BCRP gene polymorphisms are associated with susceptibility and survival of diffuse large B-cell lymphoma

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Introduction

Diffuse large B-cell lymphoma (DLBCL) is the most common lymphoid malignancy in adults. Growing evidences have shown that both genetic and environmental factors are involved in the etiology and prognosis of DLBCL (1–4). Thousands of gene polymorphisms, most often single nucleotide polymorphism (SNP), have been identified in humans. It is well demonstrated that SNPs can alter the expression and activity of the corresponding proteins and thereby influence the individual susceptibility and clinical outcome for different types of cancer (5–7).

Breast cancer resistance protein (BCRP), also known as ABCG2, is a half-molecule ABC transporter containing an ATP-binding domain and a transmembrane domain (8,9). It functions as energy-dependent efflux pump and transports a wide array of structurally divergent substrates, including toxic compounds and anticancer drugs, from the intracellular to the extracellular compartment. Therefore, BCRP plays a protective role against xenobiotics and their metabolites and can affect the intracellular concentration of anticancer drugs (10,11). The investigation on the BCRP distribution in normal tissues has revealed the highest level of BCRP in placenta, with the lower levels in the epithelium of the small intestine and colon, the liver canalicular membrane, ducts and lobules of the breast and in veiny and capillary endothelium of normal tissues (9,12). Elevated expression of the BCRP gene has been described in different types of drug-resistant cancer (11,13,14).

Naturally occurring BCRP SNPs have been reported in various ethnic populations (15–20). BCRP G34A (Val12Met) and C421A (Gln141Lys) polymorphisms occurred at high frequency in most ethnic populations and have been shown to be associated with the expression and activity of BCRP protein. The BCRP protein level in human placenta is significantly lower in homozygotes for the 421A allele than in those for the 421C allele, and heterozygotes have an intermediate value (19). Furthermore, compared with wild-type alleles, both 34A and 421A variants show decreased activity of BCRP transporter (10). Based on these evidences, we hypothesize that BCRP G34A (Val12Met) and C421A (Gln141Lys) polymorphisms should have potential effect on the susceptibility and prognosis of diseases.

To date, there have been only two studies investigating the association of BCRP genotypes with the susceptibility and survival of cancer. A case–control study has shown that the BCRP 421C homozygotes are at increased risk of developing non-gastric-mucosal type gastric carcinoma (20). In another study with survival analysis in hormone-refractory prostate cancer patients, BCRP 421A variants have shown significant association with survival beyond 15 months compared with 421CC genotype (21). To explore the potential effects of BCRP polymorphisms on the development and clinical outcome of DLBCL, the BCRP G34A and C421A polymorphisms were analyzed in 156 patients with DLBCL and 376 normal subjects in the present study. We showed that the BCRP G34A and C421A polymorphisms were associated with the risk and survival of DLBCL. Our finding warrants further investigations on the association of BCRP polymorphisms with susceptibility and clinical outcome of cancer.

Materials and methods

Study population

The case–control study consisted of 156 patients with DLBCL and 376 control subjects. All subjects were unrelated ethnic Han Chinese and residents in Guangzhou and the surrounding regions. Patients were recruited between 1998 and 2005 at the Cancer Center, Sun Yat-Sen University. All patients were diagnosed as DLBCL according to World Health Organization classification (22). There was no sex and age restriction. Control subjects, comprising 199 health check-up examinees and 177 healthy blood donors, had no history of tumors and were frequency matched to the cases on the basis of sex and age. At recruitment, informed consent was obtained from each subject. This study was approved by the Institute Research Ethics Committee at the Cancer Center, Sun Yat-Sen University.

Twenty-nine patients who received chemotherapy before they visited the Cancer Center were excluded from the survival analysis. Overall survival was calculated from the onset of treatment until the last follow-up evaluation or death from any cause. The extent of the disease was categorized according to the Ann Arbor classification, and performance status was assessed using Eastern Cooperative Oncology Group criteria. The baseline characteristics of the patients included in the survival analysis are summarized in Table 1.

Genotyping

Genomic DNA was extracted from blood samples of all subjects. Genotypes were analyzed by polymerase chain reaction (PCR)-based single-strand conformation analysis and confirmed by direct DNA sequencing. The PCR primers 5’-AAT CTC ATT TAT CTG GAC TAT CAA C and 5’-TGG ATA...
ATATTT CTT TCT CAA CTG were used to amplify the BCRP G34A site, and 5’-TCAT GTA AAT AGT GAG CAC AGG AAA AGT CCT AC for C421A site. PCR amplification was performed as follows: 25 ng genomic DNAs were amplified in 20 μl reaction volume containing 2 mM MgCl₂, 0.1 mM dNTPs, 0.1 μM of each primer and 0.5 unit Taq polymerase (Promega, Madison, WI). Thirty-five cycles of amplification were carried out with denaturation at 94°C for 1 min, annealing at 55°C for 30 s and extension at 72°C for 45 s.

