LIMITS OF DIDEOXYSEQUENCING IN THE DETECTION OF SOMATIC MUTATIONS IN GASTROINTESTINAL STROMAL TUMORS

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Abstract

Detection of mutations in cancer is particularly important in terms of proper treatment and targeted therapy. The aim of this study was the comparison of two methods: allele-specific PCR (AS-PCR) and dideoxysequencing applied for the identification of *BRAF* gene mutations in wild-type gastrointestinal stromal tumors (WT GISTs). We have optimized the conditions for the detection V600E mutation representing the c.1799 T>A substitution by AS-PCR and have used dideoxysequencing to verify our results. In nine cases, we were able to detect the mutation by AS-PCR approach; however, the mutations have been confirmed by dideoxysequencing in four cases only. AS-PCR is fast and low cost method for the detection of V600E mutation which was validated as a sensitive assay for the identification of the most common *BRAF* mutation in DNA extracted from paraffin-embedded tissue of WT GISTs.

Keywords: wild-type GIST, *BRAF*, allele-specific PCR, dideoxysequencing

INTRODUCTION

The most common mutations causing alteration of the gene function in cancer are single-base substitutions called point mutations (1). These mutations are not easy to detect because they can occur only in a small fraction of the heterogeneous cancer tissue. However, the sensitive detection of such genetic changes is important in clinical decision making concerning the administration of targeted therapy (2).

There are a variety of molecular methods which can be used to detect somatic mutations in cancer tissue. The methods used will depend on the type of mutation that is detected. We can distinguish between methods based on the DNA sequencing such as Sanger dideoxysequencing (3), next-generation deep sequencing, and pyrosequencing which are able to detect all changes within analyzed region, screening methods such as heteroduplex analysis, and methods which can detect only one specific mutation based on different allele-specific approaches.

Gastrointestinal stromal tumors are characterized by mutations in *KIT* and *PDGFRa* genes which are standardly detected by Sanger dideoxysequencing. These mutations lead to ligand-independent activation and signal transduction mediated by constitutively activated KIT or PDGFR receptor (4) and most of patients harboring these mutations respond to the targeted therapies in the form of tyrosine-kinase inhibitors. However, around 10-15% of all diagnosed GISTs are lacking the *KIT/PDGFRa* mutations and are referred as wild-type (WT) GIST. Some of the WT GISTs were showed to harbor *BRAF* V600E mutation and not to respond to the therapy with tyrosine kinase inhibitors (5). Mutations in *BRAF* have been found in <1% of WT GISTs (6), and are similar to those seen in melanoma (60%), colorectal cancer (7) or ovarian cancer (8).

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In our work, we have been developing a sensitive allele-specific PCR (AS-PCR) for the detection of the base substitution c.1799 T>A, corresponding to V600E mutation and comparing it with dideoxysequencing method.

MATERIAL AND METHODS

Patients and control samples

For the detection of somatic mutations in exon 15 of the *BRAF* gene, we have used DNA extracted from formalin-fixed paraffin-embedded (FFPE) blocks from our biopsy archive from patients tested negative for *KIT* and *PDGFR* mutations (9). The control DNA used for the optimization of the AS-PCR was isolated from the RKO cell line, (obtained from Dr. Franken, Academic Medical Center University of Amsterdam in Netherlands) which is containing heterozygous substitution V600E. Actually, 50% of the alleles represent the mutated allele (c.1799T) and 50% alleles are harboring the standard allele (c.1799A). To exclude false positive results, we have used DNA isolated from peripheral blood of healthy person as negative control and 10 anonymized blind control samples with confirmed c.1799 T>A substitution in 5 cases by a method certified for *in vitro* diagnostics (kindly provided from Martin's biopsy center).

