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ORIGINAL RESEARCH ARTICLE

Development and Validation of a Tetra-Primer Amplification Refractory Mutation System-Polymerase Chain Reaction Combined with Melting Analysis-Assay for Clinical *JAK2* V617F Mutation Detection

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Abstract

Background and objective *JAK2* V617F mutation is a molecular marker for myeloproliferative neoplasms (MPNs). As there are no China Food and Drug Administration-approved assays for the detection of *JAK2* V617F mutation in China, validation of the analytic performance of this assay is important for the clinical laboratory before its clinical implementation. We have established a method for detecting *JAK2* V617F using tetra-primer amplification refractory mutation system-polymerase chain reaction (tetra-primer ARMS-PCR) combined with melting-curve analysis.

Methods A total of 202 blood samples and 20 bone marrow aspirates were obtained from MPNs patients at Huashan Hospital, Fudan University. The accuracy, precision, reproducibility, analytical sensitivity, lower limit of detection, analytical specificity, interfering substances, ruggedness, robustness, reportable range and reporting of this assay were validated.

Results There was a close agreement between the reference method (sequencing) and melting-curve analysis ($\kappa = 0.89$). The precision was 100 % and the results of the assay were unaffected by lipoprotein (<27 mmol/L) or bilirubin (<450 μmol/L). The analytical sensitivity of the *JAK2* mutation was 1.25 %.

Conclusions Tetra-primer amplification refractory mutation system-polymerase chain reaction (tetra-primer ARMS-PCR) combined with melting-curve analysis can be used in the clinical laboratory for detection of the *JAK2* V617F mutation.

Key Points

A rapid and sensitivity assay for detecting the *JAK2* V617F mutation was developed and validated.

A major advantage of this assay is its one-step, highly sensitive and lower-cost compared with other real-time PCR with hybridization probe melting.

This assay may prove a useful tool for diagnosis and therapy monitoring of MPNs.

1 Introduction

Myeloproliferative neoplasms (MPNs) represent a heterogeneous group of clonal stem cell diseases with abnormal hematopoietic cell proliferation [1]. Somatic mutations in the *Janus kinase 2* (*JAK2*) gene at V617F occur in transformed hematopoietic precursors and have been identified in

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65–96 % of polycythemia vera (PV) specimens, 24–57 % of essential thrombocythemia (ET) specimens, and 36–57 % of myelofibrosis (MF) specimens [2–8]. The detection of *JAK2* V617F is included in the diagnostic criteria for MPNs, and specific *JAK2* inhibitors have recently been approved for the treatment of these patients [9].

There is a lack of commercial kits for detecting the *JAK2* V617F mutation in China, and we therefore developed an in-house real-time tetra-primer amplification refractory mutation system-polymerase chain reaction (tetra-primer ARMS-PCR) combined with melting-analysis assay for clinical detection of this mutation. Melting-curve analysis is a non-gel-based, automated system consisting of tetra-primer ARMS-PCR and a short melting analysis. The melting analysis with use of SYBR Green I depends on the length of the PCR product, its GC content and heterozygosity. The fluorescence intensity would change when double-stranded PCR products dissociate to single-stranded. Different PCR products generally have different melting temperatures. Mutant-type formed 279-bp product result in a higher melting point (Tm) compared with wild-type which formed 229-bp product. Heterozygous samples have two allele-specific peaks [10–13].

In this study we developed an in-house analysis assay and performed a complete analytic validation of the assay in our clinical laboratory. The following parameters were tested: accuracy, precision and reproducibility, analytical sensitivity, lower limit of detection (LoD), analytical specificity, interfering substances, ruggedness and robustness, reportable range and reporting.

2 Materials and Methods

2.1 Samples

A total of 202 blood samples and 20 bone marrow aspirates were obtained from MPNs patients at Huashan Hospital, Fudan University. Patients had clinical diagnosis of PV ($n = 49$), ET ($n = 94$), chronic idiopathic MF ($n = 11$), atypical (BCR-ABL-negative) chronic myelogenous leukemia ($n = 7$), idiopathic hypereosinophilic syndrome and chronic eosinophilia ($n = 6$), and overlap myelodysplastic syndrome/myeloproliferative disorder ($n = 35$). This study was approved by the Institutional Review Board of Huashan Hospital.

