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Research Article

Determination of the ratio of healthy and mutant forms of human DNA containing the JAK2 V617F mutation by PCR

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Abstract

The most important criterion in the diagnosis of myeloproliferative diseases is the JAK2 (Janus kinase 2) gene mutation. According to the WHO recommendations, the JAK2 V617F mutation is a marker used for primary and differential diagnosis of these diseases. In this regard, it is relevant to develop a domestic PCR diagnostic kit for the detection of JAK2 V617F by quantitative PCR analysis using fluorescently labeled allele-specific primers. In the presented work, a PCR analysis was developed using a new structure of fluorescently labeled primers to detect the JAK2 V617F mutation and determine the quantitative ratio of mutant and healthy cells, which will allow not only to detect the mutation, but also to determine the effectiveness of treatment.

Keywords: PCR, primer, fluorescent probe, amplification, chronic leukemia, DNA mutation, threshold cycle

Introduction.

The investigation relates to the field of molecular biology, medicine and pharmaceuticals, to methods for laboratory diagnosis of oncohematological diseases using PCR and can be used in laboratory and research practice to detect JAK2 (Janus kinase 2) V617F mutations when conducting molecular genetic studies in clinical diagnostic laboratories for the purpose of diagnosing chronic leukemia (Ph-negative myeloproliferative neoplasms).

In accordance with clinical guidelines [1] the main diagnostic criterion for diagnosing blood cancer of the form of Ph-negative myeloproliferative neoplasms, namely, polycythemia vera (PV), essential thrombocythemia (ET) and primary myelofibrosis (PMF) is the identification of one of the most frequent pathogenetic somatic mutations in the Januskinase-2 (V617F JAK2) genes.

Existing kits for mutation detection based on the standard polymerase chain reaction (PCR) method provide for analysis of results using gel electrophoresis, which is laborious and increases the risk of false positive results, in addition, complete laboratory-testing takes a rather long period of time and requires higher laboratory requirements.

Simultaneously study all possible mutations in DNA using DNA sequencing [2] and microchips

methods (Genome-Wide Human SNP Array 6.0. Affymetrix, Inc.) [4] is known. However, the requirements for high-tech expensive equipment and reagents, as well as the high complexity of implementation complicates the widespread use of these methods in the real practice of clinical diagnostic laboratories.

There is a known method, which consists in the use of allele-specific primers with the registration of PCR results in real time using fluorescently labeled samples. The reaction mixture includes forward primers that differ for each allele, a reverse primer common for two alleles and a fluorescent probe with a common dye for mutant and healthy DNA [3]. The disadvantage of this method is that it is impossible to carry out a PCR reaction in one sample, due to the use of the same fluorescent probe with a common FAM dye (6-Fluorescein) for both mutant and healthy forms of DNA.

Known set of reagents for the claimed method, offered by the company "Genotechnology" to detect mutations V617F in the JAK2 gene, mutations 1 and 2 types in the CALR gene and W515L and W515K mutations in the MPL gene [5] (prototype). This kit is similar to the previously discussed method and also allows you to detect mutations only in different PCR samples using different reaction mixtures, which significantly reduces the efficiency and accuracy of the analysis.

A similar method for detecting mutations is a Qiagen kit, JAK2 Muta Screen RS Kit. This method uses multiplex analysis to detect healthy and mutant DNA in a single reaction mixture. This detection method is based on the use of fluorescent probes with FAM (6-Fluorescein) dyes for mutant and HEX (5'-hexachlorofluorescein) for healthy DNA. In the process of PCR analysis, the product DNA is accumulated, regardless of the presence of a mutation (Appendix No. 1, Scheme No. 1). The probe then binds and degrades to form a fluorescent signal based on hybridization with complementary DNA. [6]

The disadvantage of this method is the use of common primers for mutant and healthy forms of DNA, which can lead to false positive results [7]. The formation of the end product, which is detected by the fluorescent signal, depends on the degree of interaction between the fluorescent probe and the DNA region being amplified. Taking into account the fact that DNA with JAK2 mutation has only a single nucleotide substitution, it is obvious that a false positive result can be obtained when registering a fluorescent signal [8,9]. And the fact that the analysis of the fluorescent signal is carried out at the end point of amplification does not allow to determine the quantitative ratio of mutant and "healthy" DNA in human cells [10].

The objective of the research is the development and production of fluorescently labeled allele-specific primers for the detection of JAK2 V617F mutations by multiplex PCR analysis in order to obtain results and diagnostic efficiency.

The essence of the research lies in the fact that the following primers are used to detect the JAK2 V617F mutation: fluorescently labeled primers consist of two oligonucleotides; Quenching Probe) containing a quencher molecule at the 3' end. Such oligonucleotides, when mixed with each other, form a double-stranded structure due to complementarities.

