CLINICAL SCIENCE

Screening for hotspot mutations in *PI3K*, *JAK2*, *FLT3* and *NPM1* in patients with myelodysplastic syndromes

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INTRODUCTION: Myelodysplastic syndromes encompass a heterogeneous group of clonal hematopoietic stem cell disorders characterized by ineffective hematopoiesis, refractory cytopenia and a tendency to progress toward acute myeloid leukemia. The accumulation of genetic alterations is closely associated with the progression of myelodysplastic syndromes toward acute myeloid leukemia.

OBJECTIVE: To investigate the presence of mutations in the points most frequent for mutations (hotspot mutations) in phosphatidylinositol-3-kinase (PI3K), Janus kinase 2 (JAK2), FMS-like tyrosine kinase 3 (FLT3) and nucleophosmin (NPM1), which are involved in leukemia and other cancers, in a population of Brazilian MDS patients.

METHODS: Fifty-one myelodysplastic syndromes patients were included in the study. According to French-American-British classification, the patients were distributed as follows: 31 with refractory anemia, 8 with refractory anemia with ringed sideroblasts, 7 with refractory anemia with excess blasts, 3 with refractory anemia with excess blasts in transformation and 2 with chronic myelomonocytic leukemia. Bone marrow samples were obtained and screened for the presence of hotspot mutations using analysis based on amplification with the polymerase chain reaction, sequencing, fragment size polymorphisms or restriction enzyme digestion. All patients were screened for mutations at the time of diagnosis, and 5 patients were also screened at the time of disease progression.

RESULTS: In the genes studied, no mutations were detected in the patients at the time of diagnosis. One patient with chronic myelomonocytic leukemia was heterozygous for a *Janus kinase 2* mutation after disease progression.

CONCLUSIONS: These results show that hotspot mutations in the *PI3K*, *JAK2*, *FLT3* and *NPM1* genes are not common in MDS patients; nevertheless, *JAK2* mutations may be present in myelodysplasia during disease progression.

KEYWORDS: Hematopoietic Disorder; Acute Leukemia; Myelodysplasia; Mutations; Bone Marrow.

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INTRODUCTION

Myelodysplastic syndromes (MDS) encompass a heterogeneous group of clonal hematopoietic stem cell disorders characterized by ineffective hematopoiesis, refractory cytopenia and a tendency to progress to acute myeloid leukemia (AML).[1] Low-risk MDS present high levels of intramedullar apoptosis, whereas high-risk MDS show a decrease in apoptosis, an increase in cell proliferation and a high frequency of evolution to AML.[2,3] The accumulation of genetic alterations is closely associated with the progression of MDS toward AML, and efforts are being made to determine the significance of various genetic aberrations in patients with MDS.[4-6] The same occurs for liver adenomatosis,[7] Rubinstein-taybi syndrome[8] and hemochromatosis.[9]

The Phosphatidylinositol-3-kinase (PI3K) and Janus kinase 2 (JAK2) signaling pathways are involved in numerous cellular processes, such as proliferation, apoptosis and differentiation.[10-12] Mutations in the catalytic subunit of PI3K are frequently observed in several cancers, including AML.[4,5,13] Hotspot mutations occur in exon 9 (E542 and E545) and in exon 20 (H1047), resulting in increased PI3K/Akt activity.[5,14-17] One somatic mutation in the JAK2 gene (V617F) has been identified in myeloproliferative disorders such as polycythemia vera (PV) and myelofibrosis.[18]

FMS-like tyrosine kinase 3 (FLT3) is a tyrosine kinase receptor that plays an important role in the proliferation and differentiation of hematopoietic progenitors.[19] Nucleophosmin (NPM1) is a key regulator of hematopoesis that shuttles

Table 1 - Patient characteristics.

