) within this LD block might alter C10orf11 transcriptional activity. C10orf11 protein, comprising 198 amino acids, is predicted to contain leucine-rich repeat domain and to have the capacity of protein binding in the SMART database (http://smart.embl-heidelberg.de/), although no report has clarified its function. It is reported that *C10orf11* region overlaps with ultraconserved elements (UCEs), perfectly constrained elements between the human, mouse and rat genomes (17–19). Their functional roles have not been completely elucidated yet; however, UCEs are thought to possess some essential functional properties. It is reported that paired box 2, encoded by *PAX2* gene on 10q24, which regulates *ERBB2* transcription and is involved in acquiring tamoxifen resistance (20), and special AT-rich sequence-binding protein-1 encoded by *SATB1* gene on 3p23, which delineates epigenetic modification and is associated with breast tumor growth and metastasis (21), are located in the UCE-rich regions (17). We further examined the association of pharmacokinetic data with *C10orf11* genotype; however, no significant difference was observed between *C10orf11* genotype and plasma levels of endoxifen and 4-hydroxytamoxifen in 98 breast cancer patients taking 20 mg/day tamoxifen (Supplementary Material, Fig. S2). According to our gene expression database (in-house), the C10orf11 transcript is expressed in breast cancer cells in clinical tissues. We examined the effects of rs10509373 on the expression levels of C10orf11 in peripheral blood mononuclear cells and brain using a public database SNPExpress (22). However, no significant associations were observed ($P = 0.63$ and 0.93, respectively) possibly because of the quite low expression of C10orf11 in these tissues. The association of *C10orf11* genotype was significant in the second replication samples, which include the patients receiving tamoxifen alone after chemotherapy (Table 2); however, neither *CYP2D6* nor *ABCC2* genotypes were significantly associated with clinical outcomes in the second replication samples as shown in our previous study (Supplementary Material, Table S5) (23). These lines of evidence might suggest that C10orf11 is involved in acquiring tamoxifen resistance or determining the characteristics of breast cancer, although further functional analysis will be needed to clarify the biological mechanisms which could have effects on the

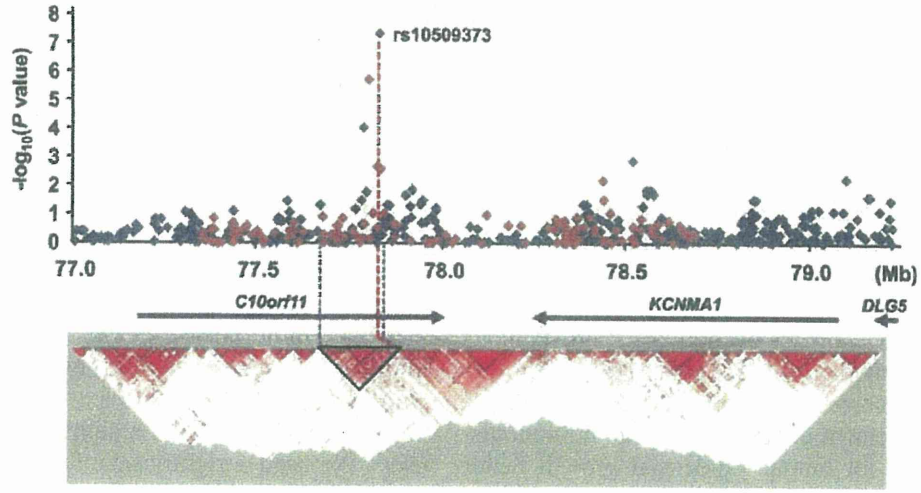
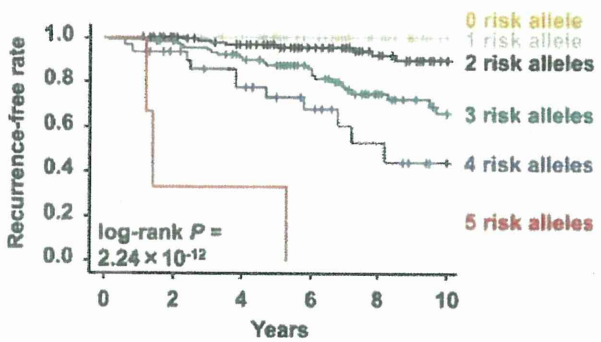


Figure 3. Association mapping and LD map of 10q22. Blue diamond dots represent $-\log_{10}$-transformed *P*-values of SNPs genotyped by Illumina Human610-Quad BeadChips in the GWAS, and red diamond dots show $-\log_{10}$-transformed *P*-values of the SNPs of fine mapping. Arrows indicate the position of known genes. The *D'*-based LD map (MAF $\geq$ 0.10) is drawn using genotype data of 240 patients with breast cancer enrolled in the GWAS.



Number at risk

0	14	14	11	10	7	3
1	54	54	45	31	20	7
2	132	120	103	67	44	23
3	107	101	80	54	33	17
4	30	24	18	11	5	1
5	3	1	0	0	0	0

Figure 4. Combined effects of *C10orf11*, *CYP2D6* and *ABCC2* genotypes on clinical outcomes of tamoxifen monotherapy. Kaplan–Meier estimates of recurrence-free survival rate for combined effects of *C10orf11* rs10509373, *CYP2D6* and *ABCC2* rs3740065 genotypes. The 345 patients receiving tamoxifen monotherapy were classified into six groups (0, 1, 2, 3, 4 and 5 risk allele groups) based on the number of risk alleles of these three genes.

clinical outcomes of breast cancer patients receiving tamoxifen therapy.

Several research groups focused on the genes involved in the pharmacokinetics of tamoxifen or its metabolites have investigated genetic variants of *CYP2C19*, *CYP3A5*, *UGT2B15* and *SULT1A1* as candidate genes associated with clinical outcomes of tamoxifen therapy (7,10,14,16). In our GWAS, no SNP in these candidate genes showed significant association with recurrence-free survival in patients treated with tamoxifen (log-rank $P = 3.14 \times 10^{-2}$–9.90×10^{-1}). According to the previous reports, the effect sizes of the above candidate genes were not so large, indicating that the sample size used in our study might not have enough power to detect associations of the SNPs with the tamoxifen efficacy.

Another group hypothesized that non-genomic steroid signaling and cross-talk with growth factor signaling pathways may contribute to the clinical outcomes of the patients treated with tamoxifen and reported that *TC21* promoter polymorphism was significantly associated with an unfavorable tamoxifen treatment outcome; however, no significant association was observed at SNPs in *TC21* gene in our GWAS (log-rank $P = 1.19 \times 10^{-1}$–9.98×10^{-1}) (24). The *P*-values of the SNPs in the *ESR1*, *ESR2* and *PGR* genes, which encode ERα, ERβ and progesterone receptor (PR), respectively, ranged from 1.33×10^{-2} to 9.88×10^{-1}, indicating no significant association.

In conclusion, our GWAS using 462 Japanese patients with breast cancer identified a new locus, containing the *C10orf11* gene, associated with the clinical outcomes of breast cancer patients treated with tamoxifen. These findings provide new insights into personalized selection of hormonal therapy for the patients with breast cancer. However, large-scale replication study and further functional analysis are required to verify our results and to clarify their biological mechanisms which have effects on the clinical outcomes of patients receiving tamoxifen therapy.

MATERIALS AND METHODS

Patients

A total of 462 patients with primary breast cancer (including the 282 patients reported previously (6,7)) were recruited at Shikoku-*10 collaborative group (Tokushima Breast Care Clinic, Yamakawa Breast Clinic, Shikoku Cancer Center, Kochi University Hospital, and Itoh Surgery and Breast Clinic), Kansai Rosai Hospital, Sapporo Breast Surgical Clinic and Sapporo Medical University Hospital. Of them, 240 patients who were recruited from September 2007 to September 2008 were used for a GWAS analysis, and the remaining 105 patients who were recruited from October 2008 to January 2010 were analyzed in a first replication study. All patients were Japanese women pathologically diagnosed with

ER-positive and/or PR-positive, invasive breast cancer who received adjuvant tamoxifen monotherapy without any other treatments after surgical treatment between 1986 and 2008. In addition, we analyzed 117 patients who had been treated with tamoxifen monotherapy after receiving chemotherapy as the second replication set (23). Data on primary breast cancer diagnosis or recurrence were confirmed from patients' medical record. Patients without recurrence were censored at the date of the last consultation. Recurrence-free survival time was defined as the time from surgical treatment to diagnosis of the recurrence of a breast cancer (locoregional, distant metastasis and contralateral breast events) or death. Patients received tamoxifen 20 mg/day for 5 years; tamoxifen was stopped at the time a recurrence was identified. ER and PR status were evaluated by enzyme immunoassay or immunohistochemistry. The cut-off for human epidermal growth factor receptor 2 overexpression was defined as 3+ immunohistochemical staining (25). Nodal status was determined according to the International Union against Cancer tumor-node-metastasis classification. This study was approved by the Institutional Review Board in the Institute of Medical Science, The University of Tokyo (Tokyo, Japan), and written informed consent was obtained from all patients.

