Cytogenetics, *JAK2* and *MPL* mutations in polycythemia vera, primary myelofibrosis and essential thrombocythemia

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Background: *The detection of molecular and cytogenetic alterations is important for the diagnosis, prognosis and classification of myeloproliferative neoplasms.*

Objectives: *The aim of this study was to detect the following mutations: JAK2 V617F, JAK2 exon 12 and MPL W515K/L, besides chromosomal abnormalities. Furthermore, molecular and cytogenetic alterations were correlated with the leukocyte and platelet counts, hemoglobin levels and age in all patients and with the degree of fibrosis in primary myelofibrosis cases.*

Methods: *Twenty cases of polycythemia vera, 17 of essential thrombocythemia and 21 of primary myelofibrosis were selected in the Hematology Department of the Universidade Federal de São Paulo (UNIFESP) between February 2008 and December 2009. The JAK2 V617F, JAK2 exon 12 mutations, MPL W515K and MPL W515L mutations were investigated by real-time PCR and direct sequencing. G-band karyotyping and fluorescence in situ hybridization were used to detect chromosomal abnormalities.*

Results: *Chromosomal abnormalities were observed only in polycythemia vera (11.8%) and primary myelofibrosis cases (17.6%), without correlation to clinical data. Chromosomal abnormalities were not detected by fluorescence in situ hybridization. The JAK2 V617F mutation was observed in polycythemia vera (90%), primary myelofibrosis (42.8%) and essential thrombocythemia (47%). Patients with JAK2 V617F-negative polycythemia vera had lower platelet and leukocyte counts compared to V617F-positive polycythemia vera (p-value = 0.0001 and p-value = 0.023, respectively). JAK2 V617F-positive and MPL W515L-positive primary myelofibrosis cases had a higher degree of fibrosis than V617F-negative cases (p-value = 0.022). JAK2 exon 12 mutations were not detected in polycythemia vera patients. The MPL W515L mutation was observed in one case of primary myelofibrosis and in one of essential thrombocythemia. The MPL W515K mutation was not found in patients with essential thrombocythemia or primary myelofibrosis. The MPL W515L-positive patient with primary myelofibrosis had more severe anemia than other patients with primary myelofibrosis.*

Conclusions: *This study demonstrates that karyotyping for JAK2 and MPL mutations is useful in the diagnosis of myeloproliferative neoplasms. The precise pathogenetic contribution of these alterations is still unclear. However, this study adds more information about the pathophysiology of polycythemia vera, essential thrombocythemia and primary myelofibrosis.*

Keywords: *Myeloproliferative disorders; Cytogenetic analysis; Karyotype; Molecular biology, Thrombocythemia, essential; Polycythemia vera*

Introduction

Polycythemia vera (PV), essential thrombocythemia (ET) and primary myelofibrosis (PMF) are clonal hematopoietic stem cell disorders and constitute a heterogeneous group of diseases characterized by increased proliferation and maturation of the erythroid, megakaryocytic or myeloid lineages.[1]

In recent years there has been renewed interest in the molecular characterization and classification of myeloproliferative neoplasms (MPN) and the targeted therapy. This interest began with the discovery of the *JAK2 V617F* mutation in *BCR-ABL1*-negative patients. However, the exact role of JAK2 mutations remains unknown, therefore it is not known yet whether the *JAK2 V617F* mutation is the first event in MPN or is secondary to other genetic and epigenetic events.[2]

The *JAK2* and *MPL* genes play important roles in cell signaling and proliferation.[3] Mutations in these genes confer constitutive activation of the JAK-STAT pathway and other pathways promoting differentiation and proliferation of different lineages.[4] The *JAK2 V617F* mutation is present in approximately 95% of patients with PV, 58% with PMF and 50% with ET.[1] The *MPL W515L* mutation is present in about 9% of patients with PMF and 5% with ET.[5] Another mutation, *MPL W515K*, is present in 5% of patients with PMF and

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1% with ET.[6] MPL mutations are not described in PV or other myeloid diseases to date.[6] In *JAK2 V617F*-negative PV cases, some mutations have been described in exon 12 of the *JAK2* gene, corresponding to approximately 3% of all PV cases. These mutations have not been described in PMF and ET.[7]

PV, PMF and ET have common clinical and biological aspects that hinder their diagnosis and thus cytogenetic and molecular studies are important tools to clarify the pathophysiological and clinical features in these diseases. Thus, the investigation of the *JAK2 V617F*, *MPL W515K* and *MPL W515L* mutations and of cytogenetic alterations using karyotyping and fluorescence in situ hybridization (FISH) may provide a more detailed understanding of the pathophysiology processes of PV, PMF and ET, as well as being important in the diagnosis, prognosis and classification of these diseases.[8]

