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Original Article

Development of ARMS-PCR Method to Detect Two Mutations of MTHFR Gene (C677T, A1298C) in Suspected Cases of Thrombosis

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ABSTRACT

Background: Thrombosis is the formation of a blood clot in a blood vessel that prevents blood flow in the blood circulatory system. Nowadays, thrombosis is one of the causes of mortality in human societies. Thrombosis can be caused by genetic disorders or environmental factors that help the formation of clots. Two common mutations of methylene tetrahydrofolate reductase gene known as C677T and A1298C are realized as the effective factors for thrombophilia. The purpose of this study is to design a proper ARMS-PCR method for the diagnosis of these two mutations of the MTHFR gene and the evaluation of its relationship with people suspected of thrombosis.

Methods: First, 50 samples suspected of thrombosis were collected from cases that had gone to laboratories for coagulation tests, then the genomic DNA of the samples was extracted using the proper commercial kit. Proper ARMS-PCR primers for C677T and A1298C mutations were designed and then synthesized. After the optimization of the ARMS-PCR reaction on the positive control sample, the reaction was carried out for all 50 samples. A number of mutated and non-mutated samples were studied for confirmation by the PCR-Sequencing method.

Results: The results from the reaction showed that from 50 samples under study, after the optimization, 13 people had the C677T mutation (26%) from which 5 people had the homozygous genotype and 8 people had the Heterozygous genotype and for the A1298C mutation, 12 positive samples were found (24%) from which 7 people had the Heterozygous genotype and 5 people had the homozygous genotype.

Conclusions: The ARMS-PCR results of this study were totally in line with the PCR-Sequencing technique. Also, the results showed that the designed ARMS-PCR system has the proper efficiency and accuracy for the recognition of these two mutations. So, based on its lower cost and time, it is suggested for diagnostic purposes in clinical centers. Also, the results of relative frequency of these two mutations in such a group of people are high that shows the necessity for the request of these two mutations in such people.

Key words: ARMS-PCR, A1298C mutation, C677T mutation, Methylene tetrahydrofolate reductase MTHFR

Introduction

Methylene Tetrahydrofolate Reductase (MTHFR) gene is responsible for enzyme coding, which catalyzes the conversion of 5, 10-methylenetetrahydrofolate to 5-methyltetrahydrofolate. 5-methyl-tetrahydrofolate is a key enzyme involved in folate metabolism. It has several functions in the body, some of which include: availability in many complex biochemical pathways such as nucleotide synthesis, DNA methylation, protein methylation, neurotransmitters and phospholipids, and conversion of homocysteine to methionine**.** The MTHFR gene is located on chromosome 1(p36.3) spanning more than 20 kb and containing a noncoding exon as well as 11 coding exons (Pepe *et al,.* 1998). In addition, the encoded enzyme catalyzes the conversion of 5,10-methyl- enetetrahydrofolate, a carbon donor in nucleotide biosyn- thesis, to 5-methyltetrahydrofolate, the key form of circulatory folate, together with providing a methyl group for the re-methylation of Hcy back to methionine (Davalos *et al*., 2005; Kaiser *et al*., 2000; Moretti and Caruso, 2019; Prasad *et al.,* 2011; Tonetti *et al*., 2000).

