Development of Molecular Beacon method to detect of JAK2 V617F mutation

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ABSTRACT

V617F mutation of JAK2 gene is a point mutation of Somatic cells leading to permanent phosphorylation and protein kinase activity of the JAK2 protein. This mutation is a valuable marker in the diagnosis of myeloid neoplasms. Development of new techniques can be effective and highly sensitive for the detection of the mutation. The aim of this study is designing and executing a molecular beacon probe-based real-time PCR detect to V617F mutation. Test results were reviewed from a set of wild type and cloned JAK2 exon 12 mutated allele genes into plasmid vector and through amplification by Real-Time PCR system. The sensitivity and specificity of Molecular Beacon probes design were tested. The results showed that the Real-Time PCR system has %100 specificity and % 0.001 sensitivity. Based on the results obtained from the molecular beacon probe-based real-time PCR, it was proved that the system is able to detect normal and mutant alleles of JAK2 V617F position with high accuracy and a short time in a closed tube system.

Keywords: Molecular Beacon Probe; JAK2V617F Mutation; Neoplasms

INTRODUCTION

Myeloproliferative neoplasms, also known as chronic myeloproliferative disorders, are a group of hematologic malignancies, derived from myeloid stem cell line which occurs mainly in adults. Among one of the major characteristics of the disorder is the existence of high accumulate of one or more hematopoietic cell lines in the bone marrow. Mature and immature cells in the bone marrow are easily imported to the blood stream, leading to an increase of white blood cell, hemoglobin, and hematocrit and platelet count [1].In 2005, four research groups managed to identify G to T single nucleotide change in the JAK2 gene, located on chromosome 9P at 1849 position (G→T base substitution) in exon 14 in a large number of patients with polycythemia vera (PV), primary thrombocythemia (ET) and Primary Myelofibrosis (PMF). Genetic analysis revealed that the patients were positive for the JAK2-V617F mutation, found in almost 90-95% of patients with PV and 50% of ET and PMF patients [2].High frequency of V617Fmutation in Polycythemia Vera (PV) has turned into a diagnostic parameter. JAK2, one of four non-receptor Janus kinase is a member of tyrosine kinase, involved as one of the components of internal cytokine signal transduction and growth factor. JAK2 protein kinase has two domains, namely (JH1 and JH2), with only the second JH1 possessing enzymatic activity. JH2 domain or false domain kinase is a negative regulator of kinase activity; through mutation V617F this inhibitory function is destroyed. This mutation occurs in the of primary stem cells and increases sensitivity to cytokines and transmits the messages independent of the cytokine transduction cascade and leads to the downstream message activation of proteins such as STAT, inositol phosphatidylinositol 3-kinase pathway, the mitogen-activated protein kinase and AKT pathways [3].So far, a different technique has been used to identify the G → T base substitution, which are most commonly included in direct sequencing, PCR-RFLP, and ARMS. However, these different molecular methods have different sensitivities, with ARMS method having a sensitivity of 0.01%, while the sensitivity of direct sequencing is between 10-40%. PCR-RFLP method can identify the presence of about 5% Mutant allele

[4].Considering to sensitivity limitation between different methods, when the amount of mutated cells in the patient is low, the exact identification is reduced. The aim of this study is designing, execution and the molecular beacon probe-based real-time PCR detect to V617F mutation.

METHODS

In order to evaluate the function of the molecular system designed, several genomic DNAs containing normal and mutant alleles of the V617F were prepared from the Tehran Noor Lab. In order to design suitable primers and probes for the implementation of Rt-PCR, gene sequence information of JAK2 exon 12 and the headquarters of the mutation were obtained, through the nucleotide NCBI site.

Then, V617F G → T mutation position was determined on the Jak2 gene sequence and a pair of primers was designed that replicated the region containing the mutation. PCR product was 115 bp. Two Molecular Beacon probes were designed for both normal and mutant alleles. Primer and probe sequences are shown in Table 1.

