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Determination of lactate dehydrogenase (LDH) and *Bcr-Abl* transcript in the follow-up of patients with chronic myeloid leukemia

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ABSTRACT. Chronic myeloid leukemia (CML) is a malignant myeloproliferative disorder that originates from a pluripotent stem cell characterized by abnormal release of the expanded, malignant stem cell clone from the bone marrow into the bloodstream. The vast majority of patients with CML present *Bcr-Abl* transcripts. Lactate dehydrogenase (LDH) is considered a biochemical marker common for tumor growth, anaerobic glycolysis and has been considered a poor prognostic factor for acute myeloid leukemia. Therefore, this study aimed to evaluate the concentration of LDH in plasma and the detection of the *Bcr-Abl* transcripts in patients with CML and healthy donors. We analyzed 22 patients demonstrably diagnosed with CML and 56 healthy donors. LDH concentration in plasma was higher in patients with CML. All patients with CML in this study were under treatment, but even so four patients had the *Bcr-Abl* (b3a2) transcript in peripheral blood. Two out of the four patients with b3a2 showed higher LDH (486 U L^{-1} and 589 U L^{-1}). Thus, although the study was conducted with small numbers of samples, it is possible to suggest therapy alteration for two patients who presented transcript b3a2 in the peripheral blood samples and whose LDH concentration was high, in order to improve the disease.

Key words: chronic myeloid leukemia, lactate dehydrogenase, *Bcr-Abl*.

RESUMO. Determinação da lactate desidrogenase (LDH) e do transcrito *Bcr-Abl* em pacientes com leucemia mielóide crônica. Leucemia mieloide crônica (LMC) é uma desordem mieloproliferativa maligna que é originada de célula-tronco pluripotente caracterizada por expansão anormal, maligna de clones de células tronco da medula óssea na circulação. A grande maioria dos pacientes com LMC apresentam transcritos *Bcr-Abl*. Lactato desidrogenase (LDH), considerado um marcador bioquímico para crescimento tumoral, glicólise anaeróbica, e tem sido considerado um fator de pior prognóstico da LMC. Portanto, este estudo visa avaliar a concentração de LDH no plasma e a detecção do transcrito *Bcr-Abl* em 22 pacientes com LMC e 56 indivíduos saudáveis. Foram avaliados 22 pacientes com LMC e 56 doadores saudáveis. A concentração de LDH no plasma foi maior nos pacientes com LMC. Todos pacientes com LMC neste estudo estavam em tratamento, mesmo assim quatro pacientes apresentavam o transcrito *Bcr-Abl* (b3a2) no sangue periférico. Dois dos quatro pacientes com o transcrito b3a2 apresentavam LDH elevado (486 U L^{-1} e 589 U L^{-1}). Embora o estudo tenha sido realizado com um pequeno número de amostras, é possível sugerir alteração de terapia para os dois pacientes que apresentam o transcrito b3a2 na amostra de sangue periférico com concentração de LDH elevada.

Palavras-chave: leucemia mieloide crônica, lactato desidrogenase, *Bcr-Abl*.

Introduction

Chronic myeloid leukemia (CML) is a myeloproliferative disease that originates in an haemopoietic stem cell as a result of t (9; 22) translocation, giving rise to the Ph (Philadelphia chromosome) and *Bcr-Abl* oncoprotein. The disease starts in a chronic phase, but as a result of genomic instability, it progresses over time to the accelerated phase and then to blast crisis, becoming increasingly resistant to therapy. The *Bcr-Abl* transcript is a constitutively active tyrosine kinase that has been targeted by tyrosine kinase inhibitors, including IM (imatinib mesylate), nilotinib and dasatinib (JORGENSEN; HOLYOAKE, 2007).

The successful development of imatinib as a therapeutic agent for CML can be attributed directly

to decades of scientific discoveries. These discoveries determined that the *Bcr-Abl* tyrosine kinase is the critical pathogenetic event in CML and an ideal target for therapy. This was confirmed in clinical trials of imatinib, with imatinib significantly improving the long-term survival of patients with CML. Continuing in this tradition of scientific discoveries leading to improved therapies, the understanding of resistance to imatinib has rapidly led to strategies to circumvent resistance (DRUKER, 2008)

The molecular pathogenesis of CML is well understood, but the mechanism that leads to gene translocation is unknown. Genetic instability as a consequence of the *Bcr-Abl* fusion might be responsible for additional chromosomal aberrations or mutations frequently seen in blast crisis (HUNTLY et al., 2002).

Myelofibrosis is a clonal myeloproliferative disorder, and osteosclerosis is the most frequently observed bone change in myelofibrosis. Jurisic et al. (2008) demonstrated an atypical case of leukemic transformation in myelofibrosis associated with diffuse osteolytic lesions and extremely elevated TNF-alpha and lactate dehydrogenase (LDH).

