Correlation between the type of *bcr-abl* transcripts and blood cell counts in chronic myeloid leukemia – a possible influence of *mdr1* gene expression

Gueorgui Balatzenko,[1] Babu Rao Vundinti,[2] Guenova Margarita[3]

[1]Laboratory of Cytogenetics and Molecular Biology, National Specialized Hospital for Active Treatment of Hematological Diseases, Sofia, Bulgaria; [2]Institute of Immunohematology, ICMR, KEM Hospital, Parel, Mumbai, India; [3]Laboratory of Hematopathology and Immunology, National Specialized Hospital for Active Treatment of Hematological Diseases, Sofia, Bulgaria

Abstract

The impact of *BCR-ABL* mRNA type (b3a2 vs. b2a2) on chronic myeloid leukemia (CML) phenotype is still a subject of controversies. We searched for a correlation between the *BCR-ABL* transcripts type and CML patients' characteristics, including *MDR1* gene expression. Ninety-eight untreated chronic phase CML patients were studied. The type of *BCR-ABL* fusion transcripts and *MDR1* gene expression were determined by reverse transcriptase polymerase chain reaction. B3a2 and b2a2 transcripts were found in 53 [54%] and 44 [45%] patients, respectively. One patient co-expressed b3a2/b2a2 and was excluded from analysis. The only difference in the clinical characteristics between the two groups was the platelets count, that was higher in $b3a2^{(+)}$ patients [791.3±441.3×10^9/L vs. 440.4±283.4×10^9/L in $b2a2^{(+)}$; P=0.007]. *MDR1* over-expression [$MDR1^{(+)}$] was observed in 48 patients (49.5%), more frequently in older patients >60 years [71% (24/34) vs. 38% (24/63) in younger; P=0.008], and was associated with a lower white blood cells (WBC) count [105.5±79.8×10^9/L vs. 143.6±96.5×10^9/L in $MDR1^{(-)}$ cases; P=0.047]. On performing the analysis only within the $MDR1^{(+)}$ group, the $b_3a_2^{(+)}$ cases were characterized with a significantly higher platelets count [908.7±470.1×10^9/L vs. 472.9±356.1×10^9/L; P=0.006] and a lower WBC count [85.4±61.2×10^9/L vs. 130±93.9×10^9/L; P=0.004) compared to $b2a2^{(+)}$ patients. No similar differences were found between $b3a2^{(+)}$ and $b2a2^{(+)}$ groups with normal *MDR1* levels. These results indicate that the type of *BCR-ABL* transcripts correlates with the hematological parameters of CML, however only in the subgroup of patients characterized by *MDR1* over-expression.

Introduction

Chronic myeloid leukemia (CML) is characterized by the consistent involvement of the Philadelphia chromosome (Ph) and the *BCR-ABL* fusion gene, which derives from the reciprocal translocation t(9;22)(q34;q11) between chromosome 9 and 22. In almost all CML patients, the breakpoint in the *BCR* gene involves the Major breakpoint cluster region (M-*bcr*). The position of the breakpoint within the M-*bcr*, after exon b2 (e13) or exon b3 (e14) determines two main types of the fused *BCR-ABL* mRNA defined as b2a2 and b3a2 transcripts differing by 75 nucleotides. These transcripts encode two 210-kDa tyrosine kinase proteins ($p210^{BCR-ABL}$), which differ by 25 amino acids respectively.[1]