2.2 DNA Extraction

DNA was extracted by using a QiaAmp DNA Blood Mini kit according to manufacturer's directions (Qiagen, Valencia, CA, USA). The human erythroleukemia (HEL) cell line (*JAK2* V617F homozygous mutant) and the acute myelocytic leukemia HL-60 cell line (*JAK2* V617F homozygous wild-type) were used as mutant and wild-type controls, respectively. DNA was quantified with a NanoDrop ND-2000 spectrophotometer (NanoDrop Technologies, Wilmington, DE, USA). All nucleic acids were stored at a concentration of 20 ng/μL prior to use in the procedure.

2.3 PCR and Melting-Curve Analysis

Tetra-primer ARMS-PCR with melting-curve analysis was carried out in a Roche LightCycler 480II (Roche Diagnostics Ltd., Switzerland). This reaction used two primer pairs to amplify the wild-type, mutant-type and non-allelic gene-specific products, respectively, in a single reaction, together with controls (Table 1). To minimize the nonallelic gene-specific product, we optimized the experiment conditions. By increasing or reducing the ratio of outer versus inner primers of their final concentration, only two PCR products for genotyping of homozygotes or heterozygotes revealed its unique melting-point curve shapes [14]. A 1:1 ratio of outer versus inner primers concentration was used in this experiment. The reaction was as follow: 1 μL (20 ng/μL) of DNA template, 10 μL of SYBR PCR Master Mix (Qiagen), 10 pmol/L of each primer (primers were synthesized by Sangon Biotech, China) and 7 μL of H_2O, The reaction started with one cycle of 50 °C for 2 min, 95 °C for 5 min, followed by 35 cycles at 94 °C for 30 s, 55 °C for 30 s, and 72 °C for 32 s. Melting-curve analysis was performed at a denaturing temperature of 60 °C for 1 min, followed by heating at a rate of 0.11 °C/s from 65 to 85 °C. Melting curves were analyzed by eye according to shape, and the Tm of the samples was

Table 1 Sequences of primers for *JAK2* V617F mutation

Primer	Sequence (5′–3′)	PCR product (bp)
Wild-type forward	GCATTTGGTTTTAAATTATGGAGTATATG	229
Outer reverse	ATTGCTTTCCTTTTTCACAAGAT	
Outer forward	TCCTCAGAACGTTGATGGCAG	279
Mutant reverse	GTTTTACTTACTCTCGTCTCCACAAAA	
Sequencing forward	AGCTTTCTCACAAGCATTTGG	150
Sequencing reverse	TGACACCTAGCTGTGATCCTG	

recorded. Melting-curve analyses were performed at the temperature-ramping and fluorescence-acquisition settings. For each assay, mutant and wild-type controls were run with the samples. All samples were analyzed in duplicate.

2.4 DNA Sequencing

We amplified all samples using the primer pairs in Table 1 and compared the outcomes with the *JAK2* reference sequence (GenBank accession number NT_008413.18). PCR products were purified using the QIAquick gel purification kit (Qiagen). DNA sequencing analysis was performed using an ABI 3500 genetic analyzer (Applied Biosystems Inc, Foster City, CA, USA).

3 Results

3.1 PCR and Melting-Curve Analysis

In the PCR reaction, the wild-type primers resulted in the creation of a 229-bp product and the mutant primers formed a 279-bp product. The mutant melted at 77.59 °C and the wild-type melted at 76.14 °C (Fig. 1). The optimal starting temperature for melting-curve analysis was determined by varying the starting temperature from 25–75 °C. Different starting temperatures resulted in different T_m values. A starting temperature of 65 °C is shown in Fig. 1. When DNA from the HL-60 and HEL cell lines were mixed, the two melting curves observed at approximately 76 °C (wild-type) and 77 °C (*JAK2* V617F mutation) could be easily resolved from each another. Similarly, comparison of the melting curves generated by amplification of pure HEL or HL-60 DNA resulted in distinct single curves (Fig. 1).

