A New and Improved Method Based on Polymerase Chain Reaction-Restriction Fragment Length Polymorphism (PCR-RFLP) for the Determination of A1298C Mutation in the Methylenetetrahydrofolate Reductase (MTHFR) Gene

Grzegorz Machnik[1,2], Małgorzata Zapała[1], Ewa Pelc[1], Monika Gąsecka-Czapla[1], Grzegorz Kaczmarczyk[1], and Bogusław Okopień[2]

[1]"Diagnostyka Sp. z o.o.", ul. Olszańska 5, Kraków, Poland and [2]Department of Pharmacology, Medical University of Silesia, Katowice, Medyków 18, Katowice, Poland.

Abstract. Intracellular folate homeostasis and metabolism is regulated by numerous genes. Among them, 5,10-methylenetetrahydrofolate reductase (MTHFR) is of special interest because of its involvement in regulation of the homocysteine level in the body as a result of folate metabolism. Moreover, some studies demonstrated that the homocysteine plasma level in individuals may be influenced by polymorphisms present in the MTHFR gene. Two common, clinically relevant mutations have been described: MTHFR C677T and MTHFR A1298C. Although several laboratory techniques allow genotyping of both polymorphisms, PCR-RFLP analysis is simple to perform, relatively cheap, and thus one of the most utilized. In the case of A1298C, the PCR-RFLP technique that utilizes MboII endonuclease class II requires an acrylamide gel electrophoresis, since agarose gel electrophoresis is unable to resolve short deoxyribonucleic acid (DNA) fragments after restriction digestion. Agarose gel electrophoresis is commonly preferred over that of acrylamide. To resolve this inconvenience, a novel PCR-RFLP, AjuI- based method to genotype A1298C alleles has been developed that can be performed on standard agarose gel.

Key words: Polymorphism, restriction fragment length, mutation, molecular biology, Methylenetetrahydrofolate reductase.

Introduction

5,10-methylenetetrahydrofolate reductase (MTHFR) is a key enzyme for intracellular folate homeostasis and metabolism. Numerous studies demonstrated that homocysteine plasma level in individual patients may be influenced by polymorphisms present in different genes involved in folate metabolism. And indeed, clinically significant polymorphisms in the MTHFR gene have been described to date, two of which are of particular importance [1]. The first polymorphism, resulting in the expression of the so-called "thermolabile" variant of the MTHFR enzyme as an effect of alanine-to-valine substitution at amino acid position 222, is a consequence of single nucleotide mutation C-to-T at nucleotide position 677 (C677T) [2]. The second clinically significant polymorphism is observed at the amino acid position 429, where the glutamic acid is substituted to alanine as a result of A-to-C mutation in the nucleic acid chain at position 1298. Generally, in both types of mutation, decreased MTHFR activity has been observed in recessive homozygous individuals, resulting in an elevated homocysteine level (homocysteinemia, homocysteinuria), which is associated with mental retardation, neural and psychiatric abnormalities, and reproductive failure such as still-birth and spontaneous abortion [3,4,5].

The MRHFR A1298C polymorphism, as previously described [3], showed a correlation with neural tube defects [6] and with susceptibility to acute leukemia in adults [7]. Moreover, an association between homozygous variant AA and the prevalence of bipolar disorders has also been documented (8). Numerous studies reveal that both common MTHRF mutations, C677T and A1298C, are

Address correspondence to Grzegorz Machnik, PhD, Department of Pharmacology, Medical University of Silesia, Katowice, Medyków 18, 40-752 Katowice, Poland; phone: +48 32 208 85 17; fax: + 48 32 252 3902; e mail: gmachnik@sum.edu.pl

Figure 1. *Restriction analysis of MTHFR gene fragment with AjuI endonuclease. Common allele (A) at the position 1298 of MTHFR gene is underlined; AjuI recognition sequence (GAANNNNNNNTTGG) is depicted by capital letters and an insertional mutation incorporated by mutagenic primer is depicted with the line above. Note, that there are two cut sites in case of an A allele at position 1298 of MTHFR gene, giving 147, 32 and 10 base pair DNA fragments after digestion. Conversely, no cut site for AjuI is found, when A is replaced by C at position 1298 giving one fragment of 189 bp. Primer sequences are in grey.*

involved in drug metabolism. Several published clinical studies have investigated the potential predictive role of MTHFR genetic variants on the toxicity and efficacy of antifolate and fluoropyrimidine agents, such as methotrexate (MTX), 5-fluorouracil (5-FU), and raltitrexed. Thus, common access to pharmacogenomics diagnostic tools may improve individualization of drug therapy [9].

