Rapid identification of *JAK2* exon 12 mutations using high resolution melting analysis

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ABSTRACT

Diverse *JAK2* exon 12 mutations have been described in patients with V617F-negative polycythemia vera. Development of a sensitive detection assay capable of identifying any of these mutations is required for medium-throughput diagnostic screens. Non-mutated and mutant *JAK2* exon 12 alleles were amplified from patient samples and cloned into plasmid vectors, then used to determine the sensitivity of a novel high-resolution melting-curve assay designed to detect all mutant *JAK2* exon 12 alleles tested. High resolution melting analysis was more sensitive than direct sequencing and capable of detecting exon 12 mutations in granulocytes at moderate levels. In a blinded analysis of DNAs from V617F-negative erythrocytosis patients, with direct sequencing and allele-specific PCR used in one laboratory and high resolution melting analysis in another, high resolution melting successfully identified *JAK2* exon 12 mutations in all 4 mutation-positive patients. High resolution melting analysis is a rapid, sensitive and high-throughput technique that is suitable for screening for *JAK2* exon 12 mutations.

Key words: *JAK2*, high resolution melting curve analysis.

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Introduction

The presence of an acquired V617F *JAK2* mutation has been reported in the majority of patients with a myeloproliferative disorder (MPD);[1-5] the mutant allele can be detected in 95% of patients with polycythemia vera (PV), and in half of those with essential thrombocythemia (ET) or primary myelofibrosis (PMF).[1,6,7] The molecular pathogenesis of the disorder in most MPD patients lacking the V617F mutation remains largely unclear. Alternate mutations are absent in the *JAK* or *STAT* gene family members in patients with V617F-negative ET or PMF,[8] although acquired mutations in *JAK2* exon 12 have been identified in cases of V617F-negative PV.[9] In contrast to patients with V617F-positive PV, patients with a *JAK2* exon 12 mutation present with erythrocytosis, but have white cell and platelet counts within the normal range. These mutations may also be detected in patients with erythrocytosis that fail to fulfill the Polycythemia Vera Study Group (PVSG) diagnostic criteria for PV,[10] and are instead classified as having idiopathic erythrocytosis (IE).[9,11]

Individual allele-specific PCR reactions have been developed to detect the first four exon 12 mutations to be described.[9] However, additional mutations have subsequently been identified,[11-16] including duplications that might not easily be identified using an allele-specific PCR strategy.[14] It is possible that additional mutant alleles exist. There is, therefore, a need to develop more generally applicable, sensitive assays to detect *JAK2* exon 12 mutations for use in a diagnostic setting.

Design and Methods

Patients

DNA samples from 50 erythrocytosis patients attending the Johns Hopkins Medical Institutes (Baltimore, MD, USA) and from exon 12 mutation-positive patients attending Addenbrooke's Hospital (Cambridge, UK) were used. Institutional Ethics Committee approval was obtained at both institutions, written informed consent obtained from each patient, and the study was carried out in accordance with the principals of the Declaration of Helsinki. Clinical features of the Baltimore patients have been published elsewhere.[13]

Mutant and wildtype JAK2 exon 12 alleles

Patient genomic DNA was prepared from density gradient-purified granulocytes using the Puregene Cell kit (Gentra Systems, Minneapolis, MN, USA). Presence of the V617F JAK2 mutation was excluded by sequencing and allele-specific PCR[1] (*data not shown*). DNA was amplified using primers flanking *JAK2* exon 12 (forward: 5'-CTCCTCTTTGGAG-

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CAATTCA-3'; reverse: 5'-GAGAACTTGGGAGTTGC-GATA-3'). PCR products were treated with ExoSAP-IT (GE Healthcare, Amersham, UK), then cloned into pGEM-T (Promega, Southampton, UK).

