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Original Article

Genetic polymorphisms of ATP-binding cassette (ABC) proteins, overall survival and drug toxicity in patients with Acute Myeloid Leukemia

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Abstract: The overall survival of patients with acute myeloid leukemia (AML) remains poor due to both intrinsic and acquired chemotherapy resistance. Over expression of ATP binding cassette (ABC) proteins in AML cells has been suggested as a putative mechanism of drug resistance. Genetic variation among individuals affecting the expression or function of these proteins may contribute to inter-individual variation in treatment outcomes. DNA from pre-treatment bone marrow or blood samples from 261 patients age 20-85 years, who received cytarabine and anthracycline-based therapy at Roswell Park Cancer Institute between 1994 and 2006, was genotyped for eight non-synonymous single nucleotide polymorphisms in the ABCB1, ABCC1 and ABCG2 drug transporter genes. Heterozygous (AG) or homozygous (AA) variant genotypes for rs2231137 (G34A) in the ABCG2 (BRCP) gene, compared to the wild type (GG) genotype were associated with both significantly improved survival (HR=0.44, 95%CI=0.25-0.79), and increased odds for toxicity (OR=8.41, 95%CI=1.10-64.28). Thus genetic polymorphisms in the ABCG2 (BRCP) gene may contribute to differential survival outcomes and toxicities in AML patients via a mechanism of decreased drug efflux in both, AML cells and normal progenitors.

Keywords: Acute myeloid leukemia, multidrug resistance, polymorphisms, survival, toxicity

Introduction

Acute myeloid leukemia (AML) is a clinically and biologically heterogeneous malignancy resulting from clonal proliferation of hematopoietic precursor cells ('blasts') which fail to differentiate, giving rise to hematopoietic insufficiency (granulocytopenia, thrombocytopenia and anemia) [1-2]. Prognosis remains poor, with many patients dying due to intrinsic drug resistance of AML cells or relapse resulting from acquired resistance to initial therapy. Over expression of ATP-binding cassette (ABC) transmembrane proteins, e.g. P-glycoprotein, which are involved in active transport of nutrients, biologically active substances and chemotherapeutic drugs across the plasma membrane [3-4] have been associated with inferior treatment outcome in AML [3, 5-7]. Genetic polymorphisms in ABC genes, which affect the level of expression or function of these transporter proteins, might thus be associated with increased toxicity and better survival in AML patients, due to decreased efflux from both somatic cells and AML cells, or decreased toxicity but poor survival, due to increased efflux from both somatic cells and AML cells,. In this study, we evaluated whether eight non-synonymous single nucleotide polymorphisms (SNPs) in three ABC genes: *ABCC1*, *ABCB1* and *ABCG2*, located on chromo-

somes 16, 7 and 4 respectively, were associated with overall survival (OS) and toxicity in a cohort of 261 AML patients treated with Cytarabine and Anthracycline-based therapy.

Materials and methods

Patients and clinical information

Patients between the ages of 20 and 85 years who were newly diagnosed with AML and received treatment at Roswell Park Cancer Institute (RPCI), Buffalo, New York, between 1994 and 2006, were included in the study sample. Morphologically confirmed cases of previously untreated primary or secondary AML, other than acute promyelocytic leukemia, by the French-American-British (FAB) criteria [8] were included in the sample. These patients received induction chemotherapy with either a standard- or high-dose regimen of cytarabine (ara-C) or an anthracycline. Two hundred and ninety three (N=293) patients met the eligibility criteria. Patients with missing genotype (n=31) or survival status (n=1) were excluded, resulting in a final sample of 261 patients.

Clinical information was obtained from the RPCI Leukemia Section Database which includes information on demographic and pre-treatment characteristics, treatment type and outcome, disease-free survival and overall survival. For information not included in the database or for missing data, information was abstracted from patients' medical records. Cytogenetic studies were performed in the RPCI Clinical Cytogenetics Laboratory and karyotypes were assigned to risk categories (favorable, intermediate, unfavorable) as described previously [9]. The study was approved by the RPCI Institutional Review Board.

