Short Communication

Chromosome 17 abnormalities and mutation of the *TP53* gene: Correlation between cytogenetics, flow cytometry and molecular analysis in three cases of chronic myeloid leukemia

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Abstract

Alterations involving the short arm of chromosome 17 (17p) during the progression of chronic myeloid leukemia (CML) have been described. This chromosomal region contains the tumor suppressor gene *TP53* that may be an important factor in the evolution of this disease. In this study, we used flow cytometry and western blotting to assess p53 protein expression and single stranded conformational polymorphism to examine *TP53* gene alterations in three patients with CML who showed alterations in 17p. Only the case with del(17)(p11) had p53 expression positive by flow cytometry and an abnormal migration pattern by SSCP analysis. The importance of the correlation between the results obtained with these techniques, as well as the clinical course of the patients, are discussed.

Key words: chronic myeloid leukemia, chromosome 17, *TP53* gene, p53 protein.

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Introduction

Chronic myeloid leukemia (CML) is a clonal, multi-lineage, myeloproliferative disorder that originates from a single, abnormal hemopoietic stem cell (Mughal *et al.,* 2001). About 95% of patients with CML characteristically have chromosome Philadelphia (Jacob *et al.,* 2002). This chromosome results the translocation t(9;22)(q34;q11) that produces the BCR/ABL (BCR: breakpoint cluster region; ABL: Abelson) gene fusion on the derivative chromosome 22 (Johansson *et al.*, 2002). The progression of CML is usually associated with the acquisition of additional cytogenetic abnormalities (Chase *et al.*, 2001), including alterations involving the short arm of chromosome 17 (17p). This region, more specifically 17p13, contains the tumor suppressor gene *TP53* (Rege-Cabrin *et al.*, 1994). This gene is often mutated in solid tumors and less often in

several types of hematological malignances (Harris *et al.*, 1993). Apparently there is a loss of the normal residual *TP53* allele during the progression of CML towards the blast crisis (Calabretta and Perrotti, 2004). About half of the cases with a loss of 17p do not show *TP53* inactivation. In these cases, the loss of 17p preceded *TP53* inactivation (Nakai and Misawa, 1995). However, the loss of 17p and *TP53* inactivation may be poor prognostic factors. In this work, we examined p53 protein expression and alterations in the *TP53* gene in three patients with 17p aberrations.

Bone marrow cells from three patients with CML diagnosed at the Hematology Service of the National Cancer Institute (Brazil) were studied. Informed consent was provided by all participants and the study was done within the guidelines of the Declaration of Helsinki. Blood samples were collected in tubes containing EDTA, except for the cytogenetic analysis, for which heparinized tubes were used. The mononuclear cells were separated by centrifugation on a Ficoll-Hypaque (Sigma) gradient and washed three times with saline solution (0.9% NaCl) before being resuspended in RPMI-1640 (Sigma) medium supple-

mented with 10% fetal calf serum (FCS; Biomast, Brazil) and then adjusted to a concentration of 10^6 cells/mL. The bone marrow samples were analyzed cytogenetically using short-term (24 h) cultures. GTG banding was done as described by Seabright (1971) and the chromosomes were identified using a standard classification (Mitelman, 1995). Partial karyotypes were prepared using the CytoVision System (Applied Imaging) for genetic image analysis. The BCR-ABL molecular analysis was done using the reverse transcription polymerase chain reaction (RT-PCR), as described by Otazú *et al.* (2000). The expression of p53 protein was detected by flow cytometry (FC) and confirmed by western blot (WB) using an anti-p53 monoclonal antibody (MoAb D07; DAKO, Carpintaria, CA, USA) that recognizes an epitope at the N-terminus of the human p53 protein and reacts with wild-type and mutant proteins (Kimura *et al.*, 1996; Filippini *et al.*, 1998).

