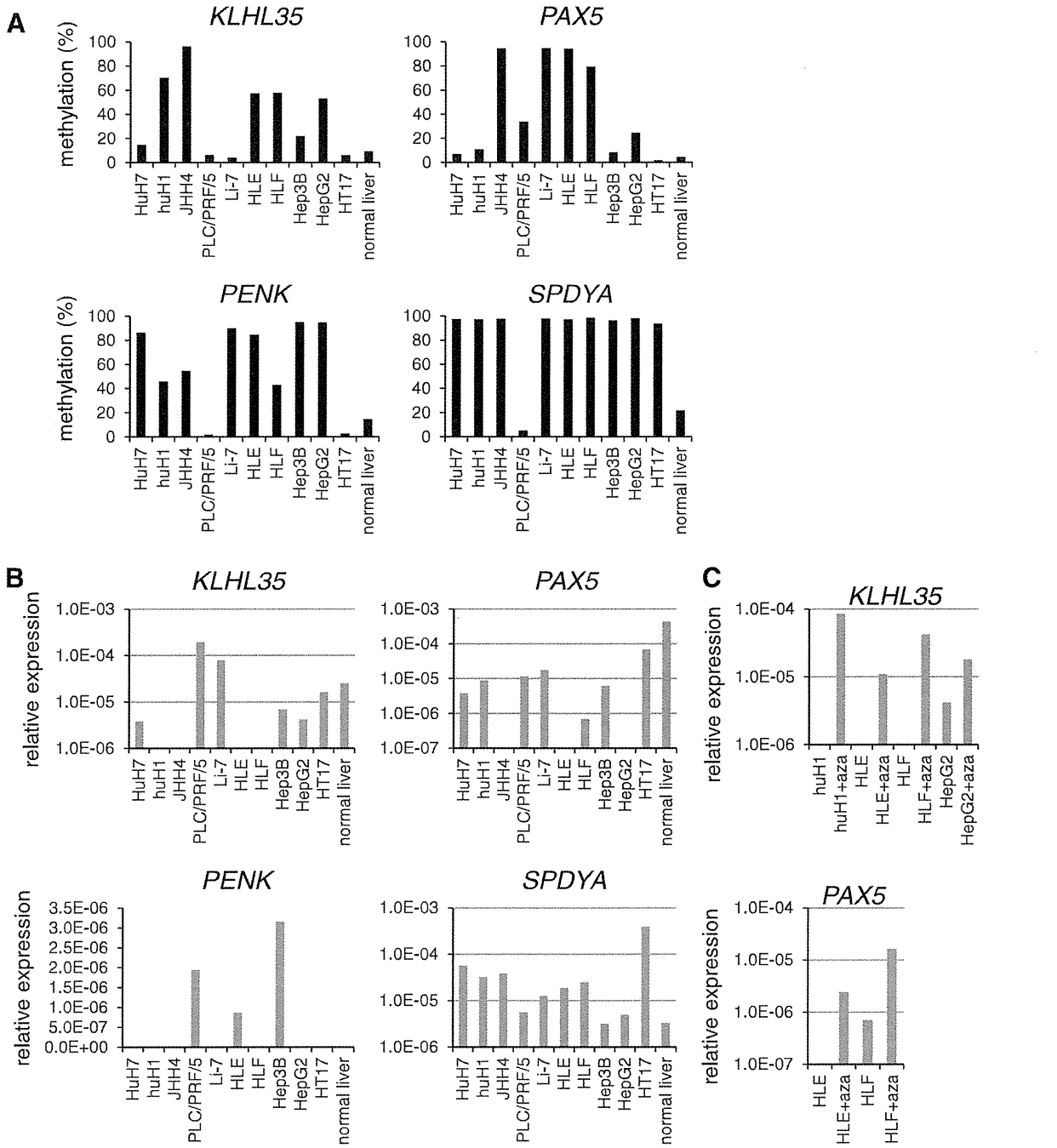

Fig. 3 Analysis of the methylation and expression of the indicated genes in HCC cell lines. **a** Bisulfite pyrosequencing of *KLHL35*, *PAX5*, *PENK*, and *SPDYA* in HCC cell lines and normal liver tissue from a healthy individual. **b** Quantitative RT-PCR of the four genes in HCC cell lines and normal liver tissue. **c** Quantitative RT-PCR of *KLHL35* and *PAX5* in HCC cell lines, with and without 5-aza-dC (*aza*) treatment

pyrosequencing, we found that levels of LINE-1 methylation were significantly lower in tumor tissues than in their noncancerous counterparts (48.5 vs. 66.8 %, $P<0.001$). LINE-1 hypomethylation was prevalent, regardless of the tumor's hepatitis virus status, but the average methylation level was lowest in the HBV/HCV-negative tumors (HBV-positive, 50.8 vs. 66.3 %, $P<0.001$; HCV-positive, 48.9 vs. 67.4 %, $P<0.001$; HBV/HCV-negative, 44.7 vs. 66.6 %, $P<0.001$; Fig. 4a). The ROC curve analysis revealed that LINE-1 methylation discriminated strongly between HCC tissue and noncancerous liver tissue (Fig. 4b), though no significant correlation was found between the levels of LINE-1 methylation and the clinicopathological characteristics of the samples (Table 1). Finally, we tested whether LINE-1 hypomethylation is linked to gene hypermethylation. We found an inverse relationship between the level of

