Comparison of *BCR-ABL* Transcript Variants Between Patients With Chronic Myeloid Leukaemia and Leukaemia Cell Lines

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Abstract. *Background/aim:* Chronic myeloid leukaemia (CML) is a myeloproliferative disorder characterized by the presence of breakpoint cluster region-Abelson murine leukemia (*BCR-ABL1*) gene fusion as a hallmark that is expressed as two major transcripts *b2a2* and *b3a2*. The aim of this study was to compare the *BCR-ABL* transcripts in the blood cells of patients with CML, and in chemoresistant and chemosensitive CML cell lines to validate their use as a good method to elucidate CML biology. *Materials and Methods:* Twelve patients with CML and CML cell lines (K562, K562-LUCENA and FEPS) were analyzed by real-time polymerase chain reaction to evaluate gene expression of *BCR-ABL* transcripts. *Results:* All patients had the same expression levels of *b2a2* and *b3a3* transcripts, however, CML cell lines presented only *b3a2* expression. There were no significant differences in absolute *b3a2* expression between patients and CML cell lines. *Conclusion:* CML cell lines provide a good *in vitro* alternative in that they have the same *BCR-ABL* expression as patients.

Chronic myeloid leukaemia (CML) represents a relevant myeloproliferative disorder with an incidence of 1-2 cases per 100,000 adults, 15% of newly-diagnosed cases of leukaemia (1). Until about a decade ago, chemotherapy of CML was

limited to non-specific agents such as hydroxyurea, busulfan and interferon-alpha. However, after the elucidation of the pathogenesis of CML, targeted therapy was developed, dramatically changing the history of the disease. One study showed the improvement of treatment response from 34.7% in a group treated with interferon-alpha plus cytarabine to 87.1% in a group given imatinib mesylate, an inhibitor of breakpoint cluster region (*BCR*)-Abelson oncogene (*ABL*)1 (2).

The central pathogenesis of CML is characterized by a reciprocal translocation of chromosome 9 and 22. This translocation results in a shortened chromosome 22 called Philadelphia (Ph+) chromosome, which presents the fused gene of *BCR-ABL* (3). The fusion of *BCR* located on chromosome 22 and *ABL* located on chromosome 9 encodes an oncoprotein that has constitutive tyrosine kinase activity, which stimulates proliferation and resistance to cell death (4).

BCR-ABL1 transcript variants depend on the site of cleavage at the *BCR* gene. Patients with CML have breaks in the major region of the *BCR* gene, between exon 13 and 15, which fuses with exon 2 of *ABL* gene, resulting in *b2a2* (e13a2) and *b3a2* (e14a2) transcripts of *BCR-ABL1* gene, which encodes a oncoprotein with 210 kDa (5). Sharma *et al.* suggested that patients with CML with *b2a2* transcripts might have higher response to imatinib mesylate treatment when compared to those with *b3a2* transcript variant (6). Therefore, the identification of transcript type for treatment management is indispensable.

The use of cell lines in experimental oncology allows for the study of the development of new antineoplastic drugs, the identification of prognostic markers and their therapeutic efficacy (7). Thus, the detailed characterization of these cell lines is essential for their validation, in addition to facilitating the understanding of the resistance and sensitivity patterns of these chemotherapies, helping in the choice of more efficient therapies for patients (8).

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It is worth noting that several leukaemia cell lines have been helpful in the molecular characterization of the disease and biological features, as well as a tool for preclinical screening of new molecules for treatment. As an example, K562, K561-Lucena and FEPS cell lines have been used as important *in vitro* models for molecular characterization of *BCR-ABL1* and for compound screening for chemotherapeutic development to improve CML treatment (9-13).

Therefore, this study aimed to analyse the transcript variant expression of *BCR-ABL1* in chemosensitive and chemoresistant CML cell lines in comparison with patients with CML to validate the cell lines as an efficient tool for understanding the biological features of this disease.

Materials and Methods

Cell lines. Leukemia cell lines K-562, K562-Lucena and FEPS were kindly provided by Dr. Vivian Rumjanek (10, 11) and were cultured in RPMI 1640 medium supplemented with 10% foetal bovine serum (FBS) at 37°C and 5% CO₂ atmosphere, with regular passages twice a week. In the case of K562-Lucena and FEPS, the medium was supplemented with vincristine sulfate at a final concentration of 60 nM and daunorubicin at a concentration of 466 nM, respectively.

Patients. The blood samples evaluated in this study were obtained from 12 patients previously diagnosed with CML at the Walter Cantídio University Hospital (Fortaleza, Ceará) from October 2017 to October 2018, who are part of the biological bank of samples from the Laboratory of Pharmacogenetics of the Federal University of Ceará. All patients' samples were collected before any therapeutic intervention.

