

Significant Effect of Polymorphisms in *CYP2D6* on Response to Tamoxifen Therapy for Breast Cancer: A Prospective Multicenter Study

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Abstract

Purpose: *CYP2D6* is the key enzyme responsible for the generation of the potent active metabolite of tamoxifen, "endoxifen." There are still controversial reports questioning the association between *CYP2D6* genotype and tamoxifen efficacy. Hence, we performed a prospective multicenter study to evaluate the clinical effect of *CYP2D6* genotype on tamoxifen therapy.

Experimental Design: We enrolled 279 patients with hormone receptor-positive and human epidermal growth factor receptor 2-negative, invasive breast cancer receiving preoperative tamoxifen monotherapy for 14 to 28 days. Ki-67 response in breast cancer tissues after tamoxifen therapy was used as a surrogate marker for response to tamoxifen. We prospectively investigated the effects of allelic variants of *CYP2D6* on Ki-67 response, pathological response, and hot flushes.

Results: Ki-67 labeling index in breast cancer tissues significantly decreased after preoperative tamoxifen monotherapy ($P = 0.000000000000013$). Moreover, proportion and Allred scores of estrogen receptor-positive cells in breast cancer tissues were significantly associated with Ki-67 response ($P = 0.0076$ and 0.0023 , respectively). Although *CYP2D6* variants were not associated with pathologic response nor hot flushes, they showed significant association with Ki-67 response after preoperative tamoxifen therapy ($P = 0.018$; between two groups, one with at least one wild-type allele and the other without a wild-type allele).

Conclusions: This is the first prospective study evaluating the relationship between *CYP2D6* variants and Ki-67 response after tamoxifen therapy. Our results suggest that genetic variation in *CYP2D6* is a key predictor for the response to tamoxifen in patients with breast cancer. *Clin Cancer Res*; 23(8); 2019–26. ©2016 AACR.

Introduction

Tamoxifen has been mainly used for the treatment or prevention of recurrence in patients with estrogen receptor (ER)-positive breast cancers. Five-year tamoxifen therapy was reported to improve the risk of its relapse at least for 15 years, particularly for estrogen receptor-positive invasive tumors in premenopausal women (1). However, in the result of the ATLAS trial (Adjuvant Tamoxifen Longer Against the Shorter), the risk of recurrence

during years 5 to 14 was >20% in the patients treated with adjuvant tamoxifen therapy (2). Despite many studies being conducted, the mechanisms underlying the response to this drug in a subset of the patients are not fully identified. 4-Hydroxytamoxifen and endoxifen (4-hydroxy-N-desmethyltamoxifen), which are representative metabolites of tamoxifen, are known to be active therapeutic moieties (3, 4). These two metabolites have 100-fold greater affinity to ER and 30- to 100-fold greater potency

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Note: Supplementary data for this article are available at Clinical Cancer Research Online (<http://clincancerres.aacrjournals.org/>).

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Translational Relevance

Although many investigations on tamoxifen pharmacogenomics have been performed, there are still inconsistencies with regard to the association results between efficacy of tamoxifen and genetic polymorphism of *CYP2D6*, which is the rate-limiting enzyme responsible for the metabolism of tamoxifen to its active metabolite, endoxifen. The data from our first prospective tamoxifen-*CYP2D6* study show that *CYP2D6* variants are significantly associated with Ki-67 response, a surrogate marker for efficacy of tamoxifen, after tamoxifen therapy. Integration of genotypes of *CYP2D6* and the other genes, which are identified as novel predictors for efficacy of tamoxifen, could be the future approach to improve the ability of physicians to select the right drug for the estrogen receptor-positive breast cancer and provide a better quality of life to patients with breast cancer.

