Association between the concentration of imatinib in bone marrow mononuclear cells, mutation status of ABCB1 and therapeutic response in patients with chronic myelogenous leukemia

CHANG-XIN YIN*, WEI-WEI CHEN*, QING-XIU ZHONG*, XUE-JIE JIANG, ZHI-XIANG WANG, XIAO-DONG LI, JIE-YU YE, RUI CAO, LI-BING LIAO, FU-QUN WU, DAN XU, JIAN-SHENG ZHONG and FAN-YI MENG

Department of Hematology, Nanfang Hospital, Southern Medical University, Guangzhou, Guangdong 510515, P.R. China

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Abstract. Low concentrations of imatinib (IM) in bone marrow cells have been linked with poor prognosis in patients with chronic myeloid leukemia (CML), which may be caused by the emergence of ATP-binding cassette transporter B1 (ABCB1) mutations. The aim of present study was to investigate how clinical outcomes vary among patients with different single nucleotide polymorphisms (SNPs) of ABCB1. A total of 48 adult patients with CML and higher than median ABCB1 mRNA levels were selected for testing of ABCB1 SNPs. In 28 of the 48 patients, the IM concentration and expression levels of human organic cation transporter 1 (hOCT1) and ABCB1 in bone marrow mononuclear cells (BMMCs) were also tested. Correlations between treatment outcomes and IM concentration or the SNP status of ABCB1 were analyzed. Patients were classified by therapeutic response as major molecular response (MMR) (n=11), complete cytogenetic response (CCyR) (n=19) and non-CCyR (n=18) groups. It was found that the concentration of IM in BMMCs of the CCyR group was significant higher than that of the resistant groups (P=0.013). In addition, the IM concentration was positively correlated with the expression of hOCT1 mRNA (R=0.456, P=0.033), but negatively correlated with the expression of ABCB1 mRNA (R=-0.491, P=0.015). Furthermore, the mRNA expression level of ABCB1 was not associated with therapeutic response, but SNPs of the ABCB1 gene were associated with the response to IM. In conclusion, the concentration of IM in BMMCs may be regulated by the ABCB1 gene, and SNPs of the ABCB1 gene predict the therapeutic response to IM in patients with CML.

Correspondence to: Professor Fan-Yi Meng, Department of Hematology, Nanfang Hospital, Southern Medical University, 1838 Dadao North Street, Guangzhou, Guangdong 510515, P.R. China
E-mail: mengfu@medmail.com.cn

*Contributed equally

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Introduction

Imatinib (IM) was the first inhibitor of breakpoint cluster region-ABL proto-oncogene 1 (BCR-ABL1) to be used in the treatment of chronic myeloid leukemia (CML), and has revolutionized clinical outcomes in comparison with conventional chemotherapeutic treatment. In previous studies, the highest major cytogenetic response (MCyR) and complete cytogenetic response (CCyR) rates for IM were observed in newly diagnosed CML patients (1,2), and the 7-year progression-free survival rate has been observed to be >80% (3). On the basis of the data of the International Randomized Study of Interferon vs. STI571 (IRIS) trial, IM has become the first-line therapy for CML patients. However, 10-25% of patients appear to be resistant to IM. Mutations in the ABL1 kinase domain (4), a gene associated with drug transport dysfunction, are considered to be a cause of resistance to IM, which results in BCR-ABL1-positive cells staying alive through reduction of the intracellular concentration of IM. As reported previously, the response to IM is particularly associated with the concentration of IM in plasma and marrow cells (5). Furthermore, the concentration of a drug in cells is dependent upon the expression levels of transporter genes such as human organic cation transporter 1 (hOCT1) and ATP-binding cassette transporter B1 (ABCB1). However, clinical outcomes continue to differ among patients with high ABCB1 transcript levels (6). In the present study, the single nucleotide polymorphisms (SNPs) of ABCB1 and IM levels in bone marrow were analyzed, in order to investigate their relevance to the treatment response in patients treated with IM.