Genotyping and quality control

Genomic DNA was extracted from peripheral blood ($n = 424$) or frozen breast tissue ($n = 38$) using Qiagen DNA Extraction Kit (Qiagen, Valencia, CA, USA). In the GWAS, 240 patients were genotyped using the Illumina Human610-Quad BeadChip (Illumina, San Diego, CA, USA). Quality control of SNPs was achieved by excluding SNPs with low call rate (<99%) and SNPs with Hardy–Weinberg equilibrium P-value $< 1.0 \times 10^{-6}$. SNPs with a minor allele frequency (MAF) <0.01 were also excluded from further analysis. A total of 470 796 SNPs passed the filters and were further analyzed. We used multiplex polymerase chain reaction-based Invader Assay (Third Wave Technologies, Madison, WI, USA) on ABI PRISM 7900HT (Applied Biosystems, Foster City, CA, USA) for the replication study and fine mapping (26). For fine mapping, tag SNPs were selected from the HapMap phase II JPT data (http://www.hapmap.org/) (27) with the following criteria: a pairwise $r^2 > 0.80$ and an MAF ≥ 0.01 using Haploview software (28).

Genotyping of *CYP2D6* and *ABCC2* rs3740065 was performed using real-time Invader (Third Wave Technologies) and TaqMan assays (Applied Biosystems) as described previously (7,29,30). To evaluate the effects of *CYP2D6* alleles, we defined all of the decreased and null alleles (including **4*, **5*, **10*, **14*, **21* and **41*, and gene-duplication alleles, **10–*10* and **36–*36*) as allele '*V*', and alleles of **1* and duplicated **1–*1* as allele '*wt*' as described previously (7).

Statistical analysis

Recurrence-free survival curves were estimated using the Kaplan–Meier method. Statistical significance of a relationship between clinical outcomes and genetic polymorphism was assessed by the trend log-rank test. The value of λ_{GC} was calculated from the median of the trend log-rank test statistics (31). Cox proportional hazards analysis was used to identify significant prognostic clinical factors and to test for an independent contribution of genetic factors to recurrence-free survival. To examine potential confounding, age was treated as a continuous variable, tumor size was treated as an ordinal variable, and the other covariates were treated as categorical variables. Genotypes were analyzed by assigning an ordinal score to each genotype (0 for homozygous non-risk alleles, 1 for heterozygous risk alleles and 2 for homozygous risk alleles). Combination effects were investigated by adding up the number of risk alleles of *CYP2D6*, *ABCC2* and *C10orf11* genes. All polymorphisms evaluated in this study were tested for deviation from Hardy–Weinberg equilibrium with the use of a χ^2-test. Statistical tests provided two-sided P-values, and a significance level of $P < 0.05$ was used. We used a significance level of $P < 1.06 \times 10^{-7}$ (0.05 of 470 796) in the GWAS and 5.56×10^{-3} (0.05 of 9) in the replication study to adjust multiple testing by the strict Bonferroni correction. Statistical analyses were carried out using SPSS (version 17.0, SPSS, Chicago, IL, USA) and the R statistical environment version 2.9.2 (http://www.r-project.org/).

SUPPLEMENTARY MATERIAL

Supplementary Material is available at *HMG* online.

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Conflict of Interest statement. None declared.

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Dose-adjustment study of tamoxifen based on *CYP2D6* genotypes in Japanese breast cancer patients

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Abstract CYP2D6 is a key enzyme responsible for the metabolism of tamoxifen to active metabolites, endoxifen, and 4-hydroxytamoxifen. The breast cancer patients who are heterozygous and homozygous for decreased-function and null alleles of *CYP2D6* showed lower plasma concentrations of endoxifen and 4-hydroxytamoxifen compared to patients with homozygous-wild-type allele, resulting in worse clinical outcome in tamoxifen therapy. We recruited 98 Japanese breast cancer patients, who had been taking 20 mg of tamoxifen daily as adjuvant setting. For the patients who have one or no normal allele of *CYP2D6*, dosages of tamoxifen were increased to 30 and 40 mg/day, respectively. The plasma concentrations of tamoxifen and its metabolites were measured at 8 weeks after dose-adjustment using liquid chromatography–tandem mass spectrometry. Association between tamoxifen dose and the incidence of adverse events during the tamoxifen treatment was investigated. In the patients with *CYP2D6*1/*10* and *CYP2D6*10/*10*, the mean plasma endoxifen levels after dose increase were 1.4- and 1.7-fold higher, respectively, than those before the increase ($P < 0.001$). These plasma concentrations of endoxifen achieved similar level of those in the *CYP2D6*1/*1* patients receiving 20 mg/day of tamoxifen. Plasma 4-hydroxytamoxifen concentrations in the patients with *CYP2D6*1/*10* and *CYP2D6*10/*10* were also significantly increased to the similar levels of the *CYP2D6*1/*1* patients according to the increasing tamoxifen dosages ($P < 0.001$). The incidence of adverse events was not significantly different between before and after dose adjustment. This study provides the evidence that dose adjustment is useful for the patients carrying *CYP2D6*10* allele to maintain the effective endoxifen level.

Keywords Endoxifen · CYP2D6 · P450 2D6 · Single nucleotide polymorphisms · SNPs

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Introduction

Tamoxifen has been widely used for the prevention of recurrence in patients with estrogen receptor (ER)-positive or progesterone receptor (PR)-positive breast cancer. However, inter-individual differences have been reported in responsiveness to tamoxifen, and 30–50% of patients with adjuvant tamoxifen therapy experience relapse and subsequently die of the disease [1, 2].

Tamoxifen is metabolized to the highly active metabolites, 4-hydroxytamoxifen and 4-hydroxy-*N*-desmethyltamoxifen (endoxifen). It is reported that these metabolites are the active therapeutic moieties, having 100-fold greater affinity to ER and 30- to 100-fold greater potency in suppressing estrogen-dependent cancer cell proliferation than those of tamoxifen [3–5]. Plasma endoxifen levels exceed plasma concentration levels of 4-hydroxytamoxifen by several folds, suggesting endoxifen to be a principal active metabolite [5–8]. Cytochrome P450 2D6 (CYP2D6) is a major enzyme responsible for the formation of endoxifen and 4-hydroxytamoxifen [9, 10]. *CYP2D6* gene is highly polymorphic, and over 80 different alleles which decrease or impair the enzymatic activity of CYP2D6 have been reported (http://www.cypalleles.ki.se/cyp2d6.htm). Subjects carrying two null alleles of *CYP2D6* are classified as poor metabolizers (PMs), and 5–10% of Caucasians are considered to be PMs [11]. The *CYP2D6*3*, *CYP2D6*4*, *CYP2D6*5*, and *CYP2D6*6* are major null alleles that cause the PM phenotype in Caucasians [12]. Although, the frequency of PMs in Asians is lower (only <1%) [13], the *CYP2D6*10* allele that causes reduction of CYP2D6 activity has been observed at a frequency of 40–50% in Asians [14].

Several research groups including us reported that the patients who show reduced and impaired activities of CYP2D6 by these genetic polymorphisms had significantly less clinical efficacy of tamoxifen therapy [15–21], because of the lower plasma concentrations of the active metabolites [7, 8, 21–24]. Therefore, the patients carrying *CYP2D6* non- or low-functional alleles would need to take an increased dosage of tamoxifen to achieve sufficient tamoxifen effects. Therefore, we designed a genotype-based dose-adjustment study of tamoxifen and determined steady-state plasma concentrations of tamoxifen and its metabolites to find the optimal dosage for the breast cancer patients with different *CYP2D6* genotypes.