Ethics. The blood sample collections were performed after the approval of the Ethics and Research Committee under protocol number 2.439.294. Assurance was provided that the use of biological materials and participation in the study would not cause any harm to nor have any negative influence on patient treatment.

RNA extraction. RNA from patients' blood samples and from K-562, K562-Lucena and FEPS cell lines were extracted using TRIzol® (Life Technologies, Carlsbad, CA, USA) according to manufacturer's instructions and reversely transcribed to cDNA using High Capacity cDNA Reverse Transcriptase (Life Technologies) in a Verity® PCR System thermal cycler (Applied Biosystems®, Foster City, CA, USA).

Real-time quantitative reverse transcription (RT-qPCR). The genes selected for expression analysis in patients and in cell lines were *BCR-ABL1* transcripts: *b2a2* (Hs03024541_ft), and *b3a2* (Hs03024541_ft), with *ABL* (Hs 99999002_mH) being used as internal control. Such genes are commercially available as TaqMan® Gene expression assays (Life Technologies). RT-qPCR was performed using *QuantStudio5 Real-Time PCR* system (Applied Biosystems®). For each sample was used concentrations following: 3 µl of cDNA, 1 µl of each primer/probe, 12.5 µl of TaqMan® Gene Expression Master Mix (Life Technologies) and 8.5 µl of Ultrapure water. Each assay was performed at least three times according to Minimum Information for Publication of Quantitative Real-Time

PCR Experiments Guidelines (14). The gene-expression levels were based on absolute and relative analyses and calculated using the $2^{-\Delta\Delta CT}$ (delta-delta threshold cycle) method, the expression level of the gene of interest is reported relative to the reference gene for each sample (15).

Statistical analysis. All groups of samples were tested for normality using Kolmogorov-Smirnov test assuming 95% of confidence intervals, indicating that the samples were normally distributed. Results are graphically shown as the mean±standard deviation (SD) for patients and cell line samples. The differences between groups were compared using *t*-test or analysis of variance (ANOVA) followed by Bonferroni post-test, assuming 95% of confidence intervals.

Results and Discussion

BCR-ABL1 fusion gene is a hallmark of CML and a crucial driver of leukaemogenesis due to its constitutive tyrosine kinase activity, which contributes to cell proliferation, resistance to cell death and deregulation of cell differentiation (16). Therefore, advances in the understanding of the molecular basis of CML, including the *BCR-ABL1* pathway, are invaluable in improving the treatment and management of CML (17).

Regarding *BCR-ABL1* studies, leukaemia cell lines are important tools in this research area, helping to understand the pathways in microenvironments. However, it is still necessary to evaluate whether *BCR-ABL1* expression in CML cell lines can be extrapolated to patients with CML.

The patients' samples were submitted to analysis of *BCR-ABL1* transcript expression. Among 34 suspected samples, only 12 (35.3%) patients were diagnosed with CML by the presence of *BCR-ABL1* gene fusion. Seven out of the 12 patients were female. The median age was 40.5 years (ranging from 24 to 74 years). However, the worldwide incidence of CML shows a median age at diagnosis of 55 to 60 years, occurring with a slight predominance in males (18, 19). Other studies in Brazil have shown the median age at diagnosis to be at least 10 years lower than that found in the international literature (20, 21). Data from the present study corroborate a study by Bortolheiro and Chiattoni (22), where patients with disease refractory to interferon-alpha had a median age at diagnosis of 40 years.

Our results showed no significant differences of *BCR-ABL1* expression between male and female patients ($p=0.8329$), nor between patients aged 40 years or more and those less than 40 years old ($p=0.7430$), consistent with previous results obtained which also did not find significant differences (23, 24).

BCR-ABL1-negative CML occurs commonly in older patients (69 years) and men (57%) (23). Given the divergent data in the literature, age and gender would not be considered important factors in *BCR-ABL1* expression analysis, but this needs to be confirmed by a study with a

Figure 1. Absolute quantitative transcript levels of breakpoint cluster region (BCR)-Abelson murine leukemia (ABL1) *b3a2* and *b2a2* in 12 patients with chronic myeloid leukaemia. Absolute expression data are represented as the mean $\pm$ SD and groups were statistically analyzed by *t*-test. NS: Not significant.

high representative number of patients. Nevertheless, men and patients with a median age of 55 years tended to be more adherent to treatment with BCR-ABL1 inhibitors than did women and younger patients (25).

Another finding of our study is that patients with CML did not present the *b2a2* breakpoint variant, while six patients (50%) presented both *b2a2* and *b3a2* transcripts, and six patients (50%) presented only *b3a2* transcript expression. Moreover, grouping patients into *b2a2* and *b3a2* groups, the absolute expression levels were not significantly different between the two groups ($p=0.3940$).