Table 1. Sequences of primers and probes

Primers and Probes	Sequence
Forward Primer (Jak-f)	AGCAGCAAGTATGATGAGCAAG
Reverse Primer (Jak-r)	GAGAAAGGCATTAGAAAGCCTGTAG
Probe Beacon Normal (Jak-PN)	6-FAM-GCTCGATTATGGAGTATGTGTCTGTGGAGACGAGC-BHQ1
Probe Beacon Mutant (Jak-PM)	JOE-GCTCGATTATGGAGTATGTTTCTGTGGAGACGAGC-BHQ1

Rt-PCR reaction mixture includes the enzyme buffer 1X, dNTP mix at a concentration of 2.0 mM, 4 mMMg2+,10pmol primers , 10 pmol probes, 3U HotstartTaq DNA Polymerase and genomic DNA with a concentration of 200 ng. In a final volume of 25 ml both in the Rotorgene (Qiagen) and Stepone (ABI) at temperature of 95°C for 10 minutes and 45 cycles in 95°C for 20 seconds and 60°C for 60 seconds by absorbing the signal in two green and yellow channels. In order to assess the sensitivity of the developed method, a3576 bp (pGH) synthetic plasmid containing normal and mutant sequence of V617F Jak2 gene was synthesized by Generay Biotech Corporation (China). Then, Serial dilution of pGH plasmid was prepared at dilutions of10^{-2}, 10^{-3},10^{-4},10^{-5} and 10^{-6}ng.Then the Real Time PCR reaction was performed on each dilution. The repeatability of the system was also verified by 10 repetitions on each dilution over a 30-day period.

RESULTS

Real-Time PCR optimization was performed on genomic DNAs with a known genotype. In this study the reaction results mentioned on both devices showed that the designed MB probes identify normal allele on wild type sequence and probes designed for the mutant allele V617FG→T, merely recognize this sequence, without any interference together. Figure 1(Part a and b) and Figure 2 (Part a and b) show the aforementioned reaction results on both devices.

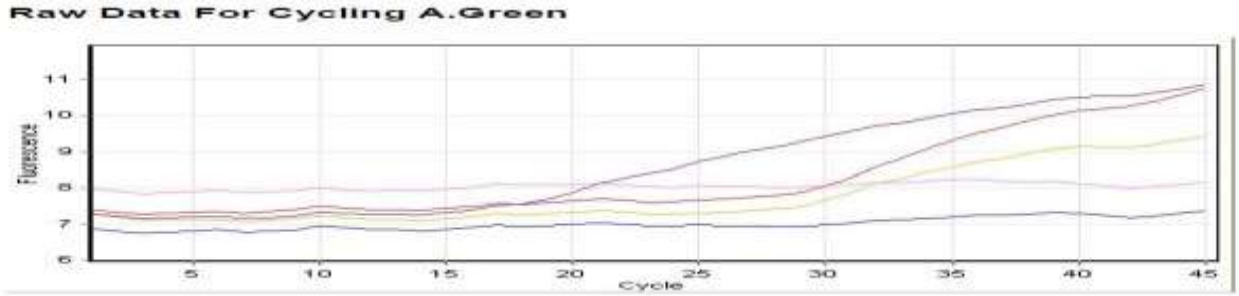

Figure 1. (a) .The result of the fluorescent in the green channel on the Rotor-Gene device. The red sample is a wild type genomic DNA, the yellow sample is a positive genomic DNA prepared from the Noor laboratory Tehran, Blue samples or Naz1 positive

control sample contain mutations that are artificially synthesized, Purple or Naz4 control samples containing the wild type sequence, the pink sample was negative controlled.

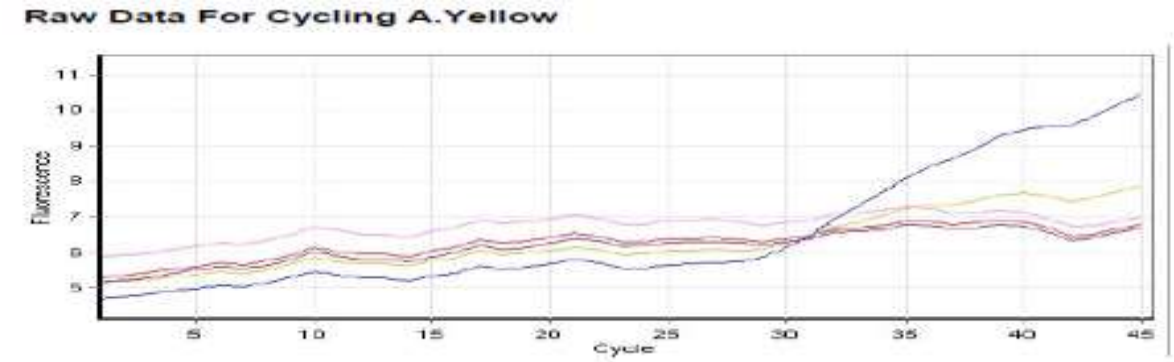

Figure 1. (b) . The result of the fluorescent in the green channel on the Rotor-Gene device. The red sample is a wild type genomic DNA, the yellow sample is a positive genomic DNA prepared from the Noor laboratory Tehran, Blue samples or Naz1 positive control sample contain mutations that are artificially synthesized, Purple or Naz4 control samples containing the wild type sequence, the pink sample was negative controlled.