Zhelyazkova et al. (2008) used a complex angiogenic assessment and determined an increased angiogenesis in chronic myeloid leukemia patients. No prognostic relevance was found for vascular endothelial growth factor plasma levels or VEGF/KDR cellular bone marrow expression. The increased cellular hepatocyte growth factor and MET expressions could be considered high-risk factors for these patients. Plasma hepatocyte growth factor and marrow micro vessel density were shown to be independent prognostic parameters for patient survival. The plasma hepatocyte growth factor correlated with all markers reflecting the tumor burden (leucocytes, blast percentage, splenomegaly and LDH) as well as with the phase of chronic myeloid leukemia and overall survival of the patients.

The present study investigated LDH concentrations and presence of *Bcr-Abl* transcript (b3a2) in the plasma from treated CML patients compared with healthy donors.

Methodology

Patients and methods

Patients

Following approval from the Human Ethics Committee of the State University of Londrina, peripheral blood was collected from 22 patients with clinical and hematological diagnosis for chronic myeloid leukemia. All patients were attended in the Cancer Institute of Londrina (ICL), Paraná, Brazil. Samples of 56 normal blood donors were obtained from University Hospital of the State University of Londrina, Paraná State, Brazil. A term of free informed consent was signed by all sample donors and researchers involved prior to blood collection.

Molecular analysis of Beta-actin mRNA

Reverse transcriptase reaction and polymerase chain reaction - Beta-actin was carried out in accordance with Amarante et al. (2005). Amplicons of 353 bp for β-actin were detected. When contaminants of genomic DNA occur, the amplification product will be up to 573 bp. In all reactions 573 bp band were not verified in the gel, indicating no contamination with genomic DNA.

Molecular analysis of *bcr-abl* mRNA

Nucleic acid preparation and reverse transcriptase reaction. Leukocytes were prepared from peripheral blood samples after using eritrocytes lysis buffer B (solution A: 0.32 M sucrose; 10 mM Tris-HCl pH-7.5; 5 mM $MgCl_2$; 1% Triton X-100). Total cellular RNA was extracted from peripheral white blood cells with Trizol (TRIzol LS – *Invitrogen*) according to the manufacturer's instructions. The RNA was resuspended in 20 μL of sterile water treated with diethylpyrocarbonate (DEPC, *Invitrogen*). cDNA was generated from 7 μL of total RNA, using specific outer anti-sense primer and a first strand cDNA synthesis kit (*Perkin Elmer GeneAmp RNA PCR kit - Part number N8 08-0017*) .

Polymerase Chain Reaction (nested) - *Bcr-abl*. The oligos were designed based on their sequence (*GenBank accession number* AJ131466) and were targeted to amplify the *Bcr-abl* gene (LM1, LM2, LM3, LM4) following reaction conditions by Oliveira et al. (2007). Amplicons of 328 pairs were analyzed by electrophoresis on a 10% acrylamide gel visualized by silver staining. A negative control (water instead of cDNA) was included in all reactions from this work. In cases where no amplification was obtained, the cDNA was tested by PCR with Beta-actin oligos, as a control, to assess the integrity of RNA molecule.

Quantitative determination of lactic dehydrogenase activity

For LDH activity determination was used the Dimension® (DADE Behring, Newark, USA) clinical chemistry system. The lactic dehydrogenase method is a modification of the enzymatic lactate to pyruvate procedure modified

by Gay et al. (1968). Reference interval at 37°C is 100 – 190 U L^{-1}.

Statistical Analysis

Statistical analysis was conducted with Student's t-test using the Scientific Graphing and Analysis Software OriginPro 8.0 (OriginLab, Northampton, MA, USA). A $p < 0,05$ value was considered statistically significant.

Results

In the present study, 22 patients were assayed (10 women and 12 men) with CML, from northern Paraná State, Brazil and 56 normal (32 women and 24 men) healthy donors. These were healthy control subjects with normal hematological values and negative cytological assay for leukemia.

Disease diagnosis was based on clinical and hematological criteria from the University Hospital of Londrina. All patients underwent blood collection during the chronic phase of the disease, after having received conventional hydroxyurea or imatinib mesylate (STI571) chemotherapy. The data for age distribution for normal individuals and patients involved in the present study are shown in Table 1.

Table 1. Age distribution between normal donors (control) and patients (CML).