Several techniques were developed in order to ascertain the particular alleles, both in C677T and in A1298 polymorphisms. These methods encompass direct sequencing, real-time fluorescent-based assays, ARMS-PCR, and polymerase chain reaction-restriction fragment length polymorphism (PCR-RFLP) [2,6,10]. RFLP is a well proven technique, often described in the literature, and is also the most convenient method, especially when compared with other techniques based on fluorescent dyes, such as TaqMan assays or sequencing analysis [11]. PCR-RFLP is composed of two steps, each of which needs optimization. Both elements, PCR and RFLP, may be performed independently, and no expensive equipment is needed. The final reaction products are usually resolved on electrophoresis gels, and the results are interpreted according to expected band patterns. In the case of the C677T polymorphism, the well-established PCR-RFLP technique described by Frosst et al [2] utilizes restriction digestion of 198-bp-length PCR product by HinfI endonuclease, and the reaction products are easily resolved on standard agarose gel electrophoresis. In this method, the band patterns are as follows: C/C alleles: 198bp (no digestion occurs); C/T alleles: 198bp, 175bp and 23bp; T/T alleles: 175bp and 23 bp. In the case of A1298C polymorphism analysis, acrylamide gel electrophoresis has been used in most PCR-RFLP methods. For example, in one of the most cited papers, van der Put and colleagues [6] describe the use of MboII endonuclease for restriction digestion of the 163 bp-length PCR fragment flanking A1298C SNP. Because there are additional MboII-specific sites in this PCR product, the analysis results in numerous bands that cannot be resolved by means of agarose gel electrophoresis (A/A alleles: 56, 31, 30, 28, 18 bp; A/C alleles: 84, 56, 31, 30, 18 bp; C/C alleles: 84, 31, 30, 18 bp).

Materials and Methods

Genomic DNA was extracted from 200µl of whole blood, using the QIAmp DNA mini kit (Qiagen, Germany), and the elution volume of DNA extracts was 40 µl in each case. Blood samples were obtained from a stored back-up set, after commercial MTHRF analysis was performed by "Diagnostyka Sp z o.o." company. Each sample was previously genotyped in order to determine the MTHFR C677T and A1298C alleles. Informed consent was obtained in every case when human genomic material was used. As the study uses no clinical information, Bioethics Committee approval was not necessary.

The genotyping of the A1298C polymorphism in the MTHFR gene was performed as previously described [6]. Briefly, polymerase chain reaction conditions were as follows: 92°C for 2 minutes; then 35 cycles of 92°C for 60sec., 51°C for 60sec., 72°C for 30sec., and a final elongation step at 72°C for 7 minutes. The reaction was performed on Mastercycler ep PCR thermocycler (Eppendorf, Germany) in the total volume of 25µl. The reaction mixture contained 1U of AmpliGold Taq polymerase (Life Technologies, Warsaw, Poland), 1x buffer A; 0.2mM of each dNTPs; 3.0 mM $MgCl_2$; 0.25µM of both MTHFR_Mbo2_F and MTHFR_Mbo2_R primers. Finally, 5µl of a sample extract of genomic DNA was added as template. Restriction digestion of 10µ of PCR product with MboII endonuclease was performed according to the manufacturer's protocol (Thermo Fermentas, Lithuania). The reaction mixture contained