Methods

Patients and samples

Twenty patients diagnosed with PV, 21 with PMF and 17 with ET according to World Health Organization (WHO) criteria (2008) were selected in the Hematology Department of the Universidade Federal de São Paulo (UNIFESP) between February 2008 and December 2009. G-band karyotyping and FISH were used to evaluate bone marrow samples (3 mL), while peripheral blood samples (3 mL) were collected to investigate the *JAK2 V617F*, *MPL W515K* and *MPL W515L* mutations. The age, gender, leukocyte count, platelet count, hemoglobin concentration, degree of marrow fibrosis and duration of disease were noted from the patients' records. Patients with unclear diagnoses or that did not agree to participate in the study were excluded. The study was approved by the Research Ethics Committee of UNIFESP.

G-band karyotyping

Briefly, 3 mL of bone marrow harvested in sodium heparin was cultivated for a short time without mitogens. Then, 70 uL of Colcemid® were added and after 30 minutes the sample was centrifuged and the supernatant discharged. Potassium chloride (0.075 mol/L) was added to the pellet for 20 minutes followed by fixing using Carnoy`s fixative three times.[9] Part of the sample (1 mL) was allocated for FISH and stored in a refrigerator at 4°C until use. Some material was dripped on glass slides, air dried, aged and banded using trypsin-Giensa (GTG). Chromosomal abnormalities were described according to the International System for Human Cytogenetic Nomenclature (ISCN - 2009).[10] The definition of a clone, from the cytogenetic point of view, was the presence of the same structural anomaly in at least two cells or the absence of the same chromosome in at least three metaphases.[9]

Fluorescence in Situ Hybridization (FISH)

The following probes were used for FISH according to the manufacturer's instructions: centromere of chromosome 8 (Alpha satellite 8 Green, CytoCell Aquarius probe), centromere of chromosome 9 (Classical satellite 9 red, Cytocell Aquarius probe), region 13q14.3 and 13qter (13q14.3 deletion probe, Cytocell Aquarius probe) and region 20q12 and 20q13.1 (del 20q, Cytocell Aquarius probe). These probes recognize the regions of the most common chromosomal alterations reported in MPN.

After informed consent, samples from three bone marrow donors were used as normal controls. These values were defined using the β-inverse function in Microsoft Excel software.[11] All cases were investigated by two observers, each of whom examined 100 cells, and the mean was calculated for each observer. The number of cells analyzed was increased to 500 when there was a discrepancy of more than 10% between the means or when the end result was very close to the cutoff point. When more than 20% of cells showed no signal, the slide was considered not analyzable and the test was repeated.

Investigation of the *JAK2 V617F*, *MPL W515K* and *MPL W515L* mutations

An investigation of the *JAK2 V617F* mutation was performed for all 58 patients and the *MPL W515K* and *MPL W515L* mutations for all 21 PMF and 17 ET patients by real-time PCR. Genomic DNA was extracted from peripheral blood collected in EDTA using the QiAmp® DNA Blood Mini kit (Qiagen).

PCR was performed using the *JAK2* MutaScreen™ kit (Ipsogen) and the *MPL* MutaScreen™ kit (Ipsogen), which contains a positive control (100% mutated alleles), negative control (0% mutated alleles), reference sample (2% of mutated alleles) and a mixture of primers and probes. For each reaction the following reagents were used: 12.5 uL of Taqman Universal PCR Master Mix (Applied Biosystems), 2.5 uL of the mixture of primers and probes and 25 ng of DNA giving a final volume of 25 uL. The reaction conditions (50°C for two minutes, followed by 95°C for 10 minutes, 50 cycles of 92°C for 15 seconds and 60°C for one minute) were according to the instructions of the manufacturer for the *JAK2* MutaScreen™ kit (Ipsogen). A Rotor-Gene™ 3000 (Corbert) thermal cycler was used.