in inhibiting estrogen-dependent cell growth compared with a parent compound, tamoxifen (3–5). Hence, it has been considered that the differences in the formation of these active metabolites could affect the interindividual variability in the response to tamoxifen. Cytochrome P450 2D6 (*CYP2D6*) is one of the key enzymes for the generation of the potent active metabolites of tamoxifen, "4-hydroxytamoxifen" and "endoxifen" (6). Many studies indicated that decreased—or null-function—alleles of *CYP2D6* were associated with poor clinical outcome of breast cancer patients treated with tamoxifen (7–12). Genotype-guided dose-adjustment study of tamoxifen provides the evidence that dose adjustment is useful for the patients carrying the reduced or null allele of *CYP2D6* to maintain the effective endoxifen level (13, 14). However, there are several reports claiming the lack of association between *CYP2D6* genotypes and tamoxifen efficacy (15–19), although these studies have been criticized for multiple issues as the cause of false-negative results, including inappropriate patients population, inappropriate DNA sources, and incomplete genotyping analysis (20). Hence, it is critically important to perform prospective studies to clarify the clinical significance of *CYP2D6* genotypes in tamoxifen therapy (21, 22).

Expression levels of Ki-67 protein, a proliferation biomarker, have been known as a predictive marker for the prognosis of cancer patients (23–25). Although clinicopathologic factors such as baseline Ki-67 and tumor size are unlikely to be associated with clinical response to tamoxifen (1, 25), higher Ki-67 expression after short-term (2 weeks) endocrine therapy is suggested to be significantly associated with lower recurrence-free survival in patients with breast cancer (24, 25). Hence, a change in the expression of Ki-67 after short-term tamoxifen therapy could be a promising surrogate biomarker of tamoxifen efficacy (24, 25). Here, we conducted the first prospective association study between Ki-67 response after short-term (14–28 days) preoperative tamoxifen therapy and *CYP2D6* variants in breast cancer patients and evaluated the effect of *CYP2D6* genotypes on tamoxifen therapy.

Materials and Methods

Patients

The primary objective of this study was to examine the association between *CYP2D6* genotypes and clinical response mea-

sured by Ki-67 expression levels in breast cancer tissues in patients who are treated with tamoxifen preoperatively. The secondary objective was to determine the effect of *CYP2D6* genotype on pathologic response and adverse event (hot flushes). According to the previous report (26), Ki-67 labeling index decreased by 59.5% and 76.0% after 2 weeks of preoperative tamoxifen and aromatase inhibitor treatment, respectively. Suppose Ki-67 response after tamoxifen therapy in patients with *CYP2D6 wt/wt* correspond to those after aromatase inhibitor therapy, sample size required in this study is approximately 280 patients under the following conditions; statistical power >80%, significance level $P < 0.05$, standard deviation (δ) = 50. Two hundred seventy nine patients with primary breast cancer were prospectively recruited from July 2012 to July 2014 at Showa University, Nippon Medical School, Tokyo Medical University, Saitama Cancer Center, Hirosaki Municipal Hospital, Sapporo Medical University, Sapporo Breast Surgical Clinic, Nakagami Hospital, Sagara Hospital, Yokohama City University Medical Center, Yokohama Minato Red Cross Hospital, St. Marianna University School of Medicine Hospital, Tan Tock Seng Hospital, and National University Cancer Institute, Singapore. All patients were women who were pathologically diagnosed with ER-positive (>10%), human epidermal growth factor receptor 2 (HER2)-negative, invasive breast cancer without distant spread. ER status was evaluated by immunohistochemistry at each site. HER2 negativity was defined as <2+ immunohistochemical staining or 2+ immunohistochemical staining without gene amplification by FISH test. After the definitive diagnosis of breast cancer, all patients received 20 mg/day of tamoxifen for 14 to 28 days (in the waiting period for radical operation) until the day before the operation for the primary breast cancer.

Core-needle biopsy samples for diagnosis of the primary tumor were obtained before the first dose of tamoxifen, and tumor tissues after tamoxifen treatment were obtained at the time of surgery. Tissue samples were fixed in 10% neutral-buffered formalin for <48 hours before being embedded in paraffin. Serial sections (4 μ m) were cut and immunostained with mouse monoclonal antibody to Ki-67 (clone Mib-1, 1/200 dilution; Dako) for 30 minutes at room temperature with an automatic immunostainer (Autostainer; Dako). Ki-67 labeling index was recorded as the percentage of immunoreactive cells over the total number of invasive neoplastic cells or over at least 2,000 tumor cells in hotspot of each of the invasive carcinoma in the core-needle biopsy and surgical specimen. Automated recognition and counting of the tumor and immunoreactive cells were carried out using the Pathology Decision Support System "e-Pathologist" (NEC Corporation, Tokyo). Pathologic response to tamoxifen was assessed by using a 6-grade scale as follows: grade 0, no response; grade 1a, mild response; grade 1b, mild to moderate response; grade 2a, moderate to marked response; grade 2b, marked to almost complete response; grade 3, almost complete response.