	Number of individuals
MDS patients	51
Age in years: median (range): 63 (26-90)	
Gender	
M/F	30/21
FAB	
RA/RARS	31/8
RAEB/RAEBt	7/3
CMML	2
WHO	
RCUD/RCMD/RARS/SMD-5q	3/24/8/3
RAEB-1/RAEB-2	4/2
CMML1	2
AML with myelodysplasia-related changes	4
MDS Unclassified	1

Abbreviations- FAB: French-American-British; RA, refractory anemia; RARS, refractory anemia with ringed sideroblasts; RAEB, refractory anemia with excess blasts; RAEBt, refractory anemia with excess blasts in transformation; CMML, chronic myelomonocytic leukemia; WHO, World Health Organization; RCUD, refractory cytopenia with unilineage dysplasia; RCMD, refractory cytopenia with multilineage dysplasia; SMD-5q, MDS associated with isolated del(5q); RAEB-1, refractory anemia with excess blasts 1; RAEB-2, refractory anemia with excess blasts 2; CMML1, chronic myelomonocytic leukemia 1; AML, acute myeloid leukemia.

between the nucleus and cytoplasm. NPM1 mutations often result in the predominant localization of the protein to the cytoplasm, leading to destabilization of p14ARF and to the inhibition of p53.[20] Internal tandem duplications (ITDs) in *FLT3* and *NPM1* mutations are frequent events in the development of AML, and are associated with prognosis. According to Gale *et al*,[21] it is possible to identify 3 prognostic groups based in the presence or absence of *FTL3* and *NPM1* mutations: good (FLT3-ITD^{-}*NPM1*$^{+}$), intermediate (FLT3-ITD^{-}*NPM1*$^{-}$ or FLT3-ITD^{+}*NPM1*$^{+}$), and poor prognosis (FLT3-ITD^{+}*NPM1*$^{-}$). Furthermore, a point mutation in exon 20 of the *FLT3* gene (FLT3-D835) has been described in a case of AML.[19]

Mutations in *PI3K*, *JAK2*, *FLT3* and *NPM1* have been described in cases of MDS; however, additional studies are necessary to clarify their role in this disease. In this context, the objective of this work was to investigate the occurrence of the hotspot mutations E542, E545 and H1047 in PI3K, V617F in JAK2, ITDs and D835 in FLT3 and exon 12 mutations in *NPM1* in MDS patients in a Brazilian population.

MATERIALS AND METHODS

Patients

DNA samples were obtained from bone marrow aspirates of 51 patients diagnosed with *de novo* MDS. According to the French-American-British (FAB) classification,[22] the patients were classified as follows: 31 cases of refractory anemia (RA), 8 cases of refractory anemia with ringed sideroblasts (RARS), 7 cases of refractory anemia with excess blasts (RAEB), 3 cases of refractory anemia with excess blasts in transformation (RAEBt), and 2 cases of chronic myelomonocytic leukemia (CMML). Using the World Health Organization (WHO) 2008 classification guidelines,[23] there were 3 cases of refractory cytopenia with unilineage dysplasia (RCUD), 23 cases of refractory cytopenia with multilineage dysplasia (RCMD), 8 cases of refractory anemia with ring sideroblasts (RARS), 3 cases of MDS associated with isolated del(5q) (MDS-5q), 7 cases of refractory anemia with excess blast-1 (RAEB-1), 3 cases of refractory anemia with excess blast-2 (RAEB-2) and 4 cases of AML with multilineage dysplasia. Samples were obtained at the time of diagnosis, and none of the patients had received any cytotoxic drugs or growth factors for MDS treatment. Patient characteristics are shown in Table 1. Additionally, among the 51 patients evaluated at the time of diagnosis, 5 patients presented disease progression and were screened for mutations after disease evolution. Patient characteristics at diagnosis and after disease progression are shown in Table 2. Samples were collected at the Hematology and Hemotherapy Center of the University of Campinas, Brazil. All patients who contributed to this study provided informed written consent, and the National Ethical Committee Board approved the study.

Nucleic acid isolation

Genomic DNA was extracted from mononuclear bone marrow cells with the GFX^{TM} Genomic Blood DNA Purification Kit (Amersham Biosciences, Piscataway, USA), according to the manufacturer's instructions.