Materials and methods

Patients

The study participants included 98 patients recruited at Tokushima Breast Care Clinic (Tokushima, Japan) as described previously [21]. Briefly, all patients were Japanese women pathologically diagnosed with hormone receptor-positive breast cancer, who had been taking 20 mg/day of tamoxifen for at least 4 weeks as adjuvant setting (patients taking selective serotonin re-uptake inhibitors were excluded). Among 74 patients who were genotyped to be heterozygous and homozygous for alleles of decreased function (**10*, **41*) or no function (**5*, **21*, **36-*36*) for *CYP2D6*, 51 patients agreed to participate in a dose-adjustment study and received increased doses of tamoxifen (30 and 40 mg/day for patients heterozygous and homozygous for alleles of decreased function or no function, respectively) for at least 8 weeks, and collection of blood samples (7 ml) was repeated (Fig. 1). The information on any grade of adverse events according to CTCAE v4.0 was collected from questionnaire and the patients' medical record (for endometrial thickening, thrombosis, and exacerbation of hepatic steatosis). ER and PR status was evaluated by enzyme immunoassay or immunohistochemistry. The cutoff for human epidermal growth factor receptor 2 overexpression was defined as 3 + immunohistochemical staining. Nodal status was determined according to the International Union Against Cancer tumor-node-metastasis classification. Written informed consent was obtained from all patients. This study was approved by the Institutional Review Board in the Institute of Medical Science, The University of Tokyo (Tokyo, Japan).

Genotyping

Genomic DNA was extracted from peripheral blood using Qiagen DNA extraction kit (Qiagen, Valencia, CA, USA). Genotyping of *CYP2D6*, including *CYP2D6*1-*1*, **4*, **5*, **6*, **10*, **14*, **18*, **21*, **36-*36*, and **41* was performed using real-time Invader (Third Wave Technologies, Madison, WI, USA) and TaqMan assays (Applied Biosystems, Foster City, CA, USA) as described previously [15, 21, 25].

Measurement of plasma concentrations of tamoxifen and its metabolites

Plasma concentrations of tamoxifen and its metabolites, endoxifen, 4-hydroxytamoxifen, and *N*-desmethyltamoxifen, were measured using a liquid chromatography–tandem mass spectrometry (LC–MS/MS) method. Tamoxifen and imipramine, an internal standard (IS), were purchased from Sigma-Aldrich (St Louis, MO, USA). (*Z*)-4-Hydroxy-*N*-desmethyltamoxifen, (*Z*)-4-hydroxytamoxifen, and *N*-desmethyltamoxifen hydrochloride were obtained from Toronto Research Chemicals Inc. (North York, ON, Canada).

Pretreatment of plasma samples was carried out by protein precipitation. Briefly, 100 µl of plasma was mixed with 250 µl of IS solution (10 ng/ml imipramine in acetonitrile). After vortex (30 s) and centrifugation (13,000 rpm, 5 min), the supernatant was directly analyzed using an autosampler. LC–MS/MS was equipped with an Acquity UPLC (Ultra Performance LC) system and a Xevo TQ MS (Waters, Milford, MA, USA). Chromatographic

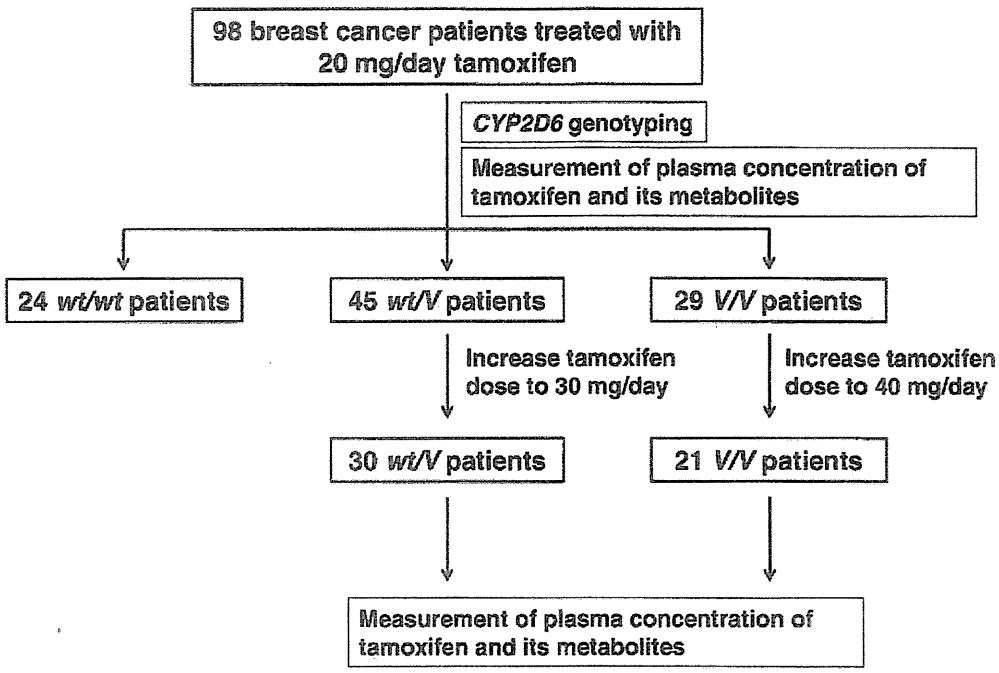

Fig. 1 Consort diagram. *wt*: **1*, *V*: **5*, **10*, **21*, **36-*36* and **41*

separations were obtained under gradient conditions using an ACQUITY UPLC BEH C18 column (100 × 2.1 mm ID, 1.7 μm particle size, Waters). The mobile phase was consisted of eluent A (10 mmol/l ammonium formate) and eluent B (acetonitrile). The flow rate was 0.4 ml/min and the gradient was as follows: 20% B for 0.2 min; 50% B at 0.3 min; 100% B at 1.3 min; 100% B for 0.6 min; and 20% B at 3.5 min. The total run time was 6 min per sample. The column temperature was 40°C, the sample temperature was 10°C, and the injection volume was 2 μl. The retention times of imipramine (IS), endoxifen, 4-hydroxytamoxifen, *N*-desmethyltamoxifen, and tamoxifen were 1.39, 1.42, 1.51, 1.79, and 1.96 min, respectively.

The mass spectrometer was run in electrospray positive, and source conditions were as follows: capillary voltage, 3 kV; cone voltage, 35 V; desolvation temperature, 500°C. A collision gas flow of 0.28 ml/min and collision energy of 5 keV were employed for creation of daughter ions. Multiple reaction monitoring mode detected the following transitions: 281.2 > 58.0, 374.3 > 58.0, 388.3 > 72.0, 358.2 > 58.0, and 372.3 > 72.0 for (IS), endoxifen, 4-hydroxytamoxifen, *N*-desmethyltamoxifen, and tamoxifen, respectively. The chromatographic data were acquired and analyzed using MassLynx software, equipped with QuanLynx (Waters).

Standard curves were prepared in the concentration range of 20–500 ng/ml for tamoxifen, 40–1000 ng/ml for *N*-desmethyltamoxifen, 4–100 ng/ml for endoxifen, and 1–25 ng/ml for 4-hydroxytamoxifen. The inter- and intra-day variabilities in precision (expressed as the coefficient of variation) for all compounds ranged from 0.6 to 10.8% and from 2.5 to 7.0%, respectively. The average accuracies for them were between 96.7 and 106.2%.

Statistical analysis

The differences between the plasma concentrations of tamoxifen and its metabolites before and after the increase of tamoxifen dose were compared using a paired *t* test. The differences in plasma concentrations of tamoxifen and its metabolites among patients with different *CYP2D6* genotypes were evaluated by a one-way ANOVA test. The associations between dose and adverse events were tested by fisher's exact test. Statistical tests provided two-sided *P* values, and significance level of less than 0.05 was used. Statistical analyses were carried out using SPSS (version 17.0, SPSS, Chicago, IL) and the R statistical environment version 2.9.2 (http://www.r-project.org/).