This gene encodes the dimeric proteins with subunits of 77 kDa and 70 kDa**.** The unstable variant (T allele) associated with reduced enzymatic activity and increased levels of plasma homocysteine as well as folate deficiency, homozygosity, and other risk factors results in the risk of venous thrombosis. Elevated plasma beta-fibrinogen levels, which increase the risk of heart attack, fibrin deposition in the internal artery and placenta thrombosis, increase the risk of abortion (Kerlin *et al*., 2004). The deficiency in the metabolism of homocysteine increases the plasma homocysteine levels and the elevated level of homocysteine is considered as a condition predisposing to the development of thrombosis MTHFR is one of the main regulatory enzymes in the metabolism of homocysteine. Mutations in MTHFR gene lead to decreased activity of enzyme and hyperhomocysteinemia, which induces platelet aggregation through promotion of endothelial oxidative damage. Although several mutations within the MTHFR gene were described, C677T and A1298C mutations are the two most common mutations (Forges *et al.,* 2007; Mtiraoui *et al.,* 2006; Trabetti, 2008). The increase in plasma homocysteine levels, which is a toxic amino acid, has negative effects on vascular endothelium, atherogenesis and coagulation factors V and VII, and it leads to the increased levels of thrombin, platelet formation and consequently to thrombosis. Therefore, by converting this amino acid into methionine, the toxic effects of it will be neutralized in normal individuals (Barlik *et al.*, 2009; Spiroski *et al*., 2008). The term thrombosis refers to the formation, from constituents of the blood, of a mass within the venous or arterial vasculature of a living animal (Kesieme *et al*., 2011). This abnormal mass or the so-called thrombus prevents blood flow through the circulatory system. Generally, this is a complication of platelet aggregation and humoral coagulation system activation. Thrombosis is a common phenomenon is injuries, except for the pathology in samples of thrombosis. Thrombosis might be the result of genetic defects or autoimmune disorders (García-Frade Ruiz *et al*., 2016; Puri *et al*., 2017).

Nearly half of the deaths from ischemic stroke are due to thrombosis. The Global Burden of Disease (GBD) (Global Burden of Diseases, Injuries, and Risk Factor) recorded 52.8 million

deaths in 2010. The noncommunicable disease has led to the death of 34.5 million people, accounting for two of every three deaths. Ischemic heart disease (7 million deaths) and stroke (5.9 deaths) collectively accounted for one in four deaths. The 7 million deaths from ischemic heart disease have increased 35% since 1990. The 2.8 million deaths from ischemic heart disease represent a 25% increase since 1990. Stroke has been reported as the first or second most common cause of annual mortality due to premature death (YLL) (years of life lost because of premature mortality) (Lozano *et al.,* 2012; Iscfwt, 2014). Apart from the mentioned factors, thrombosis is one of the main causes of recurrent abortions in women (Balasubramaniam *et al*., 2017).

Recognized as an important coagulation factor, factor XIII polymorphisms are considered as one of the candidates for thrombophilic disease that its deficiency results in serious bleeding complications, inefficient wound healing and a higher risk of abortion in women (Dossenbach-Glaninger *et al.,* 2003).

A common polymorphism in the gene for the MTHFR enzyme is known as the C677T MTHFR polymorphism. It leads to an altered amino acid sequence, and is associated with a decreased enzyme activity. A thymine (T)-to-cytosine(C) transition at nucleotide 677 in the exon 4 of MTHFR gene, leads alanine to be substituted by valine residue at position 222 in the catalytic domain of the MTHFR enzyme. The C677T polymorphism of the MTHFR gene encodes a thermolabile variant of the enzyme with reduced activity; reduced activity can lead to elevated levels of homocysteine (also called hyperhomocy-steinemia), especially when folate levels are low. As a matter of fact, recently published studies suggest that the C677T polymorphism is likely to modify the enzyme stability rather than enzyme activity. Generally, it was revealed that individuals with homozygous (677TT) genotype have 30% of the MTHFR enzymatic activity of the wild variant, while the heterozygotes (677TT) have 65% of the enzymatic activity (Nishio *et al.,* 2008; Robien *et al*., 2003).

Hyperhomocysteinemia is an independent risk factor for cardiovascular disease (Clarke *et al*., 1991), neural tube defects (Morelli *et al*., 2005) and recurrent abortions. There is a lot of evidence suggesting that the thrombosis in pairs of narrow vessels can be the cause of many abortions of unknown cause. Moreover, this polymorphism is associated with cervical cancer, breast cancer, acute lymphoblastic leukaemia, lung cancer, prostate cancer, colorectal cancer and diabetes (Liew and Gupta, 2015). Other type of MTHFR mutation is the A1129C polymorphisms occurring in exon 7 of the MTHFR gene which causes the substitution of glutamine for alanine at amino acid 429 in the protein sequences of the MTHFR enzyme (Ananth *et al*., 2007).