DNA extraction and genotyping

Bone marrow (n=217) and blood samples (n=44) were transferred to green topped tubes containing heparin and then transported to the RPCI Tissue Procurement Laboratory. Mononuclear cells were isolated by Ficoll-Hypaque density centrifugation, washed twice, counted, resuspended at a density of 1-2 x 10^7/ml in fetal calf serum with 20% dimethylsulfoxide, aliquoted into cryovials and cryopreserved at 120° C. DNA was then extracted using Gentra PureGene DNA extraction kits (Minneapolis, MN), according to the manufacturer's instructions.

Genotyping was determined using Sequenom's high-throughput matrix-assisted laser desorption/ionization time-of-flight (MALDI-TOF) mass spectrometry[10]. Functional non-synonymous SNPs were selected including rs1045642 (C3435T), rs2032582(G2677T), rs1128503 (C1236T), rs2231142(C421A), rs2231137 (G34A), rs246221(T825C), rs2230671(G4002) and rs35587(T1062C).

Initially, a locus-specific polymerase chain reaction (PCR) reaction was carried out, followed by an allele-specific primer extension reaction. PCR amplification was performed using SNP-specific primers. Controls for genotype and two-non template controls were included on each plate, as well as 10% duplicate samples. The PCR condition was 94° C for 15 minutes for hot start, followed by denaturing at 94° C for 20 seconds, annealing at 56° C for 30 seconds, extension at 72° C for 1 minute for 45 cycles, and final incubation at 72° C for 3 minutes.

Statistical analysis

OS was measured from the date of diagnosis until death from any cause, with observation censored on the date the patient was last known to be alive or at the time of allogenic hematopoietic stem cell transplantation (n=38). Treatment response was evaluated according to National Cancer Institute (NCI)-sponsored workshop guidelines (http://ctep.cancer.gov/protocolDevelopment/electronic_applications/ctc.htm). Complete remission (CR) was defined by ≤ 5% blasts in the marrow, normal peripheral blood cell counts including absolute neutrophil count ≥ 1,000/μL and platelet count ≥ 100,000/μL. Relapse was defined as a reappearance of leukemic blasts in the peripheral blood or ≥ 5% blasts in the bone marrow not attributable to any other cause. Severity of toxicities was graded 1 (mild) through 5 (fatal) according to the NCI Common Toxicity Criteria, version 3.0. Toxicities of induction chemotherapy were combined into the following categories: genitourinary, gastrointestinal, hepatic, pulmonary, cardiovascular, hemorrhage, dermatologic, neurologic and metabolic. The grade of toxicity assigned to an organ group was the maximum grade of all the specific toxicities within that group.

Distributions of patient and disease characteristics were compared among genotypes using Chi-square analysis, Fisher's exact tests, ANOVA and Kruskal-Wallis or Wilcoxon rank sum tests as appropriate. Distribution of OS was estimated by Kaplan-Meier methods and compared between genotypes by log-rank tests. Cox proportional hazards regression analysis of OS was performed to evaluate survival difference between genotype groups after adjusting for significant prognostic factors. Logistic regression models were used to examine treatment response and toxicity outcomes.

Results

Demographic characteristics of the 262 patients with AML are shown in **Table 1**. The median age at diagnosis was 65 years. The majority of the patients was Caucasian (86%) and had *de novo* onset of AML (75%), while 25% had secondary AML defined as preceded by an antecedent hematologic disorder or therapy-related. Statistically significant predictors of OS, resistant disease and complete response included age, cytogenetics, AML onset, type of induction (standard- vs. high-dose ara-C) and white blood cell (WBC) count at diagnosis (results not shown).

