Abstract. Background/aim: Chronic myeloid leukaemia (CML) is a myeloproliferative disorder characterized by the presence of breakpoint cluster region–Abelson marine 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 characterized by 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 fusioned 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 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).

This article is freely accessible online.

*These Authors contributed equally to this work.

Correspondence to: Caroline Aquino Moreira-Nunes, Laboratory of Pharmacogenetics, Drug Research and Development Center, Federal University of Fortaleza, Coronel Nunes de Melo st, n 1000, Rodolfo Teófilo, CEP: 60416-000 Fortaleza, CE, Brazil. E-mail: carolfam@gmail.com

Key Words: CML, leukaemia, BCR–ABL, cell lines.
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 Rumiñak (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 Cantidio 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 TRIzool® (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−ΔΔCT (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 Chiattone (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.
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 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-Lucena-1 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 \textit{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–ABL \(b3a2\) 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 \textit{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.

**Acknowledgements**

This study was supported by Brazilian funding agencies National Counsel of Technological and Scientific Development (CNPq; to CAMN, RCM, MEAM, MOMF and FPM) and Coordination for the Improvement of Higher Education Personnel (CAPES; to CAMN and AJSP).

**References**

1. Jabbour E and Kantarjian H: Chronic myeloid leukemia: 2014 update on diagnosis, monitoring, and management. Ann Clin Upd Hematol Malign 89: 547-556, 2014. PMID: 24727196. DOI: 10.1002/ajh.23691
2. O’Brien SG, Guilhot F, Larson RA, Gathmann I, Baccarani M, Cervantes F, Cornelissen JJ, Fischer T, Hochhaus A, Hughes T, Lechner K, Nielsen JL, Rousselot P, Reiffers J, Saglio G, Shepherd J, Simonsson B, Gratwohl A, Goldman JM, Kantarjian H, Taylor K, Verhoef G, Bolton AE, Capdeville R and Drucker BJ: Imatinib compared with interferon and low-dose cytarabine for newly diagnosed chronic-phase chronic myeloid leukemia. N Engl J Med 348: 994-1004, 2003. PMID: 12637609. DOI: 10.1056/NEJMoa022457
3. Rowley JD: Letter: A new consistent chromosomal abnormality in chronic myelogenous leukaemia identified by quinacrine fluorescence and Giemsa staining. Nature 243: 290-293, 1973. PMID: 4126434. DOI: 10.1038/243290a0
4. Pophali PA and Patnaik MM: The role of new tyrosine kinase inhibitors in chronic myeloid leukemia. Cancer JI 22: 40-50, 2016. PMID: 26841016. DOI: 10.1097/PP000000000000165
5. Deininger MW, Goldman JM and Melo JV: The molecular biology of chronic myeloid leukemia. Blood 96: 3343-3356, 2000. PMID: 11071626.
6. Sharma P, Kumar L, Mohanty S and Kochupillai V: Response to Imatinib mesylate in chronic myeloid leukemia patients with variant BCR–ABL fusion transcripts. Ann Hematol 89(3): 241–247, 2009. PMID: 19714331. DOI: 10.1007/s00277-009-0822-7
7. Ferreira D, Adega F and Chaves R: The importance of cancer cell lines as \textit{in vitro} models in cancer methyloene analysis and anticancer drugs testing. Oncogenecism and cancer proteomics-novel approaches in biomarkers discovery and therapeutic targets in cancer. First ed. InTechOpen, 2013. DOI: 10.5577/21925
8. Sharma SV, Haber DA and Settelman J: Cell line-based platforms to evaluate the therapeutic efficacy of candidate anticancer agents. Nat Rev Cancer 10(4): 241-253, 2010. PMID: 20301065. DOI: 10.1038/nrc2820
9. Uphoff CC, Habig S, Fombonne S, Matsuo Y and Drexler HG: ABL–BCR expression in the BCR–ABL-positive human leukaemia cell lines. Leuk Res 23: 1055-1060, 1999. PMID: 10576511. DOI: 10.1016/S0145-2126(99)00131-9
10. Rumjanek VM, Trindade GS, Wagner-Souza K, de-Oliverira MC, Marques-Santos LF and Maia RC: Multidrug resistance in tumour cells: Characterisation of the multidrug resistant cell line K562-Lucena 1. Anais Acad Brasil Ciências 73(1): 57-69, 2001. PMID: 11246270. DOI: 10.1590/S0001-37652001000100007
11. Rumjanek VM, Vidal RS and Maia RC: Multidrug resistance in chronic myeloid leukaemia: How much can we learn from MDR–CML cell lines? Biosci Rep 33(6): 875-888, 2013. PMID: 24070327. DOI: 10.1042/BSR20130067
Ma L, Roderick J, Kellieher MA and Green MR: High-throughput screening of tyrosine kinase inhibitor resistant genes in CML. Meth Mol Biol 1465: 159-173, 2016. PMID: 27581147. DOI: 10.1007/978-1-4939-4011-0_14

