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Differential molecular response of the transcripts B2A2 and B3A2 to imatinib mesylate in chronic myeloid leukemia

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ABSTRACT. Chronic myeloid leukemia (CML) originates from the hematopoietic stem cell and is characterized by the reciprocal translocation t(9;22)(q34;q11), which results in the *BCR-ABL* fusion gene on chromosome 22q-, also known as the Philadelphia chromosome. This chimeric gene codes for a cytoplasmic protein with constitutive tyrosine-kinase activity, responsible for cellular transformation and leukemogenesis in CML. The aim of this observational cohort study was to discriminate and quantify *BCR-ABL* transcripts in the peripheral blood of patients with CML who were treated with imatinib mesylate (Glivec®, Novartis). Twenty-two patients were followed for six months during treatment. Quantitative real time polymerase chain reaction was performed before treatment and after 3 and 6 months from treatment initiation. As compared with the third month, there was a significant decrease in *BCR-ABL* expression in the sixth month of treatment (P = 0.0002). At the sixth month, there was a significant difference in the levels of the

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two major transcripts of *BCR-ABL*, B2A2 and B3A2 (P = 0.0347), indicating that B2A2 may be more sensitive to imatinib. The results of our study indicate that imatinib is able to modify the natural history of CML, and raise the hypothesis that patients who express the B2A2 transcript may have a better prognosis.

Key words: Chronic myeloid leukemia, Imatinib mesylate, RQ-PCR

INTRODUCTION

Chronic myeloid leukemia (CML) is classified by the World Health Organization (WHO) as a chronic myeloproliferative disease (CMPD), and occupies the first place in the first chapter of the WHO classification. There are some reasons why CML is the first disease in this classification. CML was the first hematological disease to be named leukemia, the first malignancy associated with a cytogenetic marker (the Philadelphia chromosome), and the first disease in which a fusion gene (*BCR-ABL*) was found in a chromosomal translocation. This latter gene gives rise to a fusion protein that plays a fundamental role in the pathogenesis of CML (Jaffe et al., 2001). CML has become the first disease to be successfully treated with a novel therapeutic agent, imatinib mesylate (Glivec®, Novartis). Imatinib was designed to inhibit the growth of *BCR-ABL*-positive cells (Druker et al., 1999, 2001). Although imatinib is highly efficient, bone marrow transplantation is currently the only known curative treatment for CML (Deininger et al., 2000).

Patients undergoing treatment for CML need to be monitored for response. Both hematological and cytogenetic responses have been used in the assessment of imatinib efficacy (Talpaz et al., 2002). Classic cytogenetic analysis is a good predictor of the outcome of imatinib-based therapy in CML, but it is laborious and has limited sensitivity. A good monitoring test should be sensitive, easy to perform, and able to be done in peripheral blood.

Accurate techniques to detect the *BCR-ABL* transcript in blood have been developed, and have allowed the diagnosis and monitoring of minimal residual disease in CML patients (Meissner et al., 1999; Hughes and Branford, 2003). Recently, many authors have described methods to measure *BCR-ABL* expression by competitive PCR (Campanini et al., 2001) and quantitative real time polymerase chain reaction (RQ-PCR) (Saffroy et al., 2000; Barbany et al., 2000; Amabile et al., 2001). The first report on the use of RQ-PCR for monitoring CML patients undergoing treatment with imatinib was published by Stentoft et al. (2001). These authors validated the use of RQ-PCR in the evaluation of treatment responses in this setting.

Although the different types of *BCR-ABL* transcripts (B2A2 and B3A2) can be detected and quantified (Amabile et al., 2001), the clinical value of transcript discrimination is controversial and has been questioned. Some authors have found that transcript types have no prognostic value (Meissner et al., 1999; Stentoft et al., 2001), and others have associated the B3A2 transcript with increased platelet counts (Perego et al., 2000; Rosas-Cabral et al., 2003).

In the current study, we used RQ-PCR to detect and quantify B2A2 and B3A2 transcripts in CML patients during treatment with imatinib, and we were able to demonstrate their differential expression during treatment.

MATERIAL AND METHODS

Patients

Twenty-two CML patients from the Ofir Loiola Hospital in the city of Belém, Brazil, were diagnosed by clinical and hematological criteria and had disease confirmation by reverse transcriptase (RT)-PCR for *BCR-ABL*. Of these 22 patients, 15 (68%) were in chronic phase, five (23%) in accelerated phase and two (9%) in blastic phase. Eleven patients (50%) were female, and 11 (50%) were male. The mean age was 44.5 ± 11.67 years (range, 23 to 70). We defined two age groups: patients with 40 years or less (45% of the cases), and patients older than 40 years (55%). The mean period between diagnosis and inclusion in the study was 3.5 ± 2.1 years (range, 6 months to 7.8 years). All patients had been previously treated with hydroxyurea and/or interferon-alpha. According to the recommendations by the Brazilian Health Ministry, all patients were treated with single-agent imatinib, after verification of interferon intolerance and/or failure; patients in chronic phase received 400 mg/day, and patients in accelerated or blastic phases were treated with 600 mg/day.