Table 2 displays the association of polymorphisms in eight ABC transporters and overall survival. Presence of one copy of the variant allele of *ABCG2* (rs2231137/G34A) was associated with significantly reduced hazard of death (HR=0.44, 95%CI=0.25-0.79) compared to the homozygous wild type genotype (GG). The Kaplan-Meier survival probabilities for the AA/AG group are shown in **Figure 1**. While the homozygous AA genotype of rs2231142 was associated with more than four-fold increased hazard of death compared to homozygous CC genotype (HR=4.61, 95%CI=1.44-14.8), after adjusting for age, cytogenetics and AML onset, the hazard ratio was attenuated and was not statistically significant (HR=2.57, 95%CI=0.79-8.42).

We did not find any significant associations between polymorphisms in *ABCB1* and *ABCC1* and OS, either when we considered the AML population in its entirety or when restricted to patients who self-reported as Caucasian.

To test the hypothesis that SNPs associated with OS would also be associated with toxicity we analyzed the association of the rs2231137 (G34A) SNPs in the *ABCG2* gene that was found

Table1. Descriptive characteristics of 261 AML patients

Variable	N (%)/mean(STD)
Age	
Mean (STD)	61.5 (15.70)
WBC	
<100,000	224 (86)
>=100,000	37 (14)
Sex	
Male	135 (52)
Female	126 (48)
Complete response	
No	123 (47)
Yes	138 (53)
Relapse	
No	170 (65)
Yes	91 (35)
Race	
Whites	233 (89)
Other	28 (11)
FAB classification	
Acute unclassifiable leukemia (AUL)	1 (0)
M0	13 (5)
M1	61 (23)
M2	112 (43)
M4	37 (14)
M4e	7 (3)
M5a	8 (3)
M5b	4 (1)
M6	9 (3)
M7	2 (1)
UNK	3 (1)
WHO	3 (1)
Biphenotypic AML	1 (0)
Cytogenetics	
Favorable	19 (7)
Intermediate	131 (50)
Unfavorable	111 (43)
AML onset	
De novo	195 (75)
Secondary	66 (25)
Survival status	
Alive	66 (23)
Dead	145 (77)

Table 2. Association of genetic polymorphisms in ATP binding cassette proteins and overall survival in 261 AML patients at Roswell Park Cancer Institute, Buffalo, NY

Gene	SNP	Total N	Events N(%)	Unadjusted HR (95%CI)	Age Adjusted HR (95%CI)	*Final adjusted model HR(95%CI)
ABCB1	rs1045642					
	CC	79	53(29.44)	1	1	1
	CT	116	83(46.11)	1.04(0.74-1.48)	0.98(0.69-1.38)	1.04(0.73-1.47)
	TT	66	44(24.44)	0.92(0.61-1.37)	0.94(0.63-1.40)	0.90(0.60-1.35)
	rs2032582					
	GG	104	72(40.00)	1	1	1
	G/T	119	84(46.67)	0.93(0.68-1.28)	0.88(0.64-1.21)	0.89(0.65-1.23)
	TT	38	24(13.33)	0.96(0.60-1.51)	1.09(0.69-1.75)	1.06(0.66-1.70)
	rs1128503					
	CC	95	69(38.33)	1	1	1
	TC	127	85(47.22)	0.77(0.56-1.06)	0.76(0.55-1.04)	0.82(0.59-1.13)
	TT	39	26(14.44)	0.86(0.55-1.36)	1.09(0.69-1.72)	1.05(0.66-1.67)
ABCG2	rs2231142					
	CC	205	142(78.89)	1	1	1
	CA	53	35(19.44)	0.85(0.59-1.23)	0.83(0.57-1.20)	0.88(0.61-1.28)
	AA	3	3(1.67)	4.47(1.39-14.38)	3.00(0.93-9.68)	2.52(0.77-8.26)
	CA+AA	56	38(21.11)	4.61(1.44-14.80)	3.11(0.97-	2.57(0.79-8.42)
	rs2231137					
	GG	239	167(92.78)	1	1	1
	AA+AG	22	13(7.22)	0.57(0.33-1.01)	0.52(0.29-0.92)	0.44(0.25-0.79)
ABCC1	rs246221					
	TT	124	82(45.56)	1	1	1
	TC	109	78(43.33)	1.09(0.80-1.49)	1.09(0.80-1.49)	1.08(0.79-1.47)
	CC	28	20(11.11)	1.39(0.85-2.27)	1.35(0.83-2.21)	1.44(0.87-2.39)
	rs35587					
	TT	126	83(46.11)	1	1	1
	TC	108	78(43.33)	1.13(0.82-1.54)	1.11(0.81-1.51)	1.07(0.78-1.46)
	CC	27	19(10.56)	1.36(0.83-2.25)	1.33(0.81-2.19)	1.39 (0.83-2.33)
	rs2230671					
	GG	152	111(61.67)	1	1	1
	GA	94	56(31.11)	0.73(0.53-1.01)	0.75(0.54-1.04)	0.79(0.57-1.09)
	+AA	15	13(7.22)	1.02(0.57-1.82)	0.93(0.52-1.66)	0.87(0.49-1.55)
	GA+AA	109	69(38.33)	0.77(0.57-1.04)	0.78(0.58-1.05)	0.80(0.59-1.09)