International Union Against Cancer TNM classification was used to determine the tumor and nodal status. This study was approved by the Institutional Review Boards of the National Cancer Center (Tokyo, Japan) and each participating institution. Written informed consent was obtained from all patients.

Genotyping

Genomic DNA was extracted from peripheral blood using a Qiagen DNA extraction kit (Qiagen). Genotyping for key polymorphisms for *CYP2D6**4 (1846G>A), *CYP2D6**6 (1707delT), *CYP2D6**10 (100C>T), *CYP2D6**14 (1758G>A), *CYP2D6**18

(4125_4133dupGTGCCACT), *CYP2D6*21* (2573_2574insC), *CYP2D6*36* (gene conversion to *CYP2D7* in exon 9), and *CYP2D6*41* (2988G>A) was performed using Taqman Drug Metabolism Genotyping Assays (Thermo Fisher Scientific) according to the manufacturer's instructions. Determination of copy number of the *CYP2D6* gene was performed using TaqMan Copy Number Assays (Thermo Fisher Scientific). The whole-gene deletion (*CYP2D6*5*) was detected following reported protocols (27, 28). Multiplication alleles, which consisted of *CYP2D6*10* and *CYP2D6*36* (i.e., *CYP2D6*10*36* and *CYP2D6*10*36*36*), were defined as *CYP2D6*10* because the enzymatic activity of protein encoded by *CYP2D6*36* has been reported to be negligible (29, 30). To evaluate the effects of all *CYP2D6* alleles tested in this study, we defined all decreased and null alleles (*4, *5, *10, *10*10, *14, *10*18, *18, *21, and *41) as allele V, and *1 and *1*1 alleles as allele *wt*.

Statistical analysis

All polymorphisms evaluated in this study were tested for deviation from Hardy–Weinberg equilibrium with the use of a χ^2 test. The differences in the Ki-67 labeling index among *CYP2D6* genotypes were evaluated by the Kruskal–Wallis test. The Mann–Whitney *U* test was used for the evaluation of the differences in the Ki-67 labeling index before and after preoperative tamoxifen therapy, and in change of Ki-67 among the proportion of ER-positive cells. We investigated the association of the *CYP2D6* allele with pathologic response and adverse event using the Fisher exact test under allelic, dominant-inheritance, and recessive inheritance models. Statistical tests provided two-sided *P* values, and a significance level of *P* < 0.05 was used. Statistical analyses were carried out using SPSS (version 17.0; SPSS) and the Ekuseru-Toukei 2015 (Social Survey Research Information Co., Ltd.).

Results

Patient characteristics

To examine the effect of tamoxifen on change in the Ki-67 labeling index in breast cancer tissues, we recruited 279 patients receiving preoperative tamoxifen monotherapy for 14 to 28 days. Table 1 shows the characteristics of these 279 patients who were pathologically diagnosed to have an ER-positive, HER2-negative, invasive breast cancer. Their median age at the time of surgery was 56 years old (range, 25–91 years). Among the characteristics listed in Table 1, the proportion of ER-positive cells and the Allred score of ER, which is a semi-quantitative system that takes into consideration the proportion of positive cells and staining intensity, showed significant association with Ki-67 response after preoperative tamoxifen therapy in the Mann–Whitney *U* test (*P* = 0.0076 and 0.0023, respectively, Supplementary Fig. S1).

