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ORIGINAL ARTICLE

Impact of functional *ABCG2* polymorphisms on the adverse effects of gefitinib in Japanese patients with non–small-cell lung cancer

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Abstract
Purpose ABCG2 is a half-size ATP-binding cassette transporter implicated in cellular gefitinib transport. Reportedly, the c.421C > A *ABCG2* gene polymorphism was associated with gefitinib-induced diarrhea in Caucasian patients with non–small-cell lung cancer. Since c.421C > A *ABCG2*, resulting in p.Q141K substitution, is more prevalent in Asian populations, the putative relationship between gefitinib-induced adverse effects and this functional polymorphism was investigated in Japanese patients. c.376C > T, resulting in truncated, non-functional ABCG2, was also investigated.
Methods *ABCG2* gene polymorphisms were evaluated in 75 patients with non–small-cell lung cancer treated with gefitinib 250 mg/day orally, and results were correlated with treatment-related adverse effects.
Results Forty (53.3%) patients harbored c.421A *ABCG2* on at least one allele, while the remaining 35 (46.7%) were wild type for c.421C > A. No significant group difference was observed in frequency of gefitinib-related diarrhea or other adverse effects. In addition, the only one patient homozygous for the c.421A allele in this study was not affected with gefitinib-induced diarrhea or interstitial lung disease. Two patients (2.7%) were found to harbor the c.376T allele heterozygously. One of the two patients harbored both the c.376T and the c.421A genotypes on distinct alleles. Gefitinib-related interstitial lung disease and severe diarrhea were noted in neither of the two patients.
Conclusions In this Japanese population, we did not find an evident association between *ABCG2* polymorphisms, c.376C > T and c.421C > A, and susceptibility to gefitinib-induced adverse effects.

Keywords ABCG2 · Polymorphism · Lung cancer · Gefitinib · Adverse effect

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Introduction

Gefitinib (ZD1839, Iressa) is an orally active, selective epidermal growth factor receptor (EGFR) tyrosine kinase inhibitor that inhibits epidermal growth factor (EGF)-induced EGFR phosphorylation in a broad range of EGFR-expressing cells [1–4]. In two randomized double-blind phase II trials (IDEAL 1 and IDEAL 2), gefitinib was initially identified as an effective treatment for patients with advanced non–small-cell lung cancer (NSCLC) who failed to respond to platinum-based chemotherapy [5, 6]. However, in randomized, double-blind, placebo-controlled phase III trials (INTACT 1, INTACT 2), gefitinib in combination with standard chemotherapeutic agents has failed to demonstrate any added benefit compared to standard chemotherapy alone [7, 8]. Treatment with gefitinib also failed to demonstrate significant improvement in survival of advanced NSCLC patients overall, but it significantly improved survival in patients of Asian origin (ISEL) [9, 10]. Gefitinib was also reported to be non-inferior to docetaxel in improving the survival of previously treated patients with NSCLC (INTEREST) [11]. Presently, gefitinib is considered to be a valid treatment for patients with advanced NSCLC and has been approved for this use in many countries worldwide.

Gefitinib administration has proven safe for the majority of patients with NSCLC. However, occasional toxic side effects, such as diarrhea, skin rash, liver dysfunction, and pulmonary toxicities, have been observed. The former three effects usually resolve with conservative treatment, but acute interstitial pneumonia is often lethal. Of the 704 patients in the INTACT 1 and 2 trials, it was reported that treatment with oral gefitinib 250 mg/day caused diarrhea and interstitial lung disease (ILD) in 303 (43.0%) and 8 (1.1%) patients, respectively [7, 8]. In contrast, diarrhea and ILD were reported in 367 (11.0%) and 193 (5.8%) of 3,322 patients, respectively, who were administered 250 mg/day gefitinib orally in Japan [12]. These population-based differences in response to gefitinib suggested that there may be unknown ethnic factors that may predispose patients to specific adverse effects.

ABCG2 (formerly termed breast cancer resistance protein: BCRP) is a half-size ATP-binding cassette transporter that is normally expressed in a wide variety of organs and tissues, such as placenta, intestine, liver, kidney, brain, and hematopoietic stem cells [13–16]. It localizes to the apical cell membrane, where its substrates are excreted out of cells. Known physiological substrates of ABCG2 include certain chlorophyll metabolites and xenobiotics [17–20], leading to suggestions that ABCG2 plays a protective role against toxic substances and metabolites in the maternal–placental barrier, the digestive tract, and the blood–testis barrier. In addition, ABCG2 mediates concurrent resistance to chemotherapeutic agents such as mitoxantrone and SN-38 (an active metabolite of irinotecan) by pumping them out of the cell and thus reducing intracellular cytotoxic effects [13–15, 21].