Results

Patient characteristics

We recruited 98 patients with breast cancer receiving adjuvant tamoxifen therapy (Table 1). Their median age at the time of surgery was 44 years old (range, 25–69 years). We investigated *CYP2D6* genotypes of these 98 patients (Table 2). There was no significant difference in age among *CYP2D6* genotype groups (Kruskal–Wallis test $P = 0.092$).

Plasma concentrations of tamoxifen and its metabolites

As described in our previous report [21], the steady-state plasma endoxifen concentrations in patients with homozygous and heterozygous for *CYP2D6* with decreased

Table 1 Characteristics of patients

Characteristic	Total ($N = 98$) Number of patients (%)
Age at surgery, years	
Median	44
Range	29–65
Menopausal status	
Premenopause	83 (84.7)
Postmenopause	5 (5.1)
Unknown	10 (10.2)
Tumor size (cm)	
≤2	68 (69.4)
2.1–5	21 (21.4)
>5	2 (2.0)
Unknown	7 (7.1)
Nodal status	
Negative	75 (76.5)
Positive	20 (20.4)
Unknown	3 (3.1)
Estrogen receptor status	
Positive	91 (92.9)
Negative	4 (4.1)
Unknown	3 (3.1)
Progesterone receptor status	
Positive	85 (86.7)
Negative	9 (9.2)
Unknown	4 (4.1)
Her-2	
Positive[a]	4 (4.1)
Negative	89 (90.8)
Unknown	5 (5.1)

Her-2 human epidermal growth factor receptor 2

[a] Score of 3+ in immunohistochemistry

function or no function (previously described as *V/V* and *wt/V*, respectively) were 43.8 and 76.8% of those with homozygous for the wild-type allele when they were treated with 20 mg/day of tamoxifen. In this dose-adjustment study, the doses for the patients with one or no wild-type allele of *CYP2D6* were increased from 20 to 30 or 40 mg/day of tamoxifen for >8 weeks, respectively, and their steady-state plasma concentrations of endoxifen and 4-hydroxytamoxifen were measured (Fig. 2). In the patients with *CYP2D6*10/*10* genotype, the mean plasma endoxifen level after increasing tamoxifen dose to 40 mg/day was 15.8 ng/ml, which was 1.69-fold higher than that before the dose increase (9.3 ng/ml, $P < 0.001$). The *CYP2D6*1/*10* patients with 30 mg/day of tamoxifen showed 1.41-fold higher plasma concentration of endoxifen (22.4 ng/ml) than that before the dose increase ($P < 0.001$). These endoxifen plasma concentrations were comparable to that observed in the *CYP2D6*1/*1* patients receiving 20 mg/day of tamoxifen (19.7 ng/ml, $P = 0.076$; Fig. 3a). Similarly, plasma 4-hydroxytamoxifen concentrations in the patients with *CYP2D6*1/*10* ($N = 28$) and **10/*10* ($N = 17$) were significantly increased ($P < 0.001$; Fig. 2) to the similar levels of the *CYP2D6*1/*1* patients by increasing tamoxifen doses ($P = 0.11$; Fig. 3b). These results suggest that 30 and 40 mg/day of tamoxifen are necessary for the patients with *CYP2D6*1/*10* and **10/*10* genotypes to maintain the plasma levels of active metabolites of tamoxifen observed in the patients with *CYP2D6*1/*1* genotype. We also measured plasma concentrations of tamoxifen and *N*-desmethyltamoxifen, which are pharmacologically less active than endoxifen and 4-hydroxytamoxifen (Fig. 3c, d). The dose-dependent increases were observed in the plasma levels of tamoxifen and *N*-desmethyltamoxifen ($P < 0.001$).

In addition, the patients who are heterozygous carriers of *CYP2D6*10* and null allele, including **5*, **21*, and **36-*36*, showed similar degree of increase (1.94-fold) of plasma levels of endoxifen and 4-hydroxytamoxifen to those with *CYP2D6*10/*10* (1.69-fold; Fig. 4), although number of patients carrying null alleles was small.

Toxicities

We investigated the influences of the increased dose of tamoxifen on the incidence of adverse events according to CTCAE v4.0 (Table 3). Although, several adverse events were observed during tamoxifen treatment, there were no significant differences in the incidences of adverse events between before and after the adjustment of tamoxifen dose. In addition, no significant difference was observed in the patients treated with adjusted doses, when compared to the patients with *CYP2D6*1/*1*, who were administrated with 20 mg tamoxifen daily, except for hyperhidrosis. However, hyperhidrosis was observed less frequently in patients heterozygous and homozygous for decreased-function and null allele receiving higher dose of tamoxifen than in the *CYP2D6*1/*1* patients ($P = 0.032$).

Discussion

Adjuvant tamoxifen treatment substantially improves the 10-year survival of ER positive breast cancer patients with a significant reduction in breast cancer recurrence and in mortality [1, 2]. However, as reported by several research groups including us, the patients who showed decreased and impaired activities of CYP2D6 by the genetic polymorphisms showed significantly less response to tamoxifen therapy [15–21]. Thus, the investigation finding an optimal dose of tamoxifen for the patients with each *CYP2D6*

Table 2 Genotype frequency of *CYP2D6*

CYP2D6 genotype	Number of patients (%)
**1/*1*	24 (24.5)
**1/*5*	5 (5.1)
**1/*10*	40 (40.8)
**5/*10*	4 (4.1)
**10/*10*	22 (22.4)
**10/*21*	1 (1.0)
**10/*36-*36*	1 (1.0)
**21/*41*	1 (1.0)

genotype is required. We herein reported the results of a dose-adjustment study of tamoxifen based on the individual *CYP2D6* genotypes. We clarified that the increase of tamoxifen dose was able to increase the endoxifen plasma concentration, and expected to improve the prognosis of the tamoxifen-treated patients who show decreased CYP2D6 activity by genetic polymorphisms. However, the association between *CYP2D6* genotype and tamoxifen efficacy remains controversial as suggested by two recent studies [26, 27]. Therefore, a prospective large-scale study is required to investigate relationship between tamoxifen dose-adjustment based on *CYP2D6* genotype and clinical outcome of the patients with breast cancer.

As shown in Figs. 2, 3, 1.5-fold higher dosage (30 mg/day) of tamoxifen for the patients with *CYP2D6*1/*10* or **1*/null genotype was likely to be enough to achieve similar plasma levels of active metabolites to those of the patients with *CYP2D6*1/*1* genotype who received 20 mg/day of tamoxifen. In addition, in the *CYP2D6*10/*10*, **10*/null, or **41*/null patients who were treated with 40 mg/day of tamoxifen, the plasma concentrations of endoxifen and 4-hydrotamoxifen were comparable to those in the *CYP2D6*1/*1* patients administrated with 20 mg/day of tamoxifen. The ratios of endoxifen/tamoxifen and 4-hydoroxytamoxifen/tamoxifen were not significantly different between the groups before and after dose adjustment in both *CYP2D6*1/*10* or **1*/null and *CYP2D6*10/*10* or **10*/null patients ($P \geq 0.23$; Supplementary Fig. 1). Together with the data of Fig. 4, these data suggest low possibility of saturation of CYP2D6 metabolic capacity in these patients. These results indicate that appropriate