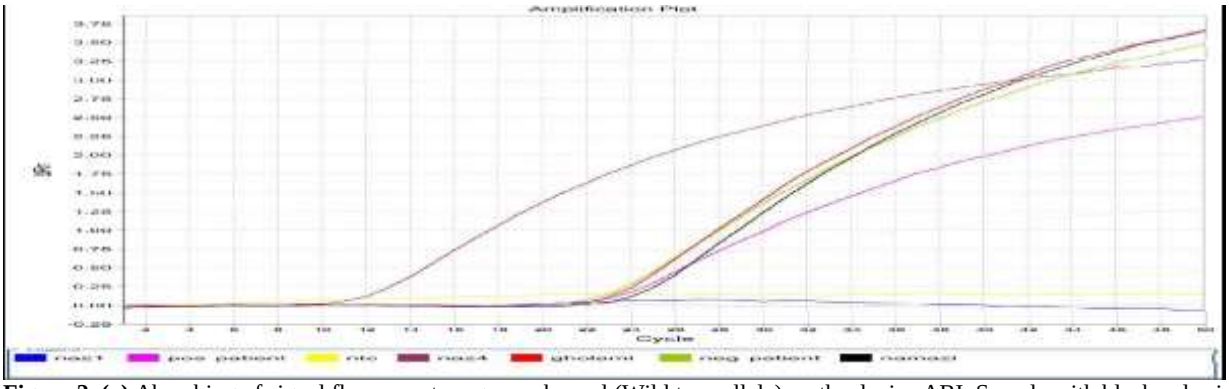

Figure 2. (a). Absorbing of signal fluorescent on green channel (Wild type allele) on the device ABI. Sample with black color is a wild type genomic DNA, The green color is a unknown sample (without mutation), red sample is a wild type genomic DNA, pink sample is a unknown sample (with mutation), Blue Sample or Naz1 positive control samples contain mutations that are artificially synthesized, Brown Sample or Naz4 control contain wild type sequence , yellow Sample was negative.

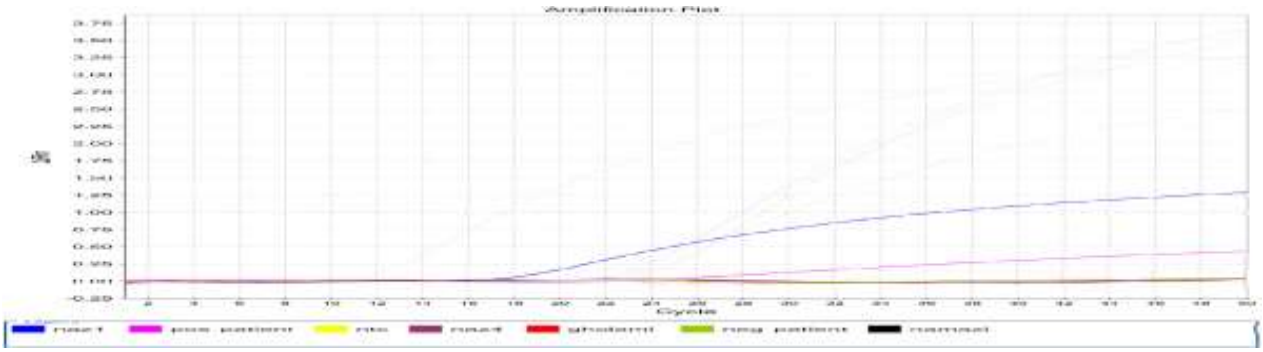

Figure 2. (b). Absorbing of signal fluorescent on Yellow channel (Mutant allele) on the device ABI. Sample with black color is a wild type genomic DNA, The green color is a unknown sample (without mutation), red sample is a wild type genomic DNA, pink sample is a unknown sample (with mutation), Blue Sample or Naz1 positive control samples contain mutations that are artificially synthesized, Brown Sample or Naz4 control contain wild type sequence, yellow Sample was negative.

Likewise, our results indicate that the MB probes have superior performance on the ABI compared to the Rotor-Gene device as the fluorescence signal absorption of each channels is higher in ABI device. Average sensitivity was measured after evaluating the sensitivity of normal and mutant probes, were prepared by serial dilution of genomic DNA from healthy individuals and plasmids containing Jak2 V617F mutation for 10^{-2}, 10^{-3}, 10^{-4}, 10^{-5} and 10^{-6} ng dilution, by a factor of deviation 2.3 % (CV). Moreover, the wild type and mutant probes are capable of identification respectively of a total of 25 copies of the normal allele in 33 Ct and the same number of copies of the mutant allele in Ct 40 in a simultaneous reaction. Besides, the fluorescent signal generated by the Natural probe was 5 times higher than the mutant allele (Table 2).