3.2 Accuracy

Qualitative *JAK2* V617F mutation assay results were compared against independent molecular data obtained from reference methods based on genotyping sequencing results. All the samples, including 117 *JAK2* V617F mutation samples and 85 *JAK2* V617F wild-type samples were subjected to direct sequencing (Table 2). The melting-curve analysis and genotyping sequencing data were analyzed by κ consistency tests. A κ value >0.75 signified excellent agreement, κ <0.40 signified poor agreement, κ between 0.40 and ≤0.75 signified fair to good agreement. In terms of the current investigation, we considered κ values of 0.89 to signify excellent agreement.

3.3 Precision and Reproducibility

Precision is the degree to which repeated replicate measurements of a sample show the same results. Precision can be affected by several variables, including template quality and amount, PCR conditions (e.g. annealing temperature), reagent-lot variability, instrumentation, and technical/mechanical errors. The reproducibility of the *JAK2* V617F assay was assessed by repeating the tetra-primer ARMS-PCR and melting-curve analysis within-run, between-run, and between-technologists using HEL and HL-60 DNA. Other variables, including PCR conditions, reagent-lot variability, and instrumentation, are considered under Sect. 3.7. Reproducibility was tested within runs by repeating 20 separate times using HEL and HL-60 DNA; reproducibility between runs were tested by repeating runs four times a day for 5 days (by different technologists). The concordance of ratios within-run, between-run, and between-technologists were all 100 % (data not shown). In terms of

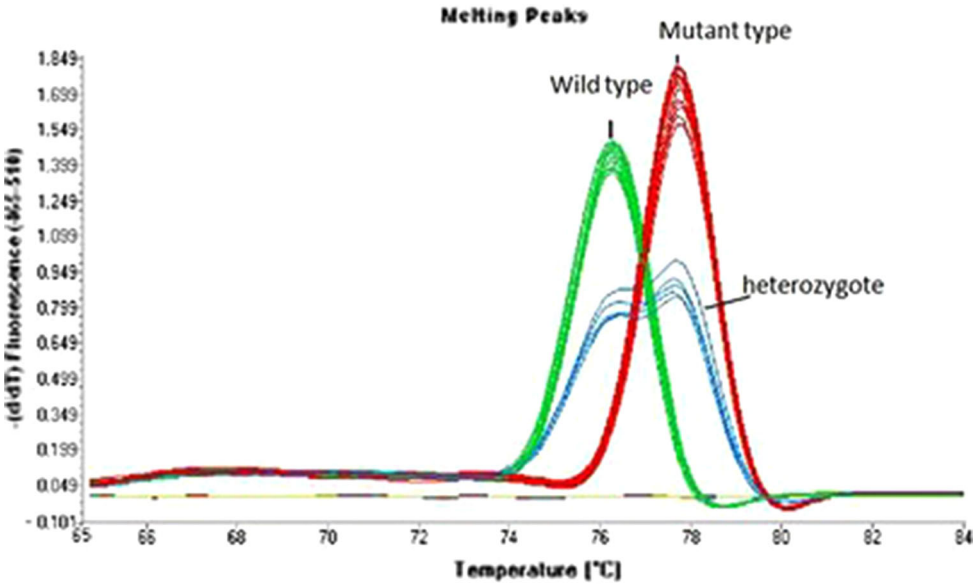

Fig. 1 Tetra-primer ARMS-PCR with melting-curve detection of *JAK2* V617F mutation. The melting curves consisted of two melting regions. The wild-type melting region is to the left (*green*) and the mutant melting curve is to the right (*red*). Wild-type samples (*green*) had a lower T_m of 76 °C, while mutant samples (*red*) had a T_m of 77 °C. Heterozygous samples (*blue*) combined the melting peaks of both alleles

Table 2 Comparison between melting-curve analysis and sequencing

Melting curve analysis	Genotyping sequencing (comparative test)		Total	κ consistency tests
	Mutation	Wild-type		
Mutation	106	11	117	
Wild-type	0	85	85	
Total	106	96	202	0.89

molecular genetic tests, technologies are often highly precision and a validation target for precision approaching 100 % is considered suitable for diagnostic purposes.