PCR product was mixed with 4-fold volume of loading buffer (95% formamide, 0.05% xylene cyanol and bromophenol blue), denatured at 95°C for 8 min and quenched on ice. The denatured mixture was then resolved at 4°C in a non-denaturing 8–10% polyacrylamide gel. DNA was visualized using silver staining. The samples representing different patterns of electrophoretic mobility were applied to direct DNA sequencing to validate the genotype.

**Statistical analysis**

Pearson χ² test was used to examine the genotype distribution differences between case patients and control subjects. Association between polymorphisms and the risk for DLBCL was estimated by unconditional logistic regression. Hardy–Weinberg equilibrium was tested by a goodness-of-fit test. Overall survival was computed using the Kaplan–Meier method and compared between groups by the log-rank tests. Association between the genotypes or characteristics of patients and the overall survival was analyzed by univariate Cox regression analysis. A P value of <0.05 was used as the criterion of statistical significance, and all statistical tests were two-sided. All analyses were performed using SPSS software (version 13.0, SPSS, Chicago, IL).

### Results

**Association between the BCRP polymorphisms and the risk of DLBCL**

To investigate the association between BCRP polymorphisms and susceptibility to DLBCL, a case–control study including 156 case patients and 376 control subjects was conducted. The age and sex distributions in patients were not significantly different from those in controls (P = 0.781 and 0.755, respectively), suggesting that the frequency matching is adequate. Two BCRP SNPs, G343A (rs2231137) and G421A (rs2231142), were genotyped in all subjects. Genotype distributions were compared between patients and controls. Unconditional logistic regression analysis was used to estimate the association between the genotype frequency and the risk of developing DLBCL.

The observed genotype distributions in both control and case patients agreed with the predicted distribution (P > 0.05), indicating that the genotypes conformed to Hardy–Weinberg equilibrium. As shown in Table II, the genotype distribution was significantly different between cases and controls for BCRP C421A but not for G343A polymorphism (data not shown). The results showed that 51.3% of the cases were 421CA heterozygotes, which was significantly higher than that of the controls (43.1%, χ² = 3.89, P = 0.049). However, the percentage of AA homozygotes in the case group was only slightly higher than that of the control group (10.3% versus 8.8%, P = 0.262), which may be due to the small number of subjects with this genotype in our study population (Table II). Therefore, the AA genotype was combined with the CA genotype for subsequent estimation of DLBCL risk. As expected, we found that the combined 421CA and AA genotypes were statistically significantly associated with increased risk of DLBCL [odds ratio = 1.49, 95% confidence interval (CI) 1.02–2.17, P = 0.042, Table II].

When further stratification for age and sex was performed, the increased risk conferred by the combined 421CA/AA genotypes was more evident in patients who were younger (<50 years) at diagnosis (odds ratio = 2.14, 95% CI 1.25–3.68, P = 0.006, Table II), whereas no significant association was observed for the group older than 50 years old (data not shown). In an attempt to clarify the potential combined effect of the G343A and C421A genotypes on the risk of DLBCL, no significant interaction between these two polymorphisms was found (data not shown).

**Association between the BCRP genotypes and the survival of DLBCL**

Among 156 patients employed in the above case–control study, 127 cases with meticulous follow-up record and without previous treatment history in other hospitals were included in the survival analysis. The characteristics of the patients are summarized in Table I. The overall survival was analyzed using Kaplan–Meier curve, log-rank test and univariate Cox regression. It is well known that clinical
variables can affect the prognosis of cancer patients. Therefore, association between the overall survival and the clinical variables was first analyzed. The results showed that age, involvement of extra-nodal site and erythrocyte sedimentation rate value could affect the survival in our study cohort, while other parameters were not the influential factors (Table I). No significant association was identified between the BCRP polymorphisms and the characteristics of patients listed in Table I (data not shown).

Next, the effect of BCRP genotypes on the overall survival of DLBCL patients was further investigated. We found that patients with 34AA genotype displayed worse survival compared with those carrying the G allele [hazard ratio (HR) = 3.69, 95% CI 1.56–8.71, \( P = 0.001 \), Figure 1]. To clarify whether the strength of this association differed among patients with different characteristics, further stratification for the clinical variables listed in Table I was performed. Interestingly, the poorer survival associated with 34AA genotype was more pronounced among patients with involvement of extra-nodal site (HR = 6.70, 95% CI 2.24–20.05, \( P < 0.001 \)), with intermediate or high international prognostic index score (IPI score =2–5, HR = 6.05, 95% CI 1.89–19.35, \( P = 0.001 \)), with B symptom (HR = 7.24, 95% CI 2.09–25.07, \( P < 0.001 \)) or performance status \( \geq 2 \) (HR = 8.36, 95% CI 1.38–50.78, \( P = 0.005 \)) at diagnosis.