Results and discussion

As a result, an oligonucleotide containing a quencher molecule suppresses non-specific fluorescence in the absence of a DNA site with a JAK mutation in the PCR sample. In the presence of a JAK2-mutated DNA region, the amplification process and the formation of a specific fluorescent signal begin. (Appendix No. 1, Scheme No. 2) When using these two fluorescently labeled primers differing in the nucleotide sequence at the 3' end (JAK2 mutation) having different fluorescent molecules covalently linked to each of the primers, it is possible to determine a single nucleotide JAK2 mutation and diagnose chronic leukemias (Ph-negative myeloproliferative neoplasms).

As a result of the use of our designed primers occurs the simultaneous detection of mutant and "healthy" DNA types in one sample by PCR.

The distinguishing features of the proposed invention are the use of two fluorescently labeled forward primers in the structure of which various dyes are covalently attached, allowing to specifically detect the JAK2 mutation V617F and a complementary oligonucleotide containing the BHQ molecule (Black Hole Quencher) suppressing nonspecific fluorescence:

Primer for amplification of the target region of "healthy" DNA

JAK2 WP HEX-5′-CGTCAGGTTTGGTTTTAGATTATGGAGTATAT**G**-3′
JAK2 QP BHQ1-3'-GCAGTCCAAACCAAAATCTAATACC-5′
Primer for amplification of the target region of the mutant DNA
JAK2 MP FAM-5′-CGTCAGGTTTGGTTTTAGATTATGGAGTATAT**T**-3′
JAK2 QP BHQ1-3'-GCAGTCCAAACCAAAATCTAATACC-5′
Reverse primer
JAK2 reverse 5′-GAATAGTCCTACAGTGTTTTCAGTTTCA-3′
WP (Wild Probe), MP (Mutant Probe)

The method makes it possible to efficiently and specifically determine this type of mutation, since the synthesis of the product DNA depends on the presence or absence of a mutation due to the complementarity of the interaction of the 3' end of the fluorescently labeled forward primers. This method can be applied in medical practice. The developed method is illustrated by examples:

The results of the study to identify the V617F mutation in the JAK2 gene are shown in Figure 1 and Table 1.

Figure №1 (a)

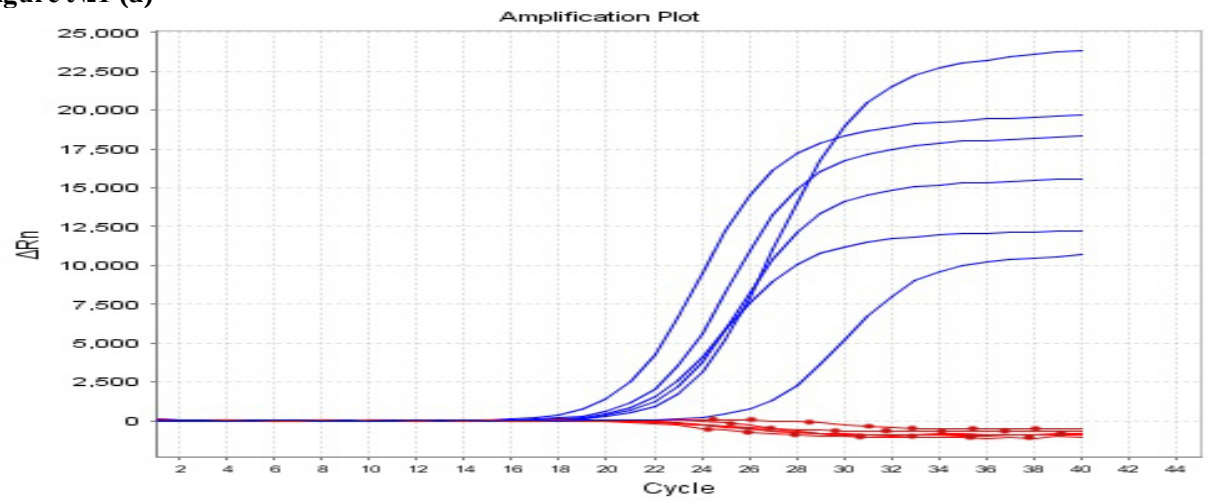

Figure №1 (b)