Associations of *CYP2D6* genotypes with pathological response and adverse events

We determined *CYP2D6* genotypes of these 279 patients (Table 2). The allele frequency of *CYP2D6*10*, which is considered to have decreased enzymatic activity and is known to be present at a relatively high frequency in Asian populations, was 32.3%. The frequencies of the alleles observed in this study were comparable to those reported previously (28, 30). We defined all of the *CYP2D6* decreased and null alleles as a "V" allele and *1 as a "*wt*" allele as described in "Materials and Methods." We then

Table 1. Patient demographics and clinical characteristics

Characteristic	Total (N = 279) No. of patients (%)
Age at registration, years	
Median	56
Range	25–91
Menopausal status	
Premenopausal	121 (43.4)
Postmenopausal	156 (55.9)
Unknown	2 (0.7)
Tumor size, cm	
≤2	163 (58.4)
>2	108 (38.7)
Unknown	8 (2.9)
Nodal status	
Negative	238 (85.3)
Positive	36 (12.9)
Unknown	5 (1.8)
ER status	
<1%	0 (0)
1%–10%	0 (0)
10%–32%	6 (2.1)
33%–67%	15 (5.4)
>67%	258 (92.5)
Allred score (ER) ^a	
<8	16 (5.8)
8	129 (46.2)
Unknown	134 (48.0)
PR status	
<1%	30 (10.8)
1%–10%	21 (7.5)
11%–32%	34 (12.2)
33%–67%	26 (9.3)
>67%	160 (57.3)
Unknown	8 (2.9)
Her-2	
Negative	89 (31.9)
1+	146 (52.3)
2+ (without amplification)	44 (15.8)

^aComposite of the percentage of cells that stained (scored on a scale of 0–5) and the intensity of their staining (scored on a scale of 0–3).

examined association of *CYP2D6* genotypes with pathologic response and hot flushes in breast cancer patients who received short-term (14–28 days) preoperative tamoxifen treatment (Table 3). We observed no significant association of *CYP2D6* genotypes with pathological response or hot flushes.

Associations between Ki-67 response and tumor response after preoperative tamoxifen therapy

As a primary endpoint of this study, we used Ki-67 response which could be a promising surrogate biomarker of tamoxifen efficacy because duration of the preoperative treatment is very short (14–28 days) for accurate evaluation of tamoxifen efficacy by the tumor size change by an ultrasound test. Ki-67 labeling index was measured by using an automated recognition and counting system as described in Materials and Methods,⁸ and representative Ki-67–stained images in patients with *CYP2D6 wt/wt* and *CYP2D6 V/V* are shown in Fig. 1. Ki-67 labeling index in post-treatment tissues (median, 4.6% (0–83.5%)) was significantly lower than that in baseline tissues (median: 9.9% (0.1–78.9%)) as shown in Fig. 2A (*P* = 0.0000000000000013). The changes in Ki-67 for patients with *wt/wt*, *wt/V*, and *V/V* of *CYP2D6* before and after preoperative tamoxifen treatment are shown in Fig. 2B, respectively. Of the 279 patients enrolled in this study, 224 patients showed a decrease of Ki-67 index, and 55 patients

Table 2. Genotype frequency of *CYP2D6*

<i>CYP2D6</i> genotype	N (%)
*1/*1	96 (34.4)
*1/*5	24 (8.6)
*1/*10	97 (34.8)
*1/*10- ⁺ 10	1 (0.4)
*1/*10- ⁺ 18	1 (0.4)
*1/*14	2 (0.7)
*1/*18	1 (0.4)
*1/*21	1 (0.4)
*1/*41	9 (3.2)
*5/*10	7 (2.5)
*5/*21	1 (0.4)
*41/*41	1 (0.4)
*10/*10	35 (12.5)
*10/*10- ⁺ 10	1 (0.4)
*10/*41	2 (0.7)

showed an increase. We investigated the association between Ki-67 response and tumor response measured by ultrasound. However, we could not observe a significant association between them ($R = -0.12$; Supplementary Fig. S2). We also investigated the association between Ki-67 response and pathological response after pre-operative tamoxifen treatment. Patients without any pathological response (grade 0) showed significantly poorer Ki-67 response compared to those showing \geq grade 1a pathological response ($P = 0.029$; Supplementary Fig. S3).