Study design

Peripheral blood samples were obtained at three different time points: day zero (DZ) before the treatment and at 3 (3M) and 6 months (6M) after the initiation of treatment.

RNA isolation

Total RNA was isolated from 100 µL of peripheral blood leukocytes with guanidinium isothiocyanate (Trizol LS™ - Invitrogen), according to the protocol provided by the manufacturer.

Polymerase chain reaction

RQ-PCR was performed in the ABI Prism 7000 (Applied Biosystems) thermocycler by the RT-PCR TaqMan® system (Applied Biosystems), according to the protocol provided by the manufacturer. Five microliters RNA was added to the reactions, which were incubated at 50°C for 2 min, 60°C for 30 min and 95°C for 10 min, followed by 50 cycles at 95°C for 15 s and 60°C for 1 min.

Primer and probe design

The following regions were amplified: exon A2 of the *ABL* housekeeping gene, as an endogenous control, and the junction *BCR-ABL*.

The follow primers and probes were used: i) Endogenous control *ABL*: cA2F (GGCCA GTAGCATCTGACTTTGA), cA2R (GTCCAGCGAGAAGGTTTTCCT) and cA2P (FAM-CCTCAGGGTCTGAGTGAAGCCGCTC-TAMRA). ii) Junction *BCR-ABL*: jB2F (TGACCA ACTCGTGTGTGAAACTC), jB3F (CCACTGGATTTAAGCAGAGTTCAA), jA2R (TCAGAC CCTGAGGCTCAAAGTC), and jA2P (VIC-AGCCCTTCAGCGGCCAGTAGCATC-TAMRA).

Measurement of *BCR-ABL* expression and interpretation

The quantification unit in the RQ-PCR is the cycle threshold (CT), which is the point in the cycle at which the log phase begins. This value was used to calculate the relative quantification. The measurement of *BCR-ABL* expression was defined as the ratio of *BCR-ABL* to *ABL*, according to the follow equation:

$$\frac{2^{-\mathrm{CT}(BCR\text{-}ABL)}}{2^{-\mathrm{CT}(ABL)}} \times 10^6$$

The quantification was thus defined as the number of copies of *BCR-ABL* per 10^6 copies of *ABL*, which was transformed to a logarithm for analysis and interpretation.

Statistical analysis

The data were divided into two groups: paired samples and independent samples. Analysis of variance (ANOVA) was used for the comparison of *BCR-ABL* expression in paired samples between the following time points: DZ and 3M, DZ and 6M, and 3M and 6M. The Student *t*-test was used for the analyses of independent samples, which were compared at the time points DZ, 3M and 6M for the following characteristics: sex (female and male), age groups (≤40 and older than 40 years), disease phase (chronic and accelerated/blastic), and transcripts (B2A2 and B3A2). In order to support the results of traditional statistical tests, bootstrapping was performed with 10,000 simulations for the *t*-test. P values <0.05 were considered significant.

RESULTS

The demographic and clinical characteristics of the 22 patients are shown in Table 1. All patients achieved complete hematological remission, and none of them underwent bone marrow aspiration for cytogenetic analysis.

Although mean *BCR-ABL* expression at the third month was slightly higher than on day zero, there was a significant decrease at six months (P = 0.0002), as shown in Figure 1. Most (73%) of the patients had a decreased expression of *BCR-ABL*, with a mean reduction of 1.73 log corresponding to approximately 80%; only 27% of the patients had an increased expression of *BCR-ABL* , with a mean of 1.67 log (Table 2).

Mean *BCR-ABL* expression was compared between categories according to sex, age group and phase of the disease at all time points, and no significant differences were found (P > 0.05). The same analysis was done between the transcripts B2A2 and B3A2, and a statistically significant difference was found for the levels of expression of transcripts B2A2 and B3A2 at six months (Figure 2 and Table 3).

DISCUSSION

Cytogenetic analysis remains an essential tool in the diagnosis of clonal evolution, but this method has a low sensitivity. RQ-PCR is a relatively simple technique that could become

Table 1. Patients' characteristics.