In the ARMS technique, a PCR comprises one allele-specific oligonucleotide primer at 5′-end and a common primer at 3′-end. The outer primers amplify a large fragment of the target gene contains variant nucleotide as a control fragment and smaller allele-specific amplicons with different sizes. The product can easily be discriminated on gel electrophoresis either as homozygous or heterozygous. A deliberate mismatch at position -2 or -3 from the 3' terminal end of the inner primers can improve allele specificity.

A PCR comprises one allele-specific oligonucleotide primer at 5′-end and a common primer at 3′-end. As you know, the most important nucleotide in primers is the '3' end, which if not paired with the pattern string, the polymerase enzyme will not be able to replicate and we will not see any PCR products. We use this principle in primer design for the ARMS technique. Under the right conditions, oligonucleotides that are not complementary to the target sequence at the end of '3' will not be able to bind to the target sequence, and as a result we

will not have a multiplied product. Sometimes, even if the '3' end is not complete, the primer will still connect and the desired sequence will be multiplied. To solve this problem, we add another mismatch in a position close to the end of '3, which increases the specificity of the reaction. This extra mismatch is often in the penultimate nucleotide. If the mismatch for the SNP is strong, the extra mismatch should be weak before the end, and vice versa.

If the presence of an amplified mutant is detected by agarose gel electrophoresis, it indicates that the target sequence contains the mutant allele. Similarly, if the result suggests an absence of the amplified mutant, it shows the presence of the normal DNA sequence at that specific point. Similarly, a normal primer at 5′-end together with a common primer at 3′-end was employed in another PCR. If there are both normal and mutant amplified products, then it shows a heterozygous gene variant by the existence of both normal and mutant DNA sequences within an individual (Hassan *et al*., 2013; Hanafi *et al*., 2014; Yang *et al.,* 2018; Ye *et al.,* 2001). The Amplification Refractory Mutation System PCR (ARM-PCR) is an amplification strategy for a specific SNP that can distinguish single nucleotide polymorphisms based on amplification. It is an extremely useful method for identification of point mutations. In this method, mutated, and normal primers are used in two separate tubes.

The purpose of this study is to design ARMS-PCR method for two commonly polymorphisms (C677T and A1298C) of the MTHFR gene which will be later employed in suspected cases of thrombosis.

Materials and methods

The samples in this experimental study were selected from patients suspected with thrombosis referred to a laboratory in North of Iran for biochemical examination of factors 2.5, S, PTT and C proteins. The samples were collected during 8 months. The medical ethics were observed and the samples were collected with patients' consent.

Sample preparation

At first, the samples were melted and vortexes extract the DNA. Then, DNA was extracted, according to the manufacturer's instruction using the commercial kit (Viogene) for GG2001. In general, DNA can be kept for repeated use at a temperature of 4 °C, but DNA was kept at 20 °C to prevent enzymatic damage.

The ARMS-PCR primer designing and synthesis

The basis of the ARMS-PCR method is on the difference in the 3' terminal nucleotide of allele-specific primer. The ARMS primers reaction is designed based on genomic DNA sequence**.** It was examined using Oligo Analyzer Software and have an optimal length of about 30 nucleotides. The minimum difference of TM between the two primers is also a feature of ARMS primer. In order to increase the power of differentiation between the alleles in reaction to design the primer, incompatible bases were inserted mainly in the combination of 3 end primers. Neglecting the details would decrease the efficiency of the reaction. Inserting the highly incompatible base at the 3 end would make it more unstable. Common and control primers were used to determine the absence of complementary regions at the 3 end of specific-primers of the alleles.

ARMS-PCR reaction optimization

ARMS-PCR thermal cycling for M1HFR A1298C and M1HFR C677T mutations was studied under different conditions to identify the optimal response for each of these mutations.

Results

The population of the study

The total number of 50 cases suspected with thrombosis were studied and the results are shown in Table 1.

