ABCB1 Polymorphism as a Predictive Biomarker for Amrubicin-induced Neutropenia

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Abstract. *Background: Amrubicin is a promising therapy for lung cancer, but is associated with a high incidence of severe neutropenia. The present study assessed the utility of ABCB1 and NAD(P)H quinone oxidoreductase 1 (NQO1) polymorphism as a predictor of amrubicin-induced neutropenia. Materials and Methods: Fifty-four Japanese lung cancer patients who received amrubicin chemotherapy were consecutively recruited and toxicities and SNPs (MDR1; C1236T, C3435T and G2677T/A, NQO1; C609T) were evaluated. Results: The incidence of neutropenia was higher in patients treated with 40 mg/m^2 of amrubicin (n=32) compared to patients treated with 35 mg/m^2 of amrubicin (n=22) (53.1% vs. 22.7%). Patients who were homogenous for the wild-type allele of C3435T were at significantly higher risk of neutropenia compared to patients with other genotypes. By contrast, the C609T genotype of NQO1 was not related to neutropenia. Conclusion: C3435T polymorphisms of ABCB1 might be able to predict severe amrubicin-induced neutropenia.*

Lung cancer is the leading cause of cancer-related death and can be sub-classified as small-cell lung cancer (SCLC; 15% of incidence) and non-SCLC (NSCLC; 85%). Patients with SCLC have poor outcomes, with a median survival time (MST) for extensive-stage SCLC of less than 10 months (1). Amrubicin is a synthetic 9-aminoanthracycline anticancer drug that has been approved for the treatment of lung cancer in Japan. This drug is effective in patients with either SCLC or NSCLC, but has a particularly beneficial effect in patients with previously treated SCLC (2). Phase II studies of SCLC patients who have failed first-line chemotherapy have demonstrated that amrubicin is superior to topotecan, which is the standard treatment for these patients (3, 4). However, amrubicin has been associated with a high incidence of severe hematological adverse events, with 53-84% of patients experiencing grade 4 neutropenia, and >10% of patients experiencing febrile neutropenia (FN) (3, 4). Several studies demonstrated that the first-cycle absolute neutrophil count (ANC) nadir can be used to predict the risk of subsequent FN (5, 6). Identification of biomarkers predicting severe neutropenia would be of benefit to help guide treatment and monitore decisions.

The serum area under the curve (AUC) for plasma concentration value of amrubicinol (the active metabolite of amrubicin) correlates with the degree of amrubicin-induced myelosuppression (7). Therefore, serum concentrations of amrubicinol might be related to the subsequent degree of neutropenia. Amrubicinol is a substrate for ABCB1 (MDR1; P-glycoprotein (P-gp)), a major adenosine triphosphate (ATP)–binding cassette transporter (8). Among single-nucleotide polymorphisms (SNPs) of the *ABCB1* gene, C3435T, G2677T/A and C1236T are the most characterized, and these SNPs influence expression and function of ABCB1 (9, 10). NAD(P)H quinone oxidoreductase 1 (NQO1) is a major enzyme that inactivates amrubicin and amrubicinol (11). The C609T SNP of the *NQO1* gene, known as *NQO1*2*, is associated with a loss of enzyme activity due to instability of the protein product (12, 13), and thereby influences *in vitro* amrubicinol activity (14). The goal of the present study was to analyze the relationship between genotypes of *ABCB1* and *NQO* SNPs and amrubicin-induced neutropenia in a Japanese population with lung cancer.

Materials and Methods

Study population. From April 2010 to April 2013, patients with lung cancer who had received chemotherapy with amrubicin were consecutively recruited for the study (n=54). Written informed

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consent was obtained from all patients, and the protocol was approved by the Institutional Ethics Committee at the Nagoya City University Hospital. Inclusion criteria consisted of an adequate bone marrow (ANC >1500/μL, platelet count >50,000/μL), hepatic function (aspartate amino transferase (AST) and alanine amino transferase (ALT) level ≤2.5 and total bilirubin ≤2.0-times the upper normal limit) and renal function (creatinine ≤2.0 mg/dL). Patients with serious co-morbidities, including poorly-controlled diabetes mellitus, ischemic heart disease, active infection, or performance status of 4 on the Eastern Cooperative Oncology Group scale, were excluded from the study.