Since our first report of naturally occurring variants in the *ABCG2* gene [22], an association between the c.421C > A polymorphism and diarrhea caused by standard chemotherapeutic agents has been reported [23, 24]. An association between the c.421C > A polymorphism and susceptibility to specific diseases has also been reported [25, 26]. This polymorphism in the *ABCG2* gene results in low protein expression levels, as a result of increased protein degradation [22, 27]. Interestingly, previous in vitro studies suggested that gefitinib could also be a substrate for ABCG2 mediated-transport [28–30]. We therefore hypothesized that cellular ABCG2 levels might affect the frequency and severity of gefitinib-induced adverse effects in patients with NSCLC. In fact, Cusatis et al. recently reported that the c.421C > A polymorphism was associated with gefitinib-induced diarrhea in Caucasian patients with NSCLC [31]. Among 124 patients treated with oral gefitinib 250 mg/day, treatment-emergent diarrhea was noted in 7 (44%) of 16 patients heterozygous for *ABCG2* c.421A, but only in 13 (12%) of 108 patients homozygous for the wild-type allele.

Based on the preceding reports that the c.421C > A polymorphism is seen with a higher incidence in the Asian population than in the Caucasian population [22, 32], we investigated the possible relationship between gefitinib-induced adverse effects and two functional *ABCG2* polymorphisms, c.421C > A and loss-of-function c.376C > T, in Japanese patients with NSCLC.

Patients and methods

Patients and treatment

ABCG2 was genotyped in 75 patients with advanced and/or recurrent NSCLC who were treated with gefitinib in either one of two hospitals, Dokkyo Medical University Koshigaya Hospital, Saitama, Japan and Ibaraki Prefectural Central Hospital, Ibaraki, Japan, between July 2002 and March 2008. Full assessment of toxicity and response was available in 75 and 66 patients, respectively. All patients were at least 20 years old, and they or their close relatives agreed to participate in this study with written consent. Clinical information and *ABCG2* genotype were made anonymous, so that they could not be traced back to the patients of origin. This study protocol was approved by the ethical review boards of Dokkyo Medical University Koshigaya Hospital (No. 0706, 0805, and 0806) and Ibaraki Prefectural Central Hospital (No. 126 and 153).

Patients were administered gefitinib orally at a dose of 250 mg once daily until disease progression. Toxicity was assessed based on National Cancer Institute Common Toxicity Criteria Version 3.0. Diagnosis of gefitinib-induced ILD was performed based on review of clinical records, serological markers, and chest x-ray film including CT by expert pulmonary physicians and radiologists. Acute exacerbation of lung cancer was carefully excluded.

DNA extraction and polymerase chain reaction (PCR)-based direct sequencing

Genomic DNA was extracted either from archival formalin-fixed, paraffin-embedded tissue stored in the pathological division of either institute or from peripheral blood nucleated cells as described previously [22]. Exons 4 and 5, which cover the 376th nucleotide and the 421st nucleotide of the *ABCG2* gene, respectively, were first amplified by PCR with a set of appropriate primers, followed by direct sequencing. The primers used for DNA extracted from paraffin-embedded samples were E4S2(p): 5′-CTG CAA GGA AAG ATC CAA GT-3′ and E4AS2(p): 5′-GAA AAT GCA AAC CCA CTA ATA C-3′ for exon 4 (124-bp fragment), and E5S(p): 5′-GAT GTT GTG ATG GGC ACT CT-3′ and E5AS2(p): 5′-GAC CTA ACT CTT GAA TGA CC-3′ for exon 5 (127-bp fragment). Sequence primers were E4S2(p) for exon 4 and E5AS(p): 5′ - CTA ACT CTT GAA TGA CCC TG-3′ for exon 5, respectively. The primers used for DNA extracted from peripheral blood were C376T-S: 5′-CTT ATA GGT TAT TAG ACC CAC A-3′ and C376T-AS: 5′-GCA AAC CCA CTA ATA CTT ACT T-3′ for exon 4 (177-bp fragment), and C421A-S: 5′-CCT TAG TTA TGT TAT CTT TGT G-3′ and C421A -AS: 5′-GAA ACT TCT GAA TCA GAG TCA T-3′ for exon 5 (344-bp fragment). Sequence primers were C376T-Seq1: 5′-TAG GTT ATT AGA CCC ACA ACA-3′ for exon 4 and C421A-Seq1: 5′-CTA AAC AGT CAT GGT CTT AGA AA-3 for exon 5, respectively. A whole exon 4-intron 4-exon 5 (984-bp fragment) was amplified with the primers C376T-S and C421A-AS using DNA extracted from peripheral blood as a template.