Patients and methods

Patients. A total of 48 patients with higher than median ABCB1 mRNA levels were selected for investigation from 90 patients with CML treated with IM in Nanfang Hospital (Guangzhou, China). In these 48 patients, the SNP status of ABCB1, including C1236T, C3435T and G2677T

Table I. Primer and probe sequences of genes.

Gene	Primer and probe sequences	Amplified fragment length (bp)
hOCT1	Forward: 5'-ACCGAAAAGCTGAGCCCTTC-3' Reverse: 5'-GAGCACAGAGTCCGTGAACC-3' Probe: 5'-(FAM)CAGACCTGTTCCGCACGCCGC (Eclipse)-3'	102
ABCB1	Forward: 5'-GAGGAAGACATGACCAGGTATGC-3' Reverse: 5'-AGCTGCCAGGCACCAAAATG-3' Probe: 5'-(FAM)TGAATGTAAGCAGCAACCAGCACCC (Eclipse)-3'	99
GAPDH	Forward: 5'-GGACCTGACCTGCCGTCTAG-3' Reverse: 5'- TAGCCCAGGATGCCCTTGAG -3' Probe: 5'-(FAM)CCTCCGACGCCTGCTTCACCACCT (Eclipse)-3'	99

hOCT1, human organic cation transporter 1; ABCB1, ATP-binding cassette transporter B1; GAPDH, glyceraldehyde-3-phosphate dehydrogenase.

polymorphisms, was determined, and for 28 of the 48 patients, the concentration of IM and expression level of BCR-ABL1 mRNA in the bone marrow mononuclear cells (BMMCs) were also observed. The BMMCs were taken from 48 patients during IM treatment, at the median time of 12 months (range, 6-80 months). Among all 48 patients, 19 cases obtained CCyR, 11 cases obtained major molecular response (MMR) and the remaining 18 cases obtained non-CCyR. The median age of the patients was 37 years (range, 13-67 years); 32 patients were male and 16 patients were female. All patients were diagnosed according to the National Comprehensive Cancer Network (NCCN) clinical practice guidelines for CML (7); 46 patients were diagnosed with chronic phase (CP) CML and 2 patients with accelerated phase (AP) CML. The median dosage and IM therapy course was 400 mg (range, 300-800 mg) and 12 months (range, 6-80 months). Any medications that may have affected the metabolism of IM had been avoided. Routine blood tests were performed monthly. The BCR-ABL1 fusion gene was examined by fluorescence in situ hybridization (FISH) every 3 months during the first year, and every 6 or 12 months after CCyR. The mRNA transcriptional level of BCR-ABL1 fusion gene was also measured by reverse transcription-quantitative polymerase chain reaction (RT-qPCR) at the same time points. Patients were classified by therapeutic response into MMR, CCyR, partial cytogenetic response (PCyR) and resistant groups according to the NCCN clinical guidelines (7).

Samples. All samples, including 3-5 ml heparinized bone marrow and blood specimens for routine blood tests, and analysis of liver and renal function, were collected 0.5 h prior to the administration of IM on an empty stomach in the early morning. BCR-ABL1 fusion gene was measured as described above, as a tumor marker. Infection and fever were ruled out prior to sample collection. The study was approved by the Ethics Committee of the Nanfang Hospital Affiliated to Southern Medical University and written informed consent was obtained from all patients.

Detection of intracellular IM concentration. Detection of IM concentration, the mRNA expression levels of BCR-ABL1, hOCT1 and ABCB1 was performed in 28 patients after the administration of IM for a median of 12 months (range, 6-80 months). BMMCs ($5x10^9$) were washed with saline following isolation, and then mixed with 1 ml healthy human plasma. The samples were kept in a refrigerator at -20˚C for detection. When required for measurement of the intracellular IM concentration, the BMMCs were thawed and centrifuged at speed of 2,400 x g for 10 min. The supernatant was collected and the IM concentration was measured using liquid chromatography in tandem with mass spectrometry on an API 4000 mass spectrometer (Applied Biosystems; Thermo Fisher Scientific, Inc., Waltham, MA, USA). STI571-D8 (07-407002; Merck Millipore, Darmstadt, Germany) was used as an internal reference and the range of detection was 2-10,000 μg/l.