HRM reaction conditions

A 126bp amplicon was generated using primers in *JAK2* exon 12 (5'-AATGGTGTTTCTGATGTACC-3') and intron 12 (5'-AGACAGTAATGAGTATCTAATGAC-3'). Each PCR contained 20-40 ng DNA or 1×10^6 plasmid copies, 2.5 mM $MgCl_2$, 200 μM dNTPs, 0.5 μM of each primer, 1× Platinum *Taq* polymerase buffer and 0.5U Platinum Taq polymerase (Invitrogen, Paisley, UK), and 1× LC-Green Plus (Idaho Technologies, Salt Lake City, UT, USA). Duplicate PCRs and the HRM analysis were conducted on a RotorGene 6000™ real-time analyzer (Corbett Life Sciences, Mortlake, Australia). The PCR profile was: an initial hold at 95°C for 10 mins., 40 cycles of 95°C for 15 secs., 58°C for 30 secs., and 72°C for 20 secs., followed by 50°C for 30 secs., then a melt from 70°C to 95°C rising at 0.1°C/second (data acquisition was up to 1,000 data collection points per °C transition). Normalization bars were between 72-75°C for the leading range, and 92-93°C for the tailing range.

Sequencing HRM products

HRM product was treated with ExoSAP-IT, then used as the template for sequencing with the Big Dye Terminator kit (Applied Biosystems, Warrington, UK). Each 10 μL reaction mix consisted of 10 ng primer, 0.5 μL of Big Dye Terminator premix and 1.5 μL 5x sequencing buffer. Sequencing reactions were performed using 24 cycles of 96° C for 30 secs., 50° C for 15 secs., and 60° C for 2 mins. on a MJ Research Tetrad thermocycler (Biorad, Hemel Hempstead, UK). The Montage SEQ96 sequencing reaction clean-up kit (Millipore, Watford, UK) was used to purify sequencing reactions, and the resultant DNA resuspended in deionised formamide and loaded onto an ABI-3100 sequencer (Applied Biosystems). Sequences were analyzed using Mutation Surveyor 3.1 software (SoftGenetics, State College, PA, USA).

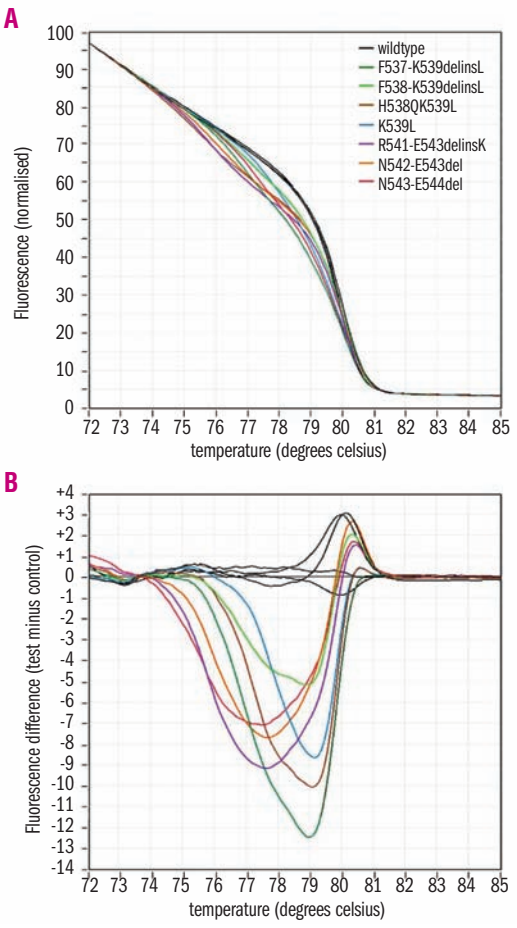

Figure 1. Development of a high-resolution melting-curve (HRM) assay to distinguish between the various *JAK2* exon 12 mutated alleles. (A) Analysis of six wildtype exon 12 samples (black line) and 50:50 mixtures of wildtype and F537-K539delinsL (green), H538-K539delinsL (lime), H538QK539L (brown), K539L (cyan), R541-E543delinsK (purple), N542-E543del (orange) or E543-D544del (red) alleles reveal that all mutant alleles had a melting curve profile distinct from that of the wildtype allele, and each with its own characteristic profile. (B) Difference plots demonstrate more clearly that each mutant allele has a characteristic melting curve, with those alleles with deletions of residues including E543 (purple, orange, red lines) having a curve distinct from those alleles with a K539L substitution (green, blue, brown lines).