The impact of M-*bcr* breakpoint position on disease phenotype and its prognosis has been a subject of controversies for a long time. Several reports have suggested that the type of the chimeric mRNA (b2a2 or b3a2) is associated with differences in the clinical and hematological characteristics of CML patients and prognosis, despite that others failed to confirm any significant correlation.[2-6] One of the most interesting finding is the association of b3a2 type of fusion transcript with a higher platelet counts with some evidence in favor[7-9] and some against.[10-12] A great deal of the discrepancy or discordance between the various studies could be due to qualitative and/or quantitative differences in patient or sample selection.[12] However, the contradictory results might also be attributed to the presence of unknown biological factors, which can affect the obtained results. This hypothesis is supported by the observation of Shepherd *et al*.,1995 who failed to detect any significant difference in the presented features at diagnosis for patients with either b2a2 or b3a2 transcripts. However, in a subgroup of patients whose presenting white blood cell (WBC) count was <100×10^9/L, those with b3a2 transcript did have a significantly higher platelet count.[13] On the other hand, it has been reported that early chronic phase CML patients with very similar characteristics (i.e. thrombocytosis, WBC count <50×10^9/L, and age >50 years) were characterized with elevated levels of *Multidrug Resistance 1* (*MDR1*) gene expression.[14] Therefore, it might be possible, that the level of *MDR1* gene expression in the individual CML patients could influence the association between the different *BCR-ABL* transcripts and disease features.

MDR1 gene, located on chromosome 7q21 encodes a 170 κD membrane transporter P-glycoprotein [P-gp] that acts as an energy-dependent, efflux pump. Over-expression of *MDR1*/P-gp confers resistance to the cytotoxic effects of a broad range of structurally unrelated compounds (i.e. anthracyclines, epipodophyllotoxins and vinca alkaloids) and is one of the most common mechanisms of so-called *multidrug resistance* (MDR) in various malignancies. Over-expression of the *MDR1* gene has been found in a significant proportion of patients with different hematological malignancies and several observations suggest its poor prognostic value.[15]

The data concerning the incidence of *MDR1* over-expression in chronic phase CML patients are controversial ranging from total absence to presence in 20-65% and up to in 100% of patients.[14,16-20] Even less is known about the relationship of the *MDR1* levels to clinical data at presentation in CML.[14] The association between the *MDR1* levels and type of *BCR-ABL* transcripts to our knowledge has not been studied yet.

In this study we analyzed the correlations between the *BCR-ABL* mRNA types and CML patients' characteristics at presentation, including the possible impact of *MDR1* mRNA levels.

Correspondence: Gueorgui Balatzenko, Laboratory of Cytogenetics and Molecular Biology, National Specialized Hospital for Active Treatment of Hematological Diseases, 6, Plovdivsko pole Str., 1756 Sofia, Bulgaria.
Tel. +3592.9701137 - Fax: +3592 +9701.107.
E-mail: balatzenko@hotmail.com

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Materials and Methods

Patients

Ninety-eight untreated consecutive chronic phase CML patients (59 males and 39 females) with a mean age 50.5±14.1 years were included in this study. All patients signed informed consent form before entry on study. The diagnosis of CML was established on the basis of the peripheral blood parameters and morphological analysis of peripheral blood and bone marrow aspirates and confirmed by the presence of Philadelphia chromosome and/or fusion *BCR-ABL* gene by conventional cytogenetics and reverse transcription polymerase chain reaction (RT-PCR) respectively.

Methods

RNA extraction and complementary DNA (cDNA) synthesis

Bone marrow and/or peripheral blood samples were collected after obtaining informed consent at the time of CML diagnosis. Mononuclear cells were separated after lysis of red blood cells with a lysis buffer (155 mM NH4Cl, 10 mM KHCO3, 0.1 mM EDTA).[21] Total cellular RNA was isolated using Trizol Reagent (Invitrogen) according to the manufacturer's protocol. cDNA was synthesized by reverse transcription of 1 μg of RNA in a reaction medium with final volume of 20 μl containing: 1x first-strand buffer, 200 U MMLV reverse transcriptase (Thermo Scientific, ABGene, UK), 1 mM of each dNTPs, 20 U RNAsin (Promega) and 5 μM random hexamers (Roche Diagnostics), by consecutive incubation of the samples at 37°C for 1 hour and at 99°C for 3 minutes. Random hexamers instead of specific primers were used so that different PCR analyses could be performed on the same cDNA sample.

