High Resolution Melting Analysis for *JAK2* Exon 14 and Exon 12 Mutations

A Diagnostic Tool for Myeloproliferative Neoplasms

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***JAK2* mutations are important criteria for the diagnosis of Philadelphia chromosome-negative myeloproliferative neoplasms. We aimed to assess *JAK2* exon 14 and exon 12 mutations by high-resolution melting (HRM) analysis, which allows variation screening. The exon 14 analysis included 163 patients with polycythemia vera, secondary erythrocytoses, essential thrombocythemia, or secondary thrombocytoses, and 126 healthy subjects. The study of exon 12 included 40 *JAK2* V617F-negative patients (nine of which had polycythemia vera, and 31 with splanchnic vein thrombosis) and 30 healthy subjects. HRM analyses of *JAK2* exons 14 and 12 gave analytical sensitivities near 1% and both intra- and interday coefficients of variation of less than 1%. For HRM analysis of *JAK2* exon 14 in polycythemia vera and essential thrombocythemia, clinical sensitivities were 93.5% and 67.9%, clinical specificities were 98.8% and 97.0%, positive predictive values were 93.5% and 79.2%, and negative predictive values were 98.8% and 94.6, respectively. Correlations were observed between the results from HRM and three commonly used analytical methods. The *JAK2* exon 12 HRM results agreed completely with those from sequencing analysis, and the three mutations in exon 12 were detected by both methods. Hence, HRM analysis of exons 14 and 12 in *JAK2* shows better diagnostic values than three other routinely used methods against which it was compared. In addition, HRM analysis has the advantage of detecting unknown mutations. (J Mol Diagn 2009, 11:155–161; DOI: 10.2353/jmoldx.2009.080110)**

lecular methods based on real-time PCR have recently been developed and allow screening of known and unknown mutations, including 1-bp substitutions. Furthermore, melting curve analysis with a high-resolution melting instrument is a simple, high performance, time saving, and low labor-intensive technique that is a sensitive and specific tool for detecting DNA variations.^{1–3}

The *JAK2* V617F mutation has become an important diagnostic criterion for Philadelphia chromosome-negative myeloproliferative neoplasms (Ph-neg MPN), especially in polycythemia vera (PV).^{4–9} Accordingly, it has been included in the World Health Organization diagnostic criteria for Ph-neg MPN.⁸ Since 2007, new exon 12 mutations involved in the pathogenesis of PV and idiopathic erythrocytosis have been described. These mutations are also important for the diagnosis of PV, as approximately 3% of PV cases have mutations in this exon.^{10,11} In addition, other *JAK2* exon 14 mutations that are distinct from *JAK2* V617F, such as C616Y, D620E, and C618R, have been detected in patients with myeloproliferative neoplasms (MPN).^{12,13} Despite this, the described *JAK2* mutations are neither specific for this group of diseases, nor are they present in all patients with classical Ph-neg MPN (especially in essential thrombocythemia [ET] and primary myelofibrosis). In addition, infrequent occurrence of the *JAK2* V617F mutation has been reported in chronic myelomonocytic leukemia, atypical MPN, myelodysplastic syndromes, systemic mastocytosis, and acute myeloid leukemia.¹⁴

Assay sensitivity should be carefully considered when interpreting test results. If low-sensitivity techniques are used, some positive cases will not be detected, whereas if the technique is highly sensitive, false positive results may arise. Highly sensitive methods used to determine the presence of the *JAK2* V617F mutation (such as denaturing high-performance liquid chromatography, pyrosequencing, and allele-specific oligonucleotide [ASO]-

Supported by grants from FIS of Spanish Ministry of Health (FIS 05/1661) and Fundación Mutua Madrileña del Automóvil and University grant CCG07-UCM/BIO-2555 (Universidad Complutense).

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Accepted for publication December 4, 2008.

