INCA

Investigation of new mutations in JAK2 and MPL in patients with BCR-ABL1 negative myeloproliferative neoplasms

80 anos

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Abstract

BCR-ABL1 negative myeloproliferative neoplasms (MPN) are diseases of clonal hematopoietic stem cell, in which there are proliferation and maturation of one or more series of myeloid cells, resulting in erythrocytosis, thrombocytosis and/or leukocytosis. MPNs are categorized in polycythemia vera (PV), primary myelofibrosis (PMF) and essential thrombocythemia (ET). Somatic mutations in *JAK2*, *CALR*, and *MPL*, represent major diagnostic criteria in combination with hematologic and morphological abnormalities. *JAK2* (V617F) mutation is the most frequently found in MPN with frequencies of 98% in PV and of 50 to 60% in ET and PMF. *JAK2* (V617F) negative PV patients may present mutations on exon 12 of the same gene, with all of them bearing a *JAK2* mutation. From 60 to 84% of *JAK2* (V617F) negative ET and PMF patients can present indels on *CALR* exon 9. MPL mutations can be found in 3% of ET patients and in 5 to 10% of PMF patients. Mutations in *JAK2*, *CALR* and *MPL* are mutually exclusive. However, about 10% of patients with ET and 5% to 10% of those with PMF have no evidence of a canonical somatic mutation in in *JAK2*, *CALR*, or *MPL* and are defined as triple negative. Recently, those cases have been screened for noncanonical mutations *JAK2* and *MPL*, and those new mutations have shown to be potentially helpful for diagnosis. Mutations outside *MPL* exon 10 and *JAK2* exons 12 and 14 have been found in approximately 20% of triple negative patients with ET or PMF. In our cohort, analysis of patients with PMF by new generation sequencing has identified four new variants of *MPL* and *JAK2* outside the classic analyzed exons. In this work, we propose the screening and validation of newly detected *JAK2* and *MPL* mutations in our cohort of triple negative patients diagnosed with ET and PMF. This work can contribute to a better characterization of triple negative patients and to the comprehension of the pathophysiology of MPN.

Patients and Ethical Aspects

This study analyzed 134 patients from Hospital Universitário Pedro Ernesto (HUPE/UERJ) and Hospital Universitário Antônio Pedro (HUAP/UFF) diagnosed with ET or PMF. Initially, samples were screened for classical *JAK2*, *CALR* and *MPL* mutations, which reduced the analysis cohort to 35 triple negative patients, of which 17 had clinical data for future correlation with molecular results. Clinical data were collected in a form elaborated by clinicians and patients signed an informed consent form. This study is approved by Ethics Comitee n° 062/08.

Material and Methods

- ✓ Analysis of JAK2 (V617F) was done by allele specific PCR.
- ✓ Detection of indels in CALR exon 9 (del 52pb and ins 5pb) was done by PCR followed by fragment analysis.
- ✓ Point mutations on MPL exon 10 (S505E, W515L/K) were detected by PCR followed by direct sequencing.
- ✓ Primers for analysis of non-classical mutations on MPL exons 3(T119I), 4 (S204P/F, E230G), 6 (R321Q, C322G) and 12 (Y591L/D); and on JAK2 exons 13(F556V), 15(V625F), 16(L696I), 17(P727T) and 21 (Y931C) were designed on Primer 3 and blasted on Primer blast on NCBI.
- ✓ PCR reactions for each exon were standardized for MgCl2 concentration and primer annealing temperature, and amplicons were analyzed by direct sequencing.
- ✓ Sequencing and fragment analysis were performed on a 3130xl (Applied Biosystems) and data were analyzed with software Mutation Surveyor 3.0 or Chimer Marker (SoftGenetics, LLC).