Age	Control (%)	Patients (%)
20-40	4 (7.14)	9 (40.91)
41-60	42 (75)	9 (40.91)
61-80	8 (14.29)	3 (13.64)
81-100	2 (3.57)	1 (4.54)
Total	56 (100)	22 (100)

Although the majority of patients were between 20 to 60 years old, the mean and median values between groups were similar. The mean for the control group was 53.39 (median 51.5), and 49.09 (median 45.5) for the patients group.

Over 14.29% of the patients were black, whose African descendent usually describes a dark-skinned Brazilian of Black African ancestry. This patient group was similar for gender, but there were more women in the control group, as shown in Table 2.

It was possible to obtain 22 samples for RNA analysis of *Bcr-abl* transcripts, which demonstrated integrity for Beta-actin mRNA expression. b3a2 mRNA was detected in samples from four CML patients (Figure 1).

Table 2. Ethnic distribution and gender of individuals.

			Gender	
Ethnicity	Control	%	Female	Male
White	41	73.21	25	16
Brown	6	10.71	2	4
Yellow	1	1.79	0	1
Black	8	14.29	5	3
Total	56	100	32	24
			Gender	
Ethnicity	CML	%	Female	Male
White	16	72.73	7	9
Brown	0	0	0	0
Yellow	1	4.55	0	1
Black	5	22.72	3	2
Total	22	100	10	12

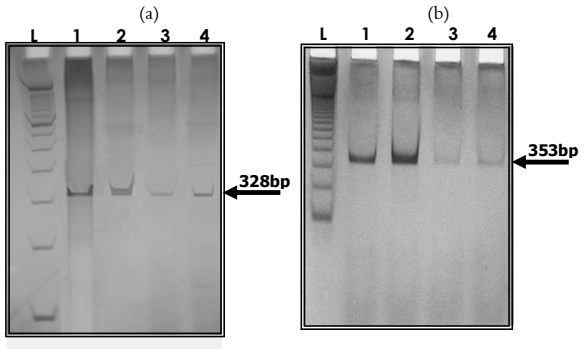

Figure 1. (a) Expression of *Bcr-abl* mRNA in peripheral blood cells from CML patients. PCR products were submitted to electrophoresis in 10% silver-stained acrylamide gels. Amplicons of 328bp correspond to b3a2 transcripts. L – ladder. (b) Integrity for RNA. Expression of beta-actin in the samples from patient Amplicons presented 353 bp, indicating no DNA contamination.

For the LDH assay, heparin anticoagulant was used with reference values of 100 to 190 U L^{-1}. This reference population consisted of 118 men and 108 women ranging in age from 20 to 65. The LDH values from patients and healthy donors are shown in Figure 2.

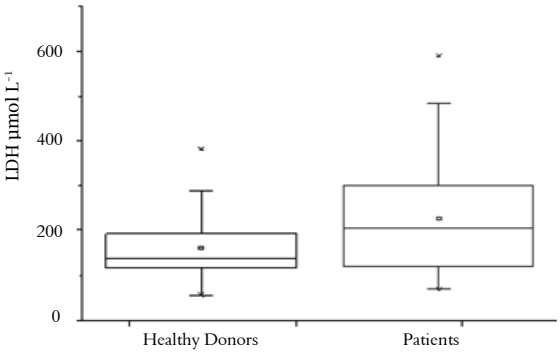

Figure 2. Quantitative determination of lactic dehydrogenase activity: The Dimension® (DADE Behring, Newark, USA) clinical chemistry system was used for LDH activity determination.

The LDH method measures L-lactate pyruvate oxidation with simultaneous reduction of nicotinamide adenine dinucleotide (NAD). The

change in absorbance at 340 nm due to the appearance of reduced NAD (NADH) is directly proportional to LDH activity, since other reactants are present in non-rate limiting quantities and is measured using a bichromatic (340, 383 nm) rate technique.

Discussion

The definition of prognostic factors is important (I) to select the optimal treatment, (II) to develop risk-adjusted treatments, (III) to adjust imbalances of treatment groups in clinical trials, and (IV) to compare outcomes of different studies. Most important is an accurate identification of the phase of the disease. In the early chronic phase, important prognostic information is derived from clinical and laboratory features (HOCHHAUS, 2008).

Minimal residual disease detection from plasma was more sensitive than from cell samples. Ma et al. (2007) suggested that absolute levels of *Bcr-abl* mRNA per unit volume of plasma may reflect tumor load.

The observation that *Bcr-abl* production is the initiating event in CML has focused attention on the survival signals triggered by this oncogene (FERNANDEZ-LUNA, 2000). The translocation results in the fusion of the *abl* gene located on the long arm of chromosome 9, with the *bcr* gene located on the long arm of chromosome 22. The *Bcr/abl* fusion gene encodes a chimeric protein with elevated tyrosine kinase activity, which plays an important role in the pathogenesis of the disease. The detection of the t (9;22)(q34;q11) translocation and *Bcr/abl* fusion gene is predictive in the diagnosis of chronic myeloid leukemia and recommended in the evaluation of the therapeutic effect (HOCHHAUS, 2008).