PCR assay for *BCR-ABL*

The presence of $P210^{BCR-ABL}$ rearrangement and the type of the respective fusion transcripts were determined by a single step RT-PCR using primers (ABL-a3B: gtttgggcttcacaccattcc/BCR-b1A: gaagtgtttcagaagcttctcc) and according to the protocol, recommended by the European BIOMED 1 Concerted Action for standardization of MRD studies in acute leukaemia.[22] Briefly, 5 μL of cDNA were amplified in a 50 μL medium containing 1x PCR buffer, 2.5 mM $MgCl_2$, 200 μM each of dNTPs, 1 U Taq polymerase (Invitrogen), and 400 nM of each of upstream and downstream primers. The mixture was incubated at 94°C for 30 sec; 65°C for 60 sec; 72°C for 60 sec with a total 35 cycles using Mastercycler Gradient (Eppendorf). Amplification products were run in a 2% agarose gel (Invitrogene) after staining with ethidium bromide, visualized after UV irradiation and photographed. For all RT-PCR reactions, K562 (b3a2) and/or BV173 (b2a2) cell lines were used as a positive control and RNA from healthy donors as a negative control.

PCR assay for *MDR1* gene expression

PCR was carried-out by simultaneous amplification of the *MDR1* and *β_2-microglobulin* (*β_2-M*) RNA, as an internal control. Briefly, 5 μl of cDNA were amplified in 50 μL medium, containing 1x PCR buffer, 1.5 mM MgCl2; 200 μM each of dNTPs; 1 U Taq polymerase (Promega) and 0.4 pM each of primers *MDR1*-TL9: ttcaaacttgtcacaatgcaga cagcagga / *MDR1*-TL10: ggttgcaggcctccatttataatggcacaa (for *MDR1*); together with B2-M1: acccccactgaaaaagatga/B2-M2: atcttcaaacctccatgatg primers (for *β2-M*) (Genset).[23] The reaction started with denaturation at 94°C for 5 min; proceeded with 25 cycles of amplification at 94°C for 30 sec; at 57°C for 30 sec; at 72°C for 30 sec; and terminated at 72°C for 10 min. Amplification products were run in a 3% agarose gel, stained with ethidium bromide and visualized after UV. The reaction conditions were chosen so that none of the RNAs analyzed reached a plateau at the end of the amplification procedure, i.e. when amplification proceeds exponentially at a constant efficiency and the yield of the RT-PCR product is proportional to the starting amount of the template. Therefore, no product of *MDR1* amplification was seen in CML samples with normal level of *MDR1* gene expression, as well as in samples from healthy donors used as negative controls. In these cases the only visible product of PCR was the intensive *band* corresponding to *β2-M* RNA (*MDR1*-negative). All samples that showed clearly visible product of *MDR1* amplification with intensity lower, equivalent or higher than *β2-M* product were considered as *MDR1*-positive (i.e. with over-expression). RNA from healthy volunteers was included as a negative control, and RNA from an acute lymphoblastic leukaemia (FAB L3 subtype) patient with manifested drug resistance and immunologically confirmed high level of P-gp expression was used as a positive control.

Strict precautions to avoid carryover contaminations were followed as recommended by Kwok & Higushi.[24]

Statistical analysis

The type of *BCR-ABL* transcripts and *MDR1* gene expression status were analyzed in correlation to age, sex and peripheral blood parameters (hemoglobin, leukocyte count and platelets). All statistical analyses were performed using the SPSS 9.0 software. The Wilcoxon Mann–Whitney test was used to compare the distributions of numerical-valued variables between patients with b3a2 and with b2a2 transcripts, as well as between subgroups of patients with normal and with elevated levels of *MDR1* gene expression. Univariate differences between categorical variables subsets were evaluated by Fisher's exact test. P-values of 0.05 or less were considered to be of statistical significance.

Results

In this study we analyzed the type of *BCR-ABL* transcripts and *MDR1* gene expression in 98 untreated adult patients with chronic phase CML and correlated the findings with clinical, hematological and molecular data (Table 1).