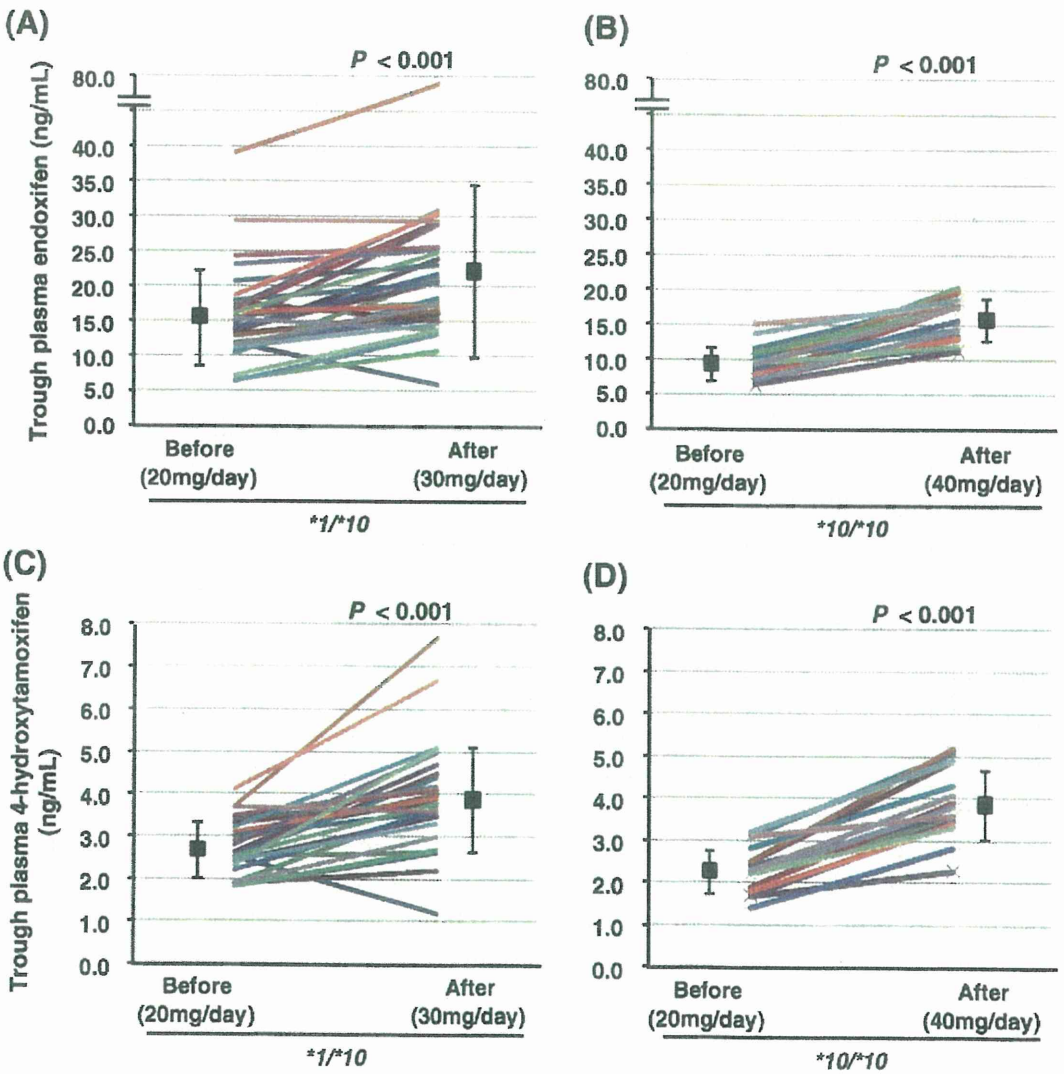

Fig. 2 Steady-state plasma concentrations of endoxifen **a**, **b** and 4-hydroxytamoxifen (**c**, **d**) before and after dose increase of tamoxifen in breast cancer patients. **a**, **c** Patients with *CYP2D6*1/*10* ($N = 28$), **b**, **d** Patients with *CYP2D6*10/*10* ($N = 17$). Data are expressed as the mean ± SD and as each individual value before and after dose increase of tamoxifen

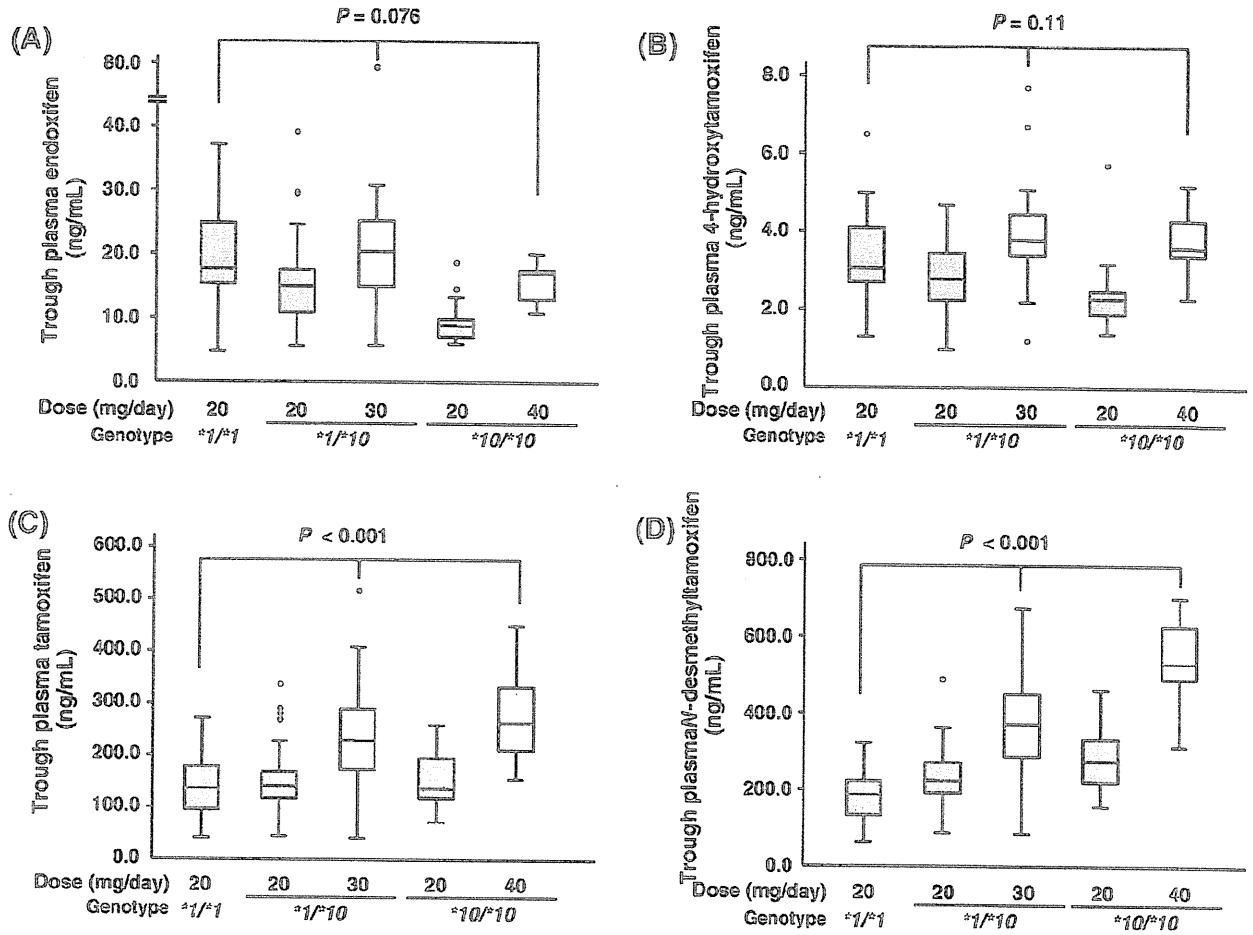

Fig. 3 Comparison of steady-state plasma concentrations of tamoxifen and its metabolites among the patients administrated with different dosages of tamoxifen. a Endoxifen, b 4-hydroxytamoxifen, c tamoxifen, and d *N*-desmethyltamoxifen. The *horizontal line* indicates the median concentration, the box covers the 25th–75th percentiles, and the maximum length of each whisker is 1.5× the interquartile range; *dots* outside the whiskers are outliers. The difference in plasma concentrations of tamoxifen and its metabolites among **1/*1* (20 mg/day), **1/*10* (30 mg/day), and **10/*10* (40 mg/day) genotypes for *CYP2D6* was evaluated by a one-way ANOVA test