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High resolution melting (HRM) analysis represents a new generation of mutation scanning technology. HRM mo-

Table 1. Oligonucleotides Sequences for *JAK2* Exons 14 and 12 Methods

			Product size (bp)
Exon 14			
HP, HP with PNA and HRM			
Forward	5'-TTCTTTGAAGCAGCAAGTATGATGA-3'		180
Reverse	5'-CTGACACCTAGCTGTGATCC-3'		
Probes	5'-LC Red640-AAAACCAATGCTTGTGAGAAAGCT-PH-3'		
	5'-TCGTCTCCACAGACATACTCCATAA-FL-3'		
PNA	5'-TCCACAGACATACT-3'		
ASO qPCR			
Forward	5'-GGTTTTAAATTATGGAGTATGTT-3'		60
Reverse	5'-AGAAAGCCTGTAGTTTACTTACTCTCG-3'		
Probe	5'-6-FAM-TGTGGAGACGAGAGTAA-MGB-3'		
Exon 12			
Sequencing PCR			
Forward	5'-CTCCTCTTTGGAGCAATTCA-3'		500
Reverse	5'-GAGAACTTGGGAGTTGCGATA-3'		
HRM			
Forward	5'-CTCCTCTTTGGAGCAATTCA-3'		280
Reverse	5'-GATTTCATTTCATGTGACATTGG-3'		

HP, hybridization probes; PH, phosphate; FL, fluorescein; FAM, 6-carboxyfluorescein; MGB, minor groove binder.

PCR) are replacing direct sequencing and are able to determine more precisely the frequency of this mutation in Ph-neg MPN.^{9,15-20}

We aimed to compare the diagnostic values of the HRM method with a number of other described techniques for detecting mutations in exon 14 and exon 12 of the *JAK2* gene to diagnose MPN. For exon 14 analysis, HRM diagnostic values were compared with three methods: two based on hybridization probes, one of which included a peptide nucleic acid probe (PNA) specific for the wild-type allele, and the third using ASO primers for the *JAK2* V617F mutant allele and a minor groove binder TaqMan probe. In the case of exon 12, dideoxynucleotide sequencing was used to confirm HRM results.

Materials and Methods

Patients

For the study of *JAK2* exon 14 mutations, 289 subjects were enrolled and peripheral blood samples were collected between January 2002 and February 2007. The samples came from three centers in Madrid (Spain) and included 31 cases of PV, 35 cases of secondary erythrocytoses or primary non-myeloproliferative erythrocytoses, 60 cases of ET, 37 cases of secondary thrombocytoses, and 126 healthy subjects with normal hemoglobin and platelet levels as controls. In the case of exon 12, 40 *JAK2* V617F-negative patients (nine of them with PV and 31 with splanchnic vein thrombosis) and 30 healthy subjects were assessed.

JAK2 mutational analysis was performed on DNA from whole peripheral blood. All samples were obtained with informed consent, and all procedures were approved by the ethics committee from our institution.

Methods

Genomic DNA was isolated from whole venous peripheral blood by using the large volume MP DNA isolation kit (Roche Applied Sciences, Mannheim, Ge).

HRM Analysis of *JAK2* Exon 12 and 14 Mutations

Primer sequences and product sizes are shown in Table 1. The primers used for HRM analysis of exon 14 were the same as those used with the hybridization probes in the LightCycler 2.0 (Roche Applied Sciences, Mannheim, Ge). The forward primer used to sequence exon 12 was the same as that used for the HRM analysis of exon 12 mutations. Amplification conditions were identical for the two exons. PCR reactions were performed in a 10- μ L volume and included 3 mmol/L MgCl₂, 5 μ L High Resolution Master Mix (Roche Applied Sciences, Mannheim, Ge), 0.3 μ mol/L primers, and 10 to 20 ng of DNA. Cycling and melting conditions were as follows: one cycle at 95°C for 10 minutes; 40 cycles at 95°C for 10 seconds, 55°C for 15 seconds, 72°C for 15 seconds; and a melt from 60°C to 95°C at 0.25°C per second.