Thirty-five cycles of PCR (92°C 30 s, 55°C 30 s, 72°C 30 s) were performed, and then the amplification products were directly sequenced except for a whole exon 4-intron 4-exon 5 as described previously [22]. PCR products of a whole exon 4-intron 4-exon 5 were TA-cloned and the resulting respective clones were separately sequenced.

Statistics

The original purpose of this study was to validate the observation that gefitinib-induced diarrhea occurred more frequently in patients heterozygous for the c.421A allele than in wild-type patients. Sample size was determined based on the previous report using the following parameters; α error, 0.05: power, 80%: gefitinib-related diarrhea in c.421A carrier, 44%: gefitinib-related diarrhea in the c.421C > A wild type, 12% [31]. A ratio of the c.421A carrier versus the wild type was assumed to be 1:1 in a Japanese population [22]. A sample size of more than 70 patients was calculated to be able to detect possible effects of a c.421C > A genotype on gefitinib-induced diarrhea using a Fisher's exact probability test. This calculation was done using G*Power 3 [33]. Clinical data were analyzed according to information available as of May 2009. With the exception of age that was analyzed with Mann–Whitney *U* test, clinical characteristics, treatment outcomes, and toxicities of gefitinib were compared using a Pearson's χ^2 test for independence according to the *ABCG2* genotype, unless an expected value was below 10 in any cell of a 2 × 2 contingency table. Otherwise, a two-sided Fisher's exact probability test was performed. Hardy–Weinberg equilibrium of the *ABCG2* genotype distribution was tested by a χ^2 goodness-of-fit test. A P value < 0.05 was required for statistical significance.

Results

Patients' characteristics relative to the *ABCG2* c.421C > A genotype

ABCG2 genotyping and full assessment of gefitinib-induced adverse effects were performed in 75 NSCLC patients. The CC, CA, and AA genotypes of the c.421C > A polymorphism were present in 35 (46.7%), 39 (52.0%), and 1 (1.3%) patients, respectively (Table 1). Unexpectedly, the observed c.421C > A allele frequency was not consistent with Hardy–Weinberg equilibrium ($P < 0.05$).

Patient clinical characteristics are presented in Table 2 according to the *ABCG2* c.421C > A genotype. No significant differences in the patient characteristics were observed between the wild-type (c.421CC) and the mutant (c.421CA and c.421AA) groups.

Table 1 Two-dimensional graph of the functional *ABCG2* variants

		c.421C > A	CC $n = 35$	CA $n = 39$	AA $n = 1$
c.376C > T					
CC	$n = 73$		34	38	1
CT	$n = 2$		1	1	0
TT	$n = 0$		0	0	0

Table 2 Clinical characteristics according to the *ABCG2* c.421C > A genotype

	Total	CC	CA, AA	*P* value
No. patients (%)	75	35 (46.7%)	40 (53.3%)	
Sex				0.404
Male	36	15	21	
Female	39	20	19	
Median age (range)	62 (36–80)	62 (36–75)	62.5 (38–80)	0.648[a]
Histology				0.742[b]
AD	65	31	34	
SQ and others	10	4	6	
TNM classification				0.168
Stage IA-IIIB	21	13	8	
Stage IV	50	22	28	
Positive smoking history				0.536
Never	40	20	20	
Ever	35	15	20	
WHO performance status				0.188
0	27	16	11	
1–4	44	19	25	
Prior radiotherapy				0.772
No	48	23	25	
Yes	27	12	15	
Prior chemotherapy				1.00[b]
No	8	4	4	
Yes	67	31	36	
Prior surgery				0.852
No	42	20	22	
Yes	33	15	18	

AD adenocarcinoma, *SQ* squamous cell carcinoma

[a] Mann–Whitney *U* test

[b] Two-sided Fisher's exact probability test

Associations between gefitinib-induced adverse effects and the *ABCG2* c.421C > A genotype