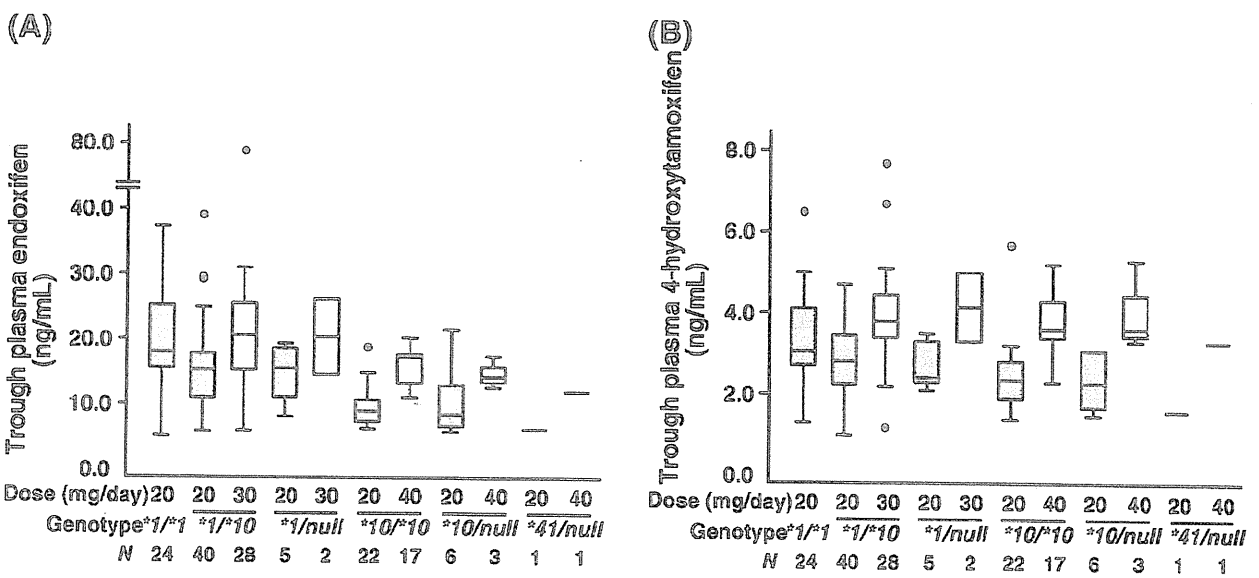

Fig. 4 Comparison of steady-state plasma concentrations of endoxifen (a) and 4-hydroxytamoxifen (b) among the patients administrated with different dosages of tamoxifen according to *CYP2D6* genotypes. The *horizontal line* indicates the median concentration, the box covers the 25th–75th percentiles, and the maximum length of each whisker is 1.5× the interquartile range; *dots* outside the whiskers are outliers. The difference in plasma concentrations of endoxifen and 4-hydroxytamoxifen between *CYP2D6*10* and null alleles was evaluated by the Student's *t* test. null: *CYP2D6*5*, **21* and **36-*36*

Table 3 Association between tamoxifen dose and incidence of adverse events (all grades according to CTCAE v4.0)

Adverse events	*CYP2D6* genotype	Event/no event, no of patients (%)		After compared to before		After compared to **1/*1*	
		Before (20 mg/day)	After (30 or 40 mg/day)	Odds ratio (95% CI)	*P* value	Odds ratio (95% CI)	*P* value
Hot flashes	**1/*1*	9/1 (90.0%)	–				
	**1/decreased and *1/null*	27/9 (75.0%)	18/9 (66.7%)	0.67 (0.22–2.00)	0.58	0.22 (0.02–2.04)	0.23
	Decreased/decreased and decreased/null	19/2 (90.5%)	19/3 (86.4%)	0.67 (0.10–4.45)	1.00	0.70 (0.06–7.74)	1.00
Hyperhidrosis	**1/*1*	9/1 (90.0%)	–				
	**1/decreased and *1/null*	21/15 (58.3%)	14/13 (51.9%)	0.77 (0.28–2.1)	0.62	0.12 (0.01–1.08)	0.056
	Decreased/decreased and decreased/null	13/8 (61.9%)	10/12 (45.5%)	0.51 (0.15–1.73)	0.36	0.09 (0.01–0.86)	0.024
Vaginal discharge	**1/*1*	7/3 (70.0%)	–				
	*1/decreased and *1/null*	30/6 (83.3%)	21/6 (77.8%)	0.70 (0.20–2.47)	0.75	1.50 (0.29–7.65)	0.68
	Decreased/decreased and decreased/null	12/9 (57.1%)	18/4 (81.8%)	3.38 (0.84–13.5)	0.10	1.93 (0.34–10.91)	0.65
Irregular menstruation	**1/*1*	1/9 (10.0%)	–				
	**1/decreased and *1/null*	3/33 (8.3%)	0/27 (0.0%)	0.17 (0.01–3.52)	0.25	0.12 (0.00–3.07)	0.27
	Decreased/decreased and decreased/null	2/19 (9.5%)	2/20 (9.1%)	0.95 (0.12–7.44)	1.00	0.90 (0.07–11.25)	1.00
Nausea or vomiting	**1/*1*	1/9 (10.0%)	–				
	**1/decreased and *1/null*	6/30 (16.7%)	3/24 (11.1%)	0.63 (0.14–2.76)	0.72	1.13 (0.1–12.27)	1.00
	Decreased/decreased and decreased/null	4/17 (19.0%)	3/19 (13.6%)	0.67 (0.13–3.44)	0.70	1.42 (0.13–15.64)	1.00
Eye disorders	**1/*1*	4/6 (40.0%)	–				
	**1/decreased and *1/null*	17/19 (47.2%)	12/15 (44.4%)	0.89 (0.33–2.44)	1.00	1.20 (0.27–5.25)	1.00
	Decreased/decreased and decreased/null	8/13 (38.1%)	11/11 (50.0%)	1.63 (0.48–5.47)	0.54	1.50 (0.33–6.83)	0.71
Malaise	**1/*1*	7/3 (70.0%)	–				
	**1/decreased and *1/null*	21/15 (58.3%)	12/15 (44.4%)	0.57 (0.21–1.57)	0.32	0.34 (0.07–1.62)	0.27
	Decreased/decreased and decreased/null	8/13 (38.1%)	7/15 (31.8%)	0.76 (0.22–2.67)	0.75	0.20 (0.04–1.01)	0.062
Reproductive system disorders-endometrial thickening	**1/*1*	0/24 (0.0%)	–				
	**1/decreased and *1/null*	2/43 (4.4%)	0/30 (0.0%)	0.28 (0.01–5.95)	0.87	–	–
	Decreased/decreased and decreased/null	0/29 (0.0%)	1/20 (4.8%)	4.12 (0.16–106.01)	0.84	3.59 (0,14–92.84)	0.84
Thromboembolic event	**1/*1*	1/23 (4.2%)	–				
	**1/decreased and *1/null*	1/44 (2.2%)	0/30 (0.0%)	0.47 (0.02–11.94)	1.00	0.26 (0.01–6.59)	0.85
	Decreased/decreased and decreased/null	0/29 (0.0%)	0/20 (0.0%)	–	–	0.36 (0.01–9.43)	1.00
Hepatobiliary disorders-exacerbation of hepatic steatosis	**1/*1*	0/24 (0.0%)	–				
	**1/decreased and *1/null*	0/45 (0.0%)	2/28 (6.7%)	7.71 (0.36–166.39)	0.74	4.30 (0.20-93.90)	0.85
	Decreased/decreased and decreased/null	0/29 (0.0%)	0/21 (0.0%)	–	–	–	–

CI confidence interval

Decreased: **10*, **41*; null: **5*, **21*, **36-*36*

increase of tamoxifen dose for the patients with *CYP2D6*1/*10*, **1*/null, **10/*10*, **10*/null, and **41*/null genotypes was an useful method to achieve the plasma levels of active metabolites of tamoxifen which was seen in the patients with *CYP2D6*1/*1* genotype.

Subjects who carry at least one decreased-function allele (*CYP2D6*10* or *CYP2D6*41*) or one null allele, remain to have a certain level of enzymatic activity although it is lower than the *CYP2D6*1/*1* genotype. Therefore, increased dose is an effective way to overcome the problem of reduced enzymatic activity and to increase the level of active metabolites for these populations. However, we could not evaluate the effects of increasing dose in the null/null patients because no null/null patient participated in this study. Recently, Irvin et al. reported that endoxifen concentration in PM patients, who were defined as homozygote for inactive alleles, was still lower after increasing tamoxifen dose to 40 mg/day (12.9 ng/ml) than that of patients classified as extensive metabolizers, who carry two alleles with normal activity (29.2 ng/ml) [28]. It should be noted that dose-adjustment strategy is useful for patients carrying at least one decreased-function allele or one null allele, while the postmenopausal patients with null/null genotype of *CYP2D6* might be more beneficial to take aromatase inhibitors instead of increased dose of tamoxifen, although further verification is required.