Results and Discussion

High-resolution melt-curve analysis assay validation and sensitivity

One drawback to employing allele-specific PCR assays to screen large numbers of DNAs has been that multiple individual PCR reactions would be needed for each sample. Moreover, novel mutations would escape detection. Given these concerns, we sought to establish a single sensitive screening method capable of detecting all possible *JAK2* exon 12 mutations; one candidate approach is high-resolution melt-curve analysis (HRM), which has been successfully applied to the detection of the V617F mutation within *JAK2* exon 14 in patients diagnosed with an MPD.[17-20] PCR primers were redesigned to generate an amplicon suitable for HRM analysis of *JAK2* exon 12. HRM involves precise monitoring of the progressive fluorescence change caused by the release of an intercalating DNA dye from a DNA duplex as that duplex is denatured by increasing temperature.[21] Base substitutions, deletions and insertions produce subtle differences in the melting behavior of individual DNA duplexes, which can be detected by the use of an appropriate fluorescent dye, such as LC-Green.[21] Since this methodology will be affected by the presence of inheritable polymorphisms, a candidate region containing *JAK2* exon 12 and intron 12 (chromosome 9: 5,059,925-5,060,831; genome build 2006) was selected on the basis of an absence of polymorphisms in the NCBI database (*http://www.ncbi.nlm.nih.gov/sites/entrez/*). However, we subsequently identified a previously unreported polymorphism within this region in 85 out of 128 tested individuals; this variation involves the presence or absence of a pentanucleotide sequence (position 5,060,231-5,060,235) that would significantly affect any HRM profile. Accordingly, this region was excluded from

the final amplicon. HRM analysis was performed on a RotorGene 6000™ real-time analyzer, and the resulting data analyzed using the associated RotorGene Series Software (V1.7.25). Figure 1 shows typical results obtained when equal amounts of wildtype and mutation-carrying plasmid were used. The data are presented in two formats: a normalized plot (Figure 1A), in which the amount of intercalating dye remaining at any temperature point is expressed as a fraction of the amount prior to data acquisition; and a difference plot, where the average HRM profile of the control samples was used by the genotype function of the machine software as the standard wildtype profile for subsequent comparison to each of the test samples (Figure 1B). Analysis of six control and seven mutant samples demonstrated that each mutant allele had its own characteristic melting curve that was distinct from those obtained when wildtype exon 12 samples were analyzed (Figure 1A). The individual nature of the mutant melting curves became more apparent when the data were represented in a difference plot (Figure 1B). Mutations that had in common a K539L substitution had similar difference plots, with the greatest divergence between mutant and wildtype profiles occurring at 79° C. Difference plots for the R541-E543delinsK, N542-E543del and E543-D544del mutants had slopes different to those associated with a K539L substitution, and the greatest divergence between mutant and wildtype occurred at 77.5° C.

The ability to detect low levels of a *JAK2* exon 12 mutation in a background of non-mutated DNA was evaluated by titrating each of the mutant alleles with wildtype exon 12 to produce a range of mutant allele dilutions. The lower limit for detecting nucleotide changes in exon 12 was calculated by the RotorGene software to be 5% for F537-K539delinsL (Figure 2) and N542-E543del alleles, 7% for the R541-E543delinsK and E543-D544del alleles, 10% for the K539L allele, and 20% for the H538-K539delinsL and H538QK539L alleles (*data not shown*). Titration data were also used to estimate the level of mutant allele in the granulocyte DNA sample of a F537-K539delinsL-positive PV patient (PT1; Figure 2).[9] Estimates obtained using HRM analysis agreed closely with those independently obtained from granulocyte DNA sequence traces.