Types of *BCR-ABL* transcripts in correlation to basic patients' characteristics at presentation

RT-PCR was used to confirm the diagnosis of CML by detection of fusion *BCR-ABL* gene, and to reveal the type of transcripts depending on the M-*bcr* region breakpoint location. All studied patients were found to be *BCR-ABL* positive. Fifty-three of them expressed b3a2 transcripts [54%], whereas forty-four of the remaining cases were positive for b2a2 transcripts [45%]. Co-expression of both types of transcripts (b2a2 (+) b3a2) was observed in one patient [1%], who was excluded from further analysis. All patients were analyzed to determine the relationship between the type of transcripts and the presenting features at diagnosis including age, sex, white blood cells (WBC) count, platelets and hemoglobin (Hb) concentration. The platelets count was statistically higher in the subgroup of patients expressing b3a2, than those with b2a2 transcript (791.3±441.3×10^9/L vs. 440.4± 283.4×10^9/L; P=0.007). The remaining variables were not significantly different in both groups (b2a2 and b3a2) (P>0.05). (Table 2).

MDR1 expression in correlation to basic patients' characteristics at presentation

The pattern of *MDR1* amplification in CML

Table 1. Basic demographic and hematological features of patients included in the study.

Total number of patients [n=]	98
Gender	
Males [n=]	59 [60%]
Females [n=]	39 [40%]
Age	
Mean ± SD [years]	50.5±14.12
WBC	
Mean ± SD [×10^9/L]	125.2±90.4
Platelets	
Mean ± SD [×10^9/L]	615.8±406.3
Hemoglobin	
Mean ± SD [g/L]	111.7±27.0

patients varied considerably from case to case with a different intensity of the reaction ranging from negative to strongly positive. RT-PCR revealed moderate or strong positive reaction, corresponding to over-expression of *MDR1* gene [*MDR1*$^{(+)}$], in 48 out of 97 patients (49.5%). No product of *MDR1* amplification, corresponding to a normal level of *MDR1* expression [*MDR1*$^{(-)}$], was seen in the remaining 49 patients (50.5%).

The mean WBC count was significantly lower in *MDR1*$^{(+)}$ patients [105.5± 79.8×10^9/L vs. 143.6±96.5 ×10^9/L (P=0.047)in *MDR1*$^{(-)}$]. In addition, *MDR1* gene over-expression was significantly more frequent in elderly patients with age >60 years - 71% (24/34), compared to that in younger patients - 38% (24/63) (P=0.008), although, the mean age did not differ between the *MDR1*$^{(+)}$ and *MDR1*$^{-}$ patients (P>0.05). No significant differences in regard to sex, type of *BCR-ABL* transcripts, platelet count and hemoglobin concentration between the two groups of patients expressing either normal or elevated *MDR1* levels were found (P>0.05) (Table 3).

The impact of *MDR1* status of patients on the association between the types of *BCR-ABL* transcripts with basic characteristics at the presentation

In order to determine the impact of *MDR1* status of patients on the association of the type of transcripts with clinical and biological characteristics, both groups were additionally divided into subgroups with normal and elevated *MDR1* levels. The analysis of the b3a2 patients revealed a lower mean WBC count in *MDR1*$^{(+)}$ cases compared to the respective value in *MDR1*$^{(-)}$ patients [85.4±61.2×10^9/L vs. 150.8±86.6 ×10^9/L$^{(-)}$(P=0.004)]. However, no differences in the patients' characteristics were found in the b2a2 group according to the *MDR1* status. Appling the same approach by stratifying the patients according to the *MDR1* status, no significant differences in patients' characteristics were observed between b3a2 and b2a2 cases with normal *MDR1* levels (Table 4). In contrast, within the group with *MDR1* over-expression, the b3a2-positve cases were characterized with a significantly higher platelets count (P=0.006) and lower WBC count (P=0.004) compared to b2a2-positive patients (Table 4)

Discussion

Data concerning the association between the type of *BCR-ABL* transcripts and CML patient characteristics remain contradictory. Therefore, we compared the main clinical fea-

Table 2. Association between clinical and laboratory data and the type of fusion *BCR-ABL* transcripts for the 97 chronic myeloid leukemia patients.