Table 1. A total number of 50 cases suspected with thrombosis

A1298C mutation	C677T mutation	Sample number
Normal	Normal	1
Normal	Normal	2
Normal	Normal	3
Normal	Normal	4
Normal	Normal	5
Normal	Normal	6
Normal	Normal	7
Normal	Normal	8
Heterozygote	Normal	9
Normal	Normal	10
Normal	Normal	11
Normal	Normal	12
Heterozygote	Normal	13
Normal	Homozygote	14
Normal	Normal	15
Heterozygote	Normal	16
Heterozygote	Normal	17
Heterozygote	Normal	18
Normal	Normal	19
Normal	Normal	20
Heterozygote	Heterozygote	21
Normal	Normal	22
Normal	Normal	23
Homozygote	Normal	24
Normal	Heterozygote	25
Normal	Homozygote	26
Homozygote	Normal	27
Normal	Normal	28
Normal	Heterozygote	29
Normal	Homozygote	30
Normal	Heterozygote	31
Normal	Normal	32
Normal	Heterozygote	33
Heterozygote	Heterozygote	34
Normal	Homozygote	35
Normal	Normal	36
Normal	Normal	37
Homozygote	Normal	38
Homozygote	Normal	39
Homozygote	Normal	40
Normal	Normal	41

Normal	Normal	42
Normal	Normal	43
Normal	Heterozygote	44
Normal	Homozygote	45
Normal	Normal	46
Normal	Normal	47
Normal	Normal	48
Normal	Normal	49
Normal	Heterozygote	50

ARMS-PCR optimization results for C677T mutation

Method ARMS-PCR was used to identify the MTHFR C677T mutation in patients suspected with thrombosis with specific primers (normal and mutant). An appropriate annealing temperature of 58 °C for 1 min and the epimdorph machine were used for the target area. Then, using the electrophoresis, transolominator and 100bp DNA ladder, the PCR products were loaded on a 2.5% agarose gel. The expected bonds were observed and two fragments of 242bp and 379bp were observed for mutant allele and a normal allele respectively (Figure 1 and Table 2).

Figure 1. ARMS-PCR reaction optimization on the first sample of C677T mutation at annealing temperature of 58 ° for 1 minute on a 2.5% gel; the desired result was not obtained

Table 2. ARMS-PCR optimization for C677T mutation

Cycle	Time	Temperature	Step PCR
1	10'	95 °C	Denaturation
32	40"	95 °C	Secondary Denaturation
32	1'	58 °C	Annealing
32	1'	72 °C	Extension
1	5'	72 °C	Secondary Extension

ARMS-PCR reaction optimization results for A1298C mutation

The ARMS-PCR technique was used to detect MTHFR A1298C mutation in cases suspected with thrombosis this with specific primers (normal and mutant) at target areas. The optimization process was done at different temperatures by changing variables such as annealing temperature, annealing time, number of cycle, a number of template, number of specific primers (normal and mutant), primary extension time. Finally, at the annealing temperature of 58 °C, the annealing time of 30 sec., using 40 cycles and using Bio Rad machine, the desired result was obtained. Then, the PCR products were loaded on 2.5% agarose gels and using electrophoresis,

translominator and 100 Ladder, sharp bonds were observed for 186bp for mutant allele and 311bp for normal allele (Figure 2 and Table 3).

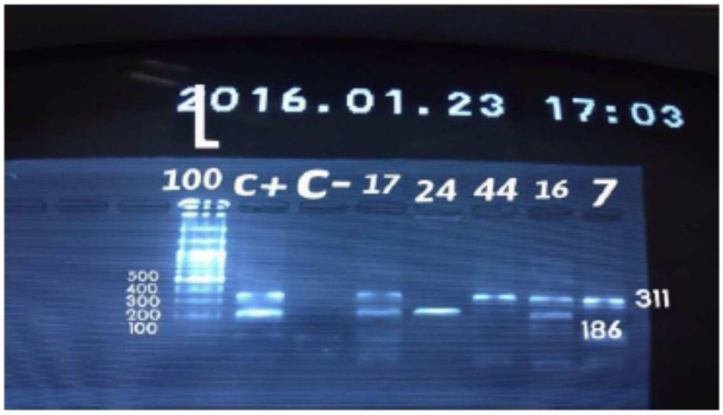

Figure 2. ARMS-PCR reaction optimization on the specimens for the A1298C mutation, which we changed the annealing temperature from 57 °C to 58 °C, and 186 mutant and 311 normal bonds were obtained on 2.5% agarose gel