Investigation of mutations in exon 12 of the *JAK2* gene

All PV patients were selected to analyze exon 12 of the *JAK2* gene by direct sequencing. A preliminary amplification of exon 12 was performed using PCR. For the PCR reaction the following reagents were used: 5 mL buffer amplifier (10x), 3 mL of $MgCl_2$ (25 mmol/L), 2 mL of dNTP

(containing 2.5 mM of each nucleotide), 1 unit Taq polymerase (Fermentas, USA), 2 mL of each primer (10 mM) and 100 ng of DNA in a final volume of 50 µL. The primers used for the amplification of exon 12 have the following sequences: 5'-CATACTTTCAGTGTATTTTGAAGTG-3' (forward) and 5'-ATGTCACATGAATGTAAATCAAG-3' (reverse). The reaction conditions were 94°C for 5 minutes, 40 cycles of 94°C for 30 seconds, 59°C for 30 seconds and 72°C for 45 seconds, with a final extension of 72°C for 7 minutes. After PCR, the amplification was checked by 1% agarose gel electrophoresis stained with ethidium bromide. After amplification of the fragments of interest by PCR, samples were purified with the QIAquick PCR Purification kit (Qiagen) according to the manufacturer's information. Direct sequencing of purified PCR products was performed for both strands, using the dideoxy chain-termination method (BigDyeTM Terminator v.3.0 Ready Reaction Cycle Sequencing Kit - Applied Biosystems). The resulting sequences were compared to those described in the GenBank and Ensembl reference sequence databases (http://www.ncbi.nlm.nih.gov/sites/entrez and http://www.ensembl.org) using the CLC Sequence Viewer program (http://www.clcbio.com/index.php?id=28) and BioEdit (http://www.mbio.ncsu.edu/BioEdit/bioedit.html).

Statistical analysis

The Statistical Package for the Social Sciences (SPSS®) version 18 was used for statistical analysis. The Shapiro-Wilk test was used to assess normal distribution of continuous variables. Student's t test was used to analyze variables with normal distribution and the Mann-Whitney test for non-normal distribution. The chi-square associated with Yates correction was used to compare degrees of fibrosis between groups with and without specific mutations (*JAK2 V617F* and *MPL W515K / L*) and between groups for disease diagnosis or monitoring. The level of significance in the tests was set for an alpha error of 5% (p-value < 0.05).

Results

G-band Karyotyping

Chromosome abnormalities were detected in 2/17 (11.8%) patients with PV, 3/17 (17.6%) with PMF and no cases with ET. The alterations identified in PV were del(13)(q14) and loss of the Y chromosome and in PMF trisomy 8 (two cases) and one case of trisomy 9 were detected (Table 1).

FISH

No cases presented abnormalities for the regions analyzed (13q14.3, 13qter, 20q12, 20q13.1 and centromeres of chromosome 8 and 9) as shown in Table 1. Figure 1 shows normal results of FISH probes for the 13q14.3 and 13qter regions.

Investigation of JAK2 V617F and MPL W515K/L

The *JAK2 V617F* mutation was detected in 18/20 (90%) cases of PV, 9/21 (42.9%) of PMF and 8/17 (47.1%) of ET. Investigations of the *MPL W515K* and *MPL W515L* were performed for all patients with ET and PMF. The *MPL W515L* mutation was found in 1/21 (4.8%) cases of PMF and 1/17 (5.9%) of ET. The *MPL W515K* mutation was not detected (Table 1). Figure 2 shows the curves drawn by the analysis program showing a JAK2 V617F-positive patient.

Investigation of mutations in exon 12 of the JAK2 gene

No patients with PV, even the two cases who did not have the *JAK2 V617F* mutation, exhibited mutations in exon 12 of the JAK2 gene (Table 1). Figure 3 shows an electropherogram from the BioEdit program; there are no anomalous peaks compared with the sequence pattern and thus no differences in the sample tested.

Number of clonal events analyzed

There were no PV patients with concomitant mutations in exon 12 of the *JAK2* gene and the *JAK2 V617F* mutation. Likewise, no PMF and ET patients had *JAK2 V617F* together with *MPL W515L* or *MPL W515K* mutations. However, one patient with PV and two with PMF had the *JAK2 V617F* mutation and abnormal karyotypes (Table 1).

Correlation with clinical data

Of the 20 patients with PV, 12 (60%) were male and 8 (40%) were female with a mean age of 60.4 ± 11.9 years old. Of the 21 patients with PMF, 11 (52.3%) were male and 10 (47.7%) were female with an average age of 64.9 ± 13.5 years old. And of the 17 patients with ET, four (23.5%) were male and 13 (76.5%) were female with an average age of 59.3 ± 14.5 years old. There were no statistical differences between the mean ages of patients with different diseases (p-value = 0.60).