HRM analysis was performed with the LightCycler 480 Gene Scanning Software (Roche Applied Sciences, Mannheim, Ge). The normalized graph and the normalized temperature-shifted difference graph (difference graph) from the gene scanning analysis were used to analyze the data (Figure 1). The normalized graph was generated by monitoring the dissociation of fluorescent dye from double-stranded DNA with an increase in temperature. The High-Resolution Melting Dye (Roche Applied Sciences, Mannheim, Ge) used in this study can only fluoresce when it is intercalated into double-stranded DNA. The normalized graph shows the degree of reduction in fluorescence over a temperature range (typically 70°C to

Figure 1. Normalized temperature-shifted difference graph showing mutation detection for *JAK2* exons 12 and 14. The melting profile of a wild-type control has been chosen as a horizontal baseline. **A:** A difference graph of *JAK2* V617F. Mutated samples with high and low allele burden and wild-type samples are shown. **B:** The difference graph of *JAK2* exon 12 shows wild-type samples and three different mutations: A, B and C. Curve A represents the duplication and curves B and C represent the two deletions.

95°C). All samples, including the wild-type, were plotted according to their melting profiles. In the difference graph, the melting profiles of each sample were compared with those of the wild-type (which were converted to a horizontal line). Significant deviations from the horizontal line were indicative of sequence changes within the amplicon. Samples with aberrant melting curves were recorded as HRM mutation positive.

Methods Used to Validate HRM Analysis of Exons 14 and 12 on *JAK2*

Mutational Analysis of *JAK2* V617F with Hybridization Probes

Analysis of the *JAK2* V617F gene mutation was performed by real-time PCR using a melting curve-based, LightCycler assay with hybridization probes. Sequences of the primers and probes are shown in Table 1. Reactions were performed with 0.5 $\mu\text{mol/L}$ of both forward and reverse primers, 0.2 $\mu\text{mol/L}$ of both sense and anchor hybridization probes, 4.5 mmol/L of MgCl_2 , 1 μL of Fast Start DNA Master Hybridization Probe (Roche Applied Sciences, Mannheim, Ge), and 50 to 100 ng of DNA, all in a final volume of 10 μL . PCR conditions were as follows: an initial hold at 95°C for 10 minutes; 35 cycles

of 95°C for 0 seconds, 58°C for 10 seconds, and 72°C for 10 seconds; and a melt from 50°C to 80°C at 0.2°C per second. Fluorescence was detected continuously. Data were analyzed using the LightCycler software v3.5 (Roche Applied Sciences, Mannheim, Ge). Mutated alleles, with melting peaks at 59°C, were distinguished from wild-type alleles, whose melting peaks occurred at 63°C.

Mutational Analysis of *JAK2* V617F with Hybridization Probes and the Addition of PNA

Amplification conditions were identical to those described above for *JAK2* analysis with hybridization probes. A specific PNA for the wild-type allele was designed and synthesized following specific rules and using Applied Biosystem's reagents (Palo Alto, CA) (Table 1). The specific PNA for the *JAK2* V617F wild-type allele was added to the reaction at a concentration of 1.25 $\mu\text{mol/L}$. Mutated and wild-type alleles were identified, as described above for PCR detection of *JAK2* V617F.

Mutational Analysis of *JAK2* V617F with Allele-Specific Primers and the Minor Groove Binder TaqMan Probe

JAK2 V617F ASO quantitative PCR (ASO qPCR) was performed with the ABI PRISM 7900 (Applied Biosystems, Palo Alto, CA), and a forward ASO primer spanning the *JAK2* V617F mutation region. A reverse primer and a minor groove binder TaqMan probe were also used (sequences are indicated in Table 1). The PCR was performed in a 10- μL reaction volume with 0.5 $\mu\text{mol/L}$ of both forward and reverse primers, 0.12 $\mu\text{mol/L}$ of the minor groove binder probe, 5 μL of TaqMan Fast Start Universal PCR Master Mix (Applied Biosystems, Palo, CA), and 10 to 20 ng of DNA. The first cycle was a 10 minutes denaturation at 95°C. The following 40 cycles included denaturation at 95°C for 1 second, and annealing at 55°C for 25 seconds.

