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GENETIC POLYMORPHISMS OF THE ADENOSINE TRIPHOSPHATE-BINDING CASSETTE TRANSPORTERS (ABCG2, ABCB1) AND GEFITINIB TOXICITY

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ABSTRACT

The purpose of this study is to investigate associations between allelic variations of *ABCG2* and *ABCB1* with skin toxicity, diarrhea, liver injury and interstitial lung disease (ILD) in gefitinib-treated patients. A prospective clinical study of 83 Japanese patients with non–small–cell lung cancer was performed. Polymorphic loci in *ABCG2* and *ABCB1* were genotyped, and their effects on gefitinib toxicities were evaluated. *ABCG2* 34G>A was statistically associated with occurrence of skin rash; 13 (42%) of the 32 patients with at least one variant *ABCG2* 34G>A allele (G/A and A/A) developed grade 2 or worse skin rash, whereas only 10 (19%) of 51 patients homozygous for the reference allele (G/G) for the wild-type sequence for both alleles did so ($P=0.046$). There was no significant association between severe toxicities and polymorphisms of *ABCG2* 421C>A nor *ABCB1* 3435C>T. The results suggested that *ABCG2* 34G>A would be useful for predicting grade 2 or worse skin rash.

Key Words: ABCG2, ABCB1, Genetic polymorphisms, Gefitinib

INTRODUCTION

Gefitinib (Iressa), an inhibitor of the epidermal growth factor receptor (EGFR) tyrosine kinase, has activity in patients with inoperable or recurrent non-small-cell lung cancer.[1,2] EGFR-TKIs inhibit the intracellular tyrosine kinase domain of the EGFR and therefore block the signal transduction pathways implicated in the proliferation and survival of cancer cells.[3,4] Recent clinical studies have demonstrated that gefitinib has consistent clinical benefit in patients with the activating mutations of the *EGFR* genes.[5,6]

Skin rash and diarrhea are prominent adverse events of gefitinib treatment, which occurred in more than half patients treated with gefitinib.[1,2] Liver injury (elevated levels of transaminases) is also a common toxicity, which is usually mild and tolerable. However, when grade 2 or especially more than grade 3 toxicities occur, the treatments might be discontinued. The etiology of these

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toxicities is unknown, but it might be caused by inhibition of EGFR signaling. In this regard, Li *et al.* reported a strong association between gefitinib steady-state plasma concentrations and the severity of diarrhea, suggesting that the high concentration of gefitinib accumulation in cells resulted in disorders of skin, intestinal mucosa and liver cells.[7] Interstitial lung disease (ILD) is relatively common in Japanese patients, and more so in older, smoking patients with preexisting ILD or poor performance status. ILD has high mortality in Japanese patients.[8] Therefore, evaluation of the risk of developing ILD and other moderate or severe toxicities is important for treatment with gefitinib.

The ATP-binding cassette (ABC) transporters are a superfamily of transmembrane proteins that transport a wide variety of substrates including anticancer drugs across extracellular and intracellular membranes.[9] In the human genome, 48 different ABC transporters have been identified and divided into seven subfamilies (A–G) based on sequence similarities. Many ABC transporter genes are associated with chemotherapeutic drug efflux. Among them, ABCG2 (BCRP: breast cancer resistance protein/MRP: mitoxantrone resistance protein) and ABCB1 (P-glycoprotein/MDR1: multidrug resistance protein 1) are demonstrated to be involved in transporting gefitinib[10].

Previous studies have shown that several naturally occurring variants in the *ABCG2* gene may affect the expression and/or function of its encoded protein. Among these variants, two major functional variants, *ABCG2* 34G>A (rs2231137), resulting in a Val12Met substitution and *ABCG2* 421C>A (rs2231142), resulting in a Glu141Lys substitution were well studied and shown to be related to the adverse effect of many drugs that were transported by ABCG2.[11,12] The *ABCG2* C421A allele has been associated with low levels of *ABCG2* expression and altered sensitivity to the anticancer drugs *in vitro*, compared with the wild type.[11,12] *In vitro* study using HEK293 human embryonic kidney cells transfected with wild-type and mutant *ABCG2* 421C>A demonstrated that HEK293 cells transfected with this variant demonstrate reduced transport of gefitinib, and the presence of the variant has been associated with greater gefitinib plasma accumulation at steady state in patients receiving gefitinib therapy.[10] And it has also been reported that the *ABCG2* G34A allele, resulting in a Val12Met substitution, causes the apical plasma membrane dislocalization of ABCG2 and produces a protein with significantly reduced ability to transport several drugs.[11,13]

The most extensively studied *ABCB1* variant is a common synonymous C to T transition at nucleotide position 3435 in exon 26 (3435C>T) (rs1045642).[14] Although this transition does not change its encoded amino acid, this variant (TT group) has been significantly associated with reducing mRNA expression[15] and stability,[16] and may have a reduced ability to transport drugs.