No correlation was found in respect to: age, the leukocyte count, platelet count, hemoglobin concentrations, and molecular cytogenetic alterations between the different groups of patients. However, PV patients without the *JAK2 V617F* mutation presented lower leukocyte and platelet counts compared to *JAK2 V617F*-positive PV patients. The average platelet counts of *JAK2 V617F*-negative and -positive PV patients were 202.5 x 10^9/L and 683.3 x 10^9/L, respectively (p-value < 0.0001), and the mean leukocyte counts of *JAK2 V617F*-negative and -positive PV patients were 8.8 x10^9/L and 13 x10^9/L, respectively (p-value = 0.023 - Table 2).

The PMF patient with the MPL W515L mutation was older (87 years) than the average age of the *JAK2 V617F*-positive and -negative PMF patients (59.2 and 67.7, respectively). In addition, her hemoglobin level (6.4 g/dL) was lower than the mean of *JAK2 V617F*-positive and -negative PMF patients (12.03 g/dL and 11.53 g/dL, respectively). The mean hemoglobin for all patients with PMF was 11.5 g/dL, the mean leukocyte count was 15.8 x10^9/L and the mean platelet count was 628 x 10^9/L.

Table 1 - Results of karyotyping and FISH, hemoglobin levels, leukocyte and platelet counts, degree of fibrosis and duration of disease of all patients