Mutational Analysis of Exon 12 *JAK2* Mutations with Sequencing

We performed bidirectional sequencing analysis to detect *JAK2* mutations in exon 12. The reaction mixture of 10 μL contained 1.25 mmol/L MgCl_2 , 1 $\mu\text{mol/L}$ of each primer (Table 1), 50 ng of genomic DNA, and 1.5 μL of Fast Start DNA Master Hybridization Probe. PCR cycling conditions were as follows: an initial denaturation at 95°C for 10 minutes; 35 cycles of 95°C for 30 seconds, 60°C for 30 seconds, 72°C for 30 seconds; and one cycle of 72°C for 10 minutes. Amplification products were purified with ExoSapIT (GE Health care, Little Chalfont, UK), followed by sequencing with Big Dye Terminator v3.1 (Applied Biosystems, Foster City, CA), according to the manufacturer's protocol. The sequencing products were run on a 3100 Genetic Analyser (Applied Biosystems, Palo Alto, CA). The sequencing data were visualized using Sequencing Analysis 5.1.1 (Applied Biosystems, Palo Alto, CA).

Table 2. Intra- and Inter-Day Coefficients of Variation ($n = 5$) of Crossing Point Cycle Values, the Number of DNA Molecules at CP, and the Number of DNA Molecules Added to the Reaction for *JAK2* Exons 14 and 12 in HRM Methods

Sample*	Intra-day CV (%)			Inter-day CV (%)		
	CP	N _{CP}	N ₀	CP	N _{CP}	N ₀
Exon 14						
1	0.61	8.9	9.6	0.66	9.9	10.2
2	0.77	12.4	13.0			
3	0.54	9.6	9.5			
4	0.41	7.4	7.7			
Exon 12						
5	0.92	13.3	13.5	0.93	13.7	14.1
6	0.51	7.4	7.0			

*JAK2 V617F allele burden in samples 1–4: 100%, 22.4%, 1.2%, and 0%. Samples 5 and 6 corresponded to *JAK2* exon 12 mutated and not mutated, respectively.

CP, crossing point cycle values; N_{CP}, the number of DNA molecules at CP; N₀, number of DNA molecules added to the reaction.

A six-point standard curve was generated for each exon as a measure of the sensitivity of all of the assays. The standard curves arose from serial dilutions of positive control DNA in negative control DNA, to obtain proportions of 1 to 10⁻⁴ in a final, constant amount of 10 ng of genomic DNA. For the assessment of HRM analysis of exon 14, *JAK2* V617F-positive DNA from homozygous human erythroleukemia cells (Deutsche Sammlung von Mikroorganismen und Zellkulturen GmbH, Braunschweig, Ge) was diluted into samples from healthy subjects, without exon 14 mutations. For the assessment of HRM analysis of exon 12, mutated DNA from a patient was used as a positive control. Each point in the dilution curve was measured in duplicate.

Intra- and interday coefficients of variation were calculated in three ways for both exons 14 and 12, ie, the crossing point (CP) cycle, and the number of DNA molecules added to reaction (N₀), and amplified at this cycle (N_{CP}).

Intraday variation of the HRM analysis of exon 14 was determined from five values obtained in the same run, in samples prepared with five different reaction mixes, and at four levels of *JAK2* V617F allele burden (100%, 22.4%, 1.2%, 0%) (Table 2). The interday precision corresponded to runs performed over five days, with a 100% allele burden. Intraday variation of the HRM analysis of exon 12 was determined from five values obtained in the same run

of mutated and non-mutated DNA samples prepared with five different reaction mixes (Table 2). The interday variation was performed over five days using a mutated patient sample.

Statistical Analyses

All data were included in a relational database, Access 2003, and analyzed by SPSS 13.0 (SPSS Inc, Chicago, Illinois).

For the three methods, clinical sensitivities, clinical specificities, positive predictive values and negative predictive values were determined by contingency tables. Receiving operating characteristic plots were performed to establish the best sensitivity and specificity levels for a quantitative diagnostic test. The best cut-off specificity level was established at over 97%, and sensitivity cut-off was assessed according to the referred specificity level. Comparisons between the diagnostic tests were performed using the kappa coefficient.