Cusatis *et al.* investigated associations between allelic variants of *ABCG2*, *ABCB1* and *EGFR* with diarrhea and skin toxicity in gefitinib-treated patients.[17] *ABCG2* 421C>A polymorphism was statistically significantly associated with the occurrence of diarrhea; seven (44%) of the 16 patients with at least one variant *ABCG2* 421C>A allele developed grade 1 or 2 diarrhea, against only 13 (12%) of 108 patients carrying the wild-type sequence for both alleles ($P = .0046$).

The frequencies of *ABCG2* or *ABCB1* variants are different among ethnic populations, which might influence clinical significance.

These previous reports led us to hypothesize that patients receiving gefitinib who have a variant of *ABCG2* and/or *ABCB1* may be especially vulnerable to toxicities of gefitinib. In the present study, we evaluated the association between three SNPs, *ABCG2* 421C>A, 34G>A and *ABCB1* 3435C>T polymorphisms and moderate or severe toxicities of gefitinib treatment in Japanese lung cancer patients.

PATIENTS AND METHODS

Study population characteristics

The study population was comprised of 83 Japanese patients with non-small cell lung cancer at Nagoya University Hospital, Aichi Cancer Center Aichi Hospital, Nagoya Ekisaikai Hospital and Aichi Cancer Center Hospital and Research Institute from August 2002 to May 2008. Patients received treatment with oral gefitinib at a dose of 250 mg once daily on a compassionate use basis until disease progression or toxicity. We defined "moderate/severe toxicity" in this research as liver injury of grade 2 or worse and/or diarrhea of grade 2 or worse and/or skin rash of grade 2 or worse and/or interstitial lung disease (ILD) of grade 1 or worse, classified in accordance with Common Terminology Criteria for Adverse Events (CTCAE v4.0) May 28, 2009 (JCOG/JSCO) (CTCAE v4.0 – JCOG). We paid close attention to adverse events (skin rash, diarrhea and liver injury) of grade 2 or worse and ILD of grade 1 or worse for which treatment may be difficult to continue. Diarrhea and skin toxicity are prominent gefitinib related adverse events that potentially limit its use. The frequency of liver injury in this study was higher than ever reported. We investigated the associations between allelic variants of ABCG2 and ABCB1 and moderate or severe toxicities of diarrhea, skin toxicity, liver injury and interstitial lung disease (ILD) in gefitinib-treated patients.

We reviewed the clinical records including patient characteristics (age, gender, primary disease, previous treatments, histological classification, American Joint Committee on Cancer (AJCC) system staging, Eastern Cooperative Oncology Group performance status and major complications) (Table 1). This study was reviewed and approved by each institutional review board (IRB) of each hospital, and signed informed consent was obtained from all patients.

Genotyping

Genomic DNA was prepared from whole blood (100–200 μl) using the QIAamp Blood Kit (QIAGEN GmbH, Hilden, Germany). The polymorphisms, *ABCG2* 421C > A and *ABCB1* 3435C

Table 1 Patient characteristics

Variable	Value
Total No. patients enrolled	83
Median age	65 y (36-86)
Sex (male/female)	35 (42%)/48 (58%)
Histological classification	
Adenocarcinoma	77 (92.8%)
Squamous cellcarcinoma	2 (2.4%)
Large-cell carcinoma	4 (4.8%)
TNM classification	
IA	2 (2.4%)
IB	1 (1.2%)
IIIA	2 (2.4%)
IIIB	15 (18%)
IV	43 (52%)
post-ope	20 (24%)

> T were genotyped by the TaqMan assay (Applied Biosystems). Genotyping was analyzed using allele discrimination plots using the SDS Software version 1.4.1 of Applied Biosystems. We sequenced *ABCG2* 34G>A after performing a single polymerase chain reaction (PCR) amplification reaction using multiplex primer sets.

the primer Sequence (5'–3') of *ABCG2* 34G>A (V12M)

S: TGCAGAAAGATAAAAACTCTCCA

AS: CTCAACTGGTTTTCGACAAGG

The amplification reaction mixture (20 μl) contained 100 ng DNA in 0.2 mmol/L each of deoxynucleoside triphosphate, 50 mmol/L KCl, 10 mmol/L Tris- HCl (pH 8.3), 1.5 mmol/L $MgCl_2$, 1.6 μmol/L of each primer, and 1.3 units of *Taq* polymerase (Takara Shuzo, Otsu, Japan). PCR was performed using a GeneAmp PCR 9700 (Applied Biosystems, Foster City, CA, USA) with an initial denaturation step of 95°C for 4 min, followed by 40 cycles of denaturation at 95°C for 30 s, annealing at 58°C for 30 s and extension at 72°C for 30 s. Cycle sequencing was performed with a dye-terminator sequence reaction (ABI Prism DNA Sequencing Kit, Perkin-Elmer, Foster City, CA) using an ABI PRISM 310 Genetic Analyzer.