Patient	Diagnosis	Karyotype	FISH	*JAK2 V617F*	JAK2 Exon 12	*MPL W515K/L*	Gender	Age	Hb	Leuk	Plate	Degree of fibrosis	Time of disease (years)
1	PV	46,XY[19]	WA	POS	NEG	-	M	46	26.0	13,100	304,000	-	3
2	PV	46,XY[19]	WA	POS	NEG	-	M	58	20.4	11,000	755,00	-	Diag.
3	PV	46,XY[13]	WA	POS	NEG	-	M	62	19.7	12,440	212,000	-	Diag.
4	PV	46,XX[15]	WA	POS	NEG	-	F	58	18.8	10,900	575,000	-	6
5	PV	46,XX,del(13)(q14)[9]/46,XX[7]	ND	POS	NEG	-	F	65	22.4	21,870	351,000	-	4
6	PV	WM	WA	POS	NEG	-	F	77	20.7	28,700	904,000	-	Diag.
7	PV	46,XY[20]	WA	POS	NEG	-	M	49	19.5	13,100	1,025,000	-	4
8	PV	46,XY[17]/46,X,-Y[3]	WA	NEG	NEG	-	M	60	19.4	8,000	202,000	-	Diag.
9	PV	46,XY[20]	WA	POS	NEG	-	M	48	16.0	11,600	533,000	-	4
10	PV	46,XY[20]	WA	NEG	NEG	-	M	55	21.0	9,600	203,000	-	15
11	PV	WM	WA	POS	NEG	-	F	74	16.5	13,000	1,400,000	-	6
12	PV	46,XY[15]	WA	POS	NEG	-	M	45	17.0	10,600	738,000	-	4
13	PV	46,XX[8]	WA	POS	NEG	-	F	73	21.1	14,000	559,000	-	11
14	PV	46,XX[20]	WA	POS	NEG	-	F	76	16.5	14,600	994,000	-	Diag.
15	PV	WM	WA	POS	NEG	-	M	64	19.9	16,200	472,000	-	Diag.
16	PV	46,XY[18]	WA	POS	NEG	-	M	74	21.0	13,000	386,000	-	Diag.
17	PV	46,XY[10]	WA	POS	NEG	-	M	71	21.7	11,600	354,000	-	Diag.
18	PV	46,XY[20]	WA	POS	NEG	-	F	64	19.4	10,300	1,031,000	-	Diag.
19	PV	46,XX[20]	WA	POS	NEG	-	F	55	21.0	35,000	650,000	-	20
20	PV	46,XY[15]	WA	POS	NEG	-	M	34	19.1	11,900	1,057,000	-	3
21	PMF	46,XY[18]	WA	POS	-	NEG	M	42	16.0	10,800	641,000	II	Diag.
22	PMF	46,XY[18]	WA	NEG	-	NEG	M	88	13.2	10,290	989,000	II	Diag.
23	PMF	47,XY,+9[14]/46,XY[4]	ND	POS	-	NEG	M	65	10.7	3,860	210,000	III	Diag.
24	PMF	47,XY,+8[20]	ND	NEG	-	NEG	M	59	13.6	42,100	33,000	I	Diag.
25	PMF	WM	WA	NEG	-	POS *MPL W515L*	F	87	6.4	8,100	300,000	III	Diag.
26	PMF	46,XY[15]	WA	POS	-	NEG	M	67	11.1	20,900	1,326,000	III	Diag.
27	PMF	46,XX[14]/47,XX,+8[3]	ND	POS	-	NEG	F	64	11.5	10,800	707,000	III	Diag.
28	PMF	46,XX[6]	WA	POS	-	NEG	F	52	13.8	8,400	834,000	III	Diag.
29	PMF	46,XY[20]	WA	POS	-	NEG	M	69	12.8	8,490	568,000	III	Diag.
30	PMF	46,XY[8]	WA	NEG	-	NEG	M	53	12.8	23,900	339,000	III	Diag.
31	PMF	46,XX[20]	WA	NEG	-	NEG	F	72	12.2	19,800	200,000	I	Diag.
32	PMF	46,XX,9ph[20]	WA	NEG	-	NEG	F	49	10.8	8,600	1,058,000	III	Diag.
33	PMF	46,XX[12]	WA	POS	-	NEG	F	44	11.0	6,700	472,000	III	7
34	PMF	46,XX[10]	WA	NEG	-	NEG	F	80	8.5	7,700	38,000	II	Diag.
35	PMF	46,XX[6]	WA	NEG	-	NEG	F	66	10.7	6,400	438,000	III	5
36	PMF	46,XY[20]	WA	NEG	-	NEG	M	85	12.1	10,000	345,000	II	Diag.
37	PMF	WM	WA	POS	-	NEG	M	70	11.6	17,300	1,134,000	III	Diag.
38	PMF	46,XX[15]	WA	NEG	-	NEG	F	50	11.3	11,500	1,644,000	II	4
39	PMF	WM	WA	POS	-	NEG	M	60	9.8	3,300	92,000	III	Diag.
40	PMF	WM	WA	NEG	-	NEG	F	75	13.2	8,900	1,120,000	III	3
41	PMF	46,XY[20]	WA	NEG	-	NEG	M	65	8.4	9,070	625,000	II	2
42	ET	46,XX[20]	WA	NEG	-	NEG	F	41	13.0	8,220	981,000	-	5
43	ET	46,XX[7]	WA	NEG	-	NEG	F	61	12.1	9,090	1,176,000	-	2
44	ET	46,XY[18]	WA	NEG	-	NEG	M	67	15.9	7,290	793,000	-	Diag.
45	ET	46,XX[20]	WA	POS	-	NEG	F	67	14.7	8,870	745,000	-	Diag.
46	ET	46,XX[20]	WA	POS	-	NEG	F	51	12.3	8,000	296,000	-	8
47	ET	46,XX[13]	WA	POS	-	NEG	F	70	15.4	48,000	1,030,000	-	Diag.
48	ET	46,XX[19]	WA	NEG	-	NEG	F	81	13.8	11,900	826,000	-	4
49	ET	WM	WA	POS	-	NEG	M	30	16.2	8,080	1,034,000	-	Diag.
50	ET	46,XX[16]	WA	POS	-	NEG	F	71	12.0	9,640	921,000	-	Diag.
51	ET	46,XX[20]	WA	NEG	-	NEG	F	34	13.0	11,700	865,000	-	4
52	ET	46,XY[20]	WA	NEG	-	NEG	M	53	13.0	11,400	1,089,000	-	7
53	ET	WM	WA	POS	-	NEG	F	70	13.2	10,600	1,565,000	-	8
54	ET	46,XX[20]	WA	NEG	-	NEG	F	54	12.4	6,090	1,773,000	-	7
55	ET	WM	WA	NEG	-	NEG	M	60	12.0	10,000	900,000	-	Diag.
56	ET	WM	WA	NEG	-	*POS W515L*	F	65	13.5	14,800	748,000	-	Diag.
57	ET	WM	WA	POS	-	NEG	F	65	13.3	17,500	626,000	-	Diag.
58	ET	WM	WA	POS	-	NEG	F	74	13.0	35,000	650,000	-	Diag.

PV: polycythemia vera; PMF: primary myelofibrosis; ET: essential thrombocythemia; WM: without metaphase; WA: without alteration; ND: not done; (-): not evaluated in this study; POS: positive; NEG: negative; M: male; F: female; Hb: hemoglobin; Leuk: Leukocytes (x 10^6/L); Plate: platelets (/mm^3); Diag: at diagnosis. FISH using probes for 20q12, 20q13.1, 13q14, 13qter and centromeres of chromosomes 8 and 9.