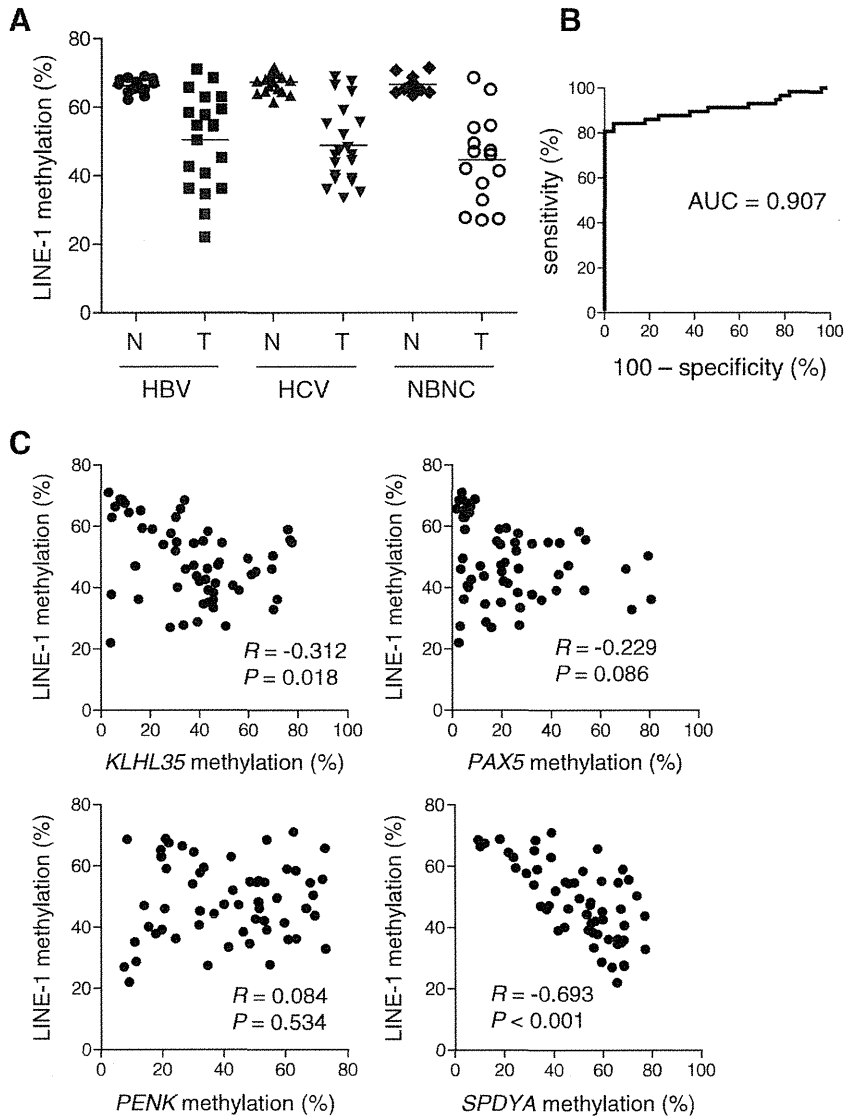

Fig. 4 Analysis of LINE-1 methylation and its association with CpG island hypermethylation in HCC. **a** Summary of bisulfite pyrosequencing analysis of LINE-1 in tumor tissue (*T*) and corresponding noncancerous liver tissue (*N*) from HBV-positive, HCV-positive, and HBV/HCB-negative (*NBNC*) HCC patients. **b** ROC curve analysis of the utility of LINE-1 methylation for distinguishing between HCC tissue and corresponding noncancerous liver tissue from the same HCC patients. **c** Correlation between the level of LINE-1 methylation and methylation of the indicated genes in HCC tissues. The Pearson correlation coefficients and *P* values are shown

LINE-1 methylation and levels of *KLHL35* and *SPDYA* methylation. On the other hand, we found no significant correlation between the LINE-1 hypomethylation and *PAX5* or *PENK* methylation (Fig. 4c).

Discussion

In the present study, we carried out high-throughput CpG island methylation profiling in a set of primary HCC tissues with and without hepatitis virus infection. MCAM analysis enabled us to evaluate the methylation status of more than 6,000 gene promoters with high specificity and sensitivity [13]. Consistent with earlier studies that showed methylation to be more abundant in the HCV-positive HCCs than in the HBV-positive or hepatitis virus-negative HCCs [15, 18], we observed the highest number of methylated genes in HCV-positive HCC tissue. However, we also noted that a large number of genes were commonly methylated among HCCs, irrespective of the hepatitis virus status, indicating that aberrant methylation of multiple genes may be involved in a common mechanism underlying hepatocarcinogenesis. Moreover, studies have also shown that aberrant methylation detected in tissues or blood samples could be a useful biomarker for early detection of HCC [19, 20]. We therefore validated the methylation status of 14 genes and identified four genes that were frequently methylated in HCC tissues but showed little or no methylation in surrounding noncancerous tissues. The high-tumor specificity suggests that methylation of these genes may not occur at precancerous stages, such as chronic hepatitis or liver cirrhosis; instead, they may be acquired during malignant transformation.