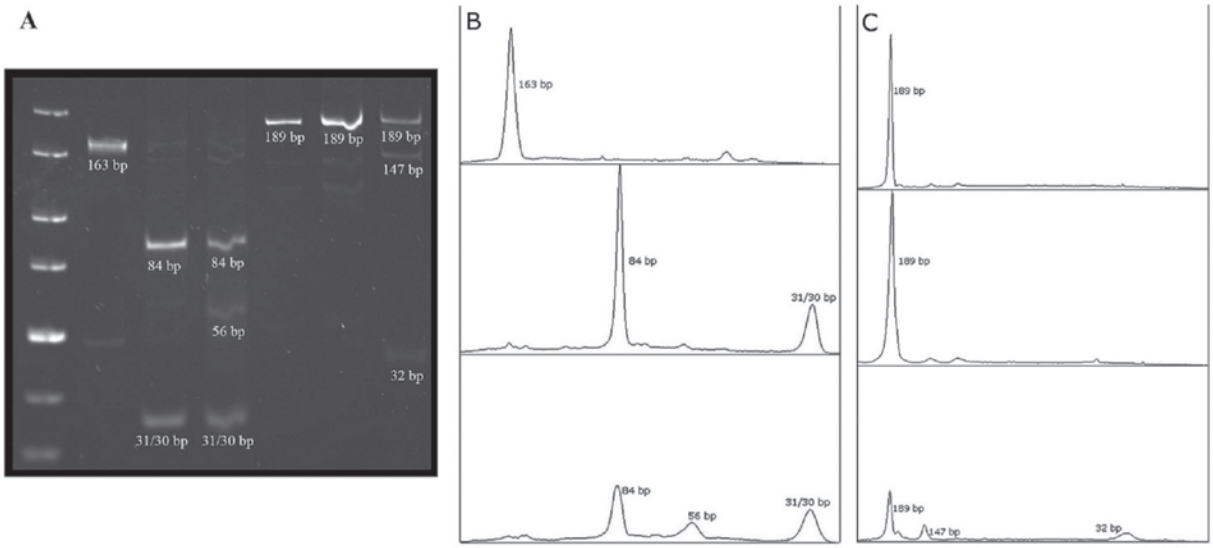

Figure 2. A. *Acrylamide gel electrophoresis of MTHFR 1298 SNP genotyping assay using MboII and AjuI endonucleases. From the left: 1.- GeneRuler Ultra Low Ladder, 1μg of, [Thermo- Fermentas, Lithuania]; 2.- PCR product for MboII analysis; 3.- C/C genotype (MboII); 4.- A/C genotype (MboII); 5.- PCR product for AjuI analysis; 6.- C/C genotype (AjuI); 7.- A/C genotype (AjuI); Appropriate product lengths are depicted below each of them.* ***B.*** *Integrated optical density (IOD) plots of the DNA bands after MTHFR 1298 SNP genotyping assay using MboII and acrylamide gel electrophoresis. From the left: 1.- IOD plot of PCR product for MboII; 2.- C/C genotype (MboII); 3.- A/C genotype genotype (MboII). The high signal-to-noise ratio that is visible on the plot, confirming the high resolution quality and enabling automatic analysis by an appropriate software. DNA fragment lengths are depicted next to each of them.* ***C.*** *Integrated optical density (IOD) plots of the DNA bands after MTHFR 1298 SNP genotyping assay using AjuI and acrylamide gel electrophoresis. From above: 1.- IOD plot of PCR product for AjuI; 2.- C/C genotype (AjuI); 3.- A/C genotype (AjuI); As in case of MboII, high signal-to-noise ratio is visible on the plot, confirming the high resolution quality and enabling automatic analysis by an appropriate software. DNA fragment lengths are depicted next to each of them.*

2 μl of 10x Buffer B and 10U of MBoII endonuclease in a total volume of 30 μl. Hydrolysis of DNA proceeded for two hours at 37°C, and the reaction was subsequently terminated by thermal inactivation for 20 minutes at 65°C.