Allele-specific PCR

Analytical sensitivity was performed on a DNA mixture consisting of the wild-type and RKO DNA in serial dilutions, according to the percentage of the V600E mutation (10%, 5%, 2.5%, 1.25%, and 0.625%) using primers specific for the mutant and wild-type allele. The allele-specific primers were designed according to reference sequence (www.ensembl.org) to detect a point mutation c.1799T> A (V600E) flanking the hotspot site of exon 15 of the *BRAF* gene (Table 1, no. 2, 3, 4, 5). The AS-PCR was performed in total volume of 25 μl using different concentrations of $MgCl_2$ with 200 mM dNPTs, (Gene Amp dNTP Mix with dTTP, Applied Biosystems, USA), 10 pM of each primer, 1U Taq polymerase (FastStart Taq DNA Polymerase, Roche Diagnostics GmbH, Germany) and 20ng of genomic DNA. The annealing temperature was 64°C. The amplification products were separated by electrophoresis on 2% agarose gel stained with GelRed Nucleic Acid (Biotinum, Inc., USA). PCR products were visualized on UV transilluminator.

	Primer type	Sequence	Annealing temperatures T_m
1.	*BRAF* exon 15 forward F	5' - tcataatgcttgctctgatagga – 3	64°C
2.	*BRAF* exon 15 AS I forward	5' - gtgattttggtctagctacagt – 3	62°C
3.	*BRAF* exon 15 AS II forward	5' - gtgattttggtctagctacaga – 3	62°C
4.	*BRAF* exon 15 AS III forward	5' - gtgattttggtctagctaccga – 3	64°C
5.	*BRAF* exon 15 reverse R	5' - ggccaaaaatttaatcagtgga – 3	64°C

Table 1 Summary of primers and temperatures used in the optimization of AS-PCR and dideoxysequencing

Dideoxysequencing

The exon 15 of *BRAF* gene was amplified by *BRAF* exon 15 forward F and *BRAF* exon 15 reverse R (Table 1, no. 1, 5). The standard PCR was performed in total volume of 25 μl using

2.5mM $MgCl_2$ with 200 mM dNPTs, (Gene Amp dNTP Mix with dTTP, Applied Biosystems, USA), 10 pM of each primer, 1U Taq polymerase (FastStart Taq DNA Polymerase, Roche Diagnostics GmbH, Germany) and 20ng of genomic DNA. The annealing temperature was 64°C. After PCR amplification, PCR products were purified by NucleoSpin Extract II kit (Macherey-Nagel, Germany) according to the manufacturer's instructions following the cycle sequencing using the forward or reverse primer and BigDye® Terminator v1.1 Cycle Sequencing Kit. (Applied Biosystems, USA). Sequencing products were purified by DyeEx 2.0 Spin Kit (Qiagen, Germany). The sequence was analyzed in 3500 Genetic Analyzer (Applied Biosystems, USA) and the sequences were compared to the corresponding reference sequence by BLAST program (http://blast.ncbi.nlm.nih.gov/Blast.cgi).

RESULTS

Optimization of AS- PCR

Hybridization of primers

Allele-specific PCR conditions were optimized. For the purpose to determinate the analytical sensitivity, AS-PCR conditions were tested in several steps. The ability of primer to anneal and subsequently to amplify the PCR product of desired length was tested in a total of 25 μl with an annealing temperature of 64 °C, 2.5 or 3 mM $MgCl_2$, and all three types of the forward primers were used (Table 1, no. 2, 3, 4) (Fig. 1). Results from RKO cell line (Fig. 1, lanes 5, 6, 11, 12) confirmed that the DNA extracted from the RKO cell line is harboring the c. 1799 T to A substitution (V600E). In control samples (Fig. 1, lanes 2, 3, 8, 9), no mutated PCR product have been seen. However, the primer ASII, differing from the wild-type primer on the 3 end by 1 nucleotide showed stronger signal (Fig. 1, lanes 5 and 11) than the other allele-specific ASIII which differ in 2 nucleotide compared with the wild-type sequence.