The paired box 5 (*PAX5*) gene is a member of the paired box-containing family of transcription factors, which are involved in the control of organ development and tissue differentiation [21]. PAX5 is also known to be a B cell-

specific activator protein that plays an essential role during B cell differentiation, neural development, and spermatogenesis. Methylation of the CpG island of *PAX5* was first discovered in breast cancer cells using the MCA technique [22]. Subsequently, methylation and downregulation of *PAX5* were found in lymphoid neoplasms [23]. In addition, while we are preparing the present manuscript, methylation of *PAX5* was reported in HCC and gastric cancer [24, 25]. Restoration of *PAX5* expression in HCC cells induced growth arrest and apoptosis through upregulation of various target genes, including p53, p21, and Fas ligand, suggesting that the PAX5 acts as a tumor suppressor [24].

The involvement of the kelch-like 35 (*KLHL35*) gene in cancer had not been reported until recently, when a genome-wide analysis of DNA methylation in renal cell carcinoma identified frequent hypermethylation of nine genes, including *KHLH35* [26]. Although the function of the gene product remains unknown, RNAi-induced knockdown of *KHLH35* in HEK293 cells promoted anchorage-independent growth, indicating its possible role in tumorigenesis [26].

The proenkephalin (*PENK*) gene encodes preproenkephalin, a precursor protein that is proteolytically cleaved to produce the endogenous opioid peptides met- and leu-enkephalin. Methylation of the CpG island of *PENK* was first identified in pancreatic cancer cells using the MCA technique [27]. Downregulated expression of *PENK* has also been reported in prostate cancer, suggesting its possible involvement in cancer development [28], and *PENK* methylation was recently identified in lung cancer, bladder cancer, and meningioma [29–31]. Although its functional role in cancer is not fully understood, a recent study showed that in response to cellular stress, PENK physically associates with p53 and RelA (p65) and regulates stress-induced apoptosis [32].

The *SPDYA* encodes Spy1, also known as Speedy, an atypical CDK activator known to promote cell survival, prevent apoptosis, and inhibit checkpoint activation in response to DNA damage [33]. The expression of *SPDYA* is upregulated in breast cancer [34], and its overexpression in a mouse model has been shown to accelerate mammary tumorigenesis [35]. Moreover, a recent study showed overexpression of *SYPDA* in HCC and its association with poor prognosis [36]. These results strongly suggest its involvement in oncogenesis. In the present study, we also observed that most of the HCC cell lines tested exhibited greater expression of *SPDYA* than normal liver tissue, regardless of the methylation status. Among the three transcription variants of *SPDYA* annotated in the NCBI Reference Sequence database, transcription start sites of variants 1 and 3 are located within the CpG island, while that of variant 2 are located approximately 5 kb downstream of the CpG island. Thus, the *SPDYA* transcript in HCC cells may be derived from the downstream transcription start site.

By analyzing the LINE-1 methylation levels, we and others have shown that global hypomethylation is a commonly observed feature of HCC [8, 9, 37]. Earlier studies have suggested that the association between global methylation and hepatitis status may be attributable to hepatitis B virus X protein, which can induce aberrant methylation of specific genes and global hypomethylation [38]. By contrast, we found in the present study that LINE-1 hypomethylation is prevalent among HCC tissues, regardless of the hepatitis virus infection, which suggests that global hypomethylation is involved in a common mechanism underlying hepatocarcinogenesis. It has been shown that the timing of global hypomethylation differs among tumor types. For example, hypomethylation is often observed during the early stages of colorectal and gastric carcinogenesis. By contrast, LINE-1 hypomethylation appears to be tumor-specific in HCC; it is rarely found in precancerous lesions such as chronic hepatitis or liver cirrhosis [8, 9]. A recent study showed that global hypomethylation is associated with a poorer prognosis in HCC patients [39]. In addition, the levels of serum LINE-1 hypomethylation in HCC patients reportedly correlate with serum HBs antigen status, large tumor size, and advanced tumor stage [40]. This suggests that hypomethylation may not occur at precancerous stages, and that LINE-1 methylation could be a useful biomarker with which to identify HCC and predict its clinical outcome.

The relationship between LINE-1 hypomethylation and CpG island hypermethylation in cancer is controversial. In one study, LINE-1 methylation levels were reduced in HCCs with the CpG island methylator phenotype, indicating a positive correlation between global hypomethylation and CpG island hypermethylation [9]. Another study showed that LINE-1 hypomethylation was positively correlated with hypermethylation of only a few genes (*p16*, *CACNA1G*, and *CDKN1C*), while methylation of a large number of genes showed inverse or no correlation with LINE-1 hypomethylation [12]. In the present study, we found that methylation of *KLHL35* and *SPYDA* correlates positively with LINE-1 hypomethylation, whereas levels of *PAX5* or *PENK* methylation are independent of LINE-1 methylation. These results suggest that the association between CpG island methylation and global hypomethylation may be site specific, and that hypomethylation of LINE-1 is a more generalized phenomenon than hypermethylation of CpG islands in HCC.

In summary, by screening targets of DNA methylation in HCC, we identified four frequently methylated genes. These genes are methylated in a cancer-specific manner and could be useful molecular markers for diagnosing HCC. In addition, we observed prevalent LINE-1 hypomethylation in HCC, irrespective of hepatitis virus infection. Identification of aberrant methylation in HCC may provide valuable information that not only contributes to our understanding of the pathogenesis

of the disease, but also to the development of new strategies for diagnosis and therapy.