Detection of FLT3-ITD and *NPM1* mutations

Identification of FLT3-ITD and *NPM1* exon 12 mutations was performed using polymerase chain reaction (PCR) and analysis of fragment size. PCR was performed in a 50-μL reaction volume consisting of 100 ng of genomic DNA, 5 μL of 10X reaction buffer, 2 μL of 50 mM $MgCl_2$, 2.5 units of Taq

Table 2 - Patient characteristics at diagnosis and after disease progression.

MDS patient	Classification at diagnosis (FAB/WHO)	Number of blasts at diagnosis	Classification after disease progression (FAB/WHO)	Number of blasts after disease progression	Mutations after disease progression
Case 1	CMML/CMML1	3%	CMML/CMML2	10%	JAKV617F mutation
Case 2	RA/RCMD	0%	RAEB/RAEB-1	12.5%	None
Case 3	RA/RCMD	3%	RAEBt/RAEB-2	10%	None
Case 4	RARS/RARS	4%	LMA/LMA with myelodysplasia-related changes	69%	None
Case 5	RA/RCMD	1%	RAEBt/RAEB-2	20%	None

Abbreviations- MDS: myelodysplastic syndromes; FAB: French-American-British; RA, refractory anemia; RARS, refractory anemia with ringed sideroblasts; RAEB, refractory anemia with excess blasts; RAEBt, refractory anemia with excess blasts in transformation; CMML, chronic myelomonocytic leukemia; WHO, World Health Organization; RCMD, refractory cytopenia with multilineage dysplasia; RAEB-1, refractory anemia with excess blasts 1; RAEB-2, refractory anemia with excess blasts 2; CMML1, chronic myelomonocytic leukemia 1; CMML2, chronic myelomonocytic leukemia 2; AML, acute myeloid leukemia.

Table 3 - Primer sequences and restriction enzymes

Gene	Mutation	Primers sequences	Restriction enzyme site
FLT3	ITD	F: 5′-GCAATTTAGGTATGAAAGCCAGC-3′ R: 5′-CTTTCAGCATTTTGACGGCAACC-3′(HEX)	
NPM1	exon 12	F: 5′-GTGGTAGAATGAAAAATAGAT-3′(FAM) R: 5′-CTTGGCAATAGAACCTGGAC-3′	
JAK2	V617F	F: 5′-GGGTTTCCTCAGAACGTTGA-3′ R: 5′-TCATTGCTTTCCTTTTTCACAA-3′	BsaXI
FLT3	D835	F: 5′-CCGCCAGGAACGTGCTTG-3′ R:5′-GCAGCCTCACATTGCCCC-3′	Eco321
PI3K	exon 9	F: 5′-TTACAGAGTAACAGACTAGC-3′ R: 5′-TTTTAGCACTTACCT GTGAC-3′	
PI3K	exon 20	F: 5′-AGCTATTCGACAGCAGTGCC-3′ R: 5′-TTGTGTGGAAGATCCAATCC-3′	

polymerase and 200 nM each of the forward and reverse primers (Table 3). The reaction conditions were set as follows: 5 minutes of denaturing at 94℃ followed by 35 cycles of 20 seconds at 92℃, 30 seconds at 57℃ and 45 seconds at 72℃, with a final step at 72℃ for 7 minutes. After dilution (1:20) in water, 1 μL of each PCR product was mixed with 9 μL of Hi-Di formamide (Applied Biosystems, Foster City, CA) and 0.5 μL of GeneScan 500-ROX size marker, and the mixture was denatured for 5 minutes at 95℃. Samples harboring the mutation were identified based on the areas under the curves representing the wild-type (FLT3:397 bp and NPM1:294 bp) and mutated alleles (FLT3-ITD>397 bp and NPM1>294 bp). AML patients with FLT3-ITD or NPM1 mutations were used as positive controls.