For flow cytometry, the samples were fixed and permeabilized as described by Faharat *et al.* (1994) and then incubated for 30 min with 10 µL of anti-p53 MoAb labeled with FITC, followed by two washes with Tween 20/PBS. The cells were then resuspended in 500 µL of PBS/formaldehyde prior to analysis. Isotype-matched MoAb was used as a negative control for each test. Flow cytometry was done with a Fluorescence Activated Cell Sorter (FACScan, San Jose, CA, USA) equipped with Cell Quest software (Cell Quest^{TM} Software, Becton Dickinson Immunocytometry Systems, San Jose, CA, USA). The p53 expression was estimated as the ratio between the arithmetic mean of the intensity of the fluorescence (MIF) in cell suspensions with anti-p53 MoAb and the MIF of cell suspensions labeled with the isotypic control (Kimura *et al.*, 1996). The samples were considered positive when the MIF ratio was greater than 1.4.

For western blot analysis, mononuclear cells were lysed in a reporter lysis buffer and separated on SDS-polyacrylamide gels, transferred to nitrocellulose membrane, and probed with anti-p53 MoAb. Western blot analysis of crude membrane preparations was done as previously described (Bonsing *et al.*, 1997; Tupeinen *et al.*, 2002).

DNA was extracted from samples using DNAzol (DNAzol® Reagents, Invitrogen Life Technologies, Carisbad, CA, USA) and 10^6 cells/patient. The oligonucleotide primers used for the PCR/single stranded conformational polymorphism (PCR/SSCP) analysis were synthesized by GIBCO-BRL for exons 5, 6, 7, and 8-9. The four fragments were amplified separately. The size of the PCR products were 190 bp for exon 5, 144 bp for exon 6, 118 bp for exon 7 and 289 bp for exon 8-9 (Klumb *et al.*, 2003). Genomic DNA was submitted to PCR in a final reaction volume of 45 µL for exons 5, 7 and 8-9 and 25 µL for exon 6 containing 25 pmol of each primer, 0.2 mM of dNTP mix, 20 mM Tris-HCl (pH 8.4), 50 mM KCl, 1.5-3.0 mM $MgCl_2$ and 1 U of *Taq* polymerase (GIBCO-BRL) in a PC-100 programable thermocycler (MJ Research Inc., Walthom, MA, USA) under the following conditions: denaturation (1 min at 94 °C), annealing (1 min at 55-58 °C) and extension (2 min at 72 °C) for 35 cycles followed by a terminal extension for 7 min at 72 °C. The PCR product (2-4 µL) was than diluted 1:1 (v/v) in a loading buffer containing formamide, 10 mM EDTA, pH 8.0, 0.1% bromophenol blue and 0.1% xylene cyanol. Samples were heated at 95 °C for 5 min, chilled on ice and immediately loaded onto an 8% or 10% polyacrylamide gel in a 0.5X Trizma base-borate-EDTA buffer (TBE), pH 8.3, with or without the addition of glycerol. The running conditions were 40-60 V for 14-19 h at room temperature. The gels were then stained with silver nitrate and air-dried (Klumb *et al.*, 2003). DNA samples from patients with a previously identified p53 mutation (Klumb *et al.*, 2003) and wild type p53 from healthy blood donors were included as positive and normal controls, respectively.

At the time of the cytogenetic analysis, one patient was in the chronic phase (case 1), another one was in the accelerated phase (case 2) and the third one was in a myeloid blast crisis (AML-M7; case 3). Molecular analysis of the BCR-ABL junction using RT-PCR was positive for b_2a_2 (case 1) and b_3a_2 (cases 2 and 3). In the cytogenetic analysis, two patients showed deletions: one in the p11 region (case 3) (Figure 1C) and the other in p12 (case 1) (Figure 1A). Additionally, an isochromosome 17q was observed in
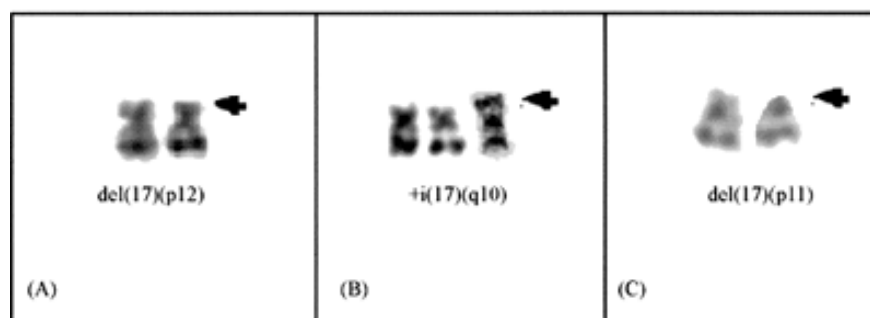

del(17)(p12)   +i(17)(q10)   del(17)(p11)