Table 1: Primers designed for amplification of JAK2 and MPL

Gene	Exon	Forward (5'>3')	Reverse (5'>3')
JAK2	13	TTC CTA CTT CGT TCT CCA TCT TT	TGA GAG CAC ATC TTT AAA CAG CA
	15	AAA GTT GTG AGT TTT GCC AAT TT	TGG CAT CCA ATT ACA GAT TTA TTTT
	16	TGC TCC AGT ACT TGT GGA CTG	CCA CTG CCC AAG TAA AGC TTA
	17	AAC CCT ACT CTG TTC GTA TCA TTT	CAA ATC ATG TGA AAG AAA TAT GAA AGT
	21	GGC AGA GTA AAA CAT TAT TTC CA	ACA CGG TTG CTT CAT CTA CA
MPL	3	CCT ATC CCA GGC AGT GAG AAG	ACA AGG GAG TCT TGG GGT G
	4	CCA GAG GCT GAG CCA TAG AC	TGG GGC AAG ATT GAA GGT AG
	6	GGA CAG GAA CTA TGT TCA GGG	CAT TGA AAG CTG GGT TTG G
	12	CTA CCA GTG TGC CAT CCC C	TGA AGC CTA ATT GTG AGG GC

Results and Discussion

JAK2 (V617F) negative patients with ET and PMF were analyzed for mutations on *MPL* exon 10 (W515L, W515K, W515A, W515R and S505N) and indels on *CALR* exon 9 (del52bp and ins5bp). The genomic DNA from the samples were amplified by PCR and visualized on 2% agarose gel.

Analysis of indels in CALR exon 9

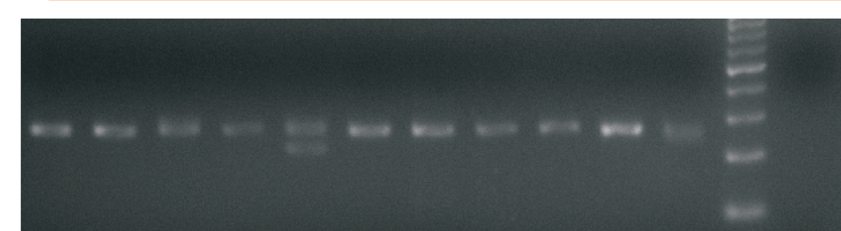

Figure 1: Analysis of PCR products of CALR in 2% agarose gel. On columns 1 to 11 samples from patients, on column 12, 100 bp DNA ladder (Invitriogen) and on column 13 negative control (water). Samples non-mutated show a single band of 260 bp and mutated samples show two bands, one in 260 and 210 bp (deletion of 52 bp) and 260 and 265 bp (insertion of 5 bp).

- ✓ PCR amplification of CALR generates a single fragment of 260 bp for non-mutated samples;
- ✓ Samples with 52 bp deletions show fragments with 260 bp, corresponding to the wild type allele, and one with 210 bp, corresponding to the mutated allele;
- ✓ Samples with 5 bp insertions, show fragments with 260 bp, corresponding to the wild type allele, and one with 265 bp, corresponding to the mutated allele;
- ✓ Samples were analyzed by fragment analysis for the confirmation of mutational status, as shown on Figure 2;ystems) and data were analyzed with software Mutation Surveyor 3.0 or Chimer Marker (SoftGenetics, LLC).