Detection of the *JAK2* V617F and *FLT3*-D835 mutations

Identification of *JAK2* and *FLT3* genotypes was performed using PCR-restriction fragment length polymorphism (PCR-RFLP) analysis. PCR was performed in a 50-μL reaction volume consisting of 100 ng of genomic DNA, 5 μL of 10X reaction buffer, 2 μL of 50 mM $MgCl_2$, 2.5 units of Taq polymerase and 200 nM each of the forward and reverse primers (Table 3). The reaction conditions were set as follows:

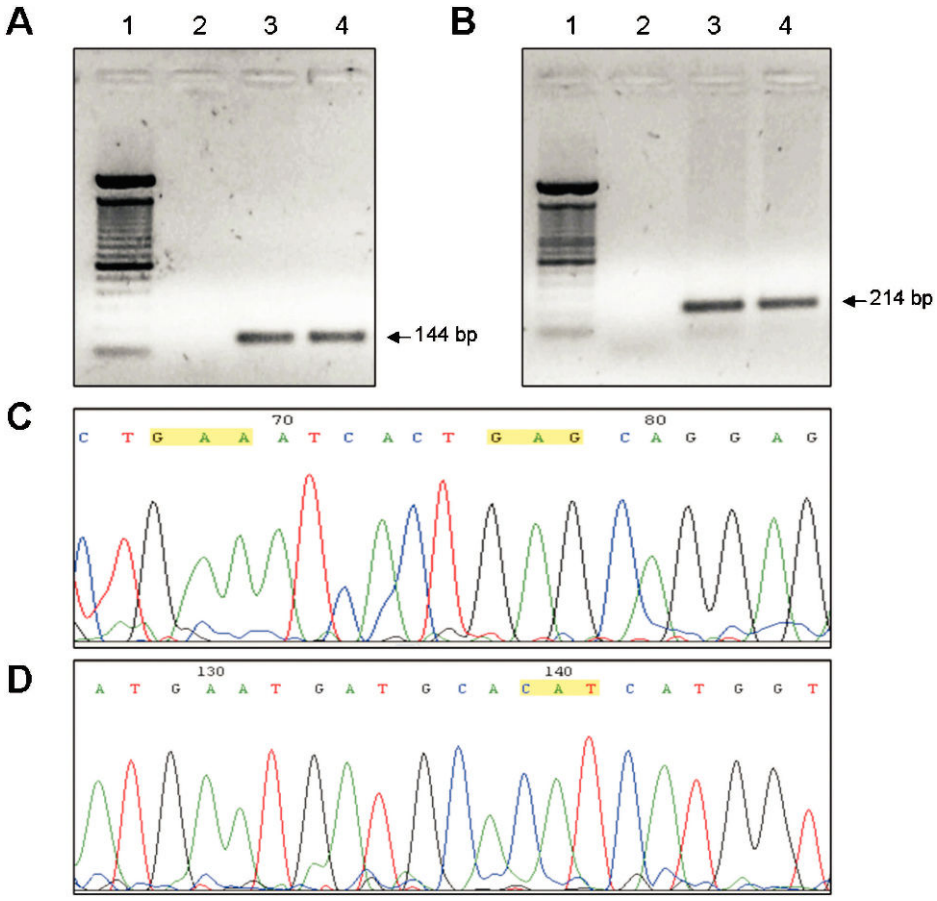

Figure 1 - PCR and Sequencing of exons 9 and 20 of *PI3K*. The fragment size of the exon 9 (A) and exon 20 (B) of PI3K are indicated in the figure. In both figures A and B, lane 1: Ladder 100bp fragments; lane 2: negative control; lanes 3 and 4: amplicons obtained from genomic DNA of patient MDS patients (RA). Representative *PI3K* sequencing from MDS patients, determined by automated sequence analysis of exon 9 (C) and 20 (D). The localization of the most frequent hotspot mutations are highlighted in the figure.