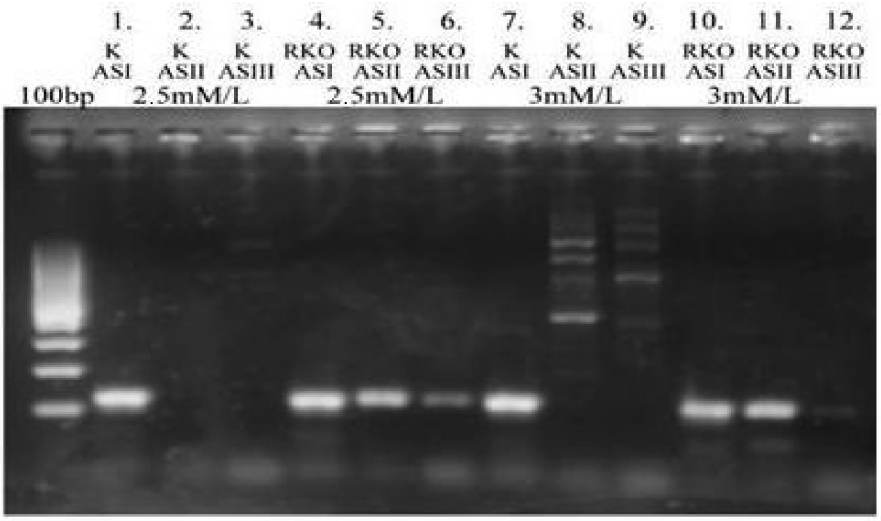

Fig. 1 Optimization of the hybridization of allele-specific primers. In lanes 1, 4, 7, 10 was used primer for the wild-type allele (ASI) and in lanes 2, 3, 5, 6, 8, 9, 11, 12 we used primers for V600E (ASII and ASIII) mutation. Length of the PCR product was 125 bp.
K- control wild-type DNA; RKO- DNA from RKO cell line harboring the V600E mutation; AS I- allele-specific primer, amplifies the standard allele; AS II- allele-specific primer, differs from the standard primer with one base, binds to the mutant allele; AS III- allele-specific primer, differs from the standard primer by one triplet, binds to the mutant allele; 100bp ladder.

Sensitivity of the PCR in serial dilutions of the RKO DNA

The ability to detect mutation using allele-specific primers in the presence of various concentrations of magnesium was tested in serial dilutions of the RKO cell line giving the 10%, 5%, 2,5% 1.25% and 0.625% of the mutant allele with an annealing temperature of 64 °C (Fig. 2). The most consistent results were obtained with the primer ASII at the 4mM $MgCl_2$ concentration (Fig. 2, D).