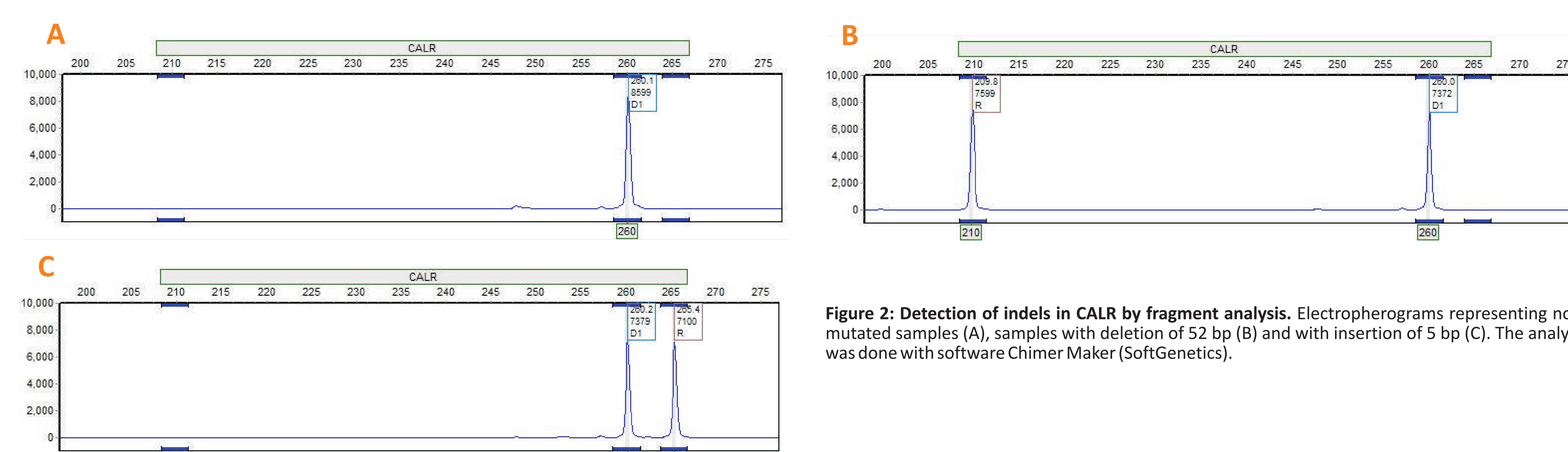

Figure 2: Detection of indels in CALR by fragment analysis. Electropherograms representing non-mutated samples (A), samples with deletion of 52 bp (B) and with insertion of 5 bp (C). The analysis was done with software Chimer Maker (SoftGenetics).

- ✓ Five samples showed indels in CALR: two with insertions and three with deletions;
- ✓ Samples positive for CALR mutations were excluded from subsequent analysis;

Analysis of mutations in MPL exon 10

- ✓ PCR for MPL generates a 200 bp amplicon (Figure 3);
- ✓ Two amplifications were necessary for a better reaction yield (Figure 4);
- ✓ Products were purified directly from the reaction (Figure 5) and submitted to direct sequencing (Figure 6)

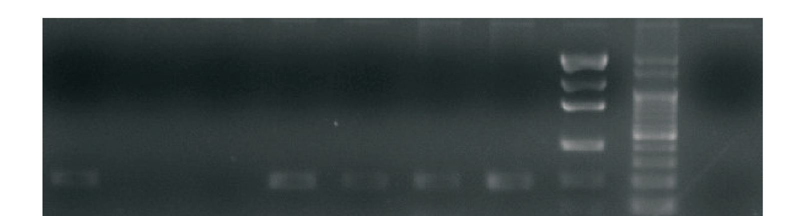

Figure 3: Analysis of MPL on 2% agarose gel. On columns 1 to 8 samples of patients, on column 9 Low Mass DNA Ladder (Thermo Fisher Scientific), on column 10 100 bp DNA Ladder (Invitrogen) and on column 11 negative control. Samples show a single band at 200 bp.

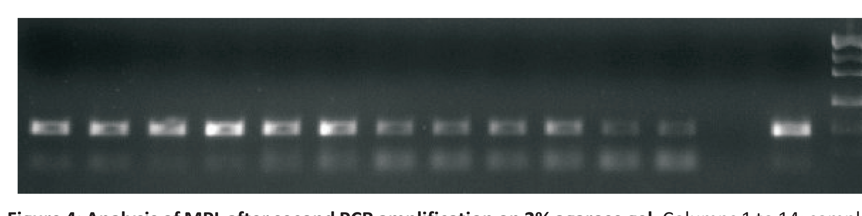

Figure 4: Analysis of MPL after second PCR amplification on 2% agarose gel. Columns 1 to 14: samples from patients and on column 15 100 bp DNA ladder (Invitrogen). Samples show a single band at 200 bp.