Patient	Sex	Age	Phase	Transcript
1	M	23	C	B2A2
2	F	46	C	B2A2
3	M	36	C	B3A2
4	M	41	C	B3A2
5	F	54	A	B3A2
6	F	45	C	B3A2
7	M	54	A	B3A2
8	M	51	A	B3A2
9	F	46	A	B3A2
10	F	37	B	B2A2
				B3A2
11	M	47	C	B3A2
12	M	70	C	B2A2
13	F	52	C	B2A2
14	M	49	C	B2A2
15	M	37	C	B3A2
16	M	38	C	B3A2
17	F	69	C	B3A2
18	F	48	C	B3A2
19	F	37	C	B3A2
20	F	38	C	B2A2
				B3A2
21	M	24	B	B2A2
22	F	36	A	B2A2

M = male, F = female, A = acute, B = blastic, C = chronic.

the method of choice for patient monitoring. RQ-PCR is very sensitive and also less invasive than cytogenetic analysis, given the fact that it is performed in peripheral blood.

To our knowledge, this is the first report on the differential expression of distinct *BCR-ABL* transcripts during imatinib treatment for CML.

We found a significant reduction in the expression of *BCR-ABL*, between the third and the sixth month of treatment (P = 0.0002), a finding that confirms the efficacy of imatinib in CML. Seventy-three percent of the patients had decreased levels of expression of *BCR-ABL*, with a mean reduction of 80% (1.73 log) in such levels. In the International Randomized Study of Interferon and STI-571 (IRIS) trial, Hughes et al. (2003) found that a 3 log reduction in the *BCR-ABL* expression predicts complete cytogenetic remissions at 12 months, given the fact that 57% of patients with these remissions had such reductions. In the present study, none of the patients had a reduction as profound as 3 log, probably because of previous reductions induced by hydroxyurea and/or interferon, and also because the analyses were limited to the first 6 months of treatment. Wang et al. (2003) reported that a reduction of 50% in *BCR-ABL* expression correlates with complete cytogenetic remissions after 6 months. Therefore, among most of patients (73%) in whom such reductions were observed in our study, only one would have had a lower likelihood of achieving a complete cytogenetic remission.

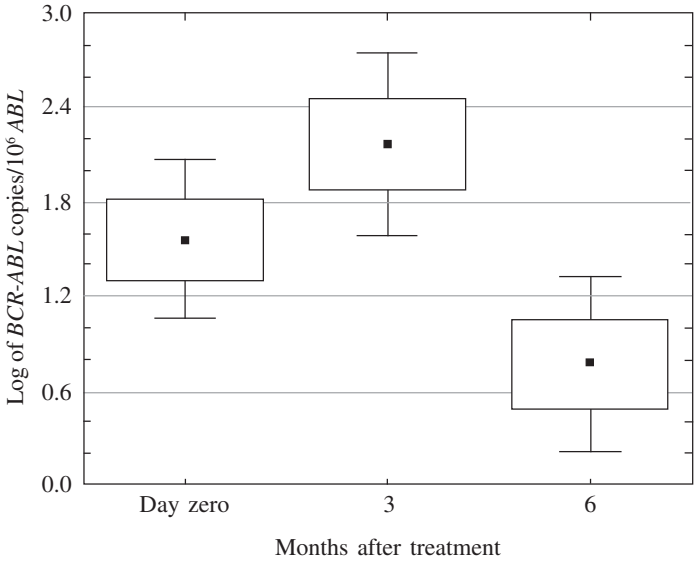

Figure 1. Mean *BCR-ABL* levels during treatment with imatinib: day zero (1.56 ± 0.84), third month (2.17 ± 0.97), sixth month (0.77 ± 0.93). Analysis of variance between the time points: day zero and 3 months (P = 0.0820); day zero and 6 months (P = 0.0182); 3 and 6 months (P = 0.0002).

Table 2. Variation of *BCR-ABL* expression during treatment.

BCR-ABL variation	% ± SD	Log ± SD
Decrease	80 ± 22	1.73 ± 0.73
Increase	380 ± 505	1.67 ± 1.38

We found increased expression of *BCR-ABL* transcripts after 6 months in 27% of our patients (Table 2). Three of these four patients had at least two-fold increases in *BCR-ABL* levels. This observation leads us to speculate that these patients may have had mutations in the kinase domain of *BCR-ABL*, with resistance to imatinib (Branford et al., 2004).

We believe that *BCR-ABL* expression should not be measured without discrimination of the various types of transcripts, since mutations and a differential kinetics between transcripts may influence response to imatinib. In this study, we found a slight difference in the mean expression of B2A2 and B3A2 transcripts at the sixth month of treatment (P = 0.0347). This significant difference was confirmed by bootstrapping (P = 0.0375).

The different *BCR-ABL* transcripts are undergoing active investigation for their roles as prognostic factors for survival and for their correlation with clinical aspects of CML. Some investigators believe such roles are still controversial (Rosas-Cabral et al., 2003), and others believe that the different transcripts have no prognostic value and no correlation with clinical characteristics (Udomsakdi-Auewarakul et al., 2000; Prejzner, 2002). However, it seems clear that B3A2 is associated with an increased platelet count, which indicates a possible influence of this transcript on thrombopoiesis (Shepherd et al., 1995; Melo, 1997; Perego et al., 2000), despite the lack of correlation with prognosis (Udomsakdi-Auewarakul et al., 2000).