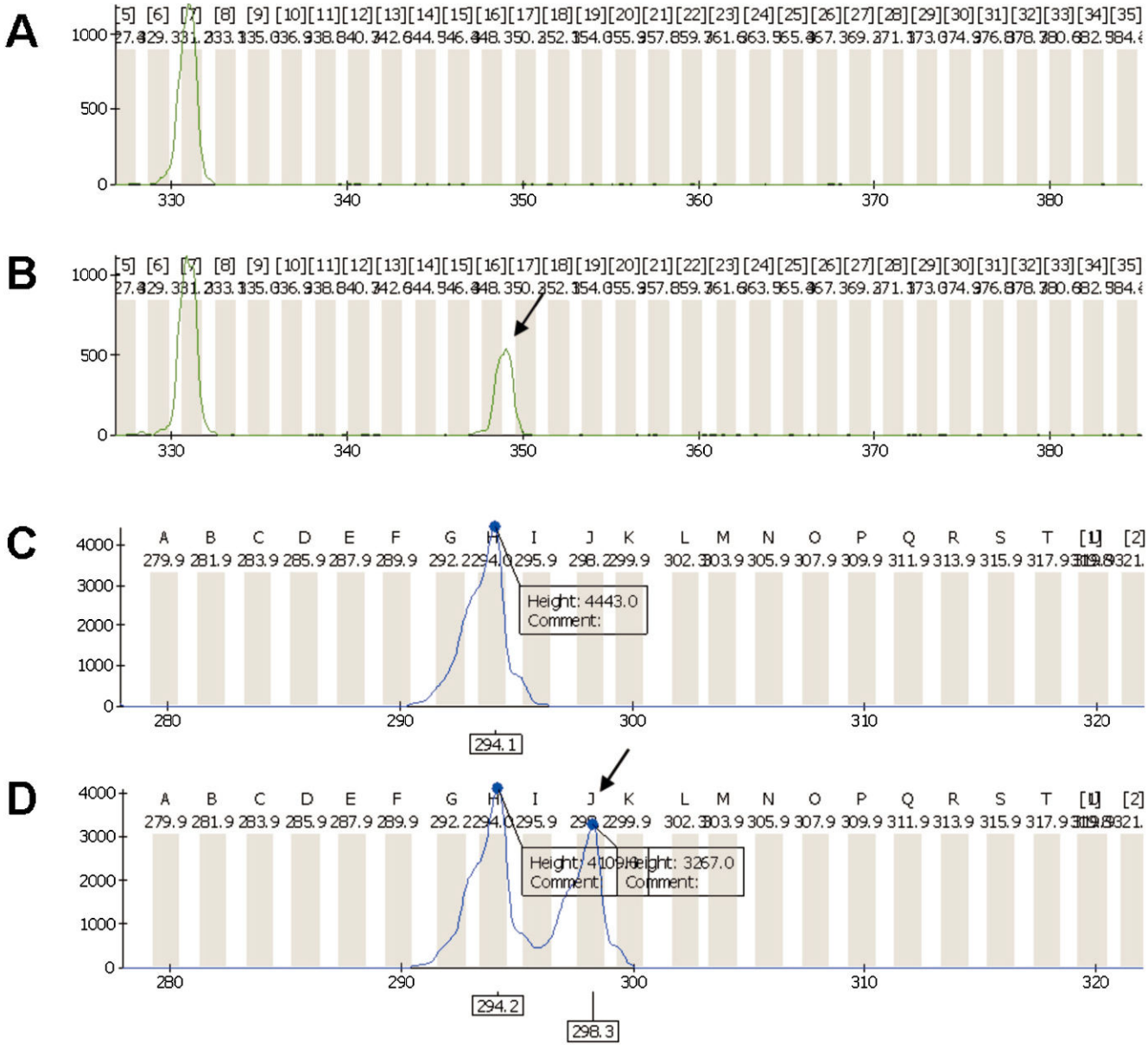

Figure 2 - Fragment analysis of *FLT3*-ITD and *NPM1* mutations. Representative fragment size analysis of a MDS patient with wild-type alleles for *FLT3* (A), an AML patient with the *FLT3*-ITD mutation (B), an MDS patient with wild-type *NPM1* (C) and an AML patient with a mutation in exon 12 of *NPM1* (D). The arrows indicate the presence of the mutant allele.

5 minutes of denaturing at 94℃ followed by 35 cycles of 30 seconds at 92℃, 30 seconds at 57℃ and 50 seconds at 72℃, with a final step at 72℃ for 7 minutes. For RFLP analysis, *JAK2* and *FLT3* PCR products were digested with BsaXI or Eco321 (New England Biolabs, Hitchin, UK), respectively, according to the manufacturer's protocol, and visualized on a 2.5% agarose gel. The normal genotype for *JAK2* was represented by a 460-bp fragment, and the heterozygous genotype was represented by 460-bp, 241-bp and 189-bp fragments, whereas the homozygous mutant genotype produced 241-bp and 189-bp fragments. For *FLT3*-D835, the normal genotype was represented by 68-bp and 46-bp fragments, and the heterozygous genotype was represented by 114-bp, 68-bp and 46-bp fragments, whereas the homozygous mutant genotype produced only a 114-bp fragment. AML or PV patients with *FLT3*-D835 and *JAK2* V617F mutations, respectively, were used as positive controls.

Detection of the *PI3K* E542, E545 and H1047 mutations

Screening for *PI3K* mutations was performed by sequencing PCR products. PCR was performed in a 50-μL reaction volume consisting of 100 ng of genomic DNA, 5 μL of 10X reaction buffer, 2 μL of 50 mM $MgCl_2$, 2.5 units of Taq polymerase and 200 nM each of the forward and reverse primers (Table 3). The reaction conditions were set as follows: 5 minutes of denaturing at 94℃ followed by 35 cycles of 30 seconds at 94℃, 50 seconds at 63℃ and 55 seconds at 72℃, with a final step at 72℃ for 7 minutes. Sequencing reactions were performed in both directions with the ABI PRISM

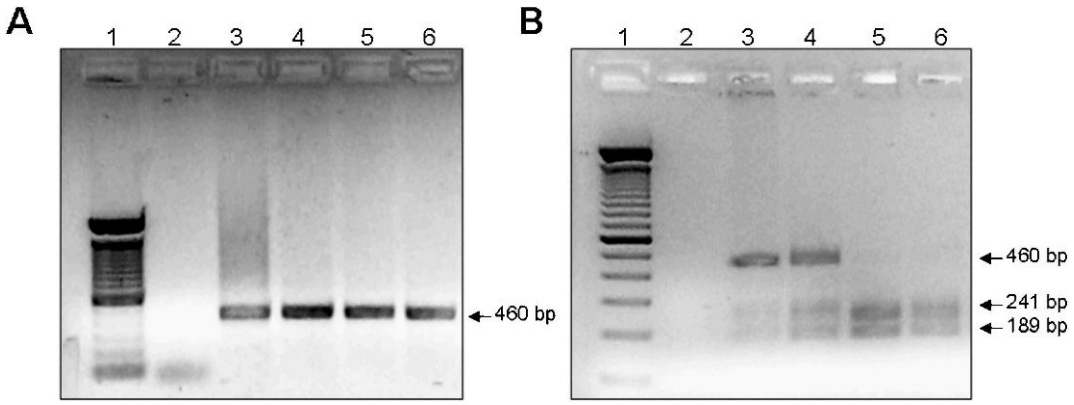

Figure 3 - *JAK2* V617F genotyping. (A) PCR amplification of *JAK2*: lane 1: 100 bp ladder; lane 2: negative control; lanes 3 to 6 – 460-bp amplicons obtained from the genomic DNA of a patient with PV (3), a CMML patient after disease progression (4) and two MDS patients (with RA) (5 and 6). (B) BsaXI digestion: lane 1: 100 bp ladder, lane 2: negative control; lanes 3 and 4: digestion pattern observed in a PV patient (3) and in the CMML patient positive for the *JAK2* V617F allele after disease progression (4); lanes 5 and 6: digestion pattern observed in two MDS patients (with RA) with wild-type *JAK2* alleles.

BigDye terminator version 3.0 cycle sequencing kit, according to the manufacturer's instructions, using either one of the primers used for amplification (Table 3). After ethanol-sodium acetate precipitation, samples were analyzed on the ABI PRISM 3100 Genetic Analyzer.

RESULTS

PI3K mutation analysis

Samples from 51 MDS patients were screened for *PI3K* mutations; all 51 samples were screened at diagnosis, and 5 were screened again after disease progression. We examined exons 9 and 20, as a previous report has shown that over 75% of the *PI3K* mutations found in a large number of cancers are present in these exons.[19] The sequencing of PCR products showed the absence of mutations in exons 9 and 20 of the *PI3K* gene in all MDS patients. PCR products and the sequences of exons 9 and 20 are presented in figure 1.

FLT3-ITD and *NPM1* mutation analysis

Forty-six MDS patients were screened for *FLT3*-ITD and *NPM1* exon 12 mutations at diagnosis, and 5 of these patients were also screened at the time of disease progression. AML patients with the *FLT3*-ITD or *NPM1* mutations were used as positive controls. Analysis of DNA samples from the MDS patients showed that all samples included fragments of normal size, indicating the absence of mutations (figure 2).

JAK2 V617F and FLT3-D835 mutation analysis

Fifty-one MDS patients were screened for *JAK2* V617F, and forty-seven were screened for *FLT3*-D835 at the time of diagnosis. Five patients were screen after disease progression. One PV patient with *JAK2* V617F and one AML patient with *FLT3*-D835 were used as positive controls. RFLP analysis showed the absence of *JAK2* and *FLT3*-D835 mutations in all MDS patients at the time of diagnosis. Interestingly, we

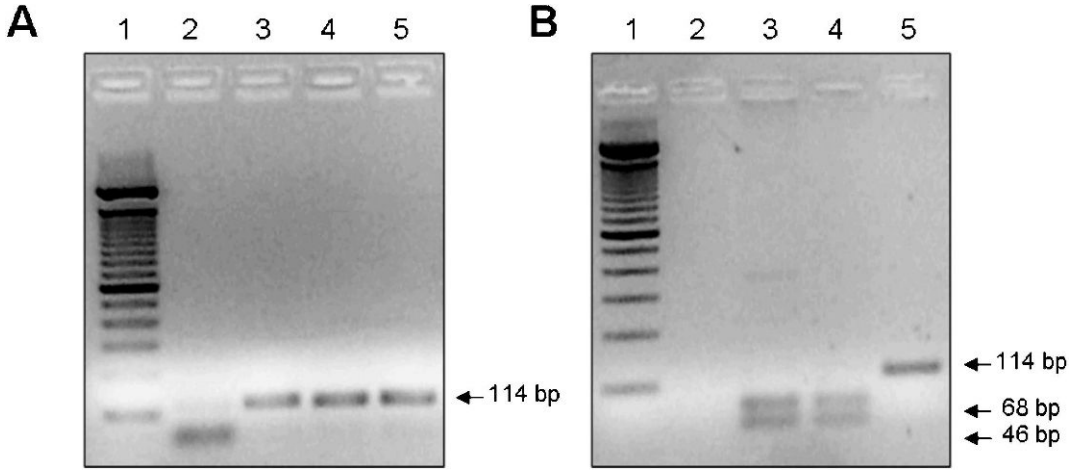

Figure 4 - *FLT3*-D835 genotyping. (A) PCR amplification of *FLT3*: lane 1: 100 bp ladder; lane 2: negative control; lanes 4 to 5: 114-bp amplicons obtained from the genomic DNA of patients with MDS (3-4) and AML (5). (B) Eco321 digestion: lane 1: 100 bp ladder, lane 2: negative control; lanes 3 and 4: digestion pattern observed in two MDS patients negative for the *FLT3*-D835 allele, lane 5: digestion pattern observed in an AML patient with the *FLT3*-D835 mutation.

observed the presence of the *JAK2* V617F mutation in one patient with CMML after disease progression (case 1; Table 2). Figures 3 and 4 represent the RFLP analysis of the *JAK2* V617 and *FLT3*-D835 mutations, respectively.