Associations between *CYP2D6* genotypes and Ki-67 response after pre-operative tamoxifen therapy

We compared the after/before ratio of the Ki-67 index (when it is below 1, the proportion of Ki-67-positive cells is decreased) in

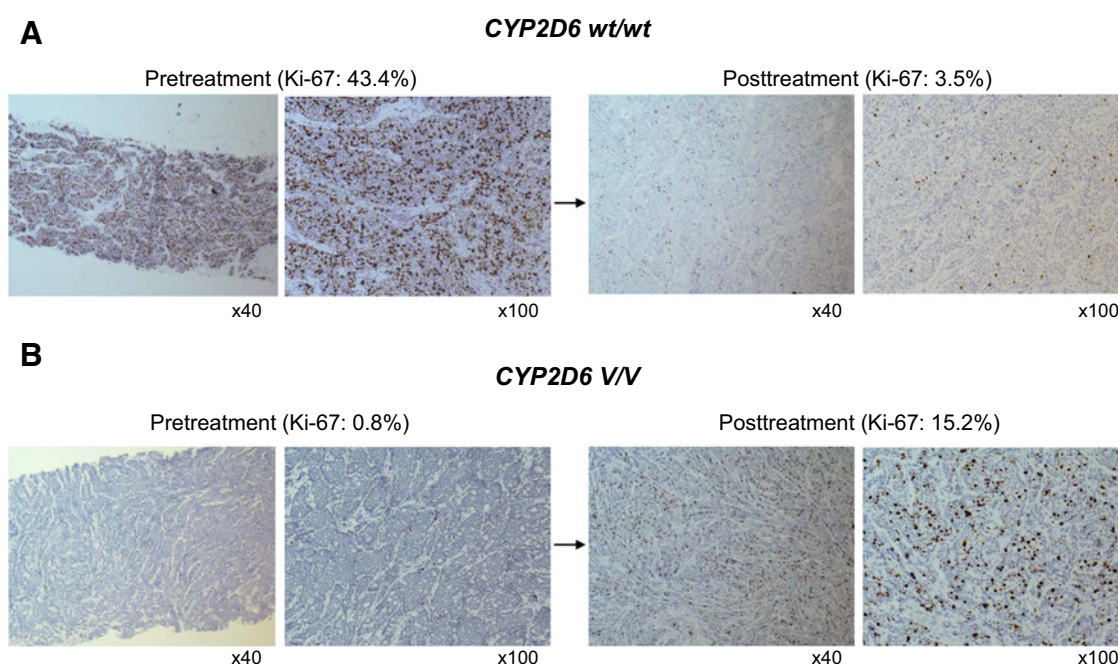
Table 3. Association of *CYP2D6* variants with pathological response and hot flush after short-term tamoxifen therapy

	Pathologic response (+) ^a	Pathologic response (-) ^a	Hot flush (+)	Hot flush (-)
Number of patients				
<i>wt/wt</i>	36 (0.44)	32 (0.33)	12 (0.32)	78 (0.34)
<i>wt/V</i>	36 (0.44)	45 (0.47)	14 (0.38)	117 (0.51)
<i>V/V</i>	10 (0.12)	19 (0.20)	11 (0.30)	36 (0.15)
Fisher test <i>P</i> values				
<i>wt/wt</i> vs	0.170		1.000	
<i>V/V</i> vs	0.220		0.059	
<i>wt</i> vs <i>V</i>	0.080		0.250	
Odds ratio (95 % CI) ^b				
<i>wt/wt</i> vs	1.57 (0.85-2.88)		0.94 (0.45-1.97)	
<i>V/V</i> vs	1.78 (0.77-4.08)		0.44 (0.19-0.96)	

^aPathological response (+), grade 1a or more; pathological response (-), grade 0.

