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Development of a Quantitative Real-Time Polymerase Chain Reaction Assay for the Detection of the *JAK2* V617F Mutation

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Achieving a specific diagnosis of polycythemia vera (PV) and other myeloproliferative disorders (MPDs) is often costly and complex. However, the recent identification of a V617F mutation in the JH2 domain of the *JAK2* gene in a high proportion of patients suffering from MPDs may provide confirmation of a diagnosis. This is an acquired mutation and, as such, may only be present in a small number of cells within a sample. There is therefore a clinical need for highly sensitive detection techniques. We have developed a sensitive real-time polymerase chain reaction (PCR)-based approach for both detection and quantification of the *JAK2* V671F mutation load, which allows determination of mutation status without the need for prior purification of granulocytes. We have performed a comparison of this assay with two previously published detection methods. Although an amplification refractory mutation system (ARMS) was shown to be slightly superior in terms of sensitivity, our real-time PCR method provides the potential for quantification of the *JAK2* V617F mutation, having potential future applications in the monitoring of minimal residual disease or predicting outcome of disease severity. *(J Mol Diagn 2007, 9:42–46; DOI: 10.2353/jmoldx.2007.060083)*

Myeloproliferative disorders (MPDs) are diseases characterized by proliferation of one or more myeloid cell lineages in the bone marrow and increased numbers of mature and immature cells in the peripheral blood. Examples include polycythemia vera (PV), essential thrombocythemia (ET), and idiopathic myelofibrosis (IMF). These conditions are reported to have an annual incidence of 2 to 3, 1.5 to 2, and 0.5 to 1.5 per 100,000 of the population, respectively.

The diagnosis of MPDs can be complex, expensive, and, in the case of ET, based solely on exclusion criteria. There is therefore a real clinical need for a faster, more specific, and more efficient test. The recent discovery of an acquired point mutation (c.1849G>T, p.V617F) within the Janus kinase 2 (*JAK2*) gene in a percentage of these patients has allowed a definitive diagnosis[1–5] by providing a specific target for genetic testing. The mutation causes a valine to phenylalanine substitution (V617F) in the JH2 domain of the protein. This region has been suggested to play a direct role in the negative regulation of *JAK2* signaling.[6] The substitution of a valine residue by the large aromatic amino acid phenylalanine is likely to disrupt this regulation. The *JAK2* V617F mutation has been shown to be present in the majority of patients with polycythemia vera (65 to 97%) and approximately half of patients with essential thrombocythemia (23 to 57%) or idiopathic myelofibrosis (43 to 50%).[1,3,5]

The identification of this acquired mutation establishes the presence of a clonal disorder and has allowed new approaches to the diagnosis and treatment of these diseases.[7] Because this acquired mutation may be present only in a small proportion of cells, sensitive detection methods are required. Previously published detection techniques include an allele-specific PCR that uses a single common reverse primer and two forward primers, one specific to the mutant allele and one that serves to generate an internal control product[1]; an amplification refractory mutation system (ARMS), which uses two primer pairs to amplify specifically the normal and mutant sequences plus a positive control band in a single reaction[5]; direct fluorescent dye chemistry sequencing[4]; pyrosequencing[5]; restriction length polymorphism[1]; and real-time assays based on melt-curve analysis.[8,9] These approaches, although sensitive, are not quantitative. A recent publication demonstrated a correlation between hematological improvement and a reduction in the proportion of the *JAK2* V617F mutant alleles.[10] An approach

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Table 1. Sequence of Primers and Probes Used in Quantitative Real-Time PCR

JAK2 forward primer	5′-AAGCTTTCTCACAAGCATTTGGTTT-3′
JAK2 reverse primer	5′-AGAAAGGCATTAGAAAGCCTGTAGTT-3′
JAK2 wild-type probe	5′-TCTCCACAGA**C**ACATAC (VIC)-3′
JAK2 mutant probe	5′-TCCACAGA**A**ACATAC (6-FAM)-3′

Genotype is determined by probes that differ only at the position of the mutation (indicated in bold and underlined). Probes specific to the wild-type (G) and mutant (T) alleles are labeled 5′ with VIC and 6-FAM, respectively.

that permits both detection and quantitation may therefore be of use in the future.