Parameter	BCR-ABL transcripts		P
	b3a2	b2a2	
Patients [n=]	53 [55%]	44 [45%]	
Age			
Mean ± SD [years]	51.0 ±13.7	49.9± 4.8	NS
Gender			
Males [n=]	28 [53%]	25 [47%]	NS
Females [n=]	31 [70%]	13 [30%]	
WBC			
Mean ± SD [×10^9/L]	119.5±81.7	132.4±100.8	NS
Platelets			
Mean ± SD [×10^9/L]	791.3±441.3	440.4±283.4	0.007
Hemoglobin			
Mean ± SD [g/L]	116.0±19.1	107.9±32.5	NS

Table 3. Clinical parameters at diagnosis of CML patients in chronic phase expressing normal and elevated levels of *MDR1* gene.

Parameter	MDR1$^{(+)}$ patients	MDR1$^{(-)}$ patients	P
Patients [n=]	48 [49.5%]	49 [50.5%]	
Age			
Mean ± SD [years]	52.4±15.4	48.7±12.6	NS
Patients			
>60 years [n=]	24/34 [71%]	10/34 [29%]	0.008
<60 years [n=]	24/63 [38%]	39/63 [62%]	
Gender			
Males [n=]	27 [56%]	32 [65%]	NS
Females [n=]	21 [44%]	17 [35%]	
BCR-ABL transcripts			
b3a2 [n=]	25 [52%]	28 [57%]	NS
b2a2 [n=]	23 [48%]	21 [43%]	
WBC			
Mean ± SD [×10^9/L]	105.5±79.8	143.6±96.5	0.047
Platelets			
Mean ± SD [×10^9/L]	647.2±447.8	591.0±381.4	NS
Hemoglobin			
Mean ± SD	110.6±38.6	112.4±17.2	NS

Table 4. Clinical and laboratory data of patients with different types of fusion *BCR-ABL* transcripts and *MDR1* expression.

Parameter	MDR1(+) patients		P	MDR1(-) patients		P
	b3a2 [n=25]	b2a2 [n=23]		b3a2 [n=28]	b2a2 [n=21]	
Age: mean±SD [years]	53.0±15.2	51.6±16.0	NS	49.3±12.1	47.9±13.6	NS
Patients						
>55 years [n=]	12 [48%]	12 [52%]	NS	7 [25%]	5 [24%]	NS
<55 years [n=]	13 [52%]	11 [48%]		21 [75%]	16 [76%]	
Gender						
Males [n=]	11 [44%]	16 [70%]	NS	17 [61%]	15 [71%]	NS
Females [n=]	14 [56%]	7 [30%]		11 [39%]	6 [29%]	
WBC: mean±SD [×10^9/L]	85.4±61.2	130±93.9	0.004	150.8±86.6	134.6±109.5	NS
Platelets: mean±SD[×10^9/L]	908.7±470.1	472.9±356.1	0.006	727.3±434.0	403.8±189.1	NS
Hemoglobin: mean±SD [g/L]	123.5±18.6	103.1±46.2	NS	112.7±19.4	112.1±15.6	NS

tures of *BCR-ABL*-positive CML patients to the type of transcripts. The only variable, that showed significant difference between the two groups, was the platelet count, which was significantly higher in patients who expressed b3a2, providing additional evidence that b3a2 transcripts seem to be associated with a higher thrombopoietic activity in CML.[7-9]

In addition, the most interesting finding in our study was that the significant correlation between the elevated platelets count and b3a2 transcripts compared to b2a2 was restricted only to the subgroup of patients with *MDR1* over-expression, Moreover, the *MDR1* over-expression status outlined a significant association between the WBC count and the type of transcripts as we observed lower counts in the b3a2-positive cases compared to b2a2. No similar differences were found between b3a2- and b2a2-positive patients within the subgroup with normal *MDR1* levels.