^bOdds ratios and confidence intervals (CI) are calculated using the *CYP2D6* *V/V* and *Wt/V* or *CYP2D6* *V/V* as reference.

two groups, one treated for less than 21 days and the other treated for 21 days or more, but found no significant difference between these two groups ($P = 0.67$, data not shown). To prospectively analyze the effects of *CYP2D6* genotypes on response to tamoxifen, we conducted an association study between *CYP2D6* genotypes and the after/before ratio of the Ki-67 index. Using the Kruskal-Wallis test, *CYP2D6* genotypes were significantly associated with Ki-67 response after tamoxifen treatment (after/before ratio of the Ki-67 index) as shown in Fig. 3A ($P = 0.045$). The patients with homozygous variant alleles (*V/V*) showed a smaller decrease of Ki-67 positivity than those carrying at least one wild-type allele ($P = 0.018$; Fig. 3B), suggesting that tumors in the

**Figure 1.**

Representative Ki-67-stained images in patients with *CYP2D6* *wt/wt* and *CYP2D6* *V/V*. **A**, Posttreatment tissues showed a lower Ki-67 labeling index (3.5%) than pretreatment tissues (43.4%) in patients with *CYP2D6* *wt/wt*. **B**, Posttreatment tissues showed higher Ki-67 labeling index (15.2%) than pretreatment tissues (0.8%) in patients with *CYP2D6* *V/V*.

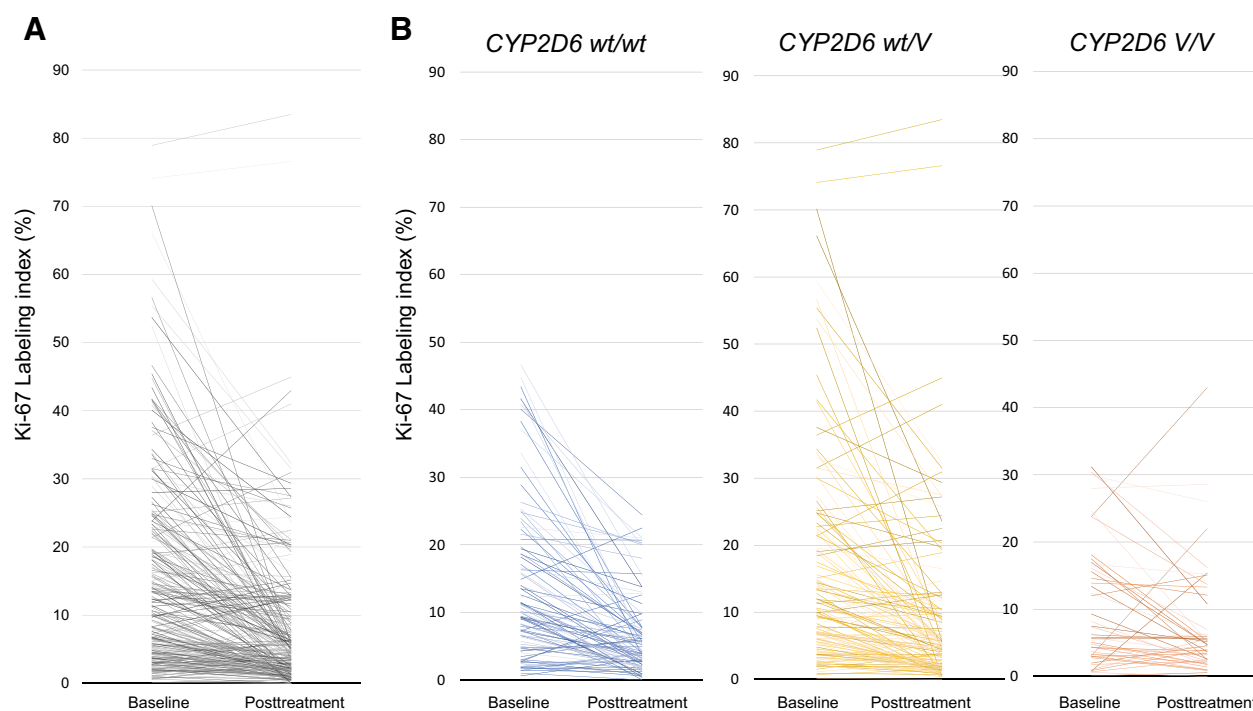


Figure 2.