It has been well known that several adverse events were observed during tamoxifen therapy [29]. Hot flash is one of the most common adverse events, which was observed in up to 80% of patients prescribed with tamoxifen, and approximately 30% of them are relatively severe [29]. In this study, no significant difference was observed in the incidence of hot flash between the groups before and after increasing tamoxifen dose (Table 3). The incidence of hot flash has been suggested to be associated with the *CYP2D6* genotypes [16, 30], implying association with plasma levels of endoxifen and 4-hydrotamoxifen. The results from our preliminary investigation suggest that dose adjustment from 20 to 30 mg/day of tamoxifen for the patients with *CYP2D6*1/*10* and **1*/null and 40 mg/day for the patients with **10/*10*, **10*/null, and **41*/null may not affect the risk of adverse events, although tamoxifen and *N*-desmethyltamoxifen showed higher plasma concentrations in the patients receiving higher tamoxifen dose than those of *CYP2D6*1/*1* patients with 20 mg/day of tamoxifen. Further analysis using a larger number of patients is required to evaluate the influences of increase of tamoxifen dose on adverse events.

In conclusion, the dose-adjustment study based on the *CYP2D6* genotypes indicated that the increase of tamoxifen dose was able to increase the endoxifen plasma concentration, and expected to improve the prognosis of the tamoxifen-treated patients who show decreased CYP2D6 activity by genetic polymorphisms. A prospective large-scale study is required to confirm our dose-adjustment strategy for improvement of tamoxifen therapy in breast cancer patients.

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Pharmacogenomics of Tamoxifen: Roles of Drug Metabolizing Enzymes and Transporters

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Summary: Tamoxifen has been widely used for the prevention of recurrence in patients with hormone receptor-positive breast cancer. Tamoxifen requires metabolic activation by cytochrome P450 (CYP) enzymes for formation of active metabolites, 4-hydroxytamoxifen and endoxifen, which have 30- to 100-fold greater affinity to the estrogen receptor and the potency to suppress estrogen-dependent breast cancer cell proliferation. CYP2D6 is a key enzyme in this metabolic activation and it has been suggested that the genetic polymorphisms of *CYP2D6* influence the plasma concentrations of active tamoxifen metabolites and clinical outcomes for breast cancer patients treated with tamoxifen. The genetic polymorphisms in the other drug-metabolizing enzymes, including other CYP isoforms, sulfotransferases and UDP-glucuronosyltransferases might contribute to individual differences in the tamoxifen metabolism and clinical outcome of tamoxifen therapy although their contributions would be small. Recently, involvement of a drug transporter in the disposition of active tamoxifen metabolites was identified. The genetic polymorphisms of transporter genes have the potential to improve the prediction of clinical outcome for the treatment of hormone receptor-positive breast cancer. This review summarizes current knowledge on the roles of polymorphisms in the drug-metabolizing enzymes and transporters in tamoxifen pharmacogenomics.

Keywords: P450 2D6; MRP2; MDR1; UGT; SULT; single nucleotide polymorphism; endoxifen; estrogen receptor

Introduction

Tamoxifen, a selective estrogen receptor (ER) modulator, has been widely used for the treatment and prevention of recurrence for patients with hormone receptor (ER or progesterone receptor)-positive breast cancers in more than 120 countries throughout the world. Since most breast cancers are hormone receptor-positive, thousands of breast cancer patients worldwide initiate endocrine treatment each year. Based on the results of the Early Breast Cancer Trialists' Collaborative Group, the standard recommendation has been 5 years of therapy with tamoxifen.[1)] In pre- and postmenopausal patients with primary breast cancer, adjuvant tamoxifen significantly decreased recurrence and breast cancer mortality for 15 years after primary diagnosis.[1)] However, 30–50% of patients with adjuvant tamoxifen therapy experience relapse and subsequently die of the disease,[1,2)] indicating individual differences in responsiveness to tamoxifen.

Tamoxifen is extensively metabolized to more active or inactive metabolites by phase I and phrase II enzymes, including cytochrome P450s (CYPs), sulfotransferases (SULTs) and UDP-glucuronosyltransferases (UGTs). Recent data support that the polymorphisms in these drug-metabolizing enzymes contribute to individual difference in plasma concentrations of active tamoxifen metabolites and tamoxifen clinical outcome. Among them, CYP2D6 is most extensively investigated. It was recently reported that drug transporters are involved in the transport of active tamoxifen metabolites and it is suggested that the polymorphisms of

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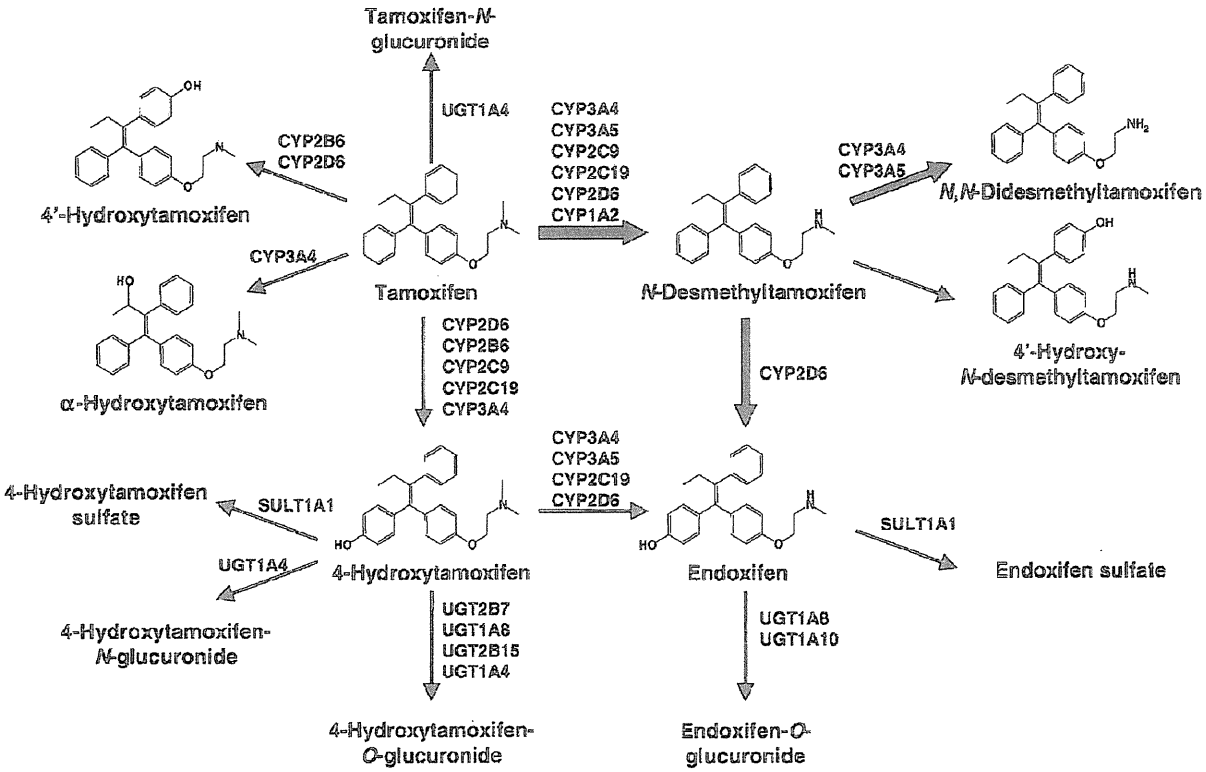

Fig. 1. Metabolic pathways of tamoxifen in humans
Major metabolic pathways are highlighted with bold arrows.

transporter genes are likely to be involved in variable clinical outcome observed in patients treated with tamoxifen. This review summarizes current data on the relationships of genetic polymorphisms of the tamoxifen-metabolizing enzymes and transporters to individual differences in tamoxifen disposition and clinical outcomes of breast cancer patients with tamoxifen treatment.