Table 3. ARMS-PCR Optimization for A1298C mutation

Cycle	Time	Temperature	Step PCR
1	10'	95 °C	Denaturation
40	35"	95 °C	Secondary Denaturation
40	30"	58 °C	Annealing
40	35"	72 °C	Extension
1	5'	72 °C	Secondary Extension

The analysis of sequencing technique results for MTHFR C677T mutation

To determine the accuracy of the reactions after the ARMS-PCR reaction, we evaluated a number of samples using the sequencing technique. Based on the analysis of the following diagram and its sequence, it was found that the ARMS-PCR technique correctly detected C677T mutation. The normal, heterozygous, and homozygous genotypes are shown in the images using the Chromas software. As seen in the image, in single nucleotide polymorphism (SNP) C was replaced with a T and created a point mutation (Figures 3-5).

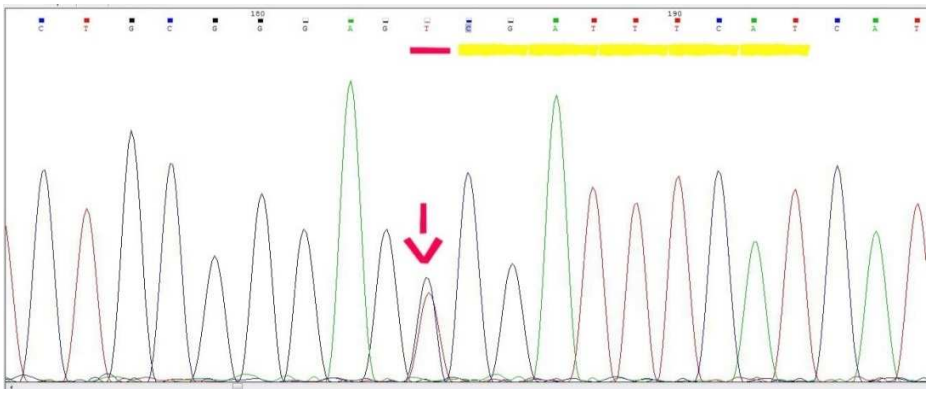

Figure 3. G1 heterozygote: The above graph is related to the C to T point mutation