JAK2 exon 12 mutation detection in V617F-negative patients with erythrocytosis

The HRM methodology established using cloned exon 12 alleles was next assessed using a set of granulocyte DNA samples obtained from 50 V167F-negative erythrocytosis patients,[13] only 10 of whom fulfilled PVSG diagnostic criteria.[10] Since exon 12 mutation-positive patients often present with an isolated erythrocytosis and might not fulfil these criteria,[9,11] this patient cohort was selected for analysis as it was considered to be similar to sample populations being assessed for the presence of an exon 12 mutation in a

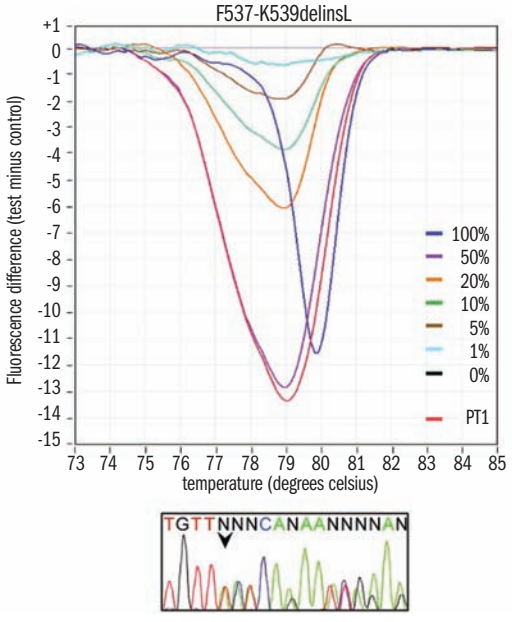

Figure 2. High resolution melting assays detect mutant *JAK2* alleles present at a low frequency. High resolution melting analysis of mixtures of wildtype and F537-K539delinsL *JAK2* exon 12 alleles (0% mutant, black line; 1%, cyan; 5%, brown; 10%, lime; 20%, orange; 50%, purple; 100%, blue) demonstrates that these assays can detect relatively low levels of mutant allele. Also included is the high resolution melting trace (red) and sequence chromatogram of a granulocyte sample from a patient previously shown to carry an exon 12 mutation.

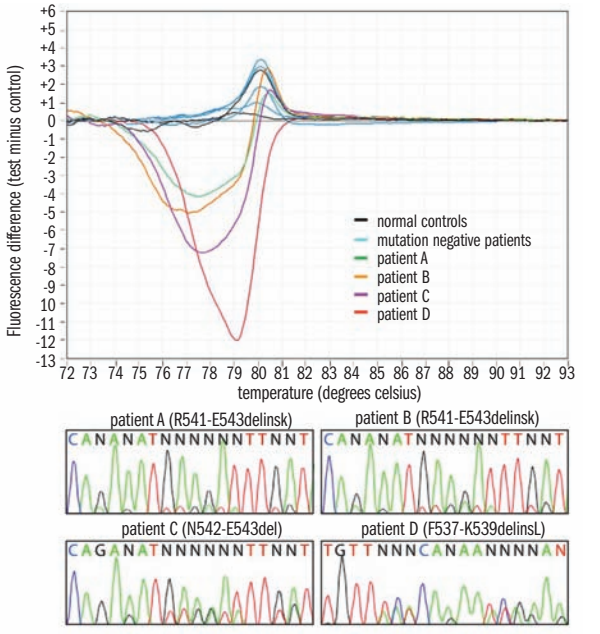

Figure 3. Screening of 50 V617F-negative patients with erythrocytosis by high resolution melting assays and sequencing independently identifies 4 mutation-positive cases. Sequencing, allele-specific PCR, and high resolution melting analysis was independently used to screen granulocyte DNAs from 50 V617F-negative erythrocytosis cases. Difference plots are shown for 4 of the 8 samples from healthy controls (black traces), 4 samples from mutation-negative patients within this cohort (cyan traces), and for the 4 samples suggestive of a *JAK2* exon 12 mutation (Patients A to D: lime, orange, purple and red lines respectively). Granulocyte DNA sequence chromatograms from each of these mutation-positive cases are also shown, with Patients A and B having an R541-E543delinsK mutation, Patient C having a N542-E543del mutation, and Patient D having a F537-K539delinsL mutation.