The *b3a2* and *b2a2* transcripts essentially had the same level of expression in the patients (Figure 1). As mentioned, several studies have shown the importance of BCR-ABL1 transcripts in treatment response, and contradictory results have been demonstrated in different studies that analysed expression of BCR-ABL1 transcript variants, showing an advantage for patients with *b3a2*. Patients with *b3a2* are more likely to achieve favourable molecular major response (MMR) because they may have lower tyrosine kinase activity than those with *b2a2*, and thus be more sensitive to imatinib treatment (26-30). In an apparent contradiction, de Lemos *et al.* reported that *b2a2* patients had better MMR at 6 months (31), whereas another study showed no significant differences between patients with *b3a2* and *b2a2* in MMR at 6 months (32).

Despite this contradiction, we also tested the expression of BCR-ABL1 transcripts in leukaemia cell lines to show how representative they are for preclinical trials or BCR-ABL1 expression analysis. The presence of *b2a2* transcript was not detected in chemosensitive and chemoresistant CML cell lines, only *b3a2* transcript expression. However, as

Figure 2. Relative breakpoint cluster region (BCR)-Abelson murine leukemia (ABL1) *b3a2* transcript expression in the chemosensitive (K562) and chemoresistant (K562 Lucena-1 and FEPS) cell lines. Relative expression data are represented as mean $\pm$ SD and groups were statistically analysed by ANOVA followed by Bonferroni post-test.

Figure 3. Comparison of breakpoint cluster region (BCR)-Abelson murine leukemia (ABL1) *b3a2* transcript expression between patients and chemosensitive (K562) and chemoresistant (K562 Lucena-1 and FEPS) leukaemia cell lines. Absolute expression data are represented as the mean $\pm$ SD and groups were statistically analysed by ANOVA followed by Bonferroni post-test. NS: Not significant.

shown in Figure 2, the relative expression indicated that chemoresistant cell lines had low transcript level compared to the parental K562 cell line ($p<0.01$), which confirmed BCR-ABL1-independent resistance that often occurs in patients with CML, such as hyperexpression of membrane transporters (17). Indeed, Moreira *et al.* have already demonstrated that the resistance mechanism of K562-Lucena1 and FEPS were mediated by membrane transporter genes ATP-binding cassette sub-family B member 1 (ABCB1) and ATP-binding cassette sub-family C member 1 (ABCC1) (33).

In order to compare the *b3a2* transcript level between patients and leukaemia cell lines, absolute quantitative expression was performed. There were no significant differences of *b3a2* transcript level between patients and chemosensitive and chemoresistant CML cell lines (Figure 3).

As described before, many studies in the literature have not yet elucidated the clinical relevance of *b2a2* and *b3a2* transcripts in patients with CML and their influence on response to treatment, thus, the *b3a2* transcript expression data presented both in patients and in the cell lines analyzed in this study, demonstrate that the similarity between the models tested may be useful in drug screening regardless of the transcript expressed by the patient.

In agreement with the present study, work performed by Ferreira *et al.* (7) and Jaeger *et al.* (34), emphasize the importance of the use of cell lines as an *in vitro* model to study the different biological aspects of cancer. They also help in the identification of biomarkers, which play a key role in the early stages of drug screening, thus reducing costs with clinical trials.

In addition, although cell lines remain an excellent model for the study of mechanisms of resistance/sensitivity of chemotherapeutics already in use in the treatment of cancer, a study recently published by Ben-David *et al.* showed that established strains, considered clonal and genetically stable, are in fact highly heterogeneous (35). Cell characterization and standardization of culture conditions between laboratories to minimize the variability of results is essential.

Conclusion

The chemosensitive and resistant CML cell lines evaluated in this study, despite presenting only the *BCR-ABL1 b3a2* transcript, showed no difference in expression of this transcript when compared to patients. In summary, the data from this study validate the use of CML cell lines as an indispensable tool for validation of molecular studies, for understanding of biological mechanisms, as well as for the elucidation of mechanisms of resistance to chemotherapy and for screening of new anticancer drugs, mimicking the molecular characteristics that give rise to the tumour model of the disease *in vivo*.

Conflicts of Interest

The Authors declare no conflicts of interest regarding this study.

Authors' Contributions

Moreira-Nunes CA, Mesquita FP, Portilho AJS and Montenegro RC performed the study design; Sales LO and Portilho AJS performed the cell culture analysis; Sales LO, Mesquita FP, Portilho AJS and Moreira-Nunes CA performed the molecular analysis; Mesquita FP, Moraes-Filho MO, Moraes MEA, Montenegro RC and Moreira-Nunes CA wrote the article. All Authors read and approved the final article.

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