Thereafter, in order to improve the analysis technique, the methylenetetrahydrofolate reductase gene, exon 7 fragment, containing the polymorphic site at position 1298 (A-to-C) was downloaded from the GenBank database (accession number: AF105983) and analyzed *in silico*. Numerous primer pairs flanking the polymorphic site were designed, using EPRIMER3 software (EMBOSS) [12]. The PCR products proposed by the software were selected according to length and localization with respect to the 1298 SNP site of the MTHFR gene. In the next step, one virtual PCR product surrounding the selected PCR primer set was chosen and analyzed by RESTRICT and REMAP software (EMBOSS). Unfortunately no restriction site for any endonuclease class II was changed in the case of 1298 A-to-C mutation stimulation, despite of that for MboII. However, subsequent silent mutation scanning (WatCut, University of Waterloo, Canada) showed that there is an AjuI –specific site after introduction of site-directed mutaganesis A-to-G at position 1313. Moreover, AjuI endonuclease possess a recognition sequence as follows: (7/12) GAANNNNNNNTTGG (11/6), giving considerably longer, and thus easier to resolve, hydrolysis products while other common endonucleases class II usually cleave at, or just next to their recognition site. A schematic view of the polymorphic MTHFR gene fragment with appropriate annotations and AjuI cut sites is shown on **Figure 1**. To create an AjuI- specific site at the position of A1298C SNP, a degenerate reverse primer has been designed. The forward and reverse primer sequences are as follows: MTHFR_Aju_F: 5'-GGA GTG TGC CCT GAC CTC-3', MTHFR_Aju_R: 5'-GAG AGG TAA AGA ACA AAG ACT CC-3', where the mutagenic nucleotide is underlined. Thermal conditions of PCR were as follows: 94°C for 10 minutes; then 40 cycles of 94°C for 60sec., 51°C for 60sec., 72°C for 30sec., and the final elongation step at 72°C for 10 minutes. We utilized the same reaction components and concentrations as in the reaction described above, i.e. 1U of AmpliGold Taq polymerase, 1x buffer A; 0.2mM of each dNTPs; 3.0 mM $MgCl_2$ and 5μl of genomic DNA as template. Primer concentrations were finally optimized to 0.2μM for both MTHFR_Aju_F and MTHFR__ Aju_R. Restriction digestion reaction of 10μl of PCR reaction mixture was set up according to the following manufacturer's protocol: 2μl of 10x Buffer R; 0.6μl of 50x S-adenosylomethionine (SAM), and 10U of AjuI endonuclease in total volume of 30μl for two hours at 37°C. Thereafter, the reaction was terminated by thermal inactivation for 20 minutes at 65°C.

The restriction digestion products of MboII and AjuI analysis were resolved on both 2.5% agarose and on 8% acrylamide gel in the presence of undigested control (PCR product). After electrophoresis, agarose as well as acrylamide gels were stained in ethidium bromide solution (0.5 μg/ml) for 20 minutes with rocking and washed briefly with distilled water. DNA bands were then visualized and digitalized using UV transilluminator and an appropriate software (Scientific Imaging System, East Kodak Company, USA). Integrated optical density (IOD) values for band pattern were calculated and plotted using ImageJ software [13].

Results

For both AjuI and MboII PCR-RFLP methods investigated in our study, the expected band patterns for each genotype were obtained. Acrylamide gel electrophoresis works well in both cases, i.e. in MboII as well as in AjuI analysis, giving clear, readable and easily interpretable results with high signal-to-noise ratio **(Figures 2A, 2B** and **2C)**. A reasonable resolution in 2.5% agarose gel was observed only in the case of AjuI **(Figures 3A** and **3B)**. For MboII restriction analysis, agarose gel electrophoresis was insufficient to adequately separate DNA fragments, even if high-resolution agarose was used (MetaPhor Agarose, Lonza). These results are shown on **Figures 4A** and **4B**. The intensities of DNA bands were also expressed as IOD values for better comparison (**Figures 2B** and **2C**, **Figure 3B** and **Figure 4B**).