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Conflicts of interest None

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Review

Pharmacogenomics of Tamoxifen: Roles of Drug Metabolizing Enzymes and Transporters

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Full text of this paper is available at http://www.jstage.jst.go.jp/browse/dmpk

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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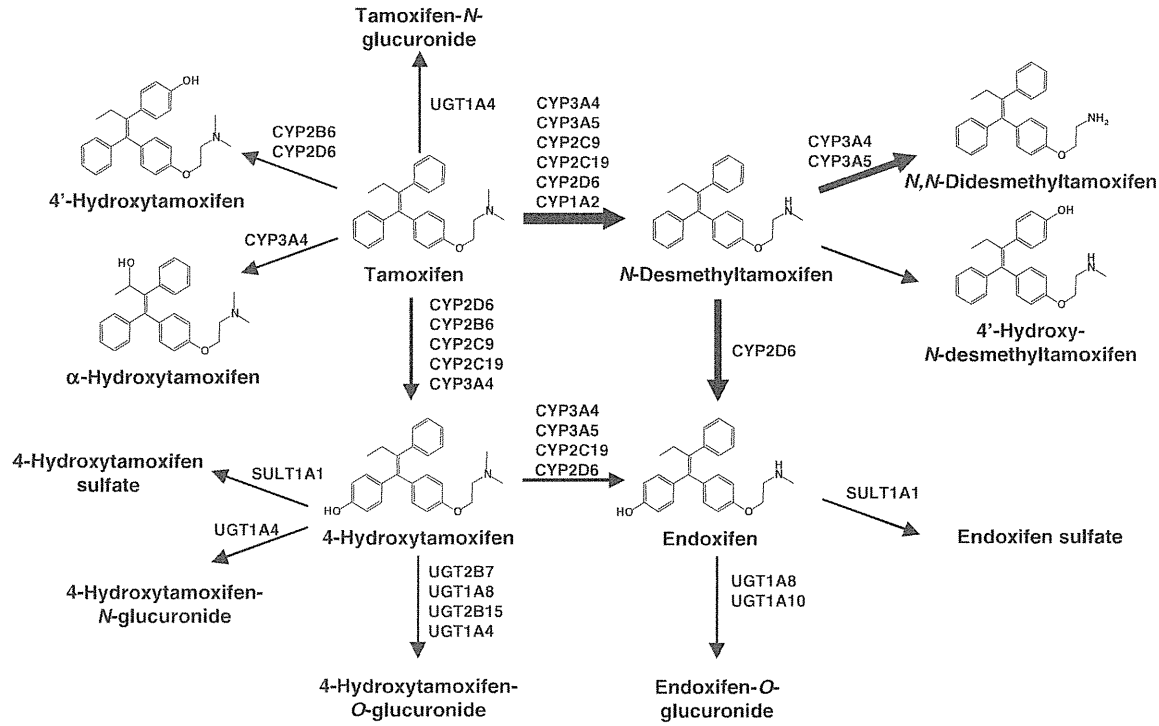

Fig. 1. Metabolic pathways of tamoxifen in humans
Major metabolic pathways are highlighted with bold arrows.

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

Table 2. Summary of studies evaluating association of *CYP2D6* genotype with response to adjuvant tamoxifen therapy

Studies	Number of patients	Tamoxifen therapy	% of monotherapy	Tamoxifen dose	Outcome[a]	Univariate		Multivariate		Comparison of *CYP2D6* genotype groups[a]
						Hazard ratio (95% CI)	p value	Hazard ratio (95% CI)	p value	
Goetz *et al.*,2005[41]	190	Monotherapy	100%	20 mg/day for 5 years	DFS	2.44 (1.22–4.90)	0.012	1.86 (0.91–3.82)	0.089	
Wegman *et al.*, 2005[48]	76	+Chemotherapy or radiation	not reported	40 mg/day for 2 years	RFS	not reported		<1.0[b]		wt/wt vs wt/*4+*4/*4
Nowell *et al.*, 2005[47]	160	+Chemotherapy or radiation	14.2%	not reported	DFS	not reported		0.67 (0.33–1.35)	0.19	*wt/wt vs wt/*4+*4/*4*
Goetz *et al.*, 2007[42]	180	Monotherapy	100%	20 mg/day for 5 years	RFS	3.20 (1.37–7.55)	0.007	not reported		*wt/wt vs* PM[c]
Wegman *et al.*, 2007[49]	103	not reported	not reported	40 mg/day for 2 years	RFS	not reported		0.87 (0.38–1.97)	0.74	*wt/wt vs wt/*4+*4/*4*
	111	not reported	not reported	40 mg/day for 5 years	RFS	not reported		0.33 (0.08–1.43)	0.14	*wt/wt vs wt/*4+*4/*4*
Schroth *et al.*, 2007[43]	206	Monotherapy	100%	not reported	RFS	not reported		2.24 (1.16–4.33)	0.02	EM *vs* decreased
Newman *et al.*, 2008[55]	115	Monotherapy or +chemotherapy and/or radiation	63.5%	20 mg/day, median duration >4 years	RFS	not reported		1.9 (0.8–4.8)	0.19	*wt/wt+wt/V vs V/V*
Kiyotani *et al.*, 2008[45]	58	Monotherapy	100%	20 mg/day for 5 years	RFS	8.67 (1.06–71.09)	0.044	10.04 (1.17–86.27)	0.036	*wt/wt vs *10/*10*
Xu *et al.*, 2008[46]	152	Monotherapy	100%		DFS	not reported		4.7 (1.1–20.0)	0.04	*100C/C+C/T vs T/T*
Okishiro *et al.*, 2009[53]	173	Monotherapy or +chemotherapy and/or goserelin	42.2%	20 mg/day, median 52 months	RFS	0.94 (0.34–2.60)	0.95	0.60 (0.18–1.92)	0.39	*100C/C+C/T vs T/T*
Schroth *et al.*, 2009[44]	1,325[d]	Monotherapy	100%	for 5 years	RFS	1.49 (1.12–2.00)	0.006	1.40 (1.04–1.90)	0.03	*wt/wt vs hetEM/IM*
						2.12 (1.28–3.50)	0.003	1.90 (1.10–3.28)	0.02	*wt/wt vs PM*
Bijl *et al.*, 2009[54]	85	not reported	not reported	not reported	Breast cancer mortality	not reported		4.1 (1.1–15.9)	0.04	*wt/wt vs *4/*4*
Kiyotani *et al.*, 2010[38]	282[d]	Monotherapy	100%	20 mg/day for 5 years	RFS	not reported		4.44 (1.31–15.00)	0.0170	*wt/wt vs wt/V*
						not reported		9.52 (2.79–32.45)	0.0032	*wt/wt vs V/V*
Ramón *et al.*, 2010[56]	91	Monotherapy or +chemotherapy	39.8%	not reported	DFS	not reported	0.016[e]	not reported	not reported	PM vs others