Amrubicin treatment. Amrubicin was dissolved in 50 mL of physiological saline and was administrated intravenously by 5-min infusion on days 1-3 every 3-4 weeks. Before treatment, all patients underwent physical examinations, hematology and serum biochemistry tests, and electrocardiography. All patients were treated with first-cycle amrubicin treatment as in-patients, and physicians assessed for the presence of symptomatic adverse effects every day. Toxicities associated with hematological tests were evaluated at least twice a week until after the nadir of blood cell counts. Toxicities were graded according to the National Cancer Institute Common Version 3.0 criteria. In this study, additional treatments for hematological toxicities, including granulocyte-colony stimulating factor (G-CSF) therapy and blood transfusion, were permitted according to the judgment of each physician after the hematological toxicity reached grade 4.

ABCB1 and NQO1 genotyping. Genomic DNA was isolated from 5 mL of peripheral blood using the Qiagen DNA extraction kit (Qiagen, Hilden, Germany). Genotypes were evaluated using a previously reported method (15). Briefly, three SNPs of the *MDR1* gene (C1236T in exon 12, C3435T in exon 26, and G2677T/A in exon 21) and C609T SNP of the *NQO1* gene were detected using the StepOnePlus Real-Time PCR System (Applied Biosystems, Foster City, CA) and Taqman Drug Metabolism Genotyping Assays (Applied Biosystems, Darmstadt, Germany), according to the manufacturer's instructions. The primer and Taqman probe set for each SNP was purchased from Applied Biosystems. The assay identification number of each SNP was as follows: C1236T; C_7586662_10, C3435T; C_7586657_20, G2677T; C_11711720D_40, G2677A; C_11711720C_30, C609T; C_2091255_30.

Statistical analysis. Data were expressed as mean±SD (standard deviation of the mean). Fisher's exact test was used to analyze associations between adverse events and genotypes or treatment doses. Differences in ANC nadir between two treatment doses were evaluated statistically with the Mann-Whitney *U*-test. Associations of genotypes with ANC nadir were evaluated statistically with analysis of variance (ANOVA) or the Mann-Whitney *U*-test.

Results

Clinical characteristics. Patients' characteristics are shown in Table I. The recommended dose of amrubicin for second-line and third-line treatment is 40 mg/m^2 or 35 mg/m^2 (16). In the study, 32 patients were treated with 40 mg/m^2 of amrubicin, and 22 patients were treated with 35 mg/m^2 of amrubicin. Patients treated with 35 mg/m^2 of amrubicin were significantly older than patients treated with 40 mg/m^2 of amrubicin (p=0.043). There was no significant difference in other patient characteristics when comparing the two groups.

Table I. *Characteristics of enrolled patients*.

Characteristics	Amrubicin dose (mg/m^2), N (%)		
	35	40	p-Value
No. of Patients	22	32	
Gender			
Male	18 (82)	27 (84)	0.804
Female	4 (17)	5 (16)	
Age, median (range)	71.1 (55-81)	66.8 (48-81)	0.043
Stage			
IIIB	4 (18)	4 (13)	0.564
IV	18 (82)	28 (88)	
Histology			
Small cell lung cancer	17 (77)	20 (63)	0.251[a]
Adenocarcinoma	1 (5)	4 (13)	
Squamous cell carcinoma	2 (9)	3 (9)	
Others	2 (9)	5 (16)	
Previous chemotherapy			0.444[b]
1	15 (68)	17 (53)	
2	4 (18)	8 (25)	
More than 3	3 (14)	7 (22)	
Performance status by ECOG[c]			0.061[d]
0-1	13 (59)	22 (69)	
2	5 (23)	9 (28)	
3	4 (18)	1 (3)	

Analysis was performed between the values of 30 or 35 mg/m^2 treatment and 40 mg/m^2 treatment using the Fisher's test. [a]Statistical analysis was performed between small cell carcinoma and other types. [b]Statistical analysis was performed between 1-2 and more than 3. [c]Eastern Cooperative Oncology Group. [d]Statistical analysis was performed between 0-2 and 3.