Discussion

Methylenetetrahydrofolate reductase gene is of special importance in the cases of several malignancies, mental retardation, neural and psychiatric abnormalities, and reproductive failure. In the latter case, it is meaningful to perform a panel of analysis, involving Leiden V factor, prothrombin G20210A gene mutation, and C677T and A1298C MTHFR gene mutations, since all of these factors may play an important pathogenic role, especially in the case of the homozygous genotypes. In fact, a number of such diagnostic screening results are in agreement with our own observations. The most widely described techniques for genotyping particular SNP-type mutations are sequencing by PCR-RFLP and by allele-specific PCR.

To meet our objectives, we have chosen PCR-RFLP because of its versatility with respect to other analyses performed in the laboratory. In high-throughput laboratories, the use of agarose gel electrophoresis is preferred over acrylamide, because it is rapid and simple to perform, more efficient, and easily scalable, allowing the simultaneous analysis of many samples.

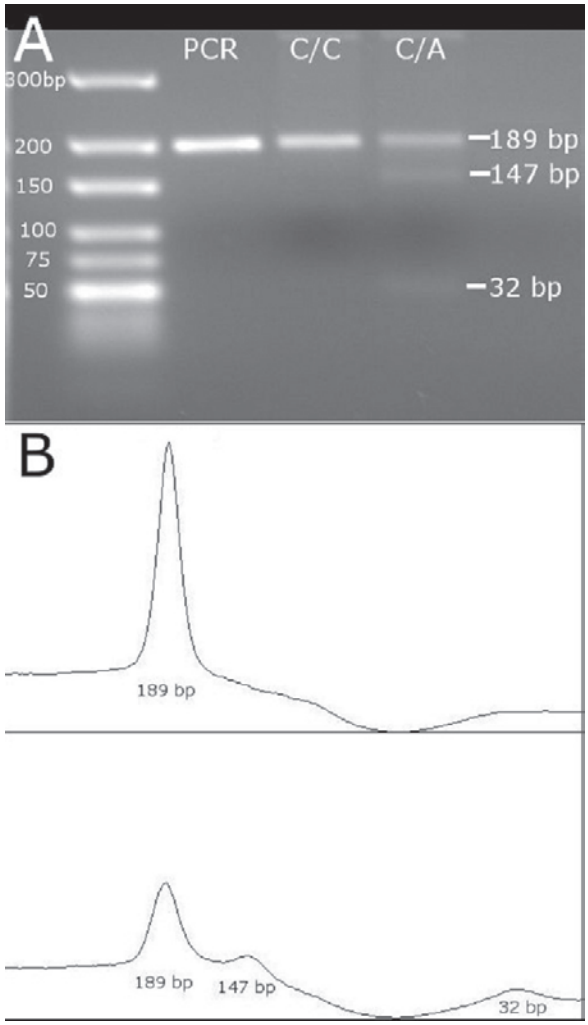

Figure 3. A. *Electrophoretogram of MTHFR 1298 SNP genotyping assay using AjuI endonuclease and agarose gel electrophoresis. From the left: 1.- GeneRuler Ultra Low Ladder, 1μg of, [Thermo- Fermentas, Lithuania]; 2.- PCR product for AjuI analysis; 3.- C/C genotype (AjuI); 4.- A/C genotype (AjuI); Appropriate product lengths are depicted on the right.* ***B.*** *Integrated optical density (IOD) plots of the DNA bands after MTHFR 1298 SNP genotyping assay using AjuI and agarose gel electrophoresis. From above: 1.- IOD plot of restriction fragments for C/C genotype (AjuI); 2.- IOD plot of restriction fragments for A/C genotype (AjuI); IOD plot of PCR product for AjuI analysis is not shown on the picture due to its very high signal strength. Please note, that the high signal-to-noise ratio is visible on the plot, confirming the high resolution quality. DNA fragment lengths are depicted beneath.*

Our goal was to develop an improved, original, and reproducible protocol for A1298C polymorphism analysis with results that will be clearly readable on agarose gel electrophoresis. The shorter the nucleic acid fragments that are resolved on agarose gel, the less staining dye they accumulate, becoming hardly