Effects of gefitinib were evaluated at least 8 weeks from the start of drug administration. Grade 1–5 ILD occurred in 6 (8.0%) of the 75 patients. One patient died of ILD (grade 5). Grade 1–2 diarrhea occurred in 22 (29.3%) of the 75 patients. Grade 1–3 liver dysfunction and skin toxicity were noted in 13 (17.3%) and 56 (74.7%) patients, respectively. The frequency of diarrhea was lower than that reported in the INTACT 1 and INTACT 2 studies [7, 8] but higher than that in previous reports on Japanese [12] and Caucasian (21 of 129 patients; 16.3%) [31] patients. The frequency of skin toxicity was similar to that previously reported in Caucasians (84 of 134 patients; 62.7%) [31]. The frequency of ILD was similar to that of a previous Japanese report [12]. The present results are summarized in Table 3. No significant differences in the frequencies of gefitinib-induced adverse effects were observed between the c.421C > A wild-type and mutant groups. The only one patient homozygous for the c.421A allele in the present study was not affected with either gefitinib-induced ILD or diarrhea. Partial response and/or complete response were noted in 14 of 32 (43.8%) c.421CC patients, while they were observed in 16 of 34 (47.1%) c.421CA, c.421AA patients. No significant difference was noted in responses to gefitinib (Table 3) and overall survival distribution (data not shown) between the two groups.

Table 3 Effects of gefitinib according to the *ABCG2* c.421C > A genotype

Effect	Total	CC	CA, AA	*P* value
Interstitial pneumonia				0.409[a]
Grade 0	69	31	38	
Grade 1–5	6	4	2	
Diarrhea				0.709
Grade 0	53	24	29	
Grade 1–4	22	11	11	
Skin toxicity				0.791[a]
Grade 0	19	8	11	
Grade 1–4	56	27	29	
Liver dysfunction				0.557[a]
Grade 0	62	30	32	
Grade 1–4	13	5	8	
Antitumor effect				0.787
PD, SD	36	18	18	
PR, CR	30	14	16	

PD progressive disease, *SD* stable disease, *PR* partial response, *CR* complete response

[a] Two-sided Fisher's exact probability test was performed

Patients' characteristics and gefitinib-induced adverse effects relative to the *ABCG2* c.376C > T genotype

The CC, CT, and TT genotypes of the c.376C > T polymorphism were present in 73 (97.3%), 2 (2.7%), and 0 (0.0%) patients, respectively (Table 1). One patient harbored both the c.376T and c.421A genotypes on distinct alleles as demonstrated by PCR-TA cloning-sequencing of the entire exon 4-intron 4-exon 5 genomic DNA. Gefitinib-related ILD and severe diarrhea (>grade 1) were noted in neither of the two patients harboring the c.376T allele. Clinical characteristics and effects of gefitinib are summarized in Table 4.

Discussion

Since our first report of the *ABCG2* functional polymorphisms, c.376C > T and c.421C > A [22], it has been suggested that the c.421C > A variant may significantly

Table 4 Clinical characteristics of the two patients harboring the c.376T allele heterozygously and effects of gefitinib

c.376T patient	Case 1	Case 2
Age	53	66
Sex	Female	Male
c.421C > A genotype	CC	CA
Histology	AD	AD
TNM classification	IV	IIIB
Positive smoking history	0	0
WHO performance status	0	0
Prior radiotherapy	No	No
Prior chemotherapy	Yes	Yes
Prior surgery	Yes	No
Interstitial pneumonia	Grade 0	Grade 0
Diarrhea	Grade 1	Grade 1
Skin toxicity	Grade 2	Grade 1
Liver dysfunction	Grade 0	Grade 0
Antitumor effect	CR	Not available