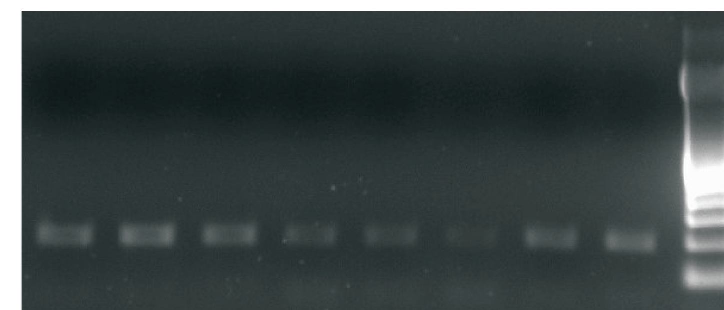

Figure 5: Analysis and quantification of purified MPL on 2% agarose gel. On columns 1 to 8 the patients' samples and on column 9 Low DNA Mass Ladder (Thermo Fisher Scientific). Samples show a single band at 200 bp.

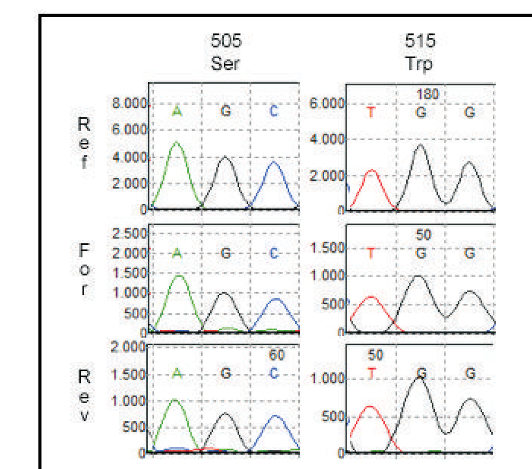

Figure 6: Representative eletropherograms of the analysis of MPL exon 10. Featuring codons 505 and 515 of non-mutated samples. Sequencing analysis was done with software Mutation Surveyor 3.0 (Soft Genetics).

- ✓ No mutations were found on exon 10 of MPL;
- ✓ 35 patients classified as triple-negatives 20% of total, against 10-15% described on literature;
- ✓ 17 patients had data for future correlation and were submitted to the analysis described below;

Primers for new mutations

✓ PCR reactions standardized for primer annealing temperature and MgCl2 concentration:

- Temperature gradient with intervals of 2 oC, from 52 oC to 58 oC;
- MgCl2 concentrations ranged from 0,75 to 2,0 mM;
- 0,75mM was enough for amplification, whereas 2,0mM was too much (Figure 7);
- For some exons, MgCl2 concentration didn't alter the results significantly (Figure 8);
- Different annealing temperatures had little or no effect on product amplification.

✓ Determined conditions for PCR reactions:

- Exon 3 of MPL, 1,0 mM of MgCl2 and 58ºC for annealing;
- Exon 4 of MPL, 0,75 mM of MgCl2 and 54ºC for annealing;
- Exon 6 of MPL, 2,0 mM of MgCl2 and 58ºC for annealing;
- Exon 12 of MPL, 1,0 mM of MgCl2 and 54ºC of annealing;
- Exon 21 of JAK2, 1,5 mM of MgCl2 and 56ºC for annealing.

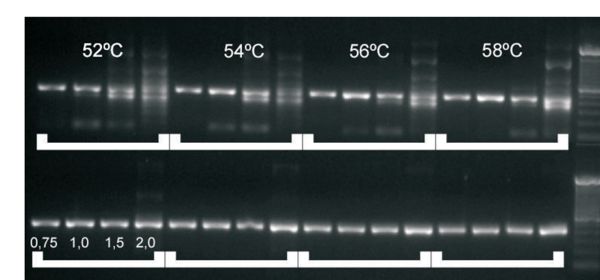

Figure 7: Analysis of PCR products of exon 3 (superior) and 4 (inferior) of MPL on 2% agarose gel. On columns 1 to 4 samples of patients with annealing temperature of 52ºC, on columns 5 to 8 of 54 ºC, on columns 9 to 12 of 56 ºC and on columns 13 to 16 of 58 ºC. In each temperature, the first column has MgCl2 concentration of 0,75 mM, the second column of 1,0 mM, the third column of 1,5 mM and the fourth of 2,0 mM. On column 17 100 bp Ladder (Invitrogen). Both exons show a single band around 400 bp.