*adjusted for age, cytogenetic and AML onset
Abbreviations: MDR1=multi drug resistance gene 1,BCRP=breast cancer resistance protein, HR=hazard's ratio, CI=confidence interval

to reduce the hazard of death. We found a significantly increased odds of toxicity associated with one copy of the variant allele in rs2231137 (G34A) when compared to wild type GG genotype (OR=8.41, 95%CI=1.10-64.28) after adjusting for confounders (results not shown).

Discussion

A large proportion of AML patients receiving chemotherapy has resistant disease or relapse following initial response due to drug resistance [11], which may be present intrinsically at diagnosis or develop secondarily [3, 11]. Identification of genetic variants associated with multidrug resistance is an important guide for tailoring chemotherapy to both improve treatment response and attenuate drug toxicity. Increased expression of ABC proteins is one of the several mechanisms of drug resistance [3] and we

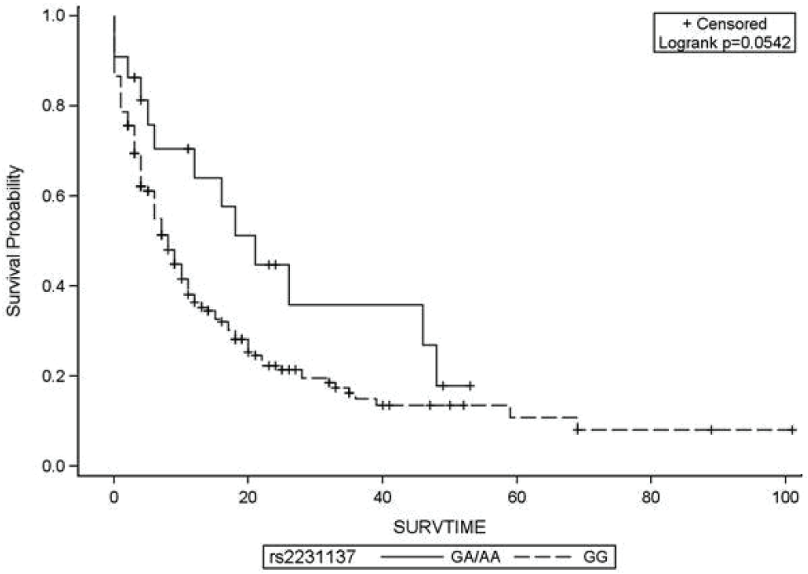

Figure 1. Kaplan Meier survival probabilities of 261 AML patients with single nucleotide polymorphism in ABCG2 gene (rs2231137). *G* and *A* refer to wild type and variant alleles, respectively. SURVTIME=survival time in months.

therefore investigated multi-drug resistance (MDR) genes including *ABCG2*, *ABCC1* and *ABCB1*.