Results

Validity study of HRM analysis of the *JAK2* exon 14 mutation (*JAK2* V617F)

PCR efficiencies were calculated from the linear regression slopes of each method. In the case of HRM, this was 1.89, with hybridization probes it was 1.93, for hybridization probes with PNA it was 1.90, and for ASO qPCR it was 1.96. Linearity in the investigated range was high for all of the methods, where $R^2 > 0.98$. For HRM analysis, analytical sensitivity obtained from the dilution curves was 1–0.5%. In the case of hybridization probes alone, analytical sensitivity was 10%, while hybridization probes with PNA gave an analytical sensitivity around 1–0.5%, and in ASO qPCR, analytical sensitivity was 0.01%.

The coefficients of variation were calculated according to CP values. In this study the intra- and interday coefficients of variation were always below 1%. When N₀ or N_{CP} coefficients of variation were determined, results were between 7.4 and 13.0% (Table 2).

We also compared the diagnostic values of the four methods for the *JAK2* V617F mutation in the most frequent MPN (PV and ET) (Table 3). In the case of HRM

Table 3. Comparison of Diagnostic Validity in PV and ET for *JAK2* V617F Mutation with PCR Methods

	HRM	HP	HP with PNA	ASO qPCR*
Polycythemia vera				
Sensitivity	93.5 (85.2–102.6)	87.1 (75.0–101.1)	93.5 (85.2–102.6)	93.5 (85.2–102.6)
Specificity	98.8 (97.1–100.5)	100 (100)	98.8 (97.1–100.5)	99.4 (98.2–100.6)
PPV	93.5 (85.2–102.6)	100 (100)	93.5 (85.2–102.6)	96.7 (90.5–103.3)
NPV	98.8 (97.1–100.5)	97.6 (95.3–100.0)	98.8 (97.1–100.5)	98.8 (97.1–100.5)
Essential thrombocythemia				
Sensitivity	67.9 (52.6–87.6)	57.1 (41.4–78.7)	67.9 (52.6–87.6)	78.6 (64.8–95.4)
Specificity	97.0 (94.4–99.7)	100 (100)	97.0 (94.4–99.7)	96.3 (93.5–99.2)
PPV	79.2 (64.5–97.2)	100 (100)	79.2 (64.5–97.2)	78.6 (64.8–95.4)
NPV	94.6 (91.3–98.1)	93.2 (89.5–97.0)	94.6 (91.3–98.1)	96.3 (93.5–99.2)

HP, hybridization probes.

*Receiving operating characteristic curve results, discriminating selected value 1.0%.

Table 4. JAK2 V617F Positive Results Obtained by the Four Methods

	N _T	HRM		HP		HP with PNA		ASO qPCR	
		N	%	N	%	N	%	N	%
Polycythemia vera	31	29	94	27	87	29	94	29	94
Secondary erythrocytoses	35	0	0	0	0	0	0	0	0
Essential thrombocythemia	60	41	68	34	57	41	68	47	78
Secondary thrombocytoses	37	3	8	0	0	3	8	3	8
Healthy subjects	126	2	2	0	0	2	2	1	1

HP, hybridization probes.

analysis of JAK2 V617F for a PV diagnosis, sensitivity was 93.5%, specificity was 98.8%, positive predictive value was 93.5%, and negative predictive value was 98.8%. In the case of an ET diagnosis, sensitivity was 67.9%, specificity was 97.0%, positive predictive value was 79.2%, and negative predictive value was 94.6%.

In the ASO quantitative analysis of the JAK2 V617F mutation, receiving operating characteristic curves were generated to select the adequate threshold level of the JAK2 V617F mutant allele burden. This was done to obtain the best relationship between sensitivity and specificity for the diagnosis of MPN. A 1% JAK2 V617F allele burden was found to be the best level with high specificity to distinguish PV or ET from secondary conditions. With this threshold value, the area under the receiving operating characteristic curve was 0.96 for PV, and 0.94 for ET (Table 3).

We correlated the HRM results with the other three analytical methods,¹⁷ and good kappa coefficients were reached. The kappa coefficient between HRM and the hybridization probe method was 0.76 (SD $\pm$ 0.05), between the HRM and the hybridization probe with PNA it was 1.00 (SD $\pm$ 0.00), and between the HRM and ASO quantitative method it was 0.91 (SD $\pm$ 0.03).