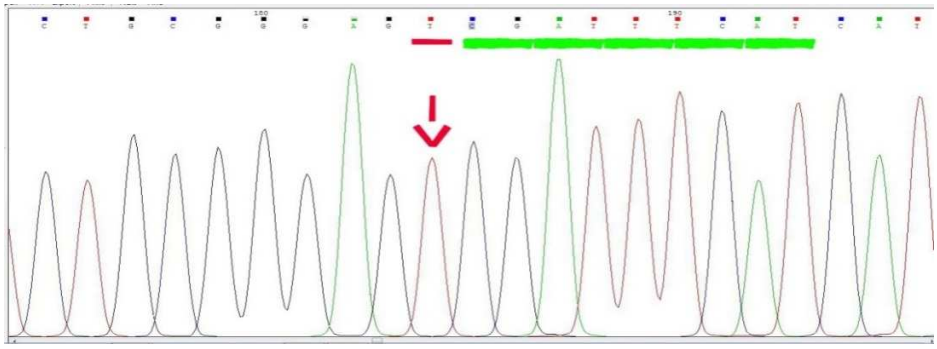

Figure 4. G2 homozygote: The above graph is related to the C to T point mutation

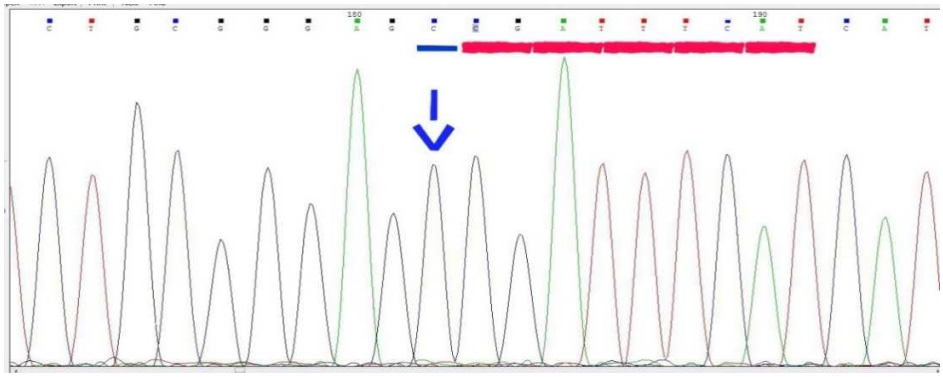

Figure 5. G3 Normal: The above graph is related to a normal person

The analysis of sequencing technique for MTHFR A1298C mutation

To determine the accuracy of the reactions after the ARMS-PCR reaction, we evaluated a number of samples using the sequencing technique. Based on the analysis of the following diagram and its sequence, it was found that the ARMS-PCR technique correctly detected the A1298C mutation. The normal, heterozygous, and homozygous genotypes are shown in the images using the Chromas software. As seen in the image, in single nucleotide polymorphism (SNP) A was replaced with C and created a point mutation (Figures 6-8).

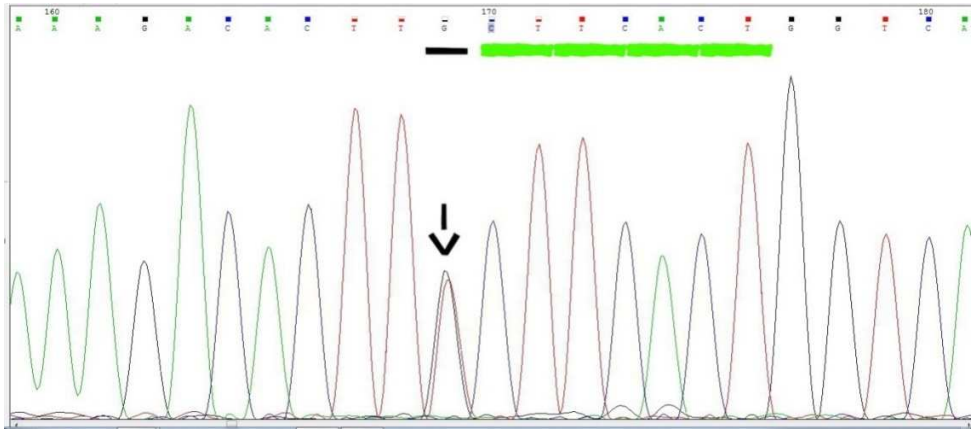

Figure 6. G4 heterozygote: The above graph is related to the A to C point mutation