The survival was not associated with BCRP C421A polymorphism when all 127 patient cases were analyzed together (HR = 1.58, 95% CI 0.76–3.29, \( P = 0.215 \)). However, in the group aged \( \leq 50 \) years, BCRP 421CC genotype was statistically significantly associated with worse survival compared with the combined AA and CA genotypes (HR = 5.80, 95% CI 1.16–28.90, \( P = 0.015 \), Figure 2A). Moreover, this association was also observed among the patients with bulky tumor (HR = 4.36, 95% CI 1.04–18.31, \( P = 0.027 \), Figure 2B).

The combined effects of BCRP G34A and C421A on the overall survival were then examined. The survival was significantly dependent on the combined genotypes (Figure 3). Compared with those carrying BCRP 34(GG + GA)421(AA + CA) genotype, the patients with 34AA421CC displayed the worst survival (HR = 7.55, 95% CI 2.36–24.17, \( P = 0.001 \)), while the 34(GG + GA)421CC and 34AA421(AA + CA) combinations showed the intermediate survival (Figure 3A). Next we compared the 34AA421CC with the group including all the other combinations, and log-rank test showed that the \( P \) value was <0.001 (HR = 5.81, 95% CI 1.98–17.03, Figure 3B).

**Discussion**

BCRP was first detected in breast cancer-resistant cell and functions as energy-dependent efflux pumps. The BCRP polymorphisms G34A and C421A
and C421A have been shown to change the expression and activity of BCRP, but their biological significance is not yet fully understood and the association between BCRP polymorphisms and cancer has only been reported in two studies (20,21). In the present investigation, we found that the 421A allele carriers were statistically significantly associated with the increased risk of DLBCL. Moreover, patients with 34AA and 421CC genotypes displayed worse survival. These results suggest that the BCRP G34A and C421A polymorphisms may be employed as biomarkers for the prediction of susceptibility and survival of DLBCL.

Our finding that 421A allele predicted the individual susceptibility to the development of DLBCL is consistent with the results from functional studies reported previously (10,19,20,23–26). BCRP transports a wide array of structurally divergent substrates including carcinogens, suggesting that BCRP may prevent the cells from accumulation of carcinogens (24–26). Furthermore, BCRP 421AA homozygotes confer a lower BCRP level and decreased BCRP activity compared with the 421CC homozygotes (10,19,23). Taken together, the individual with 421A allele may have decreased capability to exclude the carcinogens and thereby becomes susceptible to tumorigenesis.

In stratified analysis, we observed that the increased risk conferred by the combined 421CA/AA genotypes was more pronounced in subjects who were younger at diagnosis. This finding is in line with the conception that genetic susceptibility is often associated with an early onset of disease and suggests that BCRP C421A may be a useful biomarker in identifying subgroups at high risk of DLBCL. Contrast to our result that BCRP 421A allele conferred increased risk of DLBCL, a Japanese group found that BCRP 421CC homozygotes were in higher risk to develop non-papillary renal cell carcinoma (20). This difference may result from the distinct molecular mechanisms underlying the development of DLBCL and non-papillary renal cell carcinoma.

The BCRP 34AA and 421CC genotypes were associated with significantly decreased overall survival in the present study population. These results are consistent with the previous report that hormone-refractory prostate cancer patients with 421CC genotype have a shorter survival time. Mizuara et al. (10) observed that compared with the polarized LLC-PK1 cells stably transfected with wild-type BCRP gene, the ones with 34AA variant displayed dramatic decrease in 50% inhibitory concentration (IC50) values of the BCRP substrates, including various anticancer compounds such as mitoxantrone, doxorubicin and vincristine topoisomerase I inhibitors. The polarized LLC-PK1 cells stably transfected with 421AA variant also showed a decreased IC50 of anticancer compounds, but to a much lesser extent (10). Therefore, we speculate that the patients with 421A allele may be more sensitive to the chemotherapy and therefore present better survival. Considering the extremely low IC50 values for the cells with 34AA variant, the anticancer compounds may confer much higher toxic side effect to those with 34A allele and thus result in the intolerance to the treatment and worse prognosis.

In the normal subjects included in this study, the frequencies of the variant alleles in the BCRP G34A and C421A sites were 39.3 and 30.3%, respectively, which were similar to those in Han Chinese from HapMap data as well as the previous report (27). There exist ethnic differences in the allele distribution of these two SNPs (18–20,27 and HapMap data); frequency rates of the variant 34A and 421A alleles are 17–19% and 30–31%, respectively, in Japanese subjects; 2–4% and 10–12%, respectively, in Caucasians and 5–6% and 0–2%, respectively, in Africans. More analyses are required to assess the implication of these ethnic differences in the diseases including cancers.

In summary, our results suggest that the BCRP G34A and C421A polymorphisms are associated with the risk and survival of DLBCL. Clearly, additional investigations are warranted to confirm our findings before we can employ these polymorphisms in identifying the subgroup at high risk for DLBCL and in predicting the prognosis of DLBCL patients.

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References


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