3.4 Analytical Sensitivity

Sensitivity is defined as the extent to which an analyte can be identified in a mixture without interference from other components. In PCR, sensitivity can be affected by the ratio of target to background, near-neighbor contaminants in the background, the ratio of multiple target templates, the quality of individual target templates, and the amount of target template in a single assay. In particular, the potential for near-neighbor wild-type contaminants in environmental and forensic sampling is a real concern. We determined mutation sensitivity by comparing the pure template with a range of mixed amounts of wild-type DNA. The analytical sensitivity was evaluated by titration of the homozygous mutant HEL cell line DNA with increasing amounts of homozygous wild-type HL-60 cell line DNA. Serial mixtures of HEL and HL-60 in the ratios 1:1, 1:2, 1:5, 1:10, 1:20, 1:40, 1:80 and 1:160 were analyzed (Fig. 2a). The *JAK2* V617F mutation was roughly detected at a dilution of 1:80 in five replicate experiments (Table 3). As the percentage of the mutant factor increased, the relative area under the mutant curve increased (Fig. 2b).

3.5 Lower Limit of Detection

The LoD was determined as the amount of analyte that gave rise to a signal significantly different from the negative control. In this test, the LoD was detected by dilution of the homozygous mutant HEL cell line and the homozygous wild-type HL-60 cell line to determine the smallest amount of target template that could be detected by this assay. The LoD was determined to be 0.5 ng/μL in both cases (Table 4). The upper limit of the target template was not exceeded at 30 ng in either assay.

3.6 Analytical Specificity and Interfering Substances

The analytical specificity was the ability of the test to identify an entity in the presence of interfering or cross-reactive substances. In this study, the analytical specificity was determined using the Basic Local Alignment Search Tool (BLAST) to identify regions of local similarity between sequences, to check for any cross-reactivity with other regions in the genome. For interfering, we examined the association between the specific PCR product and icterus and lipemia. Nine lipoid serum samples (triglycerides: 8–27 mmol/L) and eight jaundice serum samples (total bilirubin: 156–450 μmol/L) were tested. However, the PCR product showed no significant relationship with either icterus ($t = 0.44$; $P = 0.67$) or lipemia ($t = 1.81$; $P = 0.12$) (data not shown). The College of American Pathologist Molecular Pathology Checklist (MOL.31015) requires validation studies to be performed with an adequate number of samples for each type of specimen. Presence of the *JAK2* V617F mutation was thus further evaluated in 20 fresh peripheral blood specimens and 20 bone marrow aspirate. There was no difference in expression between fresh peripheral blood specimens and bone marrow aspirate (data not shown).

3.7 Ruggedness and Robustness

Ruggedness is defined as the consistency of results when external factors, such as instruments, laboratories, analysts or reagents, are deliberately varied. We determined the ruggedness of our assays by testing 20 randomly selected samples using two different thermal cyclers, LightCycler 480II and Mastercycler ep realplex (Eppendorf, Germany), and using different reagent lots, analyzed by two different analysts. There were no differences in precision or accuracy between different reagent lots, instruments, or analysts, and all allele calls were 100 % accurate (results not shown), indicating a high degree of ruggedness.

3.8 Reportable Range and Reporting

Qualitative assays are designed to report *JAK2* as present (mutation) or absent (wild-type). The reference range is defined as the range of results expected in 95 % of the normal population, which would be wild-type (or no *JAK2* V617F mutation) in this study. The results were reported as a high diagnostic value for MPNs when combined with conventional haematological parameters.