In this study, we have developed and validated a novel real-time, quantitative allele-specific PCR assay using the TaqMan 7000 platform to detect the *JAK2* V617F mutation in a cohort of 200 patients suffering form a variety of MPDs. We have validated our approach by comparing these results with those achieved using two previously published methods in terms of sensitivity and specificity. The development of an approach that is able to quantitate *JAK2* mutation load as well as detect the presence of the mutation may prove to have clinical use in the future.

Materials and Methods

Patients

Samples were received from 200 consecutive patients initially referred for investigation of one or more of high hemoglobin, platelet count, neutrophilia, or blood film suggestive of an MPD. Patients were classified by their MPD diagnosis into five categories: idiopathic myelofibrosis ($n = 9$), polycythemia vera ($n = 74$), essential thrombocythemia ($n = 51$), secondary polycythemia ($n = 49$), apparent polycythemia ($n = 7$), or other (for example, unclassifiable myeloproliferative disease or CMML) ($n = 10$). The diagnostic criteria for polycythemia vera, apparent polycythemia, and secondary polycythemia were derived from the British Committee for Standards in Hematology Guidelines (2004) (*http://www.bcshguidelines.com/pdf/polycythaemia.pdf*). Diagnosis of essential thrombocythemia and idiopathic myelofibrosis was based on World Health Organization criteria.[11] All cases of idiopathic myelofibrosis presented *de novo*. There was nothing histologically to imply a fibrotic transformation of polycythemia vera; however, this cannot be excluded. Those cases of myelofibrosis with know antecedent PV were classified as polycythaemia vera. This study was conducted in accordance with the Declaration of Helsinki as revised in 2000.

Sample Preparation

Total DNA was extracted from peripheral blood using the QIAamp DNA mini kit (Qiagen, Paisley, UK). DNA concentration and sample absorbance at 260 and 280 nm were measured using a ND-1000 spectrophotometer (NanoDrop Technologies, Wilmington, DE). Samples with a 260:280 ratio less than 1.8 were rejected as unsuitable for analysis.

Real-Time Assay Development

Allele-specific real-time quantitative PCR was performed using the ABI Prism 7000 platform as previously described.[12] An assay specific for the *JAK2* V617F mutation was designed using the Assays-by-Design service from Applied Biosystems (Foster City, CA). The assay contains probes specific to the wild-type (G) and mutant (T) alleles labeled 5′ with VIC and 6-fluorescein (6-FAM), respectively. Our assay uses a single set of primers, so mutation discrimination is achieved by the use of probes that differ only at the position of the V617F mutation (probe and primer sequences are given in Table 1). This approach has the advantage that detection of the *JAK2* mutation is independent of amplification efficiency. The labeling of the probes with different fluorophores allows detection of the two alleles in a single-tube analysis. The presence of a probe against the wild-type sequence acts as an internal control to assess quality of the DNA and also allows quantification of mutation load by calculation of a wild-type to mutant allele ratio.

PCR reactions mixture contained 10 μl of TaqMan Universal PCR MasterMix (Applied Biosystems), 0.08 μmol/L of each probe, 0.36 μmol/L of each primer and 40 ng of DNA, in a total volume of 20 μl. PCR conditions were 2 minutes at 50°C and 10 minutes at 95°C, followed by 50 cycles of denaturation for 15 seconds at 95°C and annealing/extension for 1 minute at 60°C. The fluorescent signal intensities were recorded and analyzed during PCR in an ABI Prism 7000 sequence detector system (Applied Biosystems) using the SDS (version 1.0) software (Applied Biosystems).

The assay was validated for accuracy by the construction of standard curves using a 1:2 serial dilution of DNA containing both mutant and normal alleles. Each dilution was set up in triplicate to allow calculation of SE. The sensitivity of our real-time assay was determined by mixing experiments involving the dilution of pure V617F mutant DNA in normal DNA. Concentrations of mutant DNA ranged from 100 to 0.5%. The pure mutant *JAK2* template was generated as previously described.[1]

Comparison of Detection Methods

Patient samples were simultaneously analyzed for the *JAK2* V617F mutation by two previously described techniques[1,5] The sensitivity of the assays was compared using the χ^2 test.