The HRM PCR results matched with the results from the hybridization probe with PNA PCR, in all patients (Table 4). Two PV samples (2/31) were negative with all of the methods. In ET, 19 cases were negative with the HRM method, 13 of these were also negative with the ASO quantitative method, and the other six were slightly more than the 1% JAK2 V617F allele burden.

Three secondary thrombocytoses were positive with the HRM method, in contrast to no positive results in secondary erythrocytoses (Table 4). These secondary thrombocytoses were also positive with the hybridization probe with PNA method, and the ASO quantitative method (allele burden between 2.5% and 2.8%). Two secondary thrombocytoses were only positive by ASO qPCR (JAK2 V617F allele burden <2%). All these patients had a reactive basis to their thrombocytosis with no spleen enlargement.

Two healthy subjects were positive (1.6%, 2/127) by both the HRM and hybridization probe with PNA methods (Table 4). One of them was positive by ASO qPCR (JAK2 V617F allele burden, 4.2%). These two healthy individuals had normal blood cell counts, no spleen enlargement, and no concomitant or previous disease.

The difference plot of the high resolution melting curves for exon 14 is shown in Figure 1A. Wild-type JAK2

was selected as the baseline curve. In this figure, three cluster regions could be surmised to correspond to a wild-type, low, and high JAK2 V617F allele burden.

Validity Study of the HRM Test on JAK2 Exon 12

The efficiency of the HRM analysis of JAK2 exon 12, as determined from the slope of the linear regression of the curve CP versus log (dilution), was 1.70. A high linearity ($R^2 > 0.99$) was also observed. An analytical sensitivity of 1% was determined by diluting DNA from an exon 12-mutated sample into samples from healthy subjects with no mutation in this region.

When the assay variations were calculated with CP values, the intra- and interday coefficients of variation were below 1%. The coefficients of variation of N₀ or N_{CP} values were between 7.0% and 14.1% (Table 2).

The JAK2 exon 12 HRM results correlated 100% with sequencing results for detecting mutations in the 40 JAK2 V617F-negative patients with a clinical diagnosis of PV or splanchnic vein thrombosis, and the 30 healthy subjects. Only three JAK2 V617F-negative cases with a PV phenotype showed mutations in exon 12: two deletions of two and three amino acids and an 11-amino acid duplication. Using sequencing as a reference method, the clinical sensitivity, clinical specificity, positive predictive values, and negative predictive values of HRM for the detection of mutations within JAK2 exon 12 could be considered to be 100%.

Figure 1B shows the difference plots of the high resolution melting curves of JAK2 exon 12 in four samples. The wild-type amplicon has been selected as the baseline curve. The other three curves correspond to the two mutations where curve A represents the duplication, and curves B and C, the two deletions.

Discussion

An assessment of JAK2 mutations has become an essential and relevant tool for the diagnosis of Ph-neg MPN. Improving the methods used for detecting JAK2 mutations will significantly benefit these patients. HRM methods, which represent a new generation of real-time PCR techniques, add some advantages over the previously used methods, and could improve diagnosis performance.¹⁻³

In this study, the HRM method for detecting *JAK2* exon 14 mutations showed similar PCR characteristics to the methods with which it was compared.¹⁷ In the case of CP values, the coefficients of variation were good (below 1%), although for the N_0 or N_{CP} values, exponential transformation of the differences yielded some percentages above 10%.

Sensitivity, specificity, and predictive values for the HRM method were high in the case of a PV and ET diagnosis, and comparable with the diagnostic values obtained in the hybridization probe with PNA, and the ASO quantitative methods. Moreover, there was almost complete agreement between the results provided by these three methods. It is worth noting that analytical sensitivity of HRM was identical to that of the PCR method using hybridization probes with PNA, and to the ASO qPCR discriminating value, selected in accordance with the receiving operating characteristic curve.