Daflon-Yunes N, Pinto-Silva FE, Vidal RS, Novis BF, Berguetti T, Lopes RR, Polycarpo C and Rumjanek VM: Characterization of a multidrug-resistant chronic myeloid leukemia cell line presenting multiple resistance mechanisms. Mol Cell Biochem 383(1-2): 123-135, 2013. PMID: 23877223. DOI: 10.1007/s11010-013-1761-0

Bustn SA, Benes V, Garson JA, Helleman J, Huggett J, Kubista M, Mueller R, Nolan T, Piffall MW, Shipley GL, Vandesompele J and Wittwer CT: The MIQE Guidelines: Minimum information for publication of quantitative real-time PCR experiments. Clin Chem 55(4): 611-622, 2009. PMID: 23570709. DOI: 10.1373/clinchem.2013.206575

Schmittgen TD and Livak K: Analyzing real-time PCR data by the comparative Ct method. Nat Prot 383(1): 1451-1458, 2008. PMID: 19713230. DOI: 10.3324/haematol.2009.009134

Hanfstein B, Lauseker M, Hehlmann R, Sausselle S, Erben P, Dietz C, Fabarius A, Proetel U, Schmittgen TD, Hafeler C, Krause SW, Schubert J, Einsele H, Hanel M, Dengler J, Fulge C, Kanz L, Neubauer A, Kneba M, Stegelmann F, Pierendreich M, Waller CE, Gsiekermann K, Baerlocher GM, Pfirrmann M, Hasford J, Hofmann WK, Hauchaus A, Muller MC, SAKK and German CML Study Group: Distinct characteristics of BCR–ABL-positive chronic myeloid leukemia: The EUTOS population-based registry: incidence and clinical characteristics of 2904 CML patients in 29 European Countries. Leukemia 29(6): 1226-1231, 2015. PMID: 25006280. DOI: 10.4103/0971-5851.133707

Castagnetti F, Gugliotta G, Breccia M, Iurlo A, Levato L, Bocchia M, Soverini S, Berti P, Cervantes F, Phillips LS, Gugliumi LM, Peggio S, Cantini S, Dalla-Favera R, Rossi G, Rupoli S, Cavazzini F, Dietz C, Fabarius A, Proetel U, Schmittgen TD, Hafeler C, Krause SW, Schubert J, Einsele H, Hanel M, Dengler J, Fulge C, Kanz L, Neubauer A, Kneba M, Stegelmann F, Pierendreich M, Waller CE, Gsiekermann K, Baerlocher GM, Pfirrmann M, Hasford J, Hofmann WK, Hauchaus A, Muller MC, SAKK and German CML Study Group: Distinct characteristics of e13a2 BCR–ABL fusion transcript have inferior responses to imatinib compared to patients with the e14a2 transcript. Haematologica 94(4): 1362-1367, 2009. PMID: 19713230. DOI: 10.3324/haematol.2009.009134

De Oliveira Sales et al.: BCR-ABL Transcript Analysis in CML: Patients and Cell Lines
32 Polampalli S, Choughule A, Negi N, Shinde S, Baisane C, Amre P, Subramanian PG, Gujral S, Prabhash K and Parikh P: Analysis and comparison of clinicohematological parameters and molecular and cytogenetic response of two BCR/ABL fusion transcripts. Genet Mol Res 7: 1138-1149, 2008. PMID: 19048492.

33 Moreira MA, Bagni C, de Pinho MB, Mac-Cormick TM, dos Santos Mota M, Pinto-Silva FE, Daflon-Yunes N and Rumjanek VM: Changes in gene expression profile in two multidrug-resistant cell lines derived from a same drug sensitive cell line. Leuk Res 38(8): 983-987, 2014. PMID: 24996974. DOI: 10.1016/j.leukres.2014.06.001

34 Jaeger S, Duran-Frigola M and Aloy P: Drug sensitivity in cancer cell lines is not tissue-specific. Mol Cancer 14(1): 40, 2015. PMID: 25881072. DOI: 10.1186/s12943-015-0312-6

35 Ben-David U, Siranosian B, Ha G, Tang H, Oren Y, Hinohara K, Strathdee CA, Dempster J, Lyons NJ, Burns R, Nag A, Kugener G, Cimini B, Tsvetkov P, Maruvka YE, O’Rourke R, Garrity A, Tubelli AA, Bandopahayay P, Tsherniak A, Vazquez F, Wong B, Birger C, Ghandi M, Thorner AR, Bittker JA, Meyerson M, Getz G, Beroukhim R and Golub TR: Genetic and transcriptional evolution alters cancer cell line drug response. Nature 560(7718): 325-358, 2018. PMID: 30089904. DOI: 10.1038/s41586-018-0409-3

Received March 25, 2019
Revised April 26, 2019
Accepted April 30, 2019