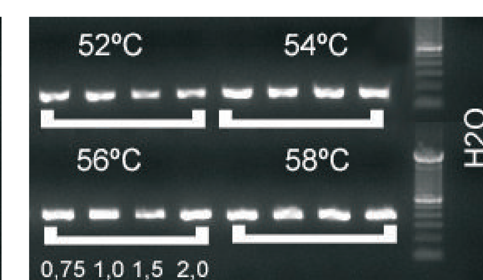

Figure 8: Analysis of PCR products of exon 6 of MPL on 2% agarose gel. On columns 1 to 4 samples of patients with annealing temperature of 52ºC, on columns 5 to 8 of 54 ºC, on columns 9 to 12 of 56 ºC ando n columns 13 to 16 of 58 ºC. In each temperature, the first column has MgCl2 concentration of 0,75 mM, the second column of 1,0 mM, the third column of 1,5 mM and the fourth of 2,0 mM. On column 9 100 bp Ladder (Invitrogen). On column 10 negative control. Exon 6 shows a single band around 400 bp.

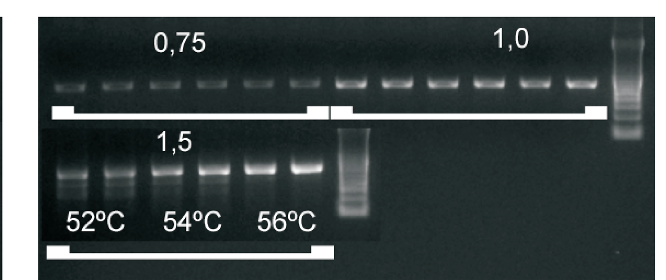

Figure 9: Analysis of PCR products of exon 12 of MPL on 2% agarose gel. On superior column 1 to 6 concentration of MgCl2 of 0,75 mM, from 7 to 12 of 1,0mM and on inferior columns 1 to 6 of 1,5 mM. In each concentration, the columns 1 and 2 had 52ºC as annealing temperature, columns 3 and 4 with 54ºC and columns 5 and 6 of 56ºC. On the superior column 13 and inferior column 7 100 bp Ladder (Invitrogen). The exon 12 shows a single band around 500 bp.

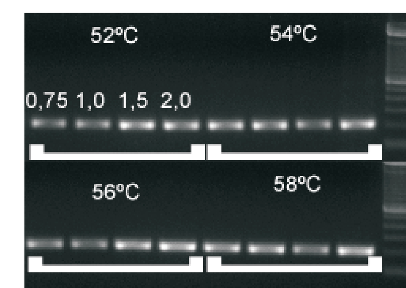

Figure 10: Analysis of PCR products of exon 21 of JAK2 on 2% agarose gel. On superior columns 1 to 4 samples of patients with annealing temperature of 52ºC, on superior columns 5 to 8 of 54 ºC, on inferior columns 1 to 4 of 56 ºC and on inferior columns 5 to 8 of 58 ºC. In each temperature, the first column has MgCl2 concentration of 0,75 mM, the second column of 1,0 mM, the third column of 1,5 mM and the fourth of 2,0 mM. On superior and inferior columns 9 100 bp Ladder (Invitrogen). Exon 21 shows a single band around 200 bp.

Investigation of new mutations

- ✓ On exons 3, 6 and 12 of MPL no mutations were found (Figure 10).
- ✓ On MPL exon 4, the mutations S204P and S204F were not found;
- ✓ The mutation p. E230G was not detected in the samples analyzed (Figure 10). However, on codon 230, two patients showed c.690A>G, which resulted in a synonym substitution (p.E230E). This variant is benign and, according to ClinVar database, and it was described on an ET patient. onal status, as shown on Figure 2;ystems) and data were analyzed with software Mutation Surveyor 3.0 or Chimer Marker (SoftGenetics, LLC).
- ✓ Mutation Y931C on JAK2 exon 21 was not found in PMF patients (Figure 11). Samples from all patients were analyzed, not only from triple-negatives. The absence of this mutation could be considered benign, since it could cause resistance to ruxolitinibe (Jakavi, Novartis).