A possible explanation is that the significant differences in the platelets and WBC count in patients with b3a2 and b2a2 transcripts only in patients with *MDR1* over-expression reflect a preferential activation of a common specific signaling cascade in the b3a2- positive cases, which is not active in the remaining patients. This might be related to variations in the level of *BCR-ABL* transcripts, since a dose-dependent hierarchy of *BCR-ABL* induced activation of signaling pathways and biological effects exists.[25] Moreover, it has been reported that b3a2 transcripts may affect the thrombopoietic activity in CML also in a dose-response manner.[7] Alternatively, it might be also possible that *MDR1* over-expression due to additional molecular abnormalities might interfere with differences in the structure of two $p210^{Bcr\text{-}Abl}$ proteins preferentially promoting the interaction of b3a2 with the cytoskeleton of megakaryocytes[8] resulting in the particular CML phenotype presented by thrombocytosis and relatively lower leukocytosis.

However, this hypothesis is only speculative and needs to be confirmed because the precise molecular mechanisms that underline an elevated *MDR1* expression in untreated CML patients are still unknown. In general, data concerning *MDR1* expression in CML are still insufficient and contradictory. Our study provides some interesting information in regard to the incidence of *MDR1* over-expression and the related patients' characteristics. We found elevated levels in approximately 50% of untreated patients. This result was in agreement with the data, reported earlier by Giles *et al.* (1999) and Weide *et al.* (1990), who using alternative methods revealed high levels of *MDR1*/P-gp expression in 57% and 41% of CML cases, respectively.[14,26] In our study, there were no differences in the prevalence of the different types of transcripts, as well as in the gender, platelets count and hemoglobin level between patients with normal and elevated levels of *MDR1*. However, the incidence of *MDR1* gene over-expression was significantly more frequent among the older patients. Similar results were observed not only in chronic phase CML patients,[14] but also in acute leukemias.[27,28] Moreover, an age-related increase of P-gp expression was found on peripheral blood cells of healthy individuals.[29] All these data suggest that common biological factors may contribute to the drug resistance in the elderly.

Additionally, we and others[14] found that *MDR1* over-expression in CML was associated with a lower WBC count compared to the patients with normal *MDR1* levels. Interestingly, in acute leukemias, the *MDR1* over-expression has been reported to be associated with hyperleukocytosis, both in AML[27] and ALL.[28] To our knowledge, no explanation for these differences has been reported so far.

Some observations suggest that the *MDR1* gene over-expression in CML might be one of multiple cellular alterations caused by the $p210^{BCR\text{-}ABL}$. Physiologically, the regulation of *MDR1* gene expression involves various signaling pathways (i.e. p53, SP1, NF-Y, AP-1, NF-kB, C/EBP and RAS).[30] A key role in this process plays Jun NH2-terminal protein kinase (JNK), which in response to stress-signals activates the heterodimeric AP-1 transcription factor, which in turn interacts with the *MDR1* gene promoters and enhances transcription.[31] Given that it has been shown that $p210^{BCR\text{-}ABL}$ may activate most of these pathways,[1] including JNK,[32] it seems possible that the hybrid oncoproteins may also affect *MDR1* expression in this manner.[31] This hypothesis is also supported by the observation that transduction of normal cells with b3a2 cDNA leads to upregulation of the expression of P-glycoprotein, the product of the *MDR1* gene.[33] Alternatively, the differences in the *MDR1* expression in CML patients might be related to defects in the methylation machinery as a distinct molecular pathway leading to malignant transformation[34] or might be a random event associated with nucleotide and/or haplotype variants of the *MDR1* gene, which seems to be important for interindividual differences of its expression.[35-38]

In conclusion, our preliminary results indicate that the type of *BCR-ABL* transcripts correlates with the hematological parameters of CML patients, however only in the subgroup of patients characterized by over-expression of *MDR1* gene. Further studies need to elucidate the molecular events underlying the relationship between the type of *BCR-ABL* transcripts and expression of *MDR1* gene in CML.

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