Toxicities of amrubicin treatment. Toxicities of the first course of amrubicin chemotherapy are summarized in Table II. The frequency of grade 4 neutropenia in patients treated with 40 mg/m^2 of amrubicin was 53%, which was significantly higher than the 23% frequency in patients treated with 35 mg/m^2 amrubicin (p=0.025). We investigated the ANC nadir after the first course of amrubicin chemotherapy in 54 patients. The mean ANC nadir was significantly lower in patients treated with 40 mg/m^2 of amrubicin than in patients treated with 35 mg/m^2 of amrubicin (607.5±584.6/μL and 803.4±276.6/μL; p=0.015) (Figure 1). Febrile neutropenia was seen in four patients, all of whom were treated with 40 mg/m^2 of amrubicin. Therefore, the rate of neutropenia was 13% in the group of patients who received 40 mg/m^2 of amrubicin, which is similar to that seen in previous reports (3, 4). Grade ≥3 thrombocytopenia was seen significantly more frequently in

Table II. *Toxicity of amrubicin treatment*.

Characteristics	Amrubicin dose, N (%)		*p*-Value
	35 mg/m^2	40 mg/m^2	
No. of Patients	22	32	
Neutropenia			0.025[a]
0-2	10 (46)	8 (25)	
3	7 (32)	7 (22)	
4	5 (23)	17 (53)	
Febrile neutropenia	0 (0)	4 (13)	0.085
Thrombnocytopenia			0.005[b]
0-2	21 (96)	20 (59)	
3	1 (5)	11 (34)	
4	0 (0)	1 (3)	
Anemia			0.140[b]
0-2	22 (100)	29 (91)	
3	0 (0)	3 (9)	
4	0 (0)	0 (0)	
Non-hematological adverse effect			0.192[c]
0	18 (82)	21 (66)	
1-2	4 (18)	11 (34)	
3-4	0 (0)	1 (3)	

Analysis was performed using the Fisher's test. [a]Analysis between 0-3 and 4 or more. [b]Analysis between 0-2 and 4 or more. [c]Analysis between 0 and 1 or more.

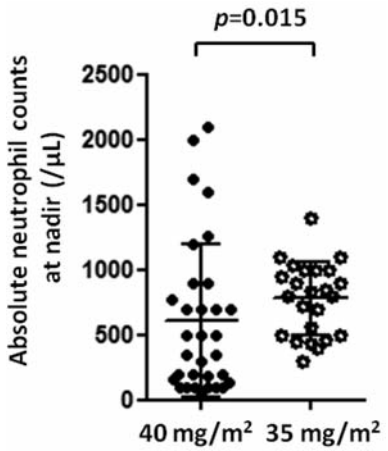

Figure 1. *Comparison of absolute neutrophil counts (ANC) at nadir in patients treated with 40 mg/m^2 and 35 mg/m^2 of amrubicin. Mean values were compared with the Mann-Whitney U-test.*

patients treated with 40 mg/m^2 of amrubicin than in those treated with 35 mg/m^2 of amrubicin. Non-hematological adverse effects grade ≥3 did not occur except for a case with grade 3 of interstitial pneumonia.

Relationship between genotypes and degree of neutropenia. Although the degree of neutropenia was more severe among patients treated with the higher dose of amrubicin than in those treated with a lower dose of amrubicin, a large variation existed within the higher dose group (Figure 1). Therefore, we assessed the relationship between the three *ABCB1* SNPs (C3435T, G2677T/A and C1236T) and one *NQO1* SNP (C609T) and the degree of neutropenia in patients treated with 40 mg/m^2 of amrubicin.

Allele frequency for each genotype is summarized in Table III. The allele frequency was similar to values seen in previous reports of the Japanese population (17, 18). The values of ANC nadir according to each genotype are shown in Figure 2. In regard to *ABCB1* SNPs, the ANC nadir of patients who were homozygous wild-type tended to be lower than that of patients with other genotypes (Figure 2A-C). There was a statistically significant association between C3435T genotypes and ANC values at nadir (p=0.047) (Figure 2A) but not between *NQO1* SNP C609T and ANC values at nadir (Figure 2D). In the assessment of the lower-dose group, similar relation were shown between genotypes of each *ABCB1* SNP and ANC values at nadir, but a statistically significant association was not shown (data not shown).

Patients were sub-categorized according the presence/absence of 3435CC and 2677GG. The mean ANC nadir value was significantly lower in patients with 3435CC than in patients with other genotypes (235.6±251.8/μL *vs*. 776.6±611.7/μL; p=0.0051) (Figure 3A). With regard to 2677GG, the mean ANC nadir value in patients with the genotype tended to be lower than that in patients with other genotypes (254.6±225.0/μL *vs*. 706.4±618.3/μL; p=0.060) (Figure 3B).