Statistical analysis

Fisher's exact tests were used to analyze the association between *ABCG2* or *ABCB1* genetic polymorphisms and toxicities. A difference was considered statistically significant when the two-tailed P value was under 0.05.

RESULTS

Gefitinib toxicities

We collected clinical information from all 83 patients (Table 1). After treatment of at least 2 months or more, we evaluated 83 patients for skin rash, diarrhea, liver injury and interstitial lung disease (ILD). Moderate or severe skin rash (grade 2 or worse) was experienced in 23 patients (27.7%). Diarrhea of grade 2 or worse was experienced in 4 patients (4.8%). Moderate or severe liver injury (grade 2 or worse) was experienced in 15 patients (18%). Interstitial lung disease (ILD) of grade 1 or worse was experienced in 5 patients (6%).

Genotyping of ABCG2 34G>A, ABCG2 421C>A and ABCB1 3435C>T

G/G allele (G/G) were found in 51 patients (61%), G/A alleles in 28 (34%), and A/A alleles in 4 (5%) for *ABCG2* 34G>A. C/C alleles were found in 45 (54%) patients, C/A alleles in 31 (38%), and A/A allele in 7 (8%) for *ABCG2* 421C>A. C/C alleles were found in 23 patients (28%), C/T alleles in 44 (53%), and T/T alleles in 16 (19%) for *ABCB1* 3435C>T. The frequencies of the *ABCG2* or *ABCB1* variants alleles were summarized in Table 2.

Table 2 Genotypes

ABCG2 34G>A	GG	GA	AA
G : A = 0.78 : 0.22	51 (61%)	28 (34%)	4 (5%)
ABCG2 421C>A	CC	CA	AA
C : A = 0.71 : 0.29	45 (54%)	31 (38%)	7 (8%)
ABCB1 3435C>T	CC	CT	TT
C : T = 0.54 : 0.46	23 (28%)	44 (53%)	16 (19%)

Association SNP of ABC transporter and gefitinib toxicity

Fisher's exact tests were performed to find any significant association between the occurrence of moderate or severe toxicities and SNPs at *ABCG2* 34G>A, *ABCG2* 421C>A and *ABCB1* 3435C>T (Table 3–5). Statistically significant associations were found between the occurrence of skin toxicity and *ABCG2* polymorphisms, 34G>A; 13 (42%) of the 32 patients with at least one variant *ABCG2* 34G>A allele (G/A and A/A) developed skin rash, against only 10 (19%) of 51 patients homozygous for the reference allele (G/G) for the wild-type sequence for both alleles did ($P=0.046$) (Table 3). Other polymorphisms were not associated with skin toxicity, liver injury, diarrhea and interstitial lung disease (ILD) (Table 3–5).

Table 3 Association between genetic polymorphisms *ABCG2* 34G>A and toxicity

	GG	GA+AA	*P value*
Skin rash			
Grade 2≦ (N=23)	10	13	P=0.046
Grade 1≧ (N=60)	41	19	
Diarrhea			
Grade 2≦ (N=4)	2	2	P=0.638
Grade 1≧ (N=79)	49	30	
Liver injury			
Grade 2≦ (N=15)	10	5	P=0.773
Grade 1≧ (N=68)	41	27	
Interstitial lung disease (ILD)			
Grade 1≦ (N=5)	4	1	P=0.644
Grade 0 (N=78)	47	31	

Table 4 Association between genetic polymorphisms *ABCG2* 421C>A and toxicity

	CC	CA+AA	*P value*
Skin rash			
Grade 2≦ (N=23)	14	9	P=0.473
Grade 1≧ (N=60)	31	29	
Diarrhea			
Grade 2≦ (N=4)	3	1	P=0.621
Grade 1≧ (N=79)	42	37	
Liver injury			
Grade 2≦ (N=15)	8	7	P=1
Grade 1≧ (N=68)	37	31	
Interstitial lung disease (ILD)			
Grade 1≦ (N=5)	4	1	P=0.369
Grade 0 (N=78)	41	37	