It is thus our opinion that these three qualitative tests for the diagnosis of PV have similar diagnostic utility. Due to the optimal results and simplicity of the HRM analysis, it may be considered the best screening test. Besides, the *JAK2* V617F ASO qPCR method is more sensitive, slightly less specific, but gives additional information regarding allele burden. Thus, if results are negative with the *JAK2* V617F HRM in patients with clinical features strongly suggestive of PV, the *JAK2* V617F ASO qPCR test should be performed, in addition to methods for detection of *JAK2* exon 12 mutations.²¹

In the case of ET, the four tests also appeared to show good validity, with an especially high specificity, although sensitivity was low. The *JAK2* V617F HRM PCR should be considered as the screening test for the differential diagnosis of thrombocytosis due to its sensitivity, specificity, and simplicity. Similarly to PV, the *JAK2* V617F ASO qPCR method, despite being less specific, is more sensitive and useful for determining the *JAK2* V617F allele burden.¹⁷ In those patients whose *JAK2* V617F HRM PCR results are negative and who show clinical features strongly suggestive of ET, the *JAK2* V617F ASO qPCR test should be performed, in addition to myeloproliferative leukemia protein gene mutations.²² Two healthy subjects, with a normal white cell and platelet count, were found to be V617F-positive, and after 3 years of follow-up they have not developed any features of MPN. Moreover, we have recently confirmed the *JAK2* V617F mutation in these healthy subjects by cloning and sequencing.¹⁷ The allele-specific quantification suggests that the mutant allele burden in one of these cases is above 4%, which was found to be sufficient to produce clinical manifestations in other reported cases. This would have to be considered a positive result given the sensitivity of the three assays. It may be explained by the previously described existence of cells with a *JAK2* V617F genotype in some healthy subjects.^{18,23}

The *JAK2* exon 12 HRM PCR quality parameters (efficiency, linearity, sensitivity, and precision) were adequate. Our remarks regarding the *JAK2* exon 14 coefficients of variation apply equally to the HRM analysis of exon 12, where precision worsened when it was calculated for the initial or crossing point number of molecules.

The analytical sensitivity attained (around 1%) allowed for the detection of frequent minor mutated clones of *JAK2* exon 12, which could not be detected by direct sequencing.¹¹ Therefore, HRM should significantly improve the diagnostic capabilities for pathologies with *JAK2* exon 12 mutations, namely PV and idiopathic erythrocytoses. Although in the three mutated cases presented here, the tumor burden was high enough for detection by sequencing.

In addition, due to the large variety of *JAK2* exon 12 mutations, the difference plot of the HRM curves showed such diverse patterns that it should certainly assist in identifying a mutation. These results are in accordance with the results of Jones et al who used a similar method based on HRM.²⁴

Thus, HRM is an ideal method to detect *JAK2* mutations on exons 12 and 14 for several reasons: 1) A low tumor burden is frequent in these patients.^{11,25} 2) The method has an intermediate analytical sensitivity, around 1%.^{4,15,17,19,20} In this sense, the HRM method is similar to the sensitivity of the ASO qPCR method which permits a potential for increasing the ability to detect mutations in PV and ET. 3) All patients studied thus far have been shown to have a mixed cellular populations, and it is thus difficult to find homozygosity for *JAK2* mutations in all cells from peripheral blood. This HRM method is designed to detect heteroduplexes and, as it is an acquired mutation, virtually all patients have heteroduplex formation.^{4-7,9} 4) HRM is capable of detecting new mutations in amplicons. Three new *JAK2* exon 14 mutations, different from V617F, have been reported in Ph-neg MPN, and more than 10 in exon 12.^{10-13,23} High throughput is possible, and up to 96 or 384 samples can be processed in the same experiment, with the possibility of detecting mutations in any of the two exons in the same run. 5) The software allows automatic classification into different genotype groups.

Conclusion

HRM methods for detecting *JAK2* exon 14 and exon 12 mutations have the same, and better, diagnostic values than the currently used methods. In addition, they have the advantage of detecting unknown mutations. HRM is a simple and fast technique for screening for mutations of the *JAK2* gene.

Acknowledgments

We thank Ian Ure for the English review and Rocio Molinero for her technical support.

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