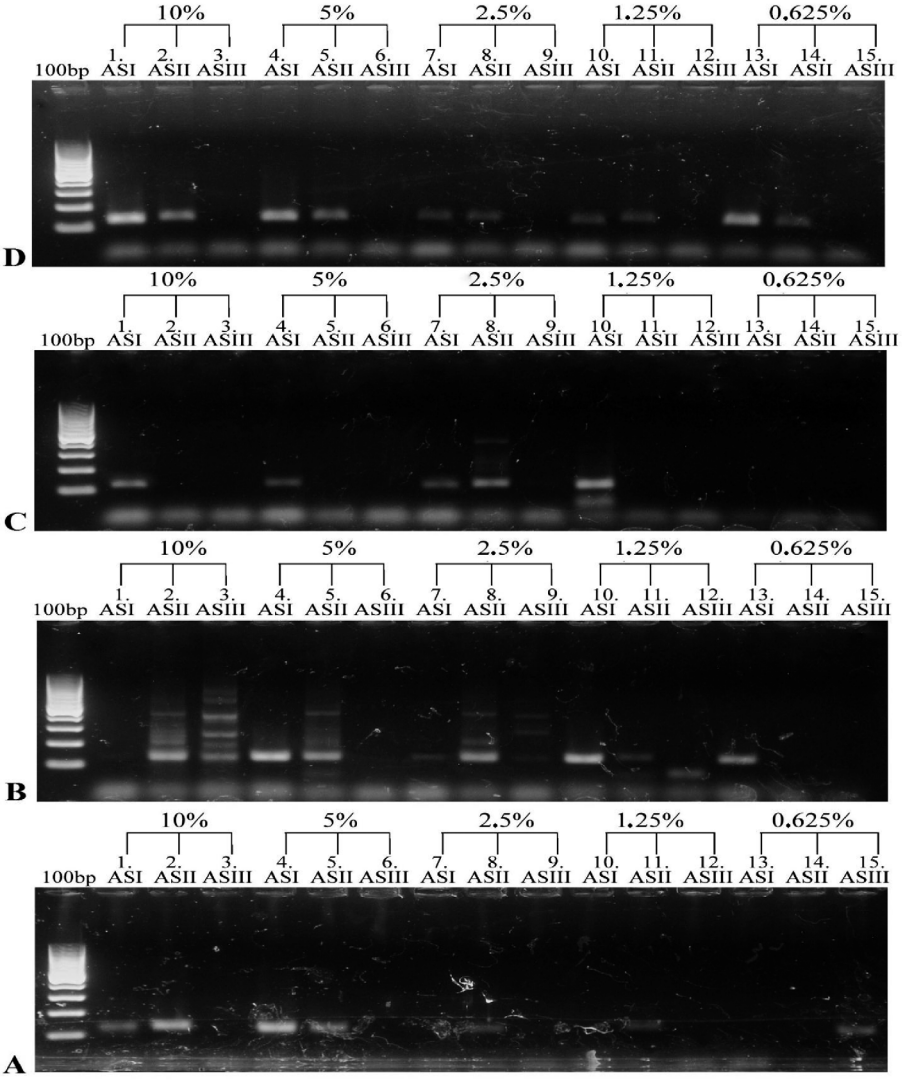

Fig. 2 The ability of allele-specific primers to detect V600E mutation in serial dilution of the RKO DNA with different percentages of mutated allele in the presence of different concentrations of magnesium ions. The V600E mutation was detected in all five control samples RKO in the presence of 4 mM $MgCl_2$ only (lanes 2D, 5D, 8D, 11D, 14D). We were unable to detect V600E mutation using the ASIII primer (lanes 3D, 6D, 9D, 12D, 15D).

AS I- allele-specific primer, amplifies the standard allele; AS II- allele-specific primer, differs from the standard primer with one base, binds to the mutant allele; AS III- allele-specific primer, differs from the standard primer by one triplet, binds to the mutant allele; 100bp ladder. **A**-in the PCR reaction is used 1.5 mM MgCl2; **B**-in the PCR reaction is used 2.5 mM MgCl2; **C**-in PCR reaction is used the 3 mM MgCl2; **D**-in PCR reaction is used the 4 mM $MgCl_2$.

Exclusion of the false positivity

The anonymized blind control samples with the c.1799 T to A substitution were tested in 1.5 mM and 4 mM $MgCl_2$ concentrations each sample using ASI (wt specific) and ASII (mutation-specific primers) (Fig. 3). The annealing temperature of both primers was 64°C. First sample is tested at 1.5 and the second at 4 mM magnesium ion concentration. We detected V600E mutation in four of five V600E positive samples. The band in case of ASII primer indicating V600E mutation has not occurred in any of the remaining WT, therefore we can confirm that the primer ASII at 4mM $MgCL_2$ does not give false positive results.