Table 5 Association between genetic polymorphisms *ABCB1* 3435C>T and toxicity

	CC	CT+TT	*P value*
Skin rash			
Grade 2≦ (N=23)	7	16	P=0.787
Grade 1≧ (N=60)	16	44	
Diarrhea			
Grade 2≦ (N=4)	1	3	P=1
Grade 1≧ (N=79)	22	57	
Liver injury			
Grade 2≦ (N=15)	3	12	P=0.542
Grade 1≧ (N=68)	20	48	
Interstitial lung disease (ILD)			
Grade 1≦ (N=5)	3	2	P=0.127
Grade 0 (N=78)	20	58	

DISCUSSION

We evaluated the clinical impact on gefitinib-related adverse effects of genetic polymorphism of ABC transporters. We examined the association of three SNPs in gefitinib-related ABC transporters with moderate or severe toxicity, since the treatment with gefitinib could be tolerable with most G1 toxicities but ILD. To our knowledge, this is the first study of the clinical impact on gefitinib-induced toxicities of *ABCG2* 34G>A. Our results suggested that *ABCG2* 34G>A would be useful for predicting grade 2 or worse skin rash. However, other genetic polymorphisms were not associated with other moderate or severe toxicities such as diarrhea, liver injury or interstitial lung disease (ILD).

Previous studies have showed that skin rash might be good predictive marker for the response to gefitinib treatment,[18] although the exact mechanism was not identified. Interestingly, a weak association between the occurrence of skin rash (G1 or more than) and the response to gefitinib was found in the present study, although it is not statistically significant (P=0.085). This result is consistent with previous studies demonstrating that occurrence of skin rash was associated with improved survival with gefitinib for recurrent NSCLC patients.[18] We also examined the association between *ABCG2* 34G>A and disease control, which means responses to gefitinib were CR, PR and SD according to the RECIST criteria v1.1.[19] The disease control rate (31 patients out of 32, 97%) among patients with at least one variant allele *ABCG2* 34G>A was significantly higher than in patients with wild type (40 patients out of 51, 78%).

Variant allele of *ABCG2* 34G>A caused apical plasma membrane dislocalization of ABCG2 and altered transporter function and sensitivity to anticancer drugs.[13] A recent study suggested that gefitinib is also an inhibitor of ABCG2 function.[20] In this regard, gefitinib may affect membrane localization of this variant type of ABCG2 more strongly than wild-type. Thus, further examination of the gefitinib effect on inhibition of ABCG2 is needed.

Cusatis *et al.* investigated the association of genetic factors with skin rash or diarrhea and indicated that *ABCG2* 421C>A was statistically significantly associated with the occurrence of diarrhea.[17] The frequencies of *ABCG2* 421C>A are reported to be 20–40% in Asian population,[21] which are higher than in Caucasians (14%) or African-Americans.[22] Although the allele frequency of the variant *ABCG2* 421C>A in our Japanese lung cancer population was higher than

in Cusatis's study,[17] we were unable to confirm the association between *ABCG2* 421C>A and diarrhea. Akasaka *et al.* also reported that the allele frequency of the variant *ABCG2* 421C>A was higher and not associated with diarrhea and skin toxicity in the Japanese patients.[23] The reason for this inconsistency remains to be investigated. One possible explanation is that other genetic factors such as polymorphisms of proteins related with EGFR signal pathway, might be more strongly involved in the adverse effects of gefitinib than this ABC transporters genotype. For example, the length of a tandem repeat (CA)n in intron 1 of *EGFR* has been inversely related to *EGFR* mRNA expression and protein levels. Several studies reported that the EGFR intron 1 polymorphism is associated with the occurrence of skin rash with gefitinib treatment.[24]

Another possible explanation might be lower allele frequency of the variant *ABCG2* 421C>A in Caucasian populations compared with two Japanese studies. In a previous positive study, 14 patients had variant alleles of *ABCG2* 421C>A and seven patients developed diarrhea, while 108 patients had only wild-type allele. There is a possibility that the significant association may be by accident due to the low frequency of variant alleles and that investigation of the larger Caucasians patients with more variant alleles might have resulted in a different conclusion. In another EGFR-TKI erlotinib study in a Caucasian population, there was no association between genetic polymorphisms of *ABCG2* 421C>A and toxicities, although *ABCG2* 16702G>A was associated with grade 2 or more skin rash.[25]

ABCB1 3435C>T has been significantly associated with reducing mRNA expression[15] and stability,[16] and may have a reduced ability to transport drugs. However, the determination of polymorphisms of *ABCB1* 3435C>T would not be useful for predicting moderate or severe toxicities induced by gefitinib in this study.

In summary, our study suggested that *ABCG2* 34G>A might be the predictor of skin toxicity in gefitinib treatments, although the effects of other variants are not consistent with previous study. Comprehensive analysis of the genes related with gefitinib metabolism is necessary for more exact prediction of its adverse effect.

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