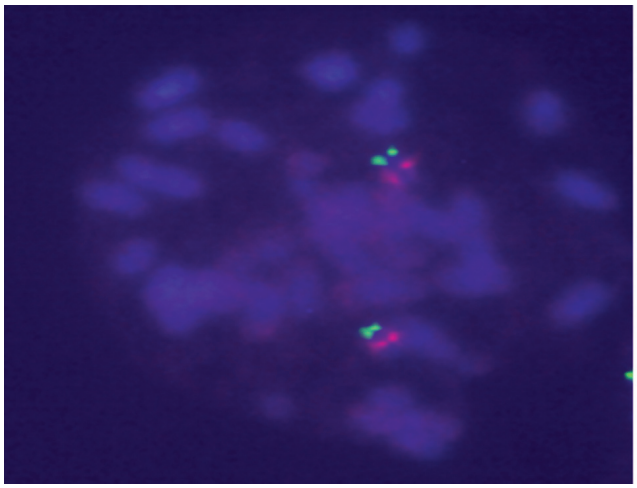

Figure 1 – Metaphase showing signals for the 13q14.3 region in red and for 13qter in green, i.e., there is no loss or gain of chromosomal material in these regions

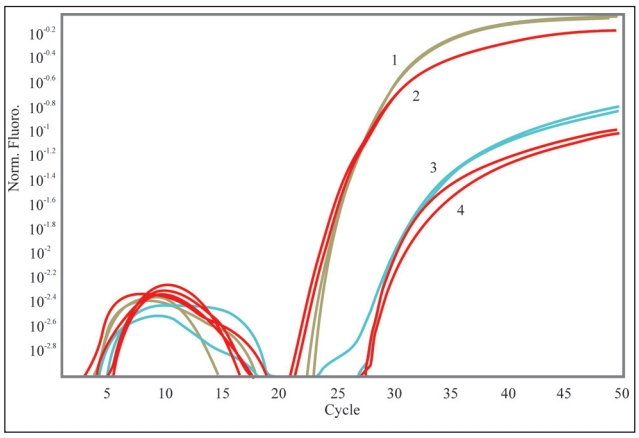

Figure 2 – *JAK2 V617F*-positive patient
1: positive control (100% of mutated alleles); 2: Sample tested; 3: Reference sample (2% of mutated alleles); 4: Negative control (0% of mutated alleles)

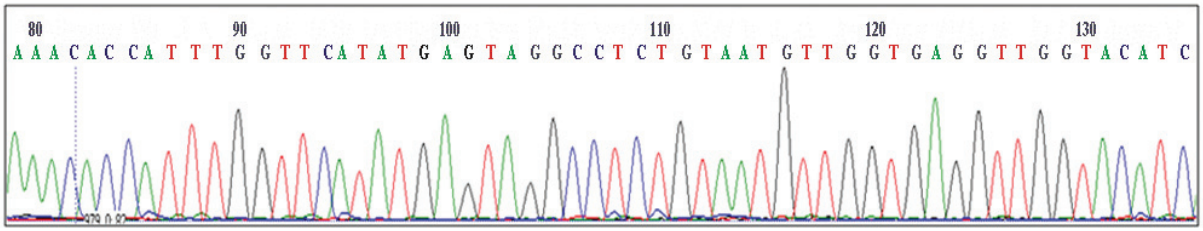

Figure 3 – Electropherogram of the BioEdit program, showing the absence of mutations in exon 12 of the *JAK2* gene

Table 2 - Polycythemia vera, primary myelofibrosis and essential thrombocythemia in respect to the *JAK2 V617F* mutation status, age, leukocyte and platelet counts and hemoglobin levels

	Polycythemia vera			Primary myelofibrosis			Essential thrombocythemia		
	Positive	Negative	p-value	Positive	Negative	p-value	Positive	Negative	p-value
JAK2 V617F mutation (%)	90	10		42.8	57.2		47	53	
Age (years)	60.7	57.5	0.44	59.2	67.7	0.16	62.3	56.4	0.44
Leukocytes (x 10^6/L)	13,000	8,800	0.02	9,645	10,000	0.83	10,120	10,000	0.50
Platelets (mm^3)	683,300	202,500	0.0001	628,400	602,818	0.68	833,000	900,000	0.30
Hemoglobin (g/dL)	19.69	20.20	0.62	10.7	11.52	0.77	14.0	13.0	0.23

For polycythemia vera and primary myelofibrosis, the data on age, platelet count and hemoglobin levels are expressed as means and the leukocyte count as a median. For essential thrombocythemia, the data on leukocyte and platelet counts and hemoglobin levels are expressed as medians and the age as a mean.