diagnostic laboratory setting. Allele-specific PCR and sequence analysis of each sample (*data not shown*) had been performed 1-2 months earlier by an investigator not involved with the HRM analysis, and four samples were found to be positive for a *JAK2* exon 12 mutation by each method. Subsequent HRM was successful in discriminating DNA samples with a wildtype *JAK2* exon 12 genotype from those including an exon 12 mutation (Figure 3), and correctly identified all positive samples in this group with no false-positive results. Direct sequencing of HRM products confirmed the presence of an exon 12 mutation in these cases. When compared with the titration experiment results (Figure 2), these data suggested mutant allele burdens of 20%, 25%, 35% and 50% respectively. All 4 patients had mild splenomegaly, and serum erythropoietin levels below the normal range, consistent with previous observations.[9,11] Two patients had not fulfilled the PVSG criteria at diagnosis; however, subsequent identification of *JAK2* exon 12 mutations permits reclassification of these cases as PV using the recently modified WHO criteria.[22] None of the 5 patients with a first- or second-degree relative with an MPD was mutation-positive.

Several mutation screening methods have been used so far to detect *JAK2* exon 12 mutations, each with its particular disadvantages. Dideoxy sequencing is rarely sensitive below a 10% mutant allele frequency, corresponding to a threshold of 20% granulocytes heterozygous for an exon 12 mutation. Allele-specific PCR requires performing multiple amplification reactions in order to ensure that all potential mutations can be excluded. A PCR-based technique with sensitivity comparable to that of allele-specific PCR has been recently described,[15] although this relies upon the presence of a deletion within exon 12, thereby precluding detection of the duplication mutations or the nucleotide substitution mutants present in some individuals. Single-strand conformation polymorphism, denaturing high performance liquid chromatography or HRM methodologies might prove useful, as they make the detection process more cost effective by reducing the amount of sequencing ultimately required. However, the HRM approach described here has the added advantage of increased simplicity and rapid turn-around time, being an in-tube method in which the melting analysis is performed immediately after PCR amplification.

Mutation analysis of granulocyte DNA samples from V617F-negative erythrocytosis patients using sequencing, allele-specific PCR and HRM allowed us to assess the sensitivity and positive predictive value of the HRM methodology. Mutations were not detected by allele-specific PCR or sequencing in samples scored as wildtype by HRM, resulting in 100% sensitivity and 100% positive predictive value for this methodology in the patient sample set tested. Four samples had aberrant melting profiles that suggested the presence of an exon 12 mutation; independently-obtained allele-specific PCR and sequencing data confirmed the presence of these alterations (Figure 3, and *data not shown*). In instances of patients with a low clonal burden, however, exon 12 mutations identified by HRM may not be confirmable by dideoxy sequencing; allele-specific PCR may be required for mutation confirmation in these instances. Alternatively, as all patients positive for *JAK2* exon 12 mutation have erythropoietin-independent erythroid colonies (EECs),[9,11] individual colonies could be analyzed by dideoxy sequencing to confirm the mutation suspected.

We observed two limitations to this HRM methodology: one was its inability to identify the H538QK539L and H538-K539delinsL *JAK2* alleles when their abundance was less than 20%. However, these are both relatively rare mutant alleles, occurring in only 4 of the 50 cases reported in the literature.[9,11-14,16,23] In contrast, the most common exon 12 mutations (F537-K539delinsL, N542-E543del, E543-D544del) were all detectable at a relative abundance of 7% or less. Secondly, patient samples with purely mutant DNA may pose a problem due to an absence of heteroduplex formation. However, homozygous exon 12 mutations are relatively rare in patients, with only 2 cases reported.[11,14] In both instances, non-mutated exon 12 sequence was apparent within the granulocyte DNA sequence trace, and we would predict that HRM analysis of these DNAs would provide a melting curve significantly different from that of a control sample.

In conclusion, we successfully used HRM to identify somatic mutations in *JAK2* exon 12 in 4 of 50 erythrocytosis patients lacking the V617F *JAK2* mutation. These results correlated exactly with allele-specific PCR and sequencing results obtained independently.

Authorship and Disclosures

AVJ designed research, performed research, analyzed data, wrote the paper; NCPC designed research, wrote the paper; HEW performed research; ARG designed research, wrote the paper; LMS designed research, performed research, analyzed data, wrote the paper.

The authors reported no potential conflicts of interest.

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