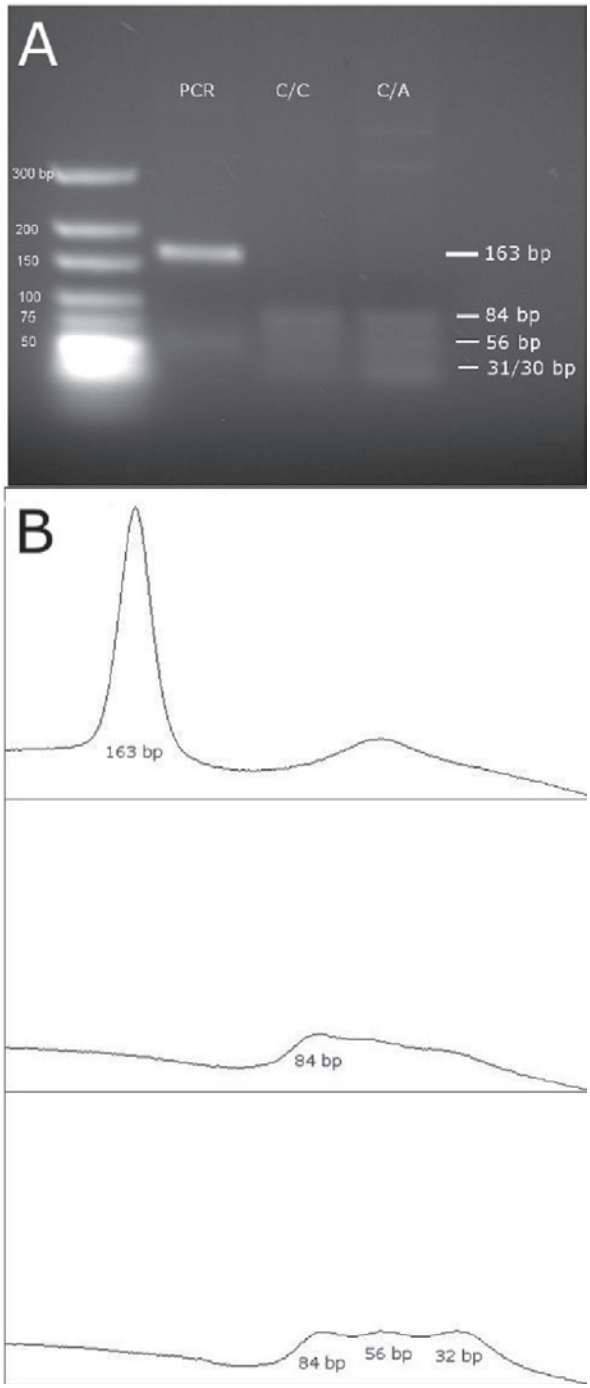

Figure 4. A. *Electrophoretogram of MTHFR 1298 SNP genotyping assay using MboII endonuclease and agarose gel electrophoresis. From the left: 1.- GeneRuler Ultra Low Ladder, 1µg of, [Thermo- Fermentas, Lithuania]; 2.- PCR product for MboII analysis; 3.- C/C genotype (MboII); 4.- A/C genotype (MboII); Appropriate product lengths are depicted on the right.* ***B.*** *Integrated optical density (IOD) plots of the DNA bands after MTHFR 1298 SNP genotyping assay using MboII and agarose gel electrophoresis. From above: 1.- IOD plot of PCR product for MboII RFLP analysis. 2.- IOD plot of restriction fragments for C/C genotype (MboII); 3.- IOD plot of restriction fragments for A/C genotype (MboII); Please note, that the weak signal-to-noise ratio is visible on the plots of restriction fragments, making the resolution quality questionable. DNA fragment lengths are depicted beneath.*

visible. Thus, according to the data given by Karcher [14] the smallest double stranded DNA size that can be used with an agarose gel is about 100 bp. If shorter DNA fragments are to be analyzed, acrylamide gel electrophoresis is recommended.