CI, confidence interval; RFS, recurrence-free survival; DFS, disease-free survival; EM, extensive metabolizer; IM, intermediate metabolizer; PM, poor metabolizer.

[a]Genotype group was reassigned using reported data.

Definition of alleles: *wt*, **1* or **1-*1*; *im*, **10*, **10-*10* or **41*; *pm*, **3*, **4*, **5*, **6*, **14*, **21* or **36-*36*; *V*, *im* or *pm*.

Definition of genotype groups: *wt/wt*, 2 *wt* alleles; EM; *wt/wt* or *wt/im*; hetEM/IM, *wt/im*, *wt/pm*, *im/im* or *im/pm*; PM, 2 *pm* alleles; decreased, *wt/pm*, *im/im*, *im/pm* or *pm/pm*.

[b]Not calculated hazard ratio according to *CYP2D6* genotypes.

[c]Genotype group defined as combination of *CYP2D6*4* and CYP2D6 inhibitors by Goetz *et al.*[42]

[d]These studies included patients reported previously.[41,42,45]

[e]log-lank test p value.

of 486 postmenopausal patients (206 of them received adjuvant tamoxifen).[43] In 2009, Schroth *et al.* subsequently published a retrospective analysis of 1,325 German and North American breast cancer patients who were at an early stage and treated with adjuvant tamoxifen, and observed that PMs revealed a higher risk of recurrence than EMs with HR of 1.90 for a time to recurrence ($p = 0.02$); however, no significant difference in overall survival was observed.[44] In Asians, Kiyotani *et al.* reported that *CYP2D6*10* was significantly associated with shorter RFS in Japanese patients receiving adjuvant tamoxifen monotherapy in 2008 (HR, 10.04; $p = 0.036$), and also confirmed significant association in a follow-up study of 282 Japanese patients receiving adjuvant tamoxifen monotherapy (HR, 9.52; $p = 0.000036$ for RFS).[38,45] The worse clinical outcome of tamoxifen therapy in the patients carrying *CYP2D6*10* was also confirmed in a Chinese population.[46] Although still based on retrospective analyses of tumor samples, the majority of these trials suggest that the presence of one or two variant *CYP2D6* alleles is associated with shorter RFS. However, several studies have reported discordant results. Two large retrospective studies reported an inverse association between *CYP2D6* genotype and breast cancer outcomes.[47,48] Nowell *et al.* reported a trend toward better overall survival with HR of 0.77 in a cohort of adjuvant tamoxifen-treated breast cancer patients with the *CYP2D6*4* genotype.[47] A Swedish trial reported the better outcome for patients with at least one *CYP2D6*4* allele who were treated with 40 mg of adjuvant tamoxifen for 2 years.[48] An independent and larger cohort study by the same group also suggested that women with ER-positive tumors who were homozygous for *CYP2D6*4* revealed no significant difference in DFS compared with those with *CYP2D6*1*.[49]

There may be several reasons for these discrepancies among the studies showing the positive and negative associations. As several reviews have pointed out,[50] considerable heterogeneity in sample collection or analysis among the studies described as follows makes it hard to compare them simply: 1) differences in dosage and duration of tamoxifen treatment, 2) incompleteness of allele determination, especially for *CYP2D6*5* allele, and most importantly 3) selection of study participants. Several reports assessed partly these confounding factors. We reported significant effects of *CYP2D6* genotypes on shorter recurrence-free survival only in patients with the tamoxifen monotherapy ($p = 0.000036$) but not in those with the combination chemotherapy ($p = 0.53$) as previous publications support this notion.[51] In addition, the importance of wide coverage of *CYP2D6* alleles was clearly demonstrated by Schroth *et al.*[52] They reported that by increasing genotyping coverage, HR for RFS and the associated power were increased.