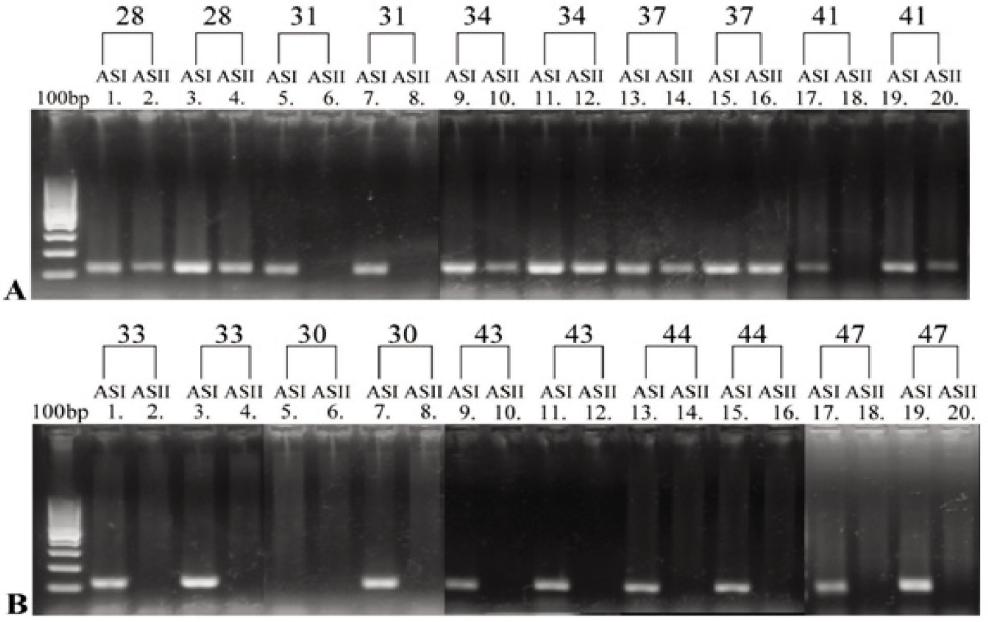

Fig. 3 The ability of ASII primer to detect hotspot *BRAF* mutation in samples with diagnosed V600E mutation in the presence of different concentrations of magnesium. The band in case of ASII primer indicating V600E mutation has not occurred in any of the WT samples (lanes 2B, 4B, 6B, 8B, 10B, 12B, 14B, 16B, 18B, 20B).

***BRAF* mutation detection by AS-PCR compared with the dideoxysequencing results.**

The AS-PCR conditions as optimized above were used for the testing of patients samples. Briefly, the patients' samples were tested using ASI (wt-specific) and ASII (mutation specific) primers at the temperature of 62°C and 4mM $MgCl_2$. From the 150 tested samples, in 9 samples was identified c.1799T A the BRAF V600E mutation (Fig. 4A). All results were verified by dideoxysequencing of PCR products (Fig. 4 B) and we could confirm the mutation by sequencing in 4 cases only.