There was a significant difference between the degree of fibrosis and presence of the *JAK2 V617F* and *MPL W515L* mutations in PMF cases (p-value = 0.022). Patients with positive markers (*JAK2 V617F* and *MPL W515L*) had a higher degree, usually grade III, of fibrosis (Figure 4). The patient with *MPL W515L* also presented with a stage of sclerosing fibrosis. There was no statistical difference between the degree of fibrosis and the duration of the disease, i.e., patients

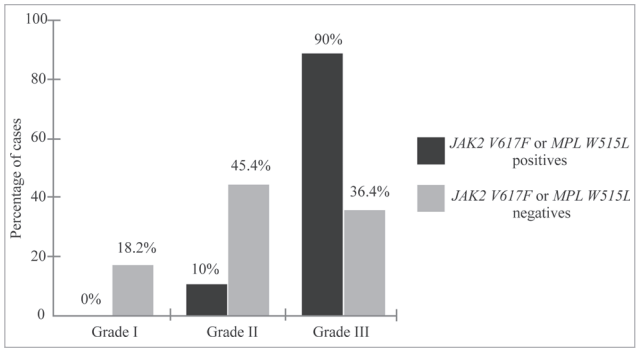

Figure 4 – Relationship between the degree of fibrosis and the presence of molecular markers (*JAK2 V617F* and *MPL W515L*) in primary myelofibrosis patients (p-value = 0.022)

with the disease longer did not have a more intense degree of fibrosis (p-value = 1).

Discussion

Chromosomal abnormalities are observed at diagnosis in between 10 to 25% of patients with PV,[12] about 30% with PMF[13] and 7% with ET.[14] Thus the incidence of chromosomal abnormalities found here was a little lower for PV and PMF than what has been reported in the literature, i.e., 11.8% of patients with PV and 17.6% with PMF. In some cases it was not possible to obtain a satisfactory mitotic index (20 metaphases). Due to fibrosis in PMF cases, it is common to have an insufficient number of metaphases and there is no way to overcome this difficulty in karyotyping. Therefore FISH is useful in these cases. However, FISH did not help in detecting chromosomal alterations in this study. In addition, patients on myelosuppressive agents may have low mitotic indexes. The alternative would be the suspension of the drug before sample collection; an option not considered due to the clinical consequences.

The del(13q) abnormality is present in 10% of cases of PMF and PV and in rare cases of ET.[12,14,15] Furthermore, this deletion is not related to changes in the clinical course of the disease.[13] Here, the patient with del(13q) showed no discrepancy regarding the mean leukocyte and platelet counts and hemoglobin levels compared to other patients with PV.

Trisomy 8 is present in about 20% of cases of PV, 6% of PMF and 10% of ET.[12,14] The prognosis conferred by the presence of trisomy 8 is not yet fully defined. One of the cases studied here presented a complicated clinical course, with thrombotic events leading to death, while the other had a clinical outcome similar to patients with normal karyotypes and no history of thrombotic events.

Trisomy 9 is present in 10% of PMF, PV and ET cases.[12,14] The prognosis of patients with this alteration is controversial in terms of survival and evolution of the disease, but more recent studies have shown that this abnormality is a sign of worse prognosis in PMF.[13,15] The patient with trisomy 9 did not show discrepancies in the mean leukocyte and platelet counts and hemoglobin levels compared to the other PMF patients.

The loss of the Y chromosome is present in 4% of PV patients and is associated with older age but has no prognostic relevance.[12] The patient in this case was a 60-year-old man who did not have the *JAK2 V617F* mutation.

The use of more sensitive techniques could extend the threshold of abnormalities detected; the thresholds for G-band karyotyping, FISH and array comparative genomic hybridization (CGH-a) are 5 Mb, 1 Mb and 50 Kb, respectively.[16] CGH-a has the potential to detect submicroscopic genomic imbalances and may be carried out with DNA without the need of metaphases and is a surrogate for cases of bone marrow fibrosis.[17] In 2009, Tefferi et al. showed that CGH-a expands the detection of chromosomal abnormalities in PMF patients.[17] However, the discovery of numerous genomic imbalances by CGH-a has no clinical significance yet; no conclusion has been reached regarding prognosis, therapeutic response and evolution of PMF related to such micro-alterations.