The change in the Ki-67 labeling index in breast cancer tissues after short-term preoperative tamoxifen therapy. **A**, Of the 279 samples, most of the breast cancer tissues showed significant decrease of Ki-67 labeling index after short-term tamoxifen treatment ($P = 1.3 \times 10^{-15}$). **B**, Ki-67 labeling index of patients with each *CYP2D6* genotype before and after short-term preoperative tamoxifen treatment.

former patients had poorer response to tamoxifen treatment. We further investigated the association of all *CYP2D6* genotypes with Ki-67 and ER in pretreatment tissue, which might be confounding factors, but observed no significant association between them (Supplementary Fig. S4 and Supplementary Table S1).

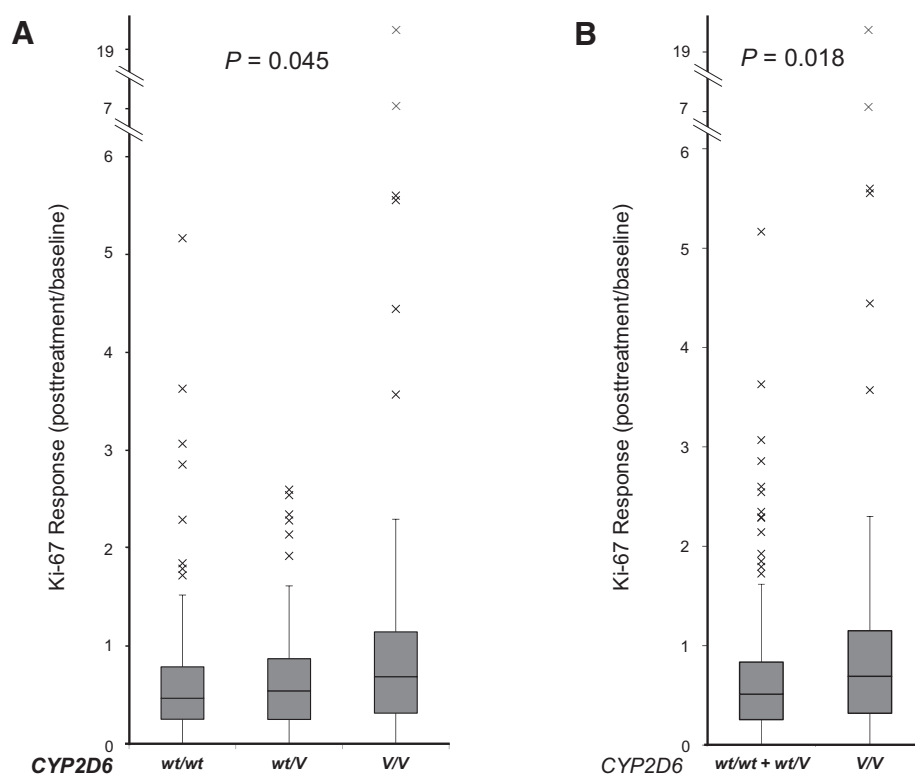
Discussion

Tamoxifen treatment significantly improves survival in patients with ER-positive breast cancer (1, 2, 31, 32). Tamoxifen has revealed inferiority to aromatase inhibitors as an adjuvant therapy for breast cancer (33); however, some reports have indicated that the risk of certain adverse events including osteoporosis is higher in patients receiving aromatase inhibitors than tamoxifen. Hence, tamoxifen keeps being a key therapeutic drug for ER-positive breast cancer. We previously reported that *CYP2D6* variant alleles, which decrease or lose its enzymatic activity, such as *4, *5, *10, *10*10, *14, *21, *36*36, and *41, were significantly associated with clinical outcome of patients with breast cancer receiving adjuvant tamoxifen monotherapy (30). Consistent with this previous report, many studies have reported a significant association between the *CYP2D6* genotypes and clinical outcome of breast cancer patients receiving the tamoxifen therapy in the adjuvant setting (7–12, 30, 34–41). However, discordant results have also been reported (15–19, 42, 43).