We compared the risk of grade 4 neutropenia among these sub-groups (Table IV). Nine of 10 patients with 3435CC had grade 4 neutropenia, the frequency of which was higher than those experienced by patients with other genotypes (90% *vs*. 36%; p=0.0048). Similarly, six of seven patients with 2677GG patients had grade 4 neutropenia, the frequency of which was marginally higher than those experienced by patients with other genotypes (86% *vs*. 44%; p=0.051). Twelve patients had either 3435CC or 2677GG, and 10 of these patients had grade 4 neutropenia, the frequency of which was higher than those experienced by patients with other genotypes (83% *vs*. 35%; p=0.0080).

Discussion

The present study demonstrated that C3435T of *ABCB1* was related to the ANC at nadir and that patients who were homozygous wild-type of this SNP had a high incidence of severe amrubicin-induced neutropenia when compared with patients with other genotypes. The results indicate that *ABCB1* SNP might be good predictor of severe amrubicin-induced neutropenia.

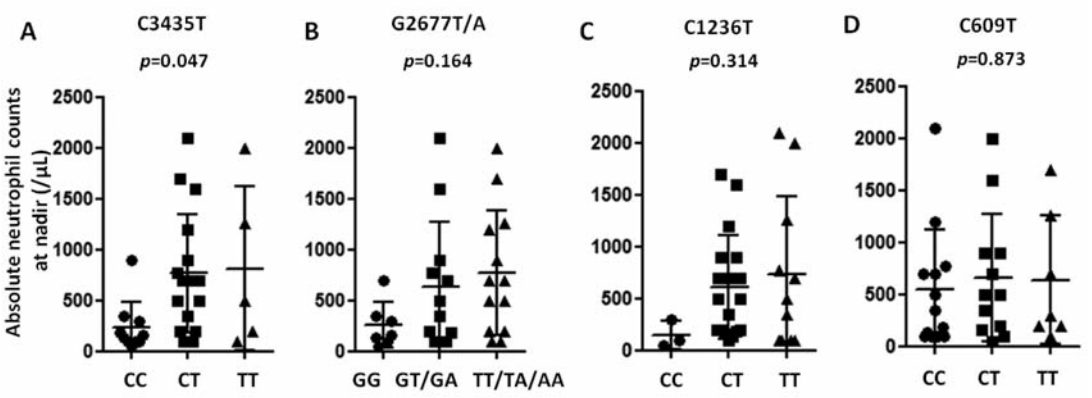

Figure 2. *Relationship between absolute neutrophil counts (ANC) at nadir and genotypes of single-nucleotide polymorphisms (SNPs) of ABCB1 and NAD(P)H quinone oxidoreductase 1. Each figure shows ANC values at nadir in patients who are homozygous wild-type, heterozygous wild- and variant-type and homozygous variant-type for each SNP in patients treated with 40 mg/m^2 of amrubicin (A; C3435T, B; G2677T/A, C; C1236T, D; C609T). Mean values are compared via ANOVA.*

Table III. *Genotype and allele frequency of ACBC1 and NAD(P)H quinone oxidoreductase 1 polymorphism in patients treated with 40 mg/m^2 of amrubicin.*

	Genotypes; N (%)						Allele frequencies		
ABCB1									
C3435T	CC	CT	TT				C	T	
	10 (31)	17 (53)	5 (15)				58	42	
G2577T/A	GG	GT	GA	TA	TT	AA	G	A	T
	7 (22)	10 (31)	2 (6)	8 (25)	4 (13)	1 (3)	41	19	41
C1236T	CC	CT	TT				C	T	
	3 (9)	18 (56)	11 (34)				38	63	
NQO1									
C609T	CC	CT	TT				C	T	
	13 (41)	12 (38)	7 (22)				59	41	

Amrubicin-induced myelosuppression correlates with the serum AUC value of amrubicinol (7). In the present study, we demonstrated that the degree of neutropenia was significantly more severe in patients treated with 40 mg/m^2 of amrubicin than in those treated with 35 mg/m^2 of amrubicin (Figure 1A). These findings support the concept that serum concentration of amrubicinol is an important factor related to the degree of neutropenia. Therefore, we examined SNPs of *ABCB1* and *NQO1* that are associated with metabolism of amrubicinol and demonstrated that the degree of neutropenia was related to the presence of *ABCB1* SNPs.