In previously described techniques, PCR products and HboII hydrolysis products were too small to be clearly visible on agarose gel, and genotypes were therefore indistinguishable. In our novel method, the use of polymerase chain reaction with standard forward and mutagenic reverse primer followed by the hydrolysis of the PCR product with an AjuI restriction enzyme allows satisfactory results even if standard 2.5% agarose gels are used.

References

1. Goyette P, Frosst P, Rosenblatt DS, Rozen R. Seven novel mutations in the methylenetetrahydrofolate reductase gene and genotype/phenotype correlations in severe methylenetetrahydrofolate reductase deficiency. Am J Hum Genet 1995;56:1052-1059.
2. Frosst P, Blom HJ, Milos R, Goyette P, Sheppard CA, Matthews RG, Boers GJH, den Heijer M, Kluijtmans LAJ, van den Heuvel LP, Rozen R. A candidate genetic risk factor for vascular disease: a common mutation in methylenetetrahydrofolate reductase. Nat Genet 1995;10:111-113.
3. van der Put NMJ, Steegers-Theunissen RPM, Frosst P, Trijbels FJM, Eskes TKAB, van den Heuvel LP, Mariman ECM, den Heyer M, Rozen R, Blom HJ. Mutated methylenetetrahydrofolate reductase as a risk factor for spina bifida. Lancet 1995;346:1070-1071.
4. Böttiger AK, Hurtig-Wennlöf A, Sjöström M, Yngve A, Nilsson TK. Association of total plasma homocysteine with methylenetetrahydrofolate reductase genotypes 677C>T, 1298A>C, and 1793G>A and the corresponding haplotypes in Swedish children and adolescents. Int J Mol Med. 2007;19:659-665.
5. Weisberg I, Tran P, Christiensen B, Sibani S, Rozen R. A second genetic polymorphism in methylenetetrahydrofolate reductase (MTHFR) associated with decreased enzyme activity. Mol Genet Metab 1998;64:169-172.
6. van der Put NM, Gabreëls F, Stevens EM, Smeitink JA, Trijbels FJ, Eskes TK, van den Heuvel LP, Blom HJ. A second common mutation in the methylenetetrahydrofolate reductase gene: an additional risk factor for neural-tube defects? Am J Hum Genet 1998;62:1044-1051.
7. Skibola CF, Smith MT, Kane E, Roman E, Rollinson S, Cartwright RA, Morgan G. Polymorphisms in the methylenetetrahydrofolate reductase gene are associated with susceptibility to acute leukemia in adults. Proc Natl Acad Sci U S A. 1999;96:12810-12805.
8. Peerbooms OL, van Os J, Drukker M, Kenis G, Hoogveld L; MTHFR in Psychiatry Group, de Hert M, Delespaul P, van Winkel R, Rutten BP. Meta-analysis of MTHFR gene variants in schizophrenia, bipolar disorder and unipolar depressive disorder: evidence for a common genetic vulnerability? Brain Behav Immun 2011;25:1530-1543.
9. De Mattia E, Toffoli G. C677T and A1298C MTHFR polymorphisms, a challenge for antifolate and fluoropyrimidine-based therapy personalisation. Eur J Cancer 2009;45:1333-1351.
10. Lajin B, Alachkar A, Sakur AA. Triplex tetra-primer ARMS-PCR method for the simultaneous detection of MTHFR c.677C>T and c.1298A>C, and MTRR c.66A>G polymorphisms of the folate-homocysteine metabolic pathway. Mol Cell Probes 2012;26:16-20.
11. Mayor-Olea A, Callejón G, Palomares AR, Jiménez AJ, Gaitán MJ, Rodríguez A, Ruiz M, Reyes-Engel A. Human genetic selection on the MTHFR 677C>T polymorphism. BMC Med Genet. 2008;9:104.
12. Rice P, Longden I, Bleasby A. EMBOSS: the European Molecular Biology Open Software Suite. Trends Genet 2000;16:276-277.
13. Schneider CA, Rasband WS, Eliceiri KW. NIH Image to ImageJ: 25 years of image analysis. Nat Methods 2012;9:671-675.
14. Karcher S.J.: Molecular biology. A project approach. Academic Press, San Diego, 1995.