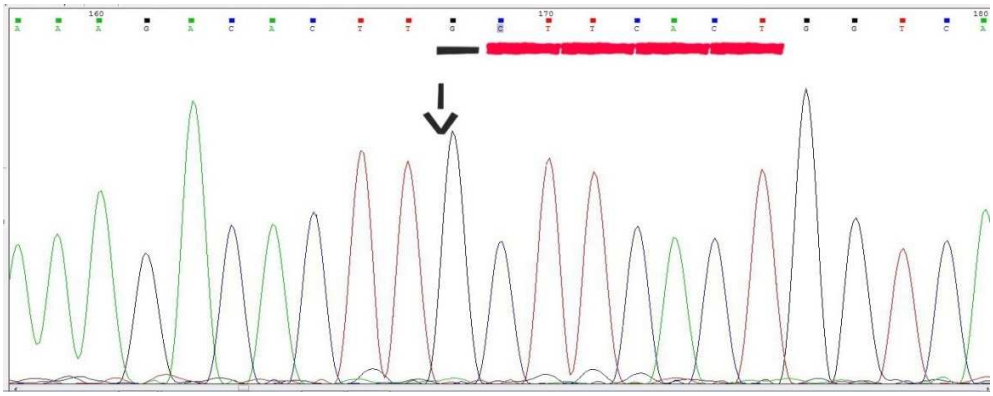

Figure 7. G5 Homozygote: The above graph is related to the A to C point mutation

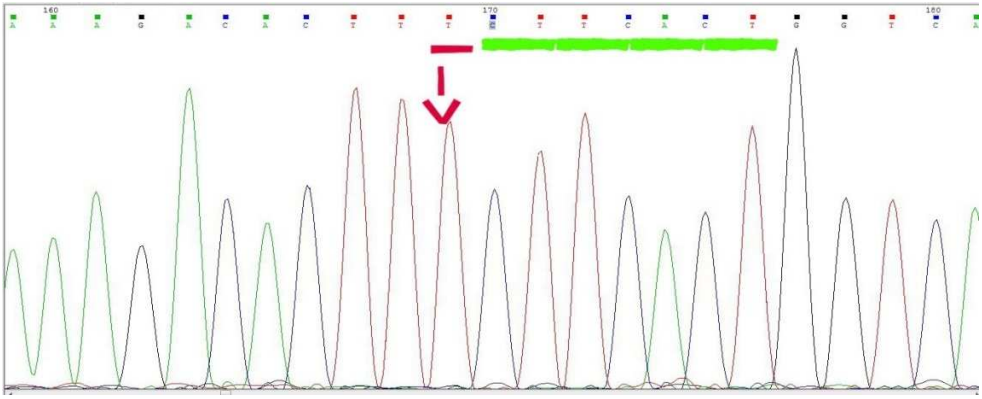

Figure 8. G6 Normal: The above graph is about the normal person's sample

Discussion

Several studies have investigated the effect of MTHFR enzyme polymorphisms on enzyme activity and its association as a risk factor in thrombophilic disease, cardiovascular disease, pair anomalies, recurrent abortions, preeclampsia using PCR based diagnostic methods; however, the studies indicated inconsistent results. Therefore, the study of these mutations would be interesting and important. In this study, we used the ARMS-PCR method which is a simple, fast and robust multiplication technique for detecting point mutation. The design of new and normal primers of mutants was performed with high precision and the PCR-Sequencing technique was used to determine the accuracy of sample detection for C677T and A1298C mutations. In the ARMS-PCR method, both normal and mutated alleles are detected simultaneously. In this method, the selection and design of primers play an important role in obtaining a desirable result.

In this study, using ARMS-PCR for C677T mutation, the number of the PCR product for the mutated fragment was 240bp and for the normal fragment was 380bp. Similarly, in the A1298C mutation, the number of PCR product for the mutated fragment was 186bp and for the normal fragment was 311bp. Using the ARMS-PCR technique and designing new primers, the mutations were identified.

The results of PCR-Sequencing for both C677T and A1298C mutations indicated the accuracy of the primers designed for the desired mutations and also the positive results of the samples. As a result, the ARMS-PCR method is an accurate, cost-efficient and fast method to identify SNPs.

We also found that choosing the appropriate melting and annealing temperature, appropriate time, the required DNA pattern, the correct method of extraction and its quality, the amount of primer required for the reaction, limiting the number of cycles and their time, and also the efficiency of the thermocycler device have significant effects on achieving the desired result.

Hcy is a well-known thrombosis factor in vascular disease. Homocysteine has been recognized as early in 1990s as a risk factor for the presence of atherosclerotic vascular disease and hypercoagulability states. Vascular diseases include the coronary artery disease (Abraham *et al*., 2006; Mallinow *et al.,*1990), heart disease (Cui *et al*., 2008), atherosclerosis (Toyoda *et al*., 2004), myocardial infarction (Eftychiou *et al*., 2012), venous thrombosis (Soltanpour *et al*., 2013), chronic kidney disease (Jamison *et al*., 2007; Holmes *et al*., 2011) and ischemic stroke (Casas *et al*., 2005; Cotlarciuc *et al*., 2014). There is growing evidence that Hcy affects the coagulation and resistance of endothelial in thrombosis (Keat Wei *et al.,* 2015). Moreover, Hcy may encounter coronary artery disease and antithrombotic function of nitric oxide (Wei *et al*., 2016). In particular, it has been reported that vascular complications in patients with homocysteinoria are more relevant to thrombosis than atherosclerosis (Jeon *et al.,* 2015; Wu *et al.,* 