(A)   (B)   (C)

Figure 1 - Partial karyotypes of cases 1 (A), 2 (B) and 3 (C).

case 2 (Figure 1B). Analysis of p53 expression by flow cytometry showed that only the patient with del(17)(p11) was positive and hand an MIF of 11.9 (Figure 2, case 3). SSCP analysis of this individual showed an abnormal migration pattern that suggested a mutation in *TP53* (Figure 3). In the two cases with no p53 expression (MIF of 1.0 and 1.3 for cases 1 and 2, respectively), SSCP analysis revealed no abnormality. Table 1 summarizes the data for the three patients.

In terms of therapy, case 1 was initially treated with hydroxyurea and, in the first acute phase, received the BFM95 protocol followed by the St. Jude's Hospital protocol for lymphoid blast crisis. Case 2 was referred for allogeneic peripheral stem cell transplantation, and case 3 was treated with imatinib mesylate. Despite this treatment, the three patients eventually died of their disease.

Mutations in the *TP53* gene occur in approximately 5% of CML cases in the chronic phase (Fenaux and Preudhomme, 1997) and in 15-23% of CML cases in blast crisis, but in some cases the cytogenetic abnormalities precede these alterations (Nakai *et al.,* 1992). These findings suggest that mutations in the *TP53* gene are a secondary event in the progression of CML (Martin, 1995). In the present study, only case 3, which was in myeloid blast crisis, showed expression of p53, as detected by flow cytometry and western blotting. The altered migration pattern in SSCP analysis suggested that this expression was the result of a mutation. In the two other cases, a deletion in the 17p12 region (case 1) and an additional isochromosome 17 (case 2) were detected. These cases were in the chronic and accelerated phases of the disease, respectively. Some authors have suggested that *TP53* mutations are more frequent in patients who develop a myeloid blast crisis, when compared with CML patients in lymphoid blast crisis (Imamura *et al.,* 1994). About 40% of the cases with i(17)(q10) show *TP53* mutations (Nakai *et al.,* 1992), although in our case we observed two normal chromosomes 17. This finding suggests that p53 function was preserved in this patient. The patient with the del(17)(p12) was in the chronic phase and, as noted by others (Fenaux and

Preudhomme, 1997), the cytogenetic alterations anteceded the *TP53* gene mutations.

The p53 protein expression detected by flow cytometry and confirmed by western blotting showed good
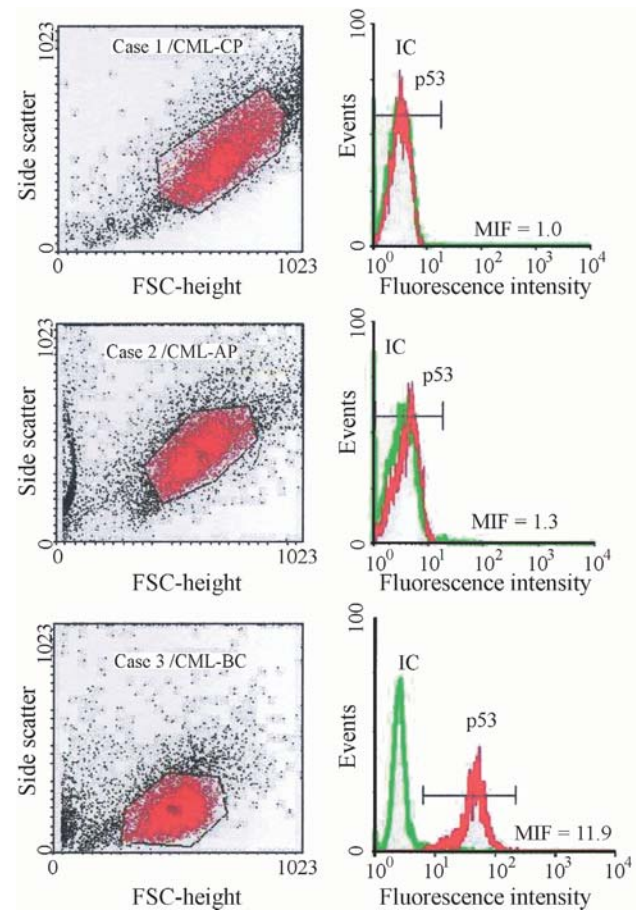