AD adenocarcinoma, *CR* complete response

impact the effects/toxicities of chemotherapeutic reagents and risk of disease [23–26]. c.376T, which results in a p.Q126Stop variant, has the most striking effect on ABCG2 function due to the resultant truncated ABCG2 that completely lacks transmembrane substrate-efflux domain. c.376T comprises approximately 1.2–1.7% of the allelic frequency in Japanese [22, 34], and this variant has also been reported in the Korean population [35]. Approximately, 20,000 homozygotes for the c.376T allele have been estimated in Japanese, but they have not yet been found to date. We had investigated expression of the c.376T variant mRNA in one healthy volunteer included in our previous study, who harbored both the c.376T and the c.421A genotype on distinct alleles [22]. *ABCG2* mRNA was amplified by reverse transcription-PCR and subcloned into TA cloning vectors. Twenty independent clones were sequenced, and it was found that six clones were the c.376T/c.421C cDNA and 14 were the c.376C/c.421A cDNA (unpublished data). The data demonstrated that the c.376T *ABCG2* was expressed in humans in vivo. It was later reported that the p.Q126Stop variant of ABCG2 expressed in Sf9 cells could not be detected by immunoblotting in plasma membrane vesicles [36]. In contrast, the c.421C > A polymorphism results in a p.Q141K variant ABCG2, of which expression level is 3- to 5-fold lower than that of the wild type in spite of similar mRNA levels [22]. Reportedly, degradation of 141K ABCG2 is enhanced via the ubiquitin–proteasome pathway, while the wild-type ABCG2 is degraded by the endosome–lysosome pathway [27]. The c.421A allele frequency is very high among Asian people, comprising approximately 25–35% of healthy populations, in contrast to about 10% prevalence in healthy Caucasians [24, 32]. Speculation that 5–10% of the Asian population would be homozygous for the c.421A allele led us to hypothesize that these individuals may be especially vulnerable to adverse effects of ABCG2-substrate anticancer agents.

The association of *ABCG2* genotype with adverse effects of some ABCG2-substrate drugs has been reported [23, 24]. The c.421C > A genotype significantly affected the pharmacokinetics of diflomotecan in white cancer patients; in five patients heterozygous for the c.421A allele, plasma mean levels after intravenous drug administration were threefold higher than those in 15 patients with the wild-type alleles [23]. In Korean patients with diffuse large B-cell lymphoma, chemotherapy (R-CHOP)-emergent diarrhea was observed more frequently in patients harboring either c.421CA or c.421AA than in patients harboring c.421CC with statistical significance. As far as we know, only one English literature of the clinical study reports the impact of the *ABCG2* variant on gefitinib-induced adverse effects [31].

Gefitinib is a relatively safe drug, but adverse effects such as diarrhea and/or skin toxicity sometimes occur. Gefitinib-induced pulmonary toxicities are particularly fatal, approximately one-third of patients that experience the complication of acute interstitial pneumonia die. Gefitinib-related ILD has been reported to occur more frequently in Japanese population than in the Caucasian population. In addition, an association between the *ABCG2* c.421C > A gene variant and the occurrence of gefitinib-induced diarrhea in Caucasian patients with NSCLC has been reported [31]. Therefore, we hypothesized that this polymorphism might be a determining factor in gefitinib-induced pulmonary toxicities as well as in drug-induced diarrhea.

However, no significant association was found between the mutant *ABCG2* alleles and any of the gefitinib-induced toxicities. ILD occurred in six of the 75 patients with NSCLC, but no statistically significant difference was found between the patients with c.421CC genotype and those with c.421CA or c.421AA genotype (4 of 35 vs. 2 of 40; $P = 0.409$). A patient homozygous for the c.421A allele was not affected with gefitinib-induced ILD. Diarrhea was observed in 22 (29.3%) of 75 patients, which was comparable to the previous reports. Unlike the report by Cusatis et al., an association between c.421C > A and diarrhea was not detected in the current study population (c.421CC, 11 of 35 vs. c.421CA or c.421AA, 11 of 40; $P = 0.709$). The reason for this discrepancy remains to be elucidated. Possible explanations would be unknown ethnic factors and/or treatment protocol variation that might have affected outcomes and the fact that the c.421A allele frequency is higher in Asians than in Caucasians. Although

only 16 of 124 patients were heterozygous for c.421A in the previous study, it would be possible that investigation of the larger patient population might have resulted in a different conclusion. Another potential explanation would be the possibility that ABCG2 contributes less to the overall elimination of gefitinib in humans in vivo than has been anticipated based on in vitro experiments in *ABCG2*-transfected cell lines. In humans, the ABCG2 function may not represent a major determinant factor of gefitinib elimination, so that loss of the function in a c.421A carrier results in minor change in the drug's pharmacokinetics. Alternatively, the existence of other non-ABCG2 elimination pathways may be able to compensate for the reduced ABCG2 activity in carriers of the c.421C > A polymorphism. In this point of view, enforced expression of ABCB1 confers weak gefitinib resistance to A431 cells [28]. Gefitinib has also been shown to interact with not only ABCG2 but also ABCB1 in the knockout mouse models [37]. Therefore, genotyping of *ABCB1* must be considered to understand the discrepancy between the previous report and the present results. The c.3435C > T variant, a common synonymous C to T transition at nucleotide position 3435 in exon 26 of *ABCB1*, has been associated with reduced mRNA expression and stability, and therefore may result in a reduced ability to transport gefitinib [38, 39]. However, the *ABCB1* c.3435C > T genotype was not fount to be associated with gefitinib-induced diarrhea or skin toxicity in the previous study by Cusatis et al. [31]. Involvement of some yet to be identified transporters or enzymes in the elimination of gefitinib in humans cannot be also excluded.