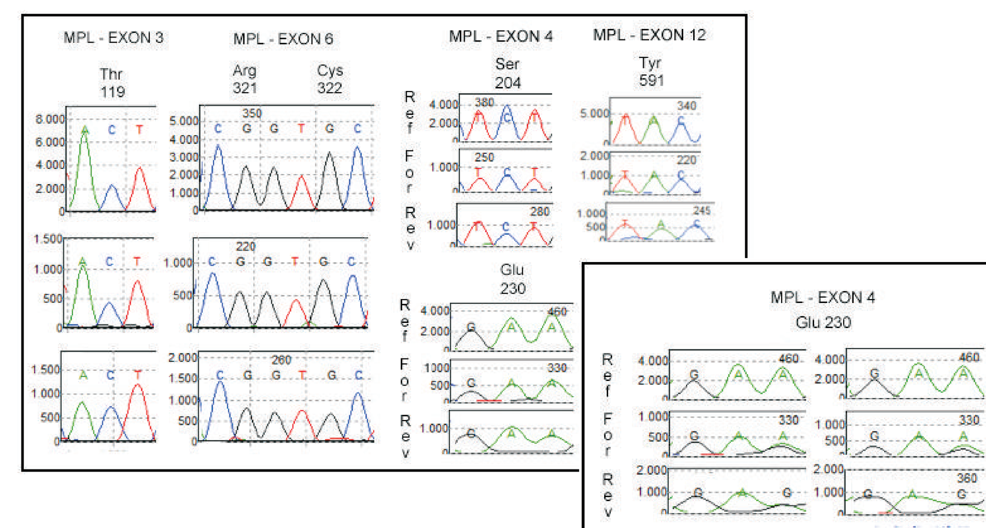

Figure 10: Eletropherograms representative of the direct sequencing of exons 3, 4, 6 and 12 of MPL. Featured the codons where mutations were described on literature with their respective reference sequences (REF). On exon 4 was detected a synonym substitution on codon 230 (p. E230E). The analysis of the sequencing was made on software Mutation Surveyor (Soft Genetics).

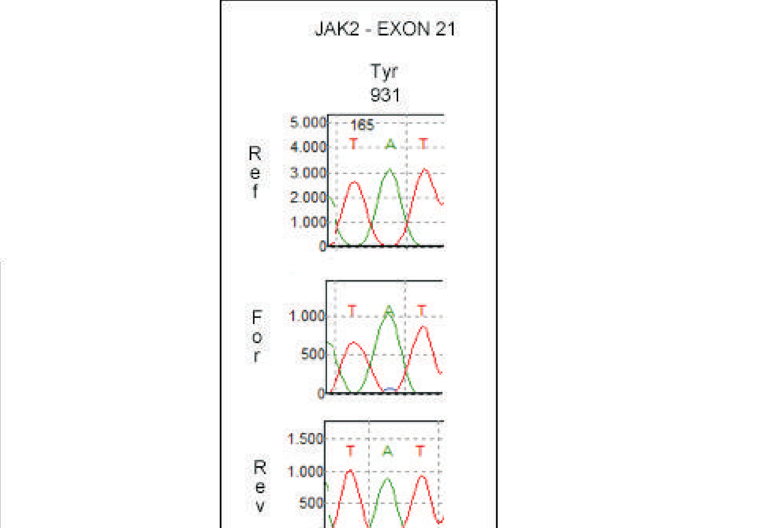

Figure 11: Eletropherograms representing the sequencing of JAK2 exon 21. Featured in detail codon 231 of a non-mutated sample. The samples were sequenced bi-directionally and aligned with the reference sequence of JAK2, obtained from NCBI. Sequencing analysis was performed on Mutation Surveyor (Soft Genetics).

Perspectives

- ✓ PCR for exons 13 and 15 of JAK2 will be standardized;
- ✓ Analysis of mutations on exons 13 and 15 of JAK2;
- ✓ Validation of mutations on exons 6 and 12 of MPL and on exons 16 and 17 of JAK2 previously detected by NGS;
- ✓ Molecular results obtained will be correlated with clinical data of patients.

Projeto Gráfico: Setor de Edição e Informação Técnico-Científica / INCA

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