Figure 2 - Analysis of p53 expression by flow cytometry. Samples were incubated with p53 monoclonal antibodies (MoAb) labeled with FITC. In the left column, plots of the cell scattering. The FSC (forward scatter) represents the size of the cells and side scatter, their internal complexity. The right column shows the p53 expression estimated as a ratio of the arithmetic mean of the intensity of fluorescence (MIF) in cells labeled with anti p53 MoAb (red histogram) and the MIF for cells labeled with the isotypic control (IC) (green histogram). The samples were considered positive when the MIF was < 1.4.

Table 1 - Clinical characteristics and cytogenetic and molecular analyses of the patients.

N.	Gender/age	Cytogenetic analysis	BCR-ABL	p53 expression		*TP53* PCR/SSCP analysis	Overall survival	Phase	Evolution
				FC	WB				
1	F/1 year and 7 months	46,XX,t(9;22)(q34;q11),del(17)(p12) [15]/46,XX,t(9;22)(q34;q11)[29]	b2a2	-	-	N	17 months	CP	Death (BC-L)
2	F/24year	47,XX,t(9;22)(q34;q11),+i(17)(q10) [10]/46,XX,t(9;22)(q34;q11)[17]	b3a2	-	-	N	38 months	AP	Death (BC-L)
3	M/28 year	46,XY,t(9;22(q34;q11),del(17)(p11) [18]/46,XY,t(9;22)(q34;q11)[12]	b3a2	+	+	A/E5	29 months	BC	Death (BC-M)

Abbreviations: M: Male; F: Female; BC: Blast crisis; CF: Chronic phase; AP: Accelerated phase FC: Flow cytometry; PCR/SSCP: Polymerase chain reaction / Single stranded conformational polymorphism; WB: Western blot; A: Abnormal; E5: Exon 5; N: Normal; BC-L: Lymphoid blast crisis; BC-M: Myeloid blast crisis.
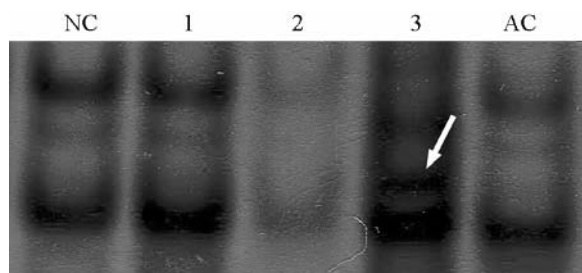
Figure 3 - PCR/SSCP analysis of exon 5 of the *TP53* gene. NC - normal control, lanes 1 and 2 - cases 1 and 2, showing the normal pattern of migration; Case 3 - 3, showing the abnormal mobility shift; AC - abnormal control.

correlation with the SSCP analysis. The specificity of SSCP is > 95% for 100-300 bp PCR fragments. In practice, not all of the changes can be resolved, although modification of the electrophoretic conditions can improve the detection of mutations in the sequence (Hayashi, 1991). The abnormal migration of case 3 was most likely resulted from a mutation in the *TP53* gene since there are no reports of polymorphism in exon 5 (IARC *TP53* DATA BASE). However, this conclusion requires confirmation by nucleotide sequencing of the *TP53* gene in this individual.

In conclusion, our results show that a combination of several techniques can be a useful tool in the follow-up of patients. The cytogenetic alterations in 17p were apparently independent of mutations in the *TP53* gene since in two patients the progression to a lymphoid blast crisis occurred precociously.

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