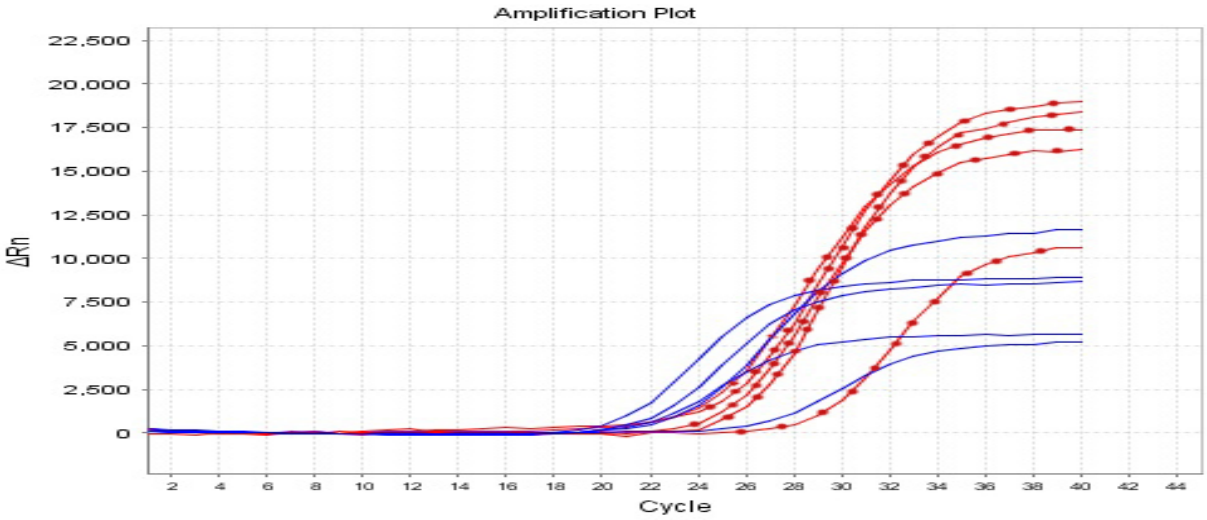

Figure 1(a-b). The results of multiplex PCR analysis of the JAK2 V617F mutation using fluorescently labeled allele-specific primers - an increase in the signal through the FAM channel (lines with dots)

indicate the presence of a mutation - JAK2 (replacement of the G nucleotide by the T nucleotide), a signal through the HEX channel (lines without dots) correspond to a "healthy" type of DNA.

Table 1: Shows data on the threshold cycle (Ct) of the rise of the fluorescent signal through the FAM channel, indicating the presence of a JAK2 mutation.

№ Samples	DNA JAK2 mutation (reading Ct* FAM/Green)	"Healthy" DNA (reading Ct* HEX/Yellow)	Results
1	23,5	20,1	Mutation
2	23,4	21,3	Mutation
3	24,0	21,2	Mutation
4	24,1	21,4	Mutation
5	27,3	25,1	Mutation
6	-	18,0	Norm
7	-	20,0	Norm
8	-	20,0	Norm
9	-	20,3	Norm
10	-	20,6	Norm
11	-	24,2	Norm

Example: Determination of the quantitative ratio of mutant and "healthy" DNA.

The quantitative ratio of JAK2 V617F mutant DNA and "healthy" DNA is obtained as a percentage. To do this, use the obtained values of Ct (threshold cycle) for each target DNA (see table № 1).

For calculation use the following formula:

$$\% \text{ mutant DNA} = \left(\frac{1}{\left(1+10^{(CtFAM-CtHEX)/3}\right)}\right) x100\%$$

The data is recorded in a table.

Table №2: Determination of the quantitative ratio of mutant and "healthy" DNA

№ Sample	DNA JAK2 mutation (reading Ct* FAM/Green)	"Healthy" DNA (reading Ct* HEX/Yellow)	Results	Percent % mutant DNA
1	23,5	20,1	Mutation	6,8 %
2	23,4	21,3	Mutation	16,6 %
3	24,0	21,2	Mutation	10,4 %
4	24,1	21,4	Mutation	11,1 %
5	27,3	25,1	Mutation	15,5 %
6	-	18,0	Norm	0 %
7	-	20,0	Norm	0 %
8	-	20,0	Norm	0 %
9	-	20,3	Norm	0 %
10	-	20,6	Norm	0 %
11	-	24,2	Norm	0 %

* If the value of the cycle threshold (Ct) of the “healthy” type of DNA is greater than 30, then the DNA extraction process was performed incorrectly.

Thus, the advantage of the claimed invention is that the developed domestic method for detecting JAK2 mutations for the diagnosis of Ph-negative myeloproliferative neoplasms, in which fluorescently labeled primers of a new design were developed to determine a single (SNP-mutation) nucleotide substitution, allowing the use of the proposed analysis to determine the quantitative the ratio of mutant and "healthy" DNA, which can be used to determine the degree of development of cancer.

Conclusion

The developed PCR analysis based on fluorescently labeled primers for detecting the JAK2 V617F mutation and determining the quantitative ratio of mutant and healthy cells containing this mutation can be effectively used not only in the diagnosis of Ph-negative myeloproliferative diseases, but also to determine the effectiveness of their treatment.

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Conflict of Interest: No potential conflict of interest declared.

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