Overall, many reports investigated the association of *CYP2D6* genotype and plasma concentration of endoxifen, and consistently clarified that patients carrying the *CYP2D6* genotype which decreased or impaired CYP2D6 function showed lower plasma levels of endoxifen than those having the homozygous wild-type genotype.[7,8,10,18,38,39] For association with clinical outcome, some, but not all,[47,48,53] of the studies showed worse clinical outcome in breast cancer patients with *CYP2D6* variant alleles who were treated with tamoxifen.[38,39,41–46,54–56] However, two large studies showed no association between the *CYP2D6* genotype and clinical outcome, which has raised concern about the *CYP2D6* genotype as a biomarker to predict tamoxifen efficacy.[57,58]

Genetic Polymorphisms in Other Drug-metabolizing Enzymes and Clinical Outcome of Tamoxifen Therapy

Other CYPs, UGTs and SULTs are involved in the metabolism of tamoxifen. Hence, there is a possibility that genetic variations in these genes may affect the efficacy or toxicity of tamoxifen therapy. The most important CYP isoforms are CYP3A4 and CYP3A5, which are involved in the metabolism of more than 40% of drugs. Several polymorphisms in the *CYP3A4* gene have been reported (http://www.cypalleles.ki.se/cyp3a4.htm), but their contribution may be small due to their low allelic frequencies. In contrast, genetic polymorphisms, particularly a *CYP3A5*3* allele, define much of the variation of CYP3A5 expression.[59] The frequency of the *CYP3A5*3* allele is higher in Caucasians (85–95%) than in Asians (74–77%). Although several studies investigated the association of *CYP3A5*3* with tamoxifen metabolism or clinical outcome of tamoxifen therapy, no significant association was observed.[8,18,41,43,60,61]

In *CYP2C9*, which catalyzes 4-hydroxylation of tamoxifen, more than 30 alleles have been reported (http://www.cypalleles.ki.se/cyp2c9.htm). Among them, *CYP2C9*2* and *CYP2C9*3* have been well investigated. These two alleles are present in approximately 35% of Caucasian individuals, but are much less common in Asian populations.[62] The carriers of *CYP2C9*2* or *CYP2C9*3* showed significantly lower concentrations of endoxifen and 4-hydroxytamoxifen,[18] but no significant association with clinical outcome of tamoxifen therapy was observed.[43]

For the *CYP2C19* gene, *CYP2C19*2* and *CYP2C19*3* are null alleles. *CYP2C19*2* is observed in 10–20% of Caucasians and in more than 20% of Asians. In contrast, *CYP2C19*3* is very rare in Caucasians, but is relatively high at 5–10% in Asian populations. As a result, in Caucasians, the frequency of PMs related to CYP2C19 is 3%, whereas the PM frequency in Asian populations is as high as 23%.[31,63] Recently, a new genetic variant in the promoter region of the *CYP2C19* gene, *CYP2C19*17*, which was associated with increased CYP2C19 activity *in vivo* (UM phenotype), was identified.[64,65] The frequencies of *CYP2C19*17* were reported to be 18–27% in Caucasian populations and 1–4% in Asians.[64,66] Schroth *et al.* found significant association with clinical outcome of tamoxifen treatment in carriers of

*CYP2C19*17*,[43] but not in the carriers of *CYP2C19*2* or *CYP2C19*3*.[43,53] As to tamoxifen metabolism, no significant association was reported in *CYP2C19* polymorphisms.[18]

Several investigations on *SULT1A1*2*, which causes reduced SULT1A1 activity, found no clear association with tamoxifen efficacy[47,49] nor with tamoxifen metabolism.[8,48] Recent reports by Gjerde *et al.* addressed the association of the *SULT1A1* genotype, including copy number variation, with tamoxifen metabolism.[19,61] They clarified that neither *SULT1A1* genotypes nor copy numbers influence the plasma concentration of tamoxifen and its metabolites. However, further analysis which takes into consideration the "allele copy number" of *SULT1A1* is required, as demonstrated in the case of *CYP2D6*.[67–69]

Genetic Polymorphisms in Drug Transporters and Clinical Efficacy of Tamoxifen Therapy

Although the biotransformation of tamoxifen to endoxifen has been well studied and documented as described above, there have been few reports investigating the involvement of drug transporters in the disposition of tamoxifen and its active metabolites, 4-hydroxytamoxifen and endoxifen.

ABCB1 (P-glycoprotein, MDR1) is an ATP-dependent, efflux transporter with broad substrate specificity widely appreciated for its role in mediating cellular resistance to many anticancer agents.[70] A number of investigators have performed clinical studies to reveal the relationship between drug pharmacokinetics and *ABCB1* polymorphisms. A synonymous single nucleotide polymorphism (SNP) 3435C>T was reported to be associated with higher digoxin levels after oral administration.[71] Several groups performed screenings for *ABCB1* polymorphisms.[72–75] These three SNPs, 1236C>T, 2667G>T and 3435C>T, and their haplotypes are considered to be important in the ABCB1 function.