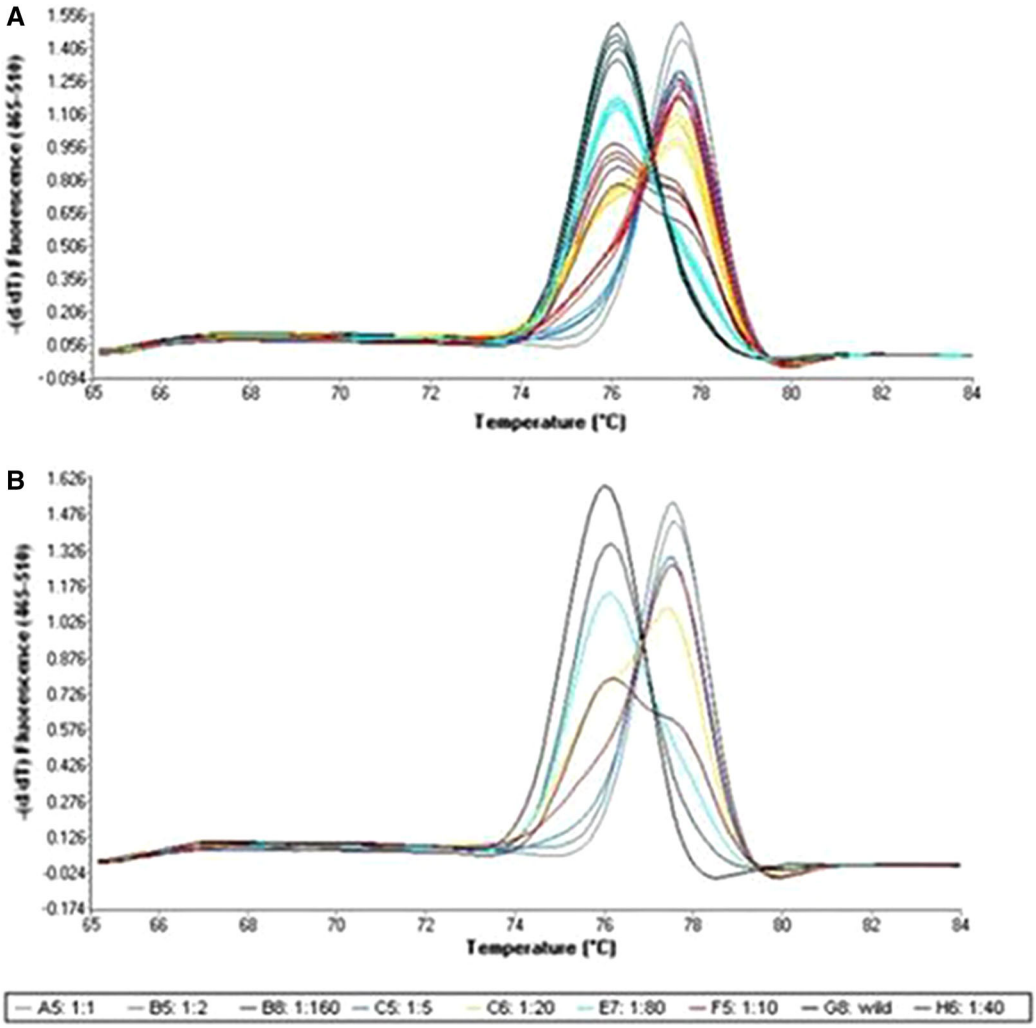

Fig. 2 Tetra-primer ARMS-PCR with melting-curve detection of *JAK2* V617F mutation. **a** Sensitivity to detect the *JAK2* V617F mutation by melting curves (all the replicates of the dilutions); **b** sensitivity to detect the *JAK2* V617F mutation by melting curves (a model of each dilution)

4 Discussion

Assessment of the *JAK2* V617F mutation has become an essential tool in the diagnosis of Ph-negative MPNs [15–18], and its detection has recently been included in the essential diagnostic criteria for PV, ET, and MF by the World Health Organization [19]. Furthermore, pharmacological *JAK2* inhibitors represent a new type of treatment for MPNs [20–22], demonstrating the need for a tool to detect *JAK2* V617F.

Different techniques have been used to detect the *JAK2* V617F mutation, but these are associated with certain disadvantages: DNA sequencing is the gold standard for mutational analysis, but is limited by its high cost and low sensitivity (20–30 %); PCR-restriction fragment length polymorphism analysis may produce false-positive results; several real-time PCR platforms require expensive fluorescence-labeled probes, including AS-PCR using fluorescence resonance energy transfer (FRET) probes or specific TaqMan probes [23–25]. Pyrosequencing needs expensive equipment; and HPLC also requires considerable initial investment in equipment [10]. We present a robust, reproducible, sensitive, and simple tetra-primer ARMS-PCR with melting-curve assay for detecting the *JAK2* V617F mutation in MPNs.