Increased expression of *ABCG2 (BCRP)* has been found to be associated with drug resistance in cell lines such as MCF7 [12]. *ABCG2* is a half-transporter and requires dimerization or oligomerization to form an active transporter [13]. Higher levels of *ABCG2* mRNA have been shown to be associated with increased incidence of refractory or relapsed AML, indicating the functional role of *ABCG2* in AML survival outcome [12]. We found a significantly reduced hazard of death in patients with GA/AA genotype compared to those with GG genotype for the SNP rs2231137 (G34A; HR=0.44, 95% CI=0.25-0.79). The variant allele was associated with a favorable survival outcome. The rs2231137 (G34A) polymorphism results in valine to methionine substitution (V12M) in the amino acid sequence of the protein and findings across studies have been contradictory regarding its functional significance. For example, in a study using LLC-KP1 cells transfected with variant vectors, no significant difference in the levels of protein expression or transport activity of ABCG2 protein between wild type and G34A variant cell types was observed [14]. However it should be noted that protein expression is influenced by factors such as chromatin changes, methylation and acetylation [15] which might be difficult to evaluate in the controlled setting of an *in vitro* study. In another study, *ABCG2* mRNA expression was found to be significantly lower in liver tissue of Hispanics who carried the G34A variant genotype [16] potentially owing to alternative splicing of mRNA in G34A variant individuals compared to that in individuals with wild genotype [16]. While it is interesting to note that our findings are in line with the toxicity effects that would be expected if this polymorphism is functional and is actually associated with improved survival, it is important that the functional significance of rs2231137(G34A) be fully evaluated and that its clinical significance be evaluated in additional appropriately sized populations.

We found a significantly increased risk of grade 3 or more toxicity in patients with AA/AG genotypes for the SNP rs2231137(G34A) compared to GG homozygotes. Thus higher survival probability is associated with greater toxicity. Conversely, AML patients who do not have the mutant ABCG2 genotype, at rs2231137(G34A), have lower survival rates and lower risk of toxicity. *ABCG2* protein expressed in normal tissues of AML patients may reduce hematological and gastrointestinal toxicity associated with chemotherapy [15] while that expressed in leukemic stem cells may be associated with drug resistance.

While Imai et al found that rs2231142 (C421A) polymorphism in the *ABCG2* gene was associated with decreased protein expression and lower levels of drug resistance compared to wild type, the mRNA expression associated with both variants was the same; which might be sugges-

tive of post-transcriptional modification in mRNA [15]. Decreased protein expression associated with variant alleles should correlate with improved survival outcome. However, we found more than two-fold increased hazard of death in patients with CA/AA variant genotype compared to wild GG genotype of rs2231142(C421A), although not statistically significant. Imai et al included a Japanese population, while our study includes predominantly Caucasians. Allele frequencies vary greatly between different ethnic groups [17] perhaps indicating differing patterns of evolutionary pressure. Clearly further research is required to elucidate the effect of polymorphism in rs2231142 (C421A) on the levels of mRNA and protein expression levels in AML patients.

The study has some limitations. The external validity of our findings is limited to predominantly Caucasian patients. We did not adjust for multiple comparisons. However, since genes analyzed in this study have been identified to be involved in drug transport, our hypothesis had a strong biological basis and hence multiple comparison bias would not be a major issue in this study.

The analyses of both survival and toxicity in a well characterized cohort in this study were appropriately powered to detect pharmacogenetic effects. Significant effect of polymorphism in *ABCG2* gene on survival as well as drug toxicity in AML patients is an interesting finding that might lead to screening high-risk patients in order to plan chemotherapy. Larger studies are required to further elucidate this association.

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