Hoffmeyer *et al*. first reported that a synonymous C3435T polymorphism was associated with reduced expression of ABCB1 (9). Kim *et al*. (19) reported that the C3435T polymorphism is linked to a non-synonymous G2677T/A (Ala893Ser) and to a synonymous C1236T polymorphism. There are numerous reports that evaluate the relationship between these SNPs and expression and function of ABCB1. However, the results of these studies are controversial, and the impact of these SNPs on clinical outcome remains incompletely characterized (17, 20). A previous study reported that 2677GG was related to low ANC in patients receiving irinotecan (21), but other studies demonstrated that the genotype of 3435TT was associated with a higher risk of neutropenia in patients receiving docetaxel treatment (22, 23). Further study would be of benefit to determine the effect of *ABCB1* SNPs on the clinical outcomes of chemotherapy which may differ in a drug-specific manner.

The active metabolite of amrubicin, amrubicinol, plays an important role in the biological effect of the parent drug (24, 25). This suggests that enzymes that metabolize amrubicin and amrubicinol could influence the clinical outcome. The present study showed there was no relationship between C609T SNP of *NQO1* and the degree of amrubicin-induced neutropenia, but

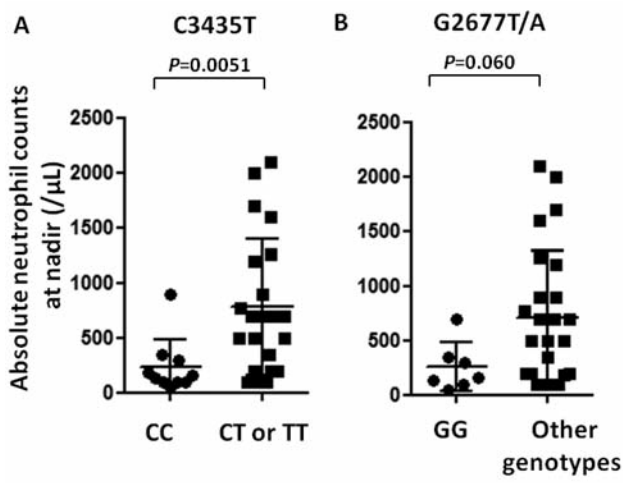

Figure 3. *Comparison of absolute neutrophil counts (ANC) at nadir between homozygous and other genotypes of C3435T and G2677T/A in patients treated with 40 mg/m^2 of amrubicin. A: ANC values at nadir in patients genotyped CC and other types of C3435T. B: ANC values at nadir in patients genotyped GG and other types of G2677T/A. Mean values are compared with the Mann-Whitney U-test.*

did show a relationship between C3435T SNP and the degree of amrubicin-induced neutropenia. A similar tendency was seen in G2677T/A, however this study did not investigate the specific mechanism that mediates this relationship. A previous study reported that the 2677GG genotype was associated with an increase in the AUC of SN-38 (a metabolite of irinotecan) and with low ANC in patients receiving irinotecan treatment (21). These observations suggest that ABCB1 SNPs may directly influence serum concentration of amrubicinol, thereby affecting the degree of neutropenia. Amrubicin has been shown to have a high affinity for bone marrow in an *in vivo* study of tissue drug distribution (26). ABCB1 is expressed in hematopoietic progenitor cells (27) and several reports have investigated the effects of *ABCB1* SNPs on P-gp activity in hematopoietic progenitor cells of the bone marrow (28, 29). Further study would be of benefit to characterize the mechanisms by which *ABCB1* SNPs influence amrubicin-induced neutropenia .

In conclusion, the present study demonstrated that *ABCB1* SNPs might be a predictive marker of severe amrubicin-induced neutropenia. Further studies would be of benefit to investigate the predictive value of these SNPs.

Conflicts of Interest

The Authors have no conflicts of interest.

Acknowledgements

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Table IV. *Association of genotypes of ABCB1 polymorphisms with grade 4 neutropenia induced by 40 mg/m^2 of amrubicin.*

	G4 neutropenia		*p*-Value
	(+)	(–)	
C3435T			0.0048
CC	9	1	
CT or TT	8	14	
G2677T/A			0.051
GG	6	1	
Other genotypes	11	14	
Combination of C3435T and G2677T/A			0.0080
CC of C3435T or GG of G2677T/A	10	2	
Other genotypes	7	13	

Analysis was performed using the Fisher's test.

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