Although several critical issues or errors described below could explain these false-negative results (20, 44, 45), it is also quite obvious that the quality of genotyping could be one of the key issues in the pharmacogenomics study. The accuracy of genotyp-

ing methods, coverage of allele (genotype; ref. 46) and source of DNA have been reported to influence the quality of genotype data (20). The studies using low-quality genomic DNA extracted from formalin-fixed paraffin-embedded tumor tissues (in some cases, DNAs were extracted from cancer cells) without genotyping *CYP2D6**5 (deletion of the entire *CYP2D6* gene) are likely to lead to the misgenotyping results (20). Moreover, most of studies showing the null association included the patients who were treated with tamoxifen combined with anticancer drugs. To adapt these essential conditions and prospectively clarify the effect of *CYP2D6* as a pharmacogenomic predictor of tamoxifen efficacy, we genotyped wide coverage of *CYP2D6* alleles using high-quality genomic DNA extracted from blood samples of the patients receiving tamoxifen monotherapy, and obtained the results which could prove the clinical significance of *CYP2D6* genotyping in tamoxifen therapy.

In this study, we observed significant decreases in the Ki-67 labeling index with short-term preoperative tamoxifen treatment. The Ki-67 response was significantly associated with the expression level of ER (Supplementary Fig. S1), which is the established target of tamoxifen and also a predictive marker for the response to tamoxifen (47). Moreover, Ki-67 response was also associated with a pathologic response in breast cancer tissues after tamoxifen treatment (Supplementary Fig. S3). Although Ki-67 response after tamoxifen treatment has not yet been a well-established predictive marker for clinical response to tamoxifen, these lines of evidence support a possibility that Ki-67 response after short-term preoperative tamoxifen treatment could be a useful surrogate marker for clinical efficacy of tamoxifen. To prospectively investigate that

**Figure 3.**

The relationship between *CYP2D6* genotypes and Ki-67 response in 279 patients treated with pre-operative tamoxifen therapy. **A**, *CYP2D6* genotypes were significantly associated with the Ki-67 response in breast cancer tissues with tamoxifen therapy ($P = 0.045$). **B**, The patients with homozygous variant alleles (*V/V*) showed lower Ki-67 response than those carrying at least one wild-type allele (*wt/V* or *wt/wt*; $P = 0.018$).

CYP2D6 genotypes could be a useful marker for prediction of the response to tamoxifen treatment, we carried out the association study of *CYP2D6* genotypes with Ki-67 response after preoperative tamoxifen therapy. Although the association was not as strong as that observed in our previous retrospective studies in which endpoint were recurrence-free survival (30, 37), *CYP2D6* genotypes were significantly associated with the Ki-67 response ($P = 0.045$; Fig. 3A), which is the primary endpoint of this prospective study. In particular, patients with homozygous variant alleles (*V/V*) showed lower Ki-67 response than those carrying at least one wild-type allele (*wt/wt* or *wt/V*; $P = 0.018$; Fig. 3B). The difference of the significance level in the above studies might be caused by the difference in study endpoints (30, 37).

As secondary endpoints of this study, we investigated the association of *CYP2D6* genotypes with pathological response and hot flushes. Although we found no significant association between *CYP2D6* genotypes and pathological response or hot flushes, it is almost certain that our experimental design was not appropriate to evaluate these parameters, probably because the number of patients was too small, and the administration and observation periods were too short. Hence, further analysis using a larger number of patients treated with longer periods of tamoxifen is required for verification of the effect of *CYP2D6* variants on these endpoints.

The pharmacogenomic information is expected to contribute to establishment of the personalized medicine system in which each patient is provided a right amount of a right drug. To reduce the medical cost with maintaining the quality of medical care, it is of special importance to use effective drugs such as tamoxifen at the lower cost on the basis of individual germline and/or somatic genetic information. In this prospective study, we concluded that

the accurate genotyping of *CYP2D6* could become an important predictor for the efficacy of tamoxifen for individual patients with breast cancer. Because novel genetic variants associated with efficacy of tamoxifen have been identified (30, 48), integration of genotypes of *CYP2D6* and other associated genes could be the future approach to improve the ability of physicians to select optimal hormonal therapy for the treatment of ER-positive breast cancer and provide better quality of lives to patients with breast cancer.

Disclosure of Potential Conflicts of Interest

No potential conflicts of interest were disclosed.

Authors' Contributions

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Administrative, technical, or material support (i.e., reporting or organizing data, constructing databases): S. Nakamura, T. Kuwayama, T. Takamaru,

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