Detection of the mRNA expression of hOCT1 and ABCB1 and gene polymorphism of ABCB1. Total RNA was extracted from the BMMC samples using TRIzol reagent (Invitrogen; Thermo Fisher Scientific, Inc.), according to the manufacturer's protocol. cDNA was synthesized from 1,000 ng total RNA using PrimerScript RT Reagent kit (RR037A; Takara Biotechnology Co., Ltd., Dalian, China). The expression of hOCT1 and ABCB1 was detected by RT-qPCR using a Premix *Ex Taq* (probe qPCR) (RR390; Takara Biotechnology Co., Ltd.). The primer and probe sequences of the genes are shown in Table I. PCR was performed using a 7500 Real-Time PCR System (Applied Biosystems; Thermo Fisher Scientific, Inc.) as follows: One cycle at 95˚C for 30 sec, followed by 40 cycles at 95˚C for 5 sec and 60˚C for 34 sec. GAPDH was used as a reference gene and the $2^{-\Delta\Delta Cq}$ method was used to quantify the data (8).

The C1236T and C3435T polymorphisms of ABCB1 were detected using PCR-restriction fragment length polymorphism, and the G2677T polymorphism was detected using sequence-specific primed PCR.

Statistical analysis. Data were analyzed as mean ± standard error, or as median (minimum-maximum) for non-normally distributed data. Nonparametric tests were performed using a χ^2 test. Correlation analysis between two variables were performed using Spearman rank analysis. Partial correlation analysis was employed for the analysis of bivariate correlation

Table II. Genotypes and alleles sequences of ABCB1 on C1236T according to therapeutic response [n(%)].

	Genotypes				Allele sequences		
Group	N	CC	CT	TT	N	C	T
Non-CCyR	18	2 (11.1)	8 (44.4)	8 (44.4)	36	12 (33.3)	24 (66.7)
CCyR	19	5 (26.4)	7 (36.8)	7 (36.8)	38	17 (44.7)	21 (55.3)
MMR	11	1 (9.1)	7 (63.6)	3 (27.3)	22	9 (40.9)	13 (59.1)

ABCB1, ATP-binding cassette transporter B1; CCyR, complete cytogenetic response; MMR, major molecular response.

Table III. Genotypes and alleles sequences of ABCB1 on C3435T according to therapeutic response [n(%)].

	Genotypes				Allele sequences		
Group	N	CC	CT	TT	N	C	T
Non-CCyR	18	10 (55.6)	6 (33.3)	2 (11.1)	36	26 (72.2)	10 (27.8)
CCyR	19	9 (47.3)	6 (31.6)	4 (21.1)	38	24 (63.2)	14 (36.8)
MMR	11	4 (36.4)	6 (54.5)	1 (9.1)	22	14 (63.6)	8 (36.4)

ABCB1, ATP-binding cassette transporter B1; CCyR, complete cytogenetic response; MMR, major molecular response.

Table IV. Genotypes and alleles sequences of ABCB1 on G2677T according to therapeutic response [n(%)].