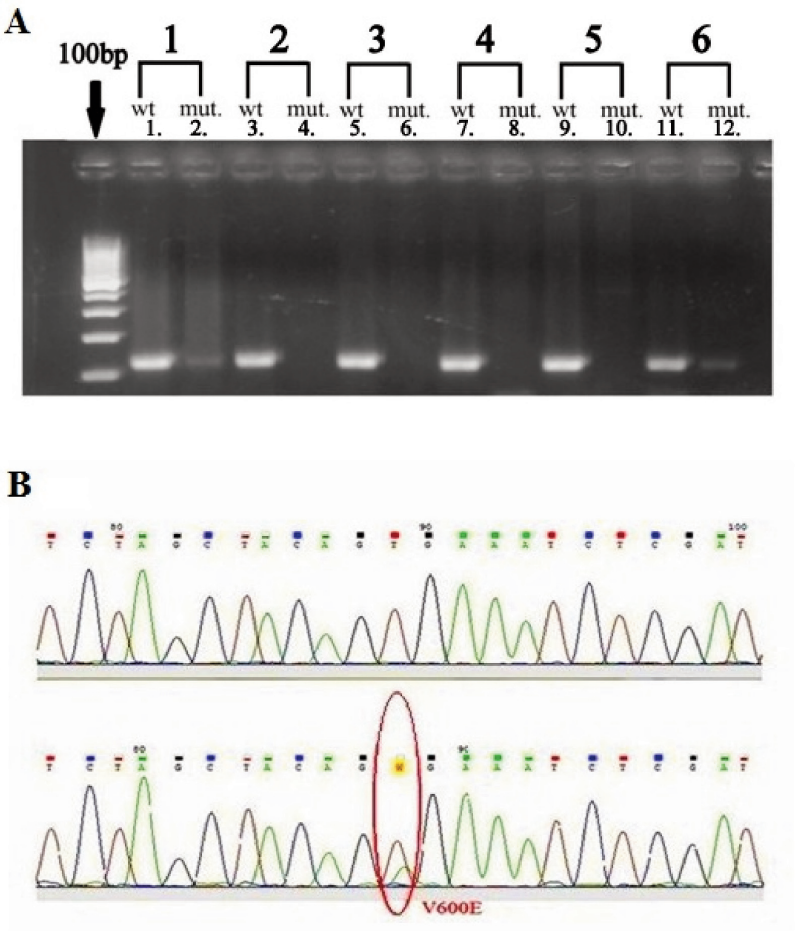

Fig. 4.
A Representative AS-PCR analysis of hotspot *BRAF* mutation in exon 15 in WT GISTs, performed in 2 tubes, in lanes 1, 3, 5, 7, 9, 11 we used allele-specific wildtype (wt) primer and in lanes 2, 4, 6, 8, 10, 12 were used allele-specific primers for detection mutant allele in exon 15 (mut), compared with the 100 bp ladder (first line on the left).
B DNA sequence of *BRAF* wild-type sequence (the electropherogram on the top) and representative patient with the mutation at hotspot site V600E (on the bottom).

DISCUSSION

Tumor growth is a result of the multistep evolution of driver mutations resulting in the disruption of the balance between the rate of cell division, cell growth and programmed cell death - apoptosis. Abnormal cell signal transduction is a driving force in the process of malignant transformation (10). Nowadays, a large attention is devoted to the analysis of tumor heterogeneity to elucidate different developments within one tumor mass. The dideoxysequencing is considered as a standard method in most cases of molecular diagnostics, in particular, the heterogeneous tumor mass with different activated pathways necessitates development of new approaches.

The allele-specific PCR assay described herein represents a sensitive and reliable solution for the comprehensive detection (11) of *BRAF* mutations in cases of GIST without *KIT* and *PDGFR* mutations to identify patients who can benefit from other than tyrosine kinase inhibitor therapies. The test detects V600E substitution mutations in an efficient two-tube format. This provides a simple and robust assay capable of detecting the vast majority of *BRAF* mutations associated WT GISTs with high analytical sensitivity less than 1% that exceeds direct sequencing (12). We were also able to demonstrate no false positivity, however, on 10 samples only. If compared with sequencing, our assay also has the advantages of being relatively inexpensive, being less labor intensive, and having a shorter processing time (12).

The sensitivity of the methods applied in the detection of mutations in heterogeneous tumors has significant influence on the results obtained by molecular analysis. Direct sequencing is considered to be standard for detection of mutations, but limits of the sensitivity of this method may lead to false negative results. Compared to Sanger sequencing, AS-PCR sensitivity in tumor tissues was significantly higher in our study and in other publications (13). This statement is supported by our results, because we were able to confirm four of nine *BRAF* positive samples by direct sequencing. We have determined that dideoxy-sequencing is not sufficiently sensitive method to detect all patients with GISTs carrying the *BRAF* mutation.

For more extensive prospective or retrospective studies, we would suggest more recent approaches such as NGS analysis or digital PCR approach, and to compare the obtained results with the patient response to therapy. Very promising approach for detection *BRAF* mutation is digital PCR, which allows detection of rare single nucleotide mutations in the population of WT sequences. It might be able to solve the just mentioned problem of lower sensitivity of some methods in detecting under-represented mutations in tumors with an admixture of large amounts of non-tumor cells.

In summary, we have validated a sensitive assay for detection of most common *BRAF* mutation in DNA extracted from paraffin-embedded tissue of WT GISTs.

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