Tamoxifen Metabolism

Tamoxifen is extensively metabolized by phase I and phase II enzymes in the human liver (Fig. 1). The parent drug itself has weak affinity to the ER, only 1.8% of the affinity of 17β-estradiol.[3] The major metabolite *N*-desmethyltamoxifen is formed by *N*-demethylation, which is catalyzed mainly by CYP3A4 and CYP3A5, with minor contribution by CYP2D6, CYP1A2, CYP2C9 and CYP2C19.[4–6] The steady state plasma concentration of *N*-desmethyltamoxifen after administration of 20 mg/day tamoxifen is approximately twice as high as that of tamoxifen.[7,8] *N*-Desmethyltamoxifen shows weak affinity to the ER, similar to that of tamoxifen.[3] 4-Hydroxytamoxifen, which is formed by 4-hydroxylation of tamoxifen, had been considered to play an important role as an active metabolite because it has 100-fold higher affinity to the ER and 30- to 100-fold greater potency than tamoxifen in suppressing estrogen-dependent breast cancer cell proliferation.[3,9–11] This conversion is catalyzed by CYP2D6, CYP2B6, CYP2C9, CYP2C19 and CYP3A4.[6,12–14] A different metabolite, 4-hydroxy-*N*-desmethyltamoxifen (endoxifen), was identified in the 1980s in humans but its role had remained unknown. Recent reports have clarified that endoxifen has a potency equivalent to 4-hydroxytamoxifen,[9,15,16] and plasma endoxifen levels exceed plasma concentration levels of 4-hydroxytamoxifen by several folds, suggesting endoxifen to be a principal active metabolite.[8–10] Although the metabolism of tamoxifen to 4-hydroxytamoxifen is catalyzed by multiple isoforms, endoxifen is formed predominantly by the CYP2D6-mediated 4-hydroxylation of *N*-desmethyltamoxifen.[17] In addition, *N*-desmethyltamoxifen can also be demethylated by CYP3A4 to form *N*,*N*-didesmethyltamoxifen. Further hydroxylation also takes place at the 4′ position, leading to 4′-hydroxytamoxifen, which is mainly mediated by CYP2B6 and CYP2D6, and to 4′-hydroxy-*N*-desmethyltamoxifen.[6] Another hydroxylated metabolite, α-hydroxytamoxifen, is produced mainly by CYP3A4.[4,5] However, except for endoxifen and 4-hydroxytamoxifen, no other highly active metabolites have been described so far.[18]

Tamoxifen and these metabolites are further metabolized by phase II enzymes, such as SULTs and UGTs. SULT1A1 is considered to be the primary SULT responsible for the sulfation of 4-hydroxytamoxifen and endoxifen.[19,20] UGT1A8, UGT1A10, UGT2B7, UGT2B15 and UGT1A4 are involved in the *O*-glucuronidation of 4-hydroxytamoxifen and endoxifen.[21–23] Tamoxifen and 4-hydroxytamoxifen are glucuronidated by UGT1A4 to the corresponding

N^+-glucuronides.[24,25] The genetic variations of these drug-metabolizing enzymes have the potential to affect tamoxifen metabolism.

Genetic Polymorphisms of *CYP2D6*

CYP2D6 is one of the most important CYP isoforms due to its central role in the metabolism of a number of clinically important drugs, including β-blockers, antiarrhythmics, antihypertensives, antipsychotics, antidepressants, opioids and others.[26] The *CYP2D6* gene is located on chromosome 22q13.1, containing two neighboring pseudogenes, *CYP2D7* and *CYP2D8*. This locus is extremely polymorphic with over 80 allelic variants, as presented at the home page of the human CYP allele nomenclature committee (http://www.cypalleles.ki.se/cyp2d6.htm), which should be one of the causes of wide inter-individual and ethnic differences in CYP2D6 activity *in vivo*. Commonly, four CYP2D6 phenotypes are observed on the basis of their metabolic capacities: extensive metabolizer (EM), poor metabolizer (PM), intermediate metabolizer (IM) and ultra-rapid metabolizer (UM).[27,28] It has been reported that the PM phenotype, which is caused by the carrying of two null alleles, reveals itself in 5–10% of Caucasians.[29] *CYP2D6*3*, *CYP2D6*4*, *CYP2D6*5* and *CYP2D6*6* are major null alleles that cause the PM phenotype and account for nearly 95% of the PMs in Caucasians (Table 1).[30] In contrast, less than 1% of Asians show the PM phenotype,[31] and most Asians are categorized as IMs due to frequent carries of *CYP2D6*10* alleles.[32,33] The *CYP2D6*14*, *CYP2D6*18*, *CYP2D6*21*, *CYP2D6*44* alleles were found as null alleles in Asian populations, although their frequencies are very low.[34–37] The frequencies of UMs, who are carriers of duplicated/multiplied *CYP2D6* gene, are 10–15% in Caucasian, whereas UMs are uncommon in Asians.

The *CYP2D6* genotype-phenotype relationship was well investigated. In the patients who are PMs or IMs, tamoxifen is not metabolized effectively to its active metabolites and therefore would provide little anti-estrogenic effect. With respect to UMs, it is important to note that such patients may be more susceptible to hot flashes during tamoxifen therapy.

CYP2D6 genotype and clinical outcome of tamoxifen therapy: In recent years, we have seen an explosion of interest in the clinical relevance of *CYP2D6* genotype on outcomes for breast cancer patients treated with tamoxifen. It has been hypothesized that patients with a lower CYP2D6 activity due to genetic variations may show low endoxifen concentration in plasma, and thus might have poorer clinical outcome.

Prospective cohort studies of adjuvant tamoxifen treatment have revealed wide inter-individual variation in the steady-state plasma concentrations of active metabolites, endoxifen and 4-hydroxytamoxifen, during tamoxifen treatment in women carrying *CYP2D6* gene variants.[7,8,10] The patients homozygous for null alleles (categorized as PM) show four-fold lower concentration of endoxifen in plasma than those carrying two normal alleles (categorized as EM). The low function alleles, including *CYP2D6*10* and *CYP2D6*41*, were also reported to cause insufficient formation of endoxifen from the data that the patients carrying two low-function alleles (categorized as IM) had two-fold lower plasma endoxifen concentration.[18,38–40] Moreover, convincing evidence has shown that selective serotonin reuptake inhibitors such as paroxetine and fluoxetine, which are known to be strong CYP2D6 inhibitors, reduced plasma endoxifen concentration.[8,10]

Table 1. Frequencies of alleles of drug-metabolizing enzymes and transporters

Gene	Allele or SNP	Activity/expression	Allelic frequency (%)	
			Asians	Caucasians
CYP2D6[30–38,89]	*3	none	0.8	1.0–3.9
	*4	none	0.5–2.8	17.5–23.0
	*5	none	5.1–6.2	1.6–7.3
	*6	none	0	0.7–1.4
	*10	decreased	38.1–70.0	1.4–3.5
	*14	none	0.18	—
	*18	none	0.7	—
	*21	none	0.39–0.71	—
	*41	decreased	1.4–2.6	8.4–10.6
CYP2C9[62]	*2	none	0–0.1	8.0–19.1
	*3	none	1.1–6.8	3.7–17.0
CYP2C19[31,63,64,65]	*2	none	23–39	10–20
	*3	none	5.0–10.0	0
	*17	increased	1–4	18–27
CYP3A5[59]	*3	none	74–77	85–95
ABCC2[38,80,81]	−1774delG	decreased	20.2–34.3	—
	−24C>T	decreased	17.4–32.6	18.1–22.5
	1249G>A	decreased	9.7–10.9	15.5–24.3

As shown in Table 2, a number of the clinical trials have reported the association between the *CYP2D6* genotype and clinical outcome of breast cancer patients having tamoxifen therapy. One of the first studies reported by Goetz *et al.* in 2005 demonstrated that homozygous carriers of a *CYP2D6*4* allele had a shorter relapse-free survival (RFS) and disease-free survival (DFS) compared with the patients heterozygous or homozygous for the wild-type allele (hazard ratio (HR), 1.85; $p = 0.18$ for RFS: HR, 1.86; $p = 0.089$ for DFS).[41] As a follow-up study, they reported that the patients classified as PMs and IMs had a significantly shorter time to recurrence (HR = 1.91; $p = 0.034$) and worse RFS (HR = 1.74; $p = 0.017$) relative to EMs.[42] Schroth *et al.* reported significantly shorter RFS (HR, 2.24; $p = 0.02$) among patients carrying the *CYP2D6*4*, *CYP2D6*5*, *CYP2D6*10* and *CYP2D6*41* alleles, compared with patients with two functional alleles in a study of a German population