	Genotypes				Allele sequences		
Group	N	GG	GT	TT	N	G	T
Non-CCyR	18	9 (50.0)	7 (38.9)	2 (11.1)	36	25 (69.4)	11 (30.6)
CCyR	19	6 (31.6)	10 (52.6)	3 (15.8)	38	22 (57.9)	16 (42.1)
MMR	11	3 (27.3)	6 (54.5)	2 (18.2)	22	12 (54.5)	10 (45.5)

ABCB1, ATP-binding cassette transporter B1; CCyR, complete cytogenetic response; MMR, major molecular response.

associated with multiple variables. Independent t-test or one-way analysis of variance was used to compare differences in 2 groups or more, respectively. Differences in non-normally distributed data were compared by Kruskal-Wallis test. All statistical analyses were performed using SPSS statistical software, version 13.0 (SPSS, Inc., Chicago, IL, USA) with the level of significance set at P<0.05 (2-tailed).

Results

Correlation of IM concentration in BMMCs with therapeutic response and with hOCT1 or ABCB1 expression. The median IM concentration in the BMMCs of the 28 patients was 8.22 (range, 2.38-111.00) μg/l. Significant differences were observed among groups with different therapeutic responses (χ^2=8.668, P=0.013). According to mean rank analysis, the IM concentration was the highest in the 14 cases in the CCyR group at 12.55 (2.43-90.40) μg/l; in the 9 cases in the resistant group the IM concentration was 2.73 (2.38~4.66) μg/l, and in the 5 cases in the PCyR group the IM concentration was 6.57 (2.61-111.00) μg/l.

The IM concentration was analyzed with respect to the mRNA expression levels of hOCT1 and ABCB1 in BMMCs at the same time point in the 28 patients. Using Spearman rank correlation analysis, the IM concentration was found to be positively associated with the mRNA expression of hOCT1 (R=0.456, P=0.033) and negatively associated with the mRNA expression of ABCB1 (R=-0.491, P=0.015).

Therapeutic response and ABCB1 gene polymorphism. In the 48 patents with higher than median expression levels of ABCB1 mRNA, which comprised 19 patients in the CCyR group, 11 patients in the MMR group and 18 patients in the non-CCyR group, the C1236T, C3435T and G2677T SNPs of ABCB1 were investigated. The genotypes and alleles are listed in Tables II-IV. No significant difference was found among

genotypes for the C1236T, C3435T and G2677T polymorphisms of ABCB1 in the CCyR, non-CCyR and MMR groups. However, in the non-CCyR group the frequency of T alleles was significantly higher than that of C alleles for C1236T (χ^2=4.00, P=0.046), while the frequency of C and G alleles was respectively higher than T alleles on C3425T (χ^2=7.111, P=0.008) and G2677T (χ^2=5.444, P=0.02).

Discussion

IM, a tyrosine kinase inhibitor, has been demonstrated to be effective and safe in a large number of patients since approval by the US Food and Drug Administration in May 2001 (9). The IRIS study with 8-year follow-up indicated that the CML-related-death free survival rate of patients in the de novo CP was 93%; relapse or side effects were rare in patients receiving IM treatment for >3 years (10). IM has been defined as the first-line therapy for CML by the NCCN since 2008 (11). However, the incidence of IM-related resistance is up to 15% in patients with CML-CP, and even higher in CML-AP or CML-blastic phase. This has created a new challenge for researchers: overcoming the resistance to IM. It has been reported that the most common cause of resistance to IM is a low drug concentration, as a result of insufficient dose of intake due to poor compliance or iatrogenic factors, reduced expression or activity of hOCT1 decreasing the retention of IM, elevated expression of ABCB1 increasing the excretion of IM (12) and specific gene mutations. Therefore, the present study was planned, with the aim of analyzing the influence of IM-concentration-related factors on therapeutic response.

It has previously been suggested that the minimum concentration (Cmin) of IM in plasma varies between individuals, and a minority of patients can reach CCyR with a Cmin <1,000 μg/l (13). Drugs in plasma have to enter a cell in order to exert a biological effect; thus the detection of intracellular IM concentration is a direct and useful method of evaluating its therapeutic response. The present study showed that the IM concentration in BMMCs was positively correlated with CCyR in 28 CML patients receiving the same dose of IM. This finding is consistent with findings from a previous study (14).