Table2. Sensitivity charts to determine of the serial dilution and Ct for any dilution of wild type and mutant alleles.

Sample	Target	Quantity (Mean)	Quantity (Std Dev)	Ct (Mean)	Ct (Std Dev)
0.000001	Mutant allele			40.47	
0.00001	Mutant allele			43.07	
0.0001	Mutant allele			24.41	
0.001	Mutant allele			22.20	
0.01	Mutant allele			28.50	
0.1	Mutant allele			18.47	
ntc	Mutant allele				
0.000001	Normal allele			32.47	
0.00001	Normal allele			29.24	
0.0001	Normal allele			25.68	
0.001	Normal allele			22.02	
0.01	Normal allele			18.95	
0.1	Normal allele			15.23	
ntc	Normal allele				

DISCUSSION

Within the past decade, great strides have been taken in molecular pathology for MPN cancer. Since the middle of the last decade and after the discovery of Jak2 mutations in MPN cancer, this molecular disorder has become one of the most important diagnostic markers in negative Philadelphia MPN cancer. All these advances in diagnosis and treatment of disease are strongly influenced by the correct identification of this mutation. Now several methods such as sequencing, PCR-RFLP, ARMS-PCR and Real-time-PCR based on fluorescent probe are available as methods for diagnosis However, PCR-RFLP and PCR-sequencing methods are challenging and time-consuming. In addition, sequencer device is expensive and not affordable in small laboratories. ARMS-PCR technique minimizes the testing time and is a powerful method for detection of single nucleotide mutations. In any case, ARMS-PCR technique based on SYBR Green dye still has false-positive results because of unwanted amplification [7, 6, 5].In 2011 Wu and his colleagues have offered an analysis method based on melting point without the use of labeled probe to detect V617F mutations. In this study, an asymmetric PCR probe with a non-fluorescent dye was released by the end of the block in the presence of fluorescent color saturation and analysis melting point offered. Their method is able to be used on three different devices and the ability to distinguish normal and mutant alleles, respectively [8].In 2014, Wu and colleagues developed a Multiplex Snapback Primer method for the simultaneous detection of V617F and MPLW515L/ K mutations. Their sensitivity of diagnostic method has been 1% with diversity coefficients being less than 5% [9].

In 2011, Zhao and his colleagues developed the dubbed restriction fragment nested allele-specific PCR method for V617F mutation detection. Their method consists of three stages: amplification of target is done with specific primers, PCR products are digested with restriction enzyme BSaXI that cuts only wild-type allele and detection Jak2V617F mutation by Allele-specific PCR which is set up in a Nested-PCR format. The sensitivity of their method was determined to be 0.001% [10]. In 2013, Zapparoli and his colleagues developed a very sensitive method without a probe for the quantitative detection ofV617F mutant allele. Quantitative threefold Allele Specific PCR method is used in a closed tube system which eliminates the possibility of contamination from previous PCR product. They form a triple mutant allele specific amplification, a deoxy 3' - prime end blocker to inhibit the amplification of false positives wild-type allele and a enhancerator reaction. In this system, the mutant allele was quantitatively detected in comparison with JAK2 exon 9. In this study, a Molecular Beacon Probes ARMS-PCR is designed into a single system based on Real-time PCR. The sensitivity achieved in our study is based on the plasmid vector containing the normal and mutant alleles, being more than 0.001%, which is similar to other researchers speculated in this study. The execution of the developed method in this study was performed on 10 positive genomic DNA and 10 negative genomic DNA prepared from the Noor laboratory in Tehran, whose positivity and negativity was confirmed as being 100% through other commercial kits. According to the conclusions drawn through the designed is able to identify both normal and mutant alleles in two distinct color channels. Despite the system being appropriate for step one ABI, this system is not effective for work on Rotor-gene devise because it is non-compliance with Molecular Beacon probe system. Similarly, in the design, amplification of the normal fragment is almost 5 times more than that of the mutant fragment, due to the amplification of both pieces in one reaction which can reduce the sensitivity of mutation detection. However, the system was able to amplify the minimal template in Ct for the mutant allele.

CONCLUSION

According to the results, it can be claimed that the system can be used as suitable close tube V617F mutation detection in the clinical laboratory which works with step one ABI devices.

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"The authors declare no conflict of interest"

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