In this study, 22 patients with chronic myeloid leukemia were evaluated. Since oligos for b3a2 transcripts were used, there was no identification of mRNA with any other junctions, nor for rare cases where the breakpoint was outside M-*bcr*. Several studies have shown that classifying CML according to mRNA type does not produce homogenous hematological data.

CML occurs in all age groups, but is most common in middle-aged and elderly patients. The annual incidence is 1 to 2 people per 100,000, slightly more prevalent among men than women. CML represents 15 to 20% of all cases of leukemia among the Western population (FADERL et al., 1999). The poor prognosis in old age among individuals with CML is well known (HERNÁDEZ-BOLUDA et al., 1999; KANTARJIAN et al., 1985). The average age among patients with chronic myeloid leukemia is 50 years, with a slight predominance of males (MORRISON, 1994). Thus, the peak incidence of CML onset is between 30-50 years of age. The prevalence of CML among 567 American patients with CML, where 52% were male (MENZIN et al., 2004); Berger et al. (2005) observed a frequency of 59% (503/856) of CML in German men. Therefore, the data obtained in this study are consistent with these authors. The predominant ethnic group for patients was Caucasian, which usually describes a Brazilian of full or predominantly European ancestry or other ancestry, such as Arab Brazilian.

The presence of exon b3 has been associated with abnormal blood parameters (SHEPHERD et al., 1995). However, a prognostic value for the type of chimeric mRNA *Bcr-abl* (b3a2) is still controversial (CABRAL et al., 2003).

Data from Andrade (2008) suggest that qualitative RT-PCRs was essential for the diagnosis and follow-up of bone marrow transplant patients, because in some samples there was no detection of Ph1 cromosome by cytogenetic assay, but it was detected by molecular qualitative RT-PCR, which is an important fact for the clinical evaluation of the patient. It was verified that of the 45 patients sequentially followed up in individual periodic evaluations, 360 samples were selected and analyzed by PCR using the qualitative technique for isoforms characteristic of p210 BCR-ABL (b3a2 and b2a2) and p190 BCR-ABL (e1a2). In their pre-bone marrow transplant study, a prevalence of isoforms characteristic of CML was observed (b3a2 and/or b2a2), a fact essential for the patients to be followed up within the CML protocol.

Multiple myeloma patients at the advanced clinical stage, with presence of osteolysis and elevated lactate dehydrogenase (LDH) had a significant difference in the serum level of TNF-alpha in comparison with those at the early stage, without osteolysis, and normal LDH. A number of previous and more recent studies have shown that an elevated serum lactate dehydrogenase (LDH) is associated with a poor prognosis in MDS (myelodysplastic syndromes) (AUL et al., 1992, 1994; WIMAZAL et al., 2001; GERMING et al., 2005). Our data demonstrate increased LDH concentration in the CML patients when compared with normal donors. Two patients out of four with detectable *Bcr-abl* transcript presented elevated LDH in the plasma.

Early recognition of disease progression in low-risk myelodysplastic syndromes is an important

decision point concerning intensive therapies. Lactate dehydrogenase (LDH) has been identified as a most suitable follow-up parameter in a screening program searching for dynamic prognostic determinants. LDH is an interesting follow-up parameter in myelodysplastic syndromes, which may assist in early recognition of disease progression and thus help in risk stratification and patient selection for interventional therapies. An elevated LDH at diagnosis was found to be associated with an increased probability of acute myeloid leukemia (AML) evolution and decreased probability of survival (WIMAZAL et al., 2008).

Lactate dehydrogenase (LDH), a common biochemical marker for tumor burden and anaerobic glycolysis (VON EYBEN, 1978), is a poor prognostic factor for AML (TENG et al., 2006).

Prognostic factors for survival following allogeneic bone marrow transplant for acute myeloid leukemia include age, disease status and cytogenetic risk classification. Lactate dehydrogenase (LDH) levels have not been studied as a potential risk factor. Kalaycio et al. (2007) included LDH at the time of admission in an analysis of prognostic factors for survival.

Conclusion

It was concluded that LDH > 330 U L^{-1} is an important adverse risk factor for survival and should be included in future studies of risk performed on larger patient groups. With regard to laboratory findings, in the patients with CML submitted for chemotherapy which presented significantly higher LDH values also detected *Bcr-abl* transcript in the peripheral blood. In the context, it is possible that the determination of LDH and *Bcr-abl* transcript in the follow-up of patients with CML could be considered a marker for therapy following.

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