The incidence of the *JAK2 V617F* mutation observed in this study (90% of cases of PV, 42.8% of PMF and 47% of ET) is similar to reported data. The incidence of the mutation can vary somewhat between studies and it is believed that one of the factors responsible for these differences is the sensitivity of the method used.[18-21]

Although the vast majority of PV patients present the *JAK2 V617F* mutation, about 5% were V617F negative, even when using more sensitive detection methods. These cases require further investigations of other mutations, such as mutations in exon 12 of the *JAK2* gene which also characterize gain of function. However, we did not identify PV patients with mutations in exon 12, suggesting that there may be other, as yet unknown, mutations. In this study, two PV cases did not have mutations in exon 12 or the *JAK2 V617F* mutation. The diagnoses of these two cases were confirmed by the WHO criteria (2008). Both had hemoglobin levels ≥ 18.5 g/dL as a major criterion, and low serum erythropoietin levels and hypercellular bone marrow biopsies with prominent erythroid, granulocytic and megakaryocytic proliferations as minor criteria. One of these patients had a clonal alteration, loss of the Y chromosome, while no abnormality was detected for the other. In respect to clinical data, these two patients had lower platelet and leukocyte counts (p-value < 0.0001 and p-value = 0.023, respectively) compared to *JAK2 V617F*-positive PV patients. These data show that the absence of *JAK2* mutations may improve the excessive proliferation of megakaryocytic and granulocytic series in PV cases.

The sensitivity of the method used also influences the detection of mutations in exon 12 of the *JAK2* gene. Recent studies show that such mutations are present in a small fraction of granulocytes of some patients. In addition, several groups have demonstrated that conventional Sanger sequencing without enrichment of the sample, as used in this study, cannot detect this type of mutation.[7,22,23] Laughlin et al. presented a technique called clamp that favors the amplification of the mutant allele, thereby increasing the analytical capabilities of testing.[24] The use of even more advanced technology, coupled with the enrichment of the sample, are significant improvements in the detection of most or all of the mutations in exon 12 of the *JAK2* gene.

The relationship between survival, clinical course, transformation of the disease and *JAK2 V617F* is not yet fully defined. In addition, the literature does not provide reports of significant associations of *V617F, MPL* mutations and degree of fibrosis in patients with PMF. Here we

demonstrated a statistical difference between patients with these molecular markers (*JAK2 V617F* and *MPL W515L*) and fibrosis (p-value = 0.003). *Most V617F*-positive patients had Grade III fibrosis, whereas most *V617F*-negative individuals had Grade II fibrosis. The fact that no statistical correlation was found between the duration of the disease and increases in the degree of fibrosis in this study (p-value = 1) reinforces the idea that the presence of molecular markers (*JAK2 V617F* and *MPL W515L*) promote greater complications in these patients. The increase in the degree of bone marrow fibrosis may promote pancytopenia (due to ineffective hematopoiesis), extramedullary hematopoiesis (spleen), production of atypical cells (atypical megakaryocytes and tear cells) and a greater likelihood of transformation to acute leukemia.

The incidence of the *MPL W515L* mutation was similar to the literature. *MPL W515K* mutations were not found. Cases of ET and PMF with MPL mutations have similar morphological and pathological bone marrow results as cases of ET and PMF with *JAK2* mutations, including hypercellularity and fibrosis.

ET patients with *MPL* mutations show no statistically significant differences in relation to platelet levels when compared to *JAK2 V617F*-positive ET patients. However, a statistical difference is detected when hemoglobin levels are compared. Thus, ET patients with *MPL* mutations have lower hemoglobin levels when compared to *JAK2 V617F*-positive ET patients.[25] The ET patient with *MPL W515L* had a platelet count similar to the mean count as *JAK2 V617F*-positive ET cases (748 x 10^9/L and 858 x 10^9/L, respectively), a slightly lower leukocyte count (14.8 x10^9/L and 18.2 x 10^9/L, respectively) and almost the same level of hemoglobin (13.5 g/dL and 13.7 g/dL, respectively).

The PMF patient with the *MPL W515L* mutation presented a different clinical course from the *JAK2 V617F*-positive PMF patients. She was an 87-year old and had severe anemia (hemoglobin 6 g/dL) that was more pronounced than in *JAK2 V617F*-positive and -negative patients. In addition, she had Grade III fibrosis, highlighting that the *MPL W515L* mutation is more aggressive compared to the *JAK2 V617F* mutation. The patient died due to abdominal thrombosis.

Thus, the results of this study show that karyotyping and analysis of *JAK2 V617F* and *MPL W515L* mutations are useful tools in the diagnosis of MNP, making it more precise. The presence of *JAK2* and *MPL* mutations demonstrates different clonal alterations, indicating the presence of different pathophysiological mechanisms in these diseases.

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