The intracellular IM concentration is regulated not only by the drug concentration in plasma, but also by drug transporters on the cell membrane, such as hOCT1 and ABCB1, and their activity (13). hOCT1 is highly expressed in hepatocytes to transfer drugs into cells under physiological conditions (15), and the gene is also expressed in other cells. In CML blasts, increases in the mRNA expression or activity of hOCT1 frequently result in a high probability of MMR and CCyR (16,17). The present study indicated that the association between hOCT1 expression and the therapeutic response was in accordance with the variation of intracellular IM concentration, which supports the conclusion reached by White *et al* in previous studies (14,16,17). The mRNA expression of hOCT1 in the PCyR group was lower than that in the resistant group; the limited number of cases may account for this. The main function of ABCB1 is to pump drugs out of cells, which plays an important role in the resistance to IM (18-20). ABCB1 induces the outflow of IM; thus, the expression and activity of ABCB1 remarkably influence the therapeutic effect (12,21-24). The present study indicated that the mRNA expression of ABCB1 in the resistant group was significantly higher than that in the CCyR and PCyR groups, which further confirmed the role of ABCB1 in IM resistance. In addition, it was also found that the IM concentration in BMMCs was positively associated with the mRNA expression level of hOCT1 and negatively associated with ABCB1, illustrating the impact of these two genes on the therapeutic response to IM.

ABCB1, a gene with a length 209 kb, is located on human chromosome 7q21.1-21.12 and encodes 1,280 amino acids by 28 exons (25). Studies on the SNPs of ABCB1 over the last 20 years have identified >50 SNPs on its exons, introns and core promoters. SNPs G2677T/A, C3435T and C1236T on exons 21, 26 and 12 are of great importance in the treatment of CML. The C1236T and C3435T mutations are silent mutations, while G2677T/A leads to amino acid substitution (26). The observation that the mRNA expression levels of ABCB1 varied among groups of patients with different therapeutic responses, suggests that the expression of ABCB1 may affect the therapeutic response and is associated with drug resistance. Nevertheless, some patients with high expression levels of ABCB1 achieved CCyR or even MMR. To investigate the mechanism, the SNPs of ABCB1 were further detected, and no significant difference in the genotypes of ABCB1 for C1236T, C3435T and G2677T polymorphisms among the non-CCyR, CCyR and MMR groups was found. However, the frequency of T alleles was significantly higher than that of C alleles for C1236T in the non-CCyR group, indicating that T alleles might be associated with a poor response to IM. By contrast, Dulucq *et al* (27) suggested that CML patients with a high frequency of T alleles were more likely to reach MMR. This discrepancy may be interpreted by the discrepancy of allele frequencies on this locus in patients of different ethnicities (28). Moreover, the present study found that the frequency of C alleles was significantly higher than that of T alleles on C3435T in the non-CCyR group, which indicated that the effect of IM was not as good in patients with C alleles as in those with T alleles. This result is consistent with that reported by Maffioli *et al* (29). However, it has been observed that the activity of ABCB1 is rather high in patients of TT genotype (30), which results in poor therapeutic response. It is uncertain that whether this distinction accords with racial differences. On G2677T, the frequency of G alleles on was significantly higher than that of T alleles in the non-CCyR group. A similar result was previously reported by Dulucq *et al* (27), and this previous study also found that patients of TT genotype had a more favorable therapeutic response and higher probability of MMR than patients of GG genotype.

Consensus has been made on the effectiveness and safety of IM in CML. However, the mechanism of drug resistance is complicated and varies among patients in clinical practice; therefore, it is hard to exclude the influence of other confounding factors in a single factor analysis. For patients resistant to IM, with the exception of testing for mutations of ABL1 and ruling out the poor compliance of patients, analysis of the gene polymorphism of ABCB1 and the mRNA expression of hOCT1 and ABCB1 in BMMCs could be a useful complementary method to rationalize the complexity, and improve the therapeutic response.

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