Another explanation would be that sufficient ABCG2 transport activity may be maintained in subjects of all genotypes. Although the c.421C > A polymorphism influences ABCG2 expression levels due to increased instability, some studies have not observed a correlation between the *ABCG2* genotype and ABCG2 protein/mRNA expressions in human tissue, including the intestine or heart [40, 41].

In addition, intrinsic gefitinib sensitivity may also affect the outcome. We observed that IC_{50} values (drug doses causing 50% inhibition of cell growth) of colon-derived cells, DLD-1 and HT-29, were >1 μM (data not shown). Since the maximum plasma concentration of gefitinib in cancer patients has been reported to be <1 μM after oral administration of clinical doses, tissue or cells of which IC_{50}s are more than 1 μM would not be sensitive to gefitinib toxicity [42]. We speculate that some of colon epithelial cells may not be subjects of strong EGFR-signaling and that ABCG2-mediated transport of gefitinib out of cells might not have affected its toxicity to colon epithelial cells.

The c.421C > A allele frequency was not consistent with Hardy–Weinberg equilibrium in the present study. c.421CC, c.421CA, and c.421AA were found in 35, 39, and 1 patients, respectively. The allele frequency for c.421A was 0.273. In our previous study in which 124 healthy Japanese volunteers were analyzed, we found c.421CC, c.421CA, and c.421AA in 67, 48, and 9 individuals, respectively, with the allele frequency for c.421A being 0.266. Although the c.421A allele frequency was similar between the two studies, the distribution was quite different. We have to say that the reason cannot be explained well at present. One possible explanation would be that low levels of ABCG2 expression in cells homozygous for the c.421A allele might be disadvantageous for malignant transformation and later survival in the lung. It has been reported that the *ABCG2* c.421C > A genotype was associated with increased risk of certain malignancies, such as diffuse large B-cell lymphoma in Chinese individuals and non-papillary type renal cell carcinoma in Japanese individuals, although the underlying mechanisms of these associations are unknown [25, 26]. We can only speculate that the c.421C > A genotype resulting in decrease of ABCG2 function might be related to morbidity rate of specific disease. Given that 421A allele frequency is 0.27 in healthy population, c.421CC and c.421CA: c.421AA is 0.927: 0.073. If c.421AA was specifically less frequent in NSCLC, c.421AA genotype frequency of 0.016 would be calculated to result in significant Hardy–Weinberg disequilibrium. In order to detect the significantly less frequent c.421AA with the following parameters, α error: 0.05 and power: 80%, a total of 460 subjects (230 controls and 230 NSCLC patients) were calculated to be necessary using G*Power 3. In the study by Cusatis et al., c.421CC: c.421CA: c.421AA was 143: 23: 1 in the enrolled patients. The allele frequency of c.421A was 0.075, and the putative distribution was calculated to be 0.856: 0.138: 0.006 under Hardy–Weinberg equilibrium. Accordingly, c.421AA genotype would be extremely rare in Caucasian populations, making it difficult to detect a significant decrease in the c.421AA genotype in at most 170 cancer patients. Although our 40 study subjects for c.421A were all heterozygous except for one, we think that the sample size in our present study would have power to detect the association between the c.421C > A variant and gefitinib-induced diarrhea based on the study by Cusatis et al. in which comparison was performed between c.421CC patients and c.421CA patients, no c.421AA patient included.

Gefitinib-induced ILD was observed in neither of the two patients harboring the c.376T allele. One patient who harbored both the c.376T and c.421A genotype on distinct alleles was affected with mild diarrhea (grade 1) but not with ILD (Table 4). Although very small in number, the present analysis could not support the hypothesis that loss-of-function of ABCG2 might be associated with ILD.

In conclusion, the *ABCG2* functional polymorphisms were found not to be associated with gefitinib-induced adverse effects in Japanese patients with NSCLC. It appears that gefitinib-induced side effects might be determined not only by the *ABCG2* genotype and/or expression levels but also by other unknown factors.

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