Callaghan and Higging reported that tamoxifen directly bound to ABCB1 and inhibited ABCB1-mediated vinblastine transport, but cellar accumulation of tamoxifen itself was not influenced by ABCB1.[76] As supporting this, it was reported that *N*-desmethyltamoxifen and 4-hydroxytamoxifen as well as tamoxifen were not substrates of this transporter in the transport assay, although 4-hydroxytamoxifen showed some tendency.[77] Recently, two studies found that ABCB1 is involved in the transport of active tamoxifen metabolites, endoxifen and 4-hydroxytamoxifen.[78,79] In both reports, P-glycoprotein knockout mice showed a tendency toward higher serum concentration of endoxifen than wild-type mice although the difference was not statistically significant, suggesting that ABCB1 does not play a major role in regulating the absorption, distribution or excretion of endoxifen. With respect to the association with the clinical outcome of tamoxifen, no single nucleotide

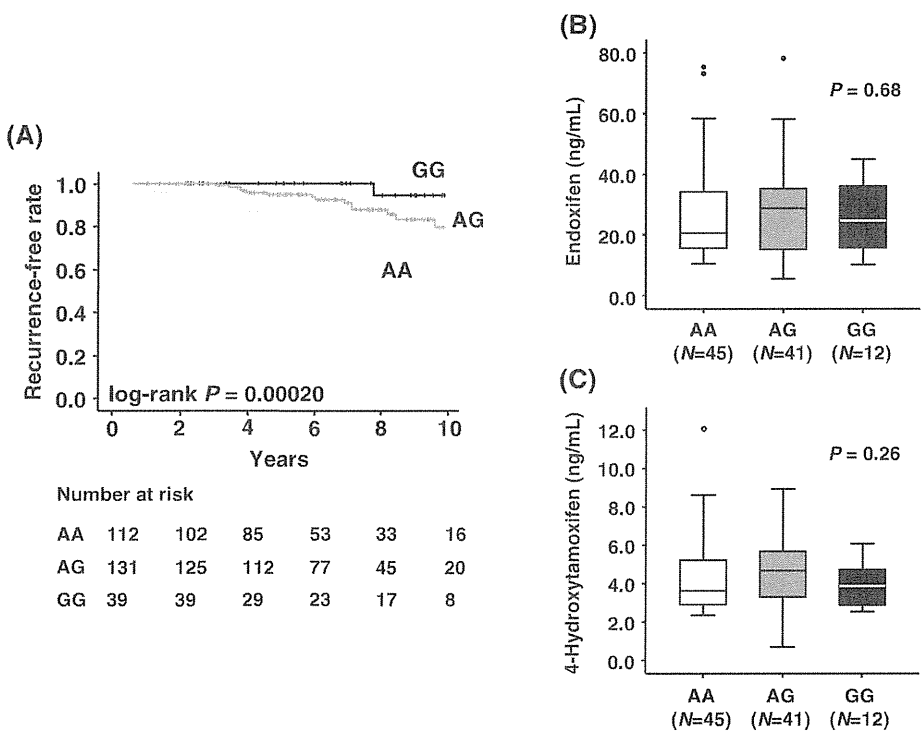

Fig. 2. Kaplan-Meier estimates of recurrent-free survival and steady-state plasma concentrations of endoxifen and 4-hydroxytamoxifen according to *ABCC2* genotype

(A) In 282 patients treated with adjuvant tamoxifen monotherapy, rs3740065 G allele was significantly associated with shorter recurrence-free survival. (B, C) Steady-state plasma concentrations of endoxifen (B) and 4-hydroxytamoxifen (C) were not significantly different among rs3740065 genotype groups.

polymorphism (SNP), which includes the SNPs described above, showed significant association in our recent report.[38]

ABCC2 (MRP2) plays an important role in the biliary excretion of conjugated drugs and xenobiotics, and also in that of some non-conjugated drugs including pravastatin and methotrexate. Tamoxifen and its metabolites are excreted into the biliary tract as glucuronides or sulfates.[16] However, there has been no report investigating the involvement of ABCC2 in the transport of tamoxifen and its active metabolites. Recent SNP screening for the *ABCC2* gene identified several common SNPs such as −1774delG (**1A*), −24C>T (**1C*) and 1249G>A (**2*).[80,81] No functional significance of 1249G>A causing Val417Ile has been shown *in vitro*,[82,83] but its *in vivo* association was reported.[84] −1774delG and −24C>T are associated with reduction of its promoter activity.[82,85] In our recent study, an intronic SNP of *ABCC2* (rs3740065) was found to be significantly associated with the clinical outcome of patients with tamoxifen therapy, whereas this SNP was not associated with plasma concentration of endoxifen or 4-hydroxytamoxifen, suggesting that the contribution of ABCC2 to biliary excretion of tamoxifen and its metabolites might be limited (**Fig. 2**).[38] An *in vitro* study reporting that ABCC2 was expressed at higher levels in tamoxifen-resistant breast cancer cells suggests the possibility that active metabolites of tamoxifen are transported by ABCC2 from breast cancer cells.[86] As described previously,[87,88] rs3740065A/G is in strong linkage disequilibrium ($r^2 = 0.89$) with −1774G/delG, which was reported to be associated with decreased ABCC2 promoter activity. Although we believe that rs3740065 has potential to predict efficacy of tamoxifen treatment, further analyses, including replication study and functional analysis to identify the causative SNP, will be required.

Conclusion

There have been several reports on the association of *CYP2D6* genotype/phenotype and clinical outcome of breast cancer patients receiving tamoxifen therapy in large numbers of subjects. The association results with tamoxifen metabolism are consistent, but still controversial in association with clinical outcome. Investigation of the combination of the *CYP2D6* genotype and other genes, which did not affect tamoxifen pharmacokinetics, may be one of the important approaches to identify the prediction marker(s) for the clinical efficacy of tamoxifen.

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CLINICAL TRIAL

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].