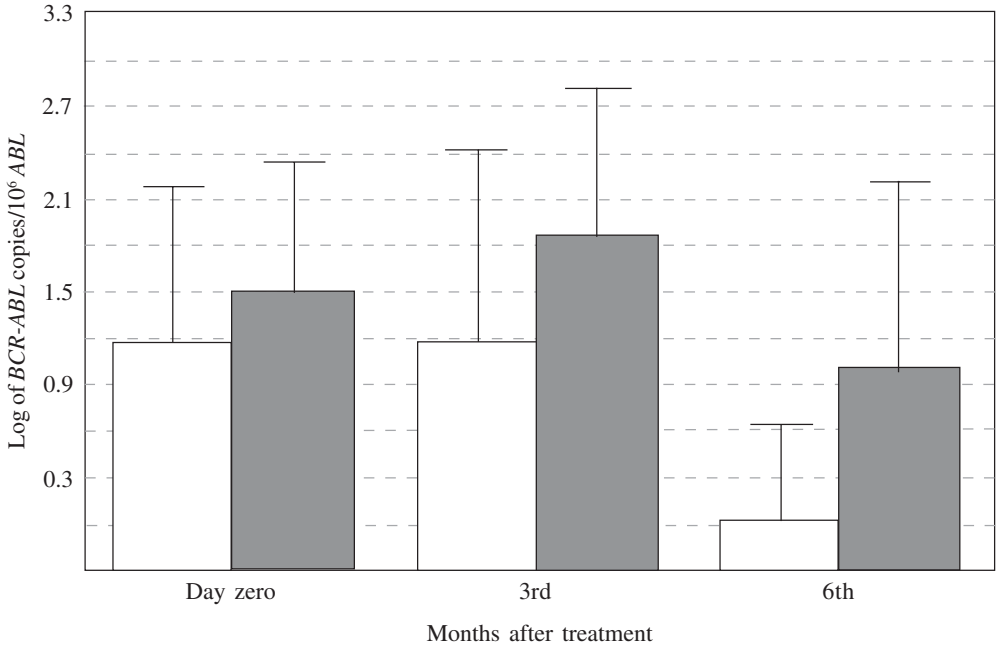

Figure 2. Difference between the levels of transcripts B2A2 (open columns) and B3A2 (filled columns) at three time points during treatment with imatinib: day zero and 3 and 6 months. There was a significant difference between the levels of both transcripts at 6 months (P = 0.0347).

Table 3. Mean levels of transcripts B2A2 and B3A2 (log of copies of *BCR-ABL*/10^6 *ABL*) at different time points during treatment with imatinib: day zero and 3 and 6 months.

Time point	Mean *BCR-ABL* levels		P value
	B2A2	B3A2	
Day zero	1.48	1.79	0.5291
3rd month	1.48	2.17	0.2712
6th month	0.33	1.32	0.0347
			0.0375*

*Bootstrap result.

There is no current indication that the different transcripts are associated with specific clinical features of CML. Nevertheless, our study raises the hypothesis that the natural history of CML may be modified during treatment with imatinib, given that the reduction in *BCR-ABL* levels may occur preferentially with the *B2A2* transcript; this preferential reduction occurred in spite of the similar levels of expression of B2A2 and B3A2 at the beginning of treatment (P = 0.5291). Thus, we believe that patients who express B2A2 should be studied further, and possibly be considered a subgroup with a better response to imatinib than patients who express B3A2 (Figure 2).

In this regard, it is important to bear in mind that 7 to 10% of patients display co-expression of B2A2 and B3A2. It is unlikely that such co-expression is due to two independent

chromosomal rearrangements, but rather to alternative splicing of a transcript that is originally of the B3A2 type (Avana-Trejo et al., 2002). Thus, not all patients with isolated expression of B2A2 may be considered potential candidates for response to imatinib, since B3A2 may be initially present at undetectable levels and increase during treatment, eventually replacing B2A2. In fact, a patient in our series seems to represent an example of this phenomenon: after 3 months of treatment, this patient appeared to be in complete molecular remission, but B3A2 was present at the sixth month.

Despite the hypothesis-generating character of our study, we believe that the main types of transcripts (B2A2 and B3A2) should be quantified in patients with CML who undergo treatment with imatinib. This quantification may predict the likelihood of achieving a cytogenetic response, since the presence of B2A2 seems to indicate a higher likelihood of response to imatinib. Our results notwithstanding, this hypothesis should be further investigated by the inclusion of larger numbers of patients.

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