We tested the tetra-primer ARMS-PCR combined with melting-curve assay in terms of several parameters: accuracy, precision and reproducibility, sensitivity, LoD, ruggedness and robustness. Ruggedness, robustness, and sensitivity are not typically examined when developing and validating molecular assays, but they were included in our validation studies, to ensure that this method would produce consistent results.

Comprehensive analytic characterization studies showed excellent agreement ($\kappa = 0.89$) between the results of melting-curve analysis and sequencing (reference methods).

Table 3 Sensitivity of assay for detecting the *JAK2* V617F mutation (T_m and peaks)

Mixture ratio	Mean T_m	Peaks
V617F mutation:V617F wild 1:1	77.58	Single
V617F mutation:V617F wild 1:2	77.52	Single
V617F mutation:V617F wild 1:5	77.47	Single
V617F mutation:V617F wild 1:10	77.38	Double
V617F mutation:V617F wild 1:20	77.04	Double
V617F mutation:V617F wild 1:40	76.64	Double
V617F mutation:V617F wild 1:80	76.35	Double
V617F mutation:V617F wild 1:160	76.17	Single

Table 4 LoD determined as the lowest amount at which all eight replicates were amplified

Amount of DNA (ng)	HEL (mutant-type)	HL-60 (wild-type)
30	8	8
20	8	8
10	8	8
5	8	8
0.5	8	8
0.25	8	7

11 discrepant results may due to the different analytical sensitivity between these two methods. For all these 11 discrepant cases were consistent with the clinical manifestations. The LoD of melting-curve analysis was detected at 0.5 ng/μL, and the analytical sensitivity was 1.25 % ($V617F^{mutation}$:$V617F^{wild}$ = 1:80 detectable), whereas the Pyrosequencing offered a sensitivity of 5–10 %. Although a lower LoD would be desirable to detect low levels of mutation, comparative validation studies would be challenging because of the lack of accepted reference methods at this level of sensitivity. Reproducible detection of the *JAK2* V617F mutation within-run and between-run experiments for a total of 40 repeats resulted in 100 % qualitative agreement. Potential interference by lipoprotein or bilirubin contaminants were ruled out, and the ruggedness and robustness were confirmed.

Previous studies using tetra-primer ARMS-PCR analysis showed high sensitivity for single-nucleotide polymorphism (SNP) detection. It has shown analytical sensitivities of 0.01–5 % [12, 26], and Ibrahim Baris et al. [13] detected the common mutations in the FV, PII, MTHFR and FGFR3 gene with a sensitivity of 0.1–100 ng genomic DNA isolated from peripheral blood samples. Our study described a rapid and sensitivity method for detecting the *JAK2* V617F mutations. The analytical sensitivity was 1:80 which was equivalent to 1.25 %. Obviously, the analytical sensitivity of this paper was lower than what is reported in the literature for most probe-based melting curve assays for *JAK2*. For tetra-primer ARMS-PCR, the most sensitive (0.01–5 %) method which we used combined with melting curve analysis has increased the analytical sensitivity.

A major advantage of the current melting-curve assay is its one-step, highly sensitive and lower-cost compared with other real-time PCR with hybridization probe melting. Although molecular studies have not yet clarified the relationship between mutation rate of somatic cells and the extent of the MPNs, it is important to be able to distinguish mutation in a lower percentage of cells from a background of wild-type cells. The current technique thus provides the potential for detecting these cells. And, using this method makes it possible on the further research about discriminating clones between heterozygous or homozygous for the *JAK2* V617F mutation.

In summary, this novel tetra-primer ARMS-PCR assay with melting-curve analysis represents a suitable molecular diagnostic method for detecting the *JAK2* V617F mutation in clinical specimens. It has a simple sample-processing step, rapid turnaround time, good sensitivity, and excellent precision and reproducibility. Furthermore, it is suitable for analyzing archived materials, and thus allows the retrospective study of *JAK2* V617F in MPNs. This method may prove a useful tool for evaluating the progression and prognosis of MPNs.

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Conflict of interest We declare that none of the authors have any conflict of interests regarding the publication of this study.

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