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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

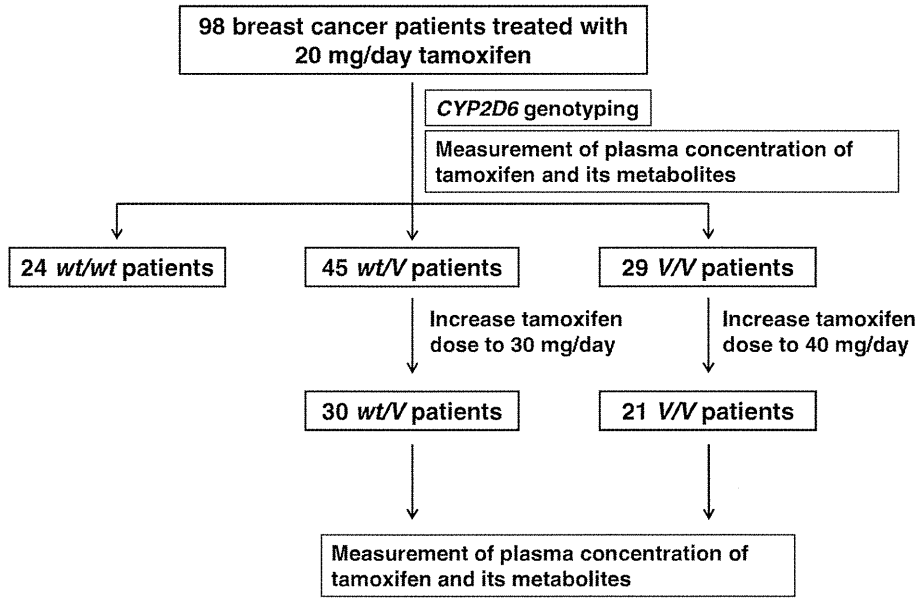

Fig. 1 Consort diagram. *wt*: **1*, *V*: **5*, **10*, **21*, **36*-**36* and **41*

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

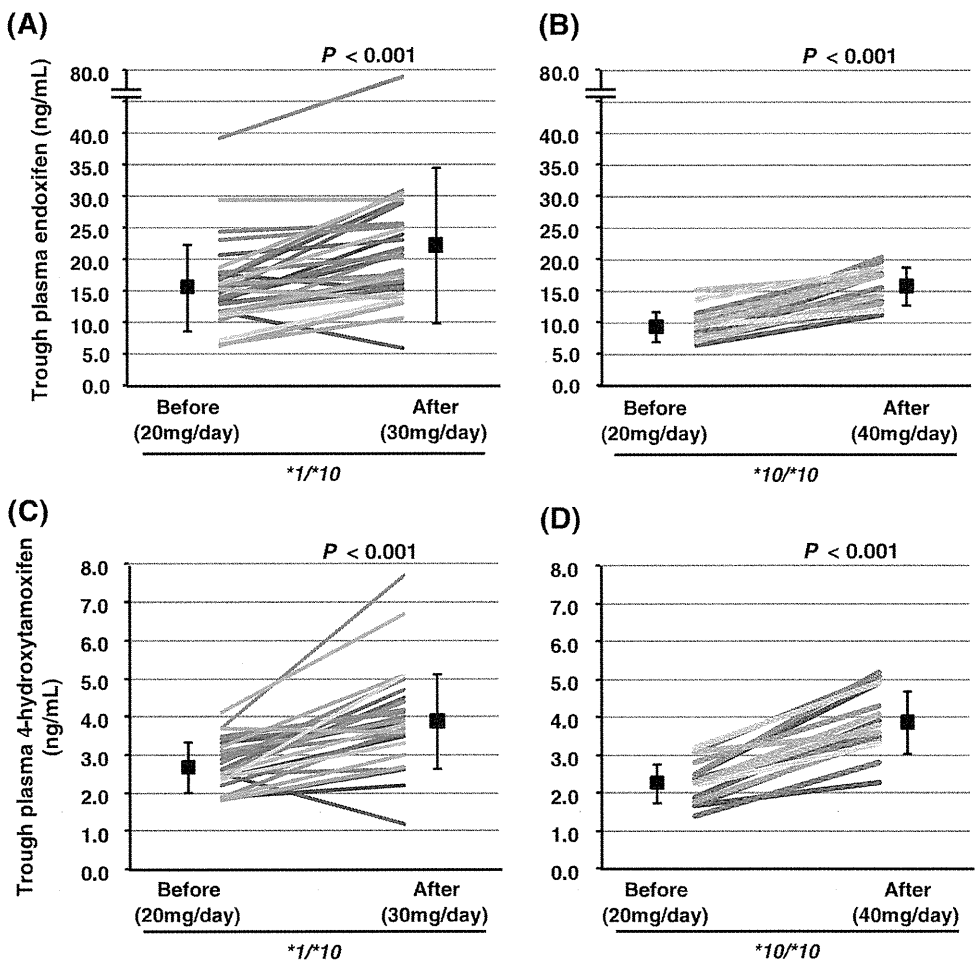

Fig. 2 Steady-state plasma concentrations of endoxifen **a**, **b** and 4-hydroxytamoxifen (**c**, **d**) before and after dose increase of tamoxifen in breast cancer patients. **a**, **c** Patients with *CYP2D6*1/*10* ($N = 28$), **b**, **d** Patients with *CYP2D6*10/*10* ($N = 17$). Data are expressed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