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

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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). 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. 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. The same occurs for liver adenomatosis, Rubinstein-taybi syndrome and hemochromatosis.

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

FMS-like tyrosine kinase 3 (FLT3) is a tyrosine kinase receptor that plays an important role in the proliferation and differentiation of hematopoietic progenitors. Nucleophosmin (NPM1) is a key regulator of hematopoiesis that shuttles...
Table 1 - Patient characteristics.

| MDS patients | Number of individuals |
|--------------|-----------------------|
| Age in years (median (range): 63 (26-90)) | 51 |
| Gender | MF 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 |
| 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.

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%                           | RAEB/RAEBt                      | 10%                                    | None                             |
| Case 4      | RARS/RARS                            | 4%                           | myelodysplasia-related changes | 69%                                    | None                             |
| Case 5      | RA/RCMD                              | 1%                           | RAEB/R/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.

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,25 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,26 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 GFXSM 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 MgCl2, 2.5 units of Taq DNA polymerase, 1 μL of each primer (5 μM), and 2.5 μL of dNTP mix (200 μM). PCR was performed in a thermal cycler (model 9700; Applied Biosystems, Foster City, CA) according to the following protocol: initial denaturation at 94°C for 3 min, followed by 35 cycles of denaturation at 94°C for 30 s, annealing at 60°C for 30 s, and extension at 72°C for 30 s, and a final extension at 72°C for 5 min. The PCR products were purified using the GFXSM PCR DNA and Gel Band Purification Kit (Amersham Biosciences) according to the manufacturer’s instructions.
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 °C followed by 35 cycles of 20 seconds at 92 °C, 30 seconds at 57 °C and 45 seconds at 72 °C, with a final step at 72 °C 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 °C. 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, 2.5 units of Taq polymerase and 200 nM each of the forward and reverse primers (Table 3). The reaction conditions were set as follows:

| Gene | Mutation | Primers sequences | Restriction enzyme site |
|------|----------|-------------------|-----------------------|
| FLT3 | ITD      | F: 5'-GCAATTAGTTGATGAAAGCCACGAC-3' <br>R: 5'-CTTTCAGTTTTGACGCAACC-3' (HEX) | |
| NPM1 | exon 12  | F: 5'-GTCGTTAGAATAAGAAATAGAT-3' (FAM) <br>R: 5'-CTGCGCAAAGATGCCTGAC-3' | |
| JAK2 | V617F    | F: 5'-GGGTGTTCTCAGAAAGGTTA-3' <br>R: 5'-TCATTGGTCTTCTTTTCTTTTCAAA-3' | BsaXI |
| FLT3 | D835     | F: 5'-CGCCAGAAAGCTGCTG-3' <br>R: 5'-GACGCCAAGCAGAAGGCCC-3' | Eco321 |
| PI3K | exon 9   | F: 5'-TTACAGAATCTACGATCC-3' <br>R: 5'-TTTAGCACTTACCTGTGAC-3' | |
| PI3K | exon 20  | F: 5'-AGGCTTGGTGAAGATCCATGCAATCC-3' <br>R: 5'-TTGTTGAAAATCGTTTTTCAAC-3' | |

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.
5 minutes of denaturing at 94°C followed by 35 cycles of 30 seconds at 92°C, 30 seconds at 57°C and 50 seconds at 72°C, with a final step at 72°C 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 homozigous 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 MgCl₂, 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°C followed by 35 cycles of 30 seconds at 94°C, 50 seconds at 63°C and 55 seconds at 72°C, with a final step at 72°C for 7 minutes. Sequencing reactions were performed in both directions with the ABI PRISM
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. 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 screened 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
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. Although FLT3 and NPM1 mutations have been described with great frequency in cases of AML, 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 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. 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. 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. A recent publication by Malcovati et al. 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 PL3K gene are frequently described in cancer. 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, and mutations in exons 9 and 20 result in constitutive activation of this protein. The presence of PI3K mutations in AML 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, 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. Other members of the PI3K signaling pathway have also been investigated as targets for leukemia treatment. Clinical studies with rapamycin analogues, which inhibit mTOR, are currently in phase II AML trials, alone or in combination with other chemotherapeutics. Furthermore, FLT3 inhibitors have shown therapeutic activity in AML patients with FLT3 mutations, and selective JAK2 inhibitors have been tested in patients with JAK2 mutations.

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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Mutations in Patients with Myelodysplastic Syndromes

Machado-Neto JA et al.

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