DISCUSSION

Acute leukemia results from a combination of mutations and changes in protein function that lead to an increase in proliferation and defects in differentiation and apoptosis.[24] Although *FLT3* and *NPM1* mutations have been described with great frequency in cases of AML,[25,26] these mutations were not detected in the MDS patients included in this study. As the presence of these mutations was investigated at the time of diagnosis, screening during disease progression could be interesting. Pinheiro and colleagues[27] have reported the acquisition of the *FLT3*-ITD mutation in 2 of 50 MDS patients included in their study one year after diagnosis. These patients later progressed toward AML, suggesting that the acquisition of this mutation may be related to leukemic transformation.

JAK2 mutations were not found in the MDS patients in this study at diagnosis. Interestingly, the *JAK2* V617F mutation was identified in one CMML patient after disease progression. Initially, this patient presented with fewer than 5% bone marrow blasts and lacked the *JAK2* V617F mutation. We observed the presence of the *JAK2* V617F mutation during disease progression, with increased white blood cell (WBC) counts and bone marrow blasts (at diagnosis: 9000 WBC/L, 3% bone marrow blasts; at disease progression: 60000 WBC/L, 10% bone marrow blasts). *JAK2* mutations occur in 10% of CMML cases and are associated with clinical and morphological features.[28] The *JAK2* V617F mutation leads to constitutive activation of the JAK2/STAT3 pathway and aberrant signaling, resulting in growth factor independence, increased proliferation and differentiation failure.[29] In light of the frequency of these events during MDS progression, our results suggest that the acquisition *of JAK2* mutations may be involved in disease progression and should be investigated in more cases of MDS evolution. This finding is in agreement with other authors.[30,31] A recent publication by Malcovati *et al*.[31] reported 3 patients who evolved from RARS with normal platelet counts and wild-type *JAK2* to RARS-T with *JAK2* mutation at the time of transformation.

Mutations in exons 9 and 20 of the *PI3K* gene are frequently described in cancer.[4,5,13] However, we did not observe the presence of these mutations in the MDS patients included in this study. Constitutive activation of PI3K occurs in AML and high-risk MDS patients at diagnosis[32-34], and mutations in exons 9 and 20 result in constitutive activation of this protein.[5,14-17] The presence of *PI3K* mutations in AML[5] justifies the evaluation of these mutations in a larger number of MDS patients, as they represent a possible factor involved in disease progression.

The presence or absence of these mutations has prognostic value in AML,[21] and therefore, the investigation of similar mutations in other myeloid diseases such as MDS could be interesting in the context of developing targeted therapies. The PI3K/Akt pathway has already been targeted in acute leukemia, and specific PI3K inhibitors, such as LY294002, have been tested *in vitro*.[35] Other members of the PI3K signaling pathway have also been investigated as targets for leukemia treatment. Clinical studies with rapamycin analogues, which inhibitor mTOR, are currently in phase II AML trials, alone or in combination with other chemotherapeutics.[36] Furthermore, FLT3 inhibitors have shown therapeutic activity in AML patients with *FLT3* mutations,[37] and selective JAK2 inhibitors have been tested in patients with *JAK2* mutations.[38]

In summary, our study has shown that mutations in the *JAK2*, *FLT3*, *NPM1* and *PI3K* genes are not common in patients with MDS at diagnosis and that *JAK2* mutations may occur in MDS during disease progression. Further studies may be helpful to understand the involvement of genetic changes and the impact of these mutations in MDS progression and in different subgroups of patients with the disease.

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Contributions: João Agostinho Machado-Neto contributed to the selection of patients, carried out all experiments and participated in the writing of the manuscript; Fabiola Traina contributed to the selection of patients, clinical follow-up of the patients, analysis of the results and the writing of the manuscript; Mariana Lazarini provided technical assistance with the experiments and participated in the writing of the manuscript; Paula de Melo Campos contributed to the selection of patients and clinical follow-up of the patients; Katia Borgia Barbosa Pagnano contributed to the clinical follow-up of the patients and with the techniques to detect the FLT3-ITD and *NPM1* mutation; Irene Lorand-Metze was responsible for the morphological diagnosis of myelodysplastic syndrome in the patients included in this study; Fernando Ferreira Costa contributed to the analysis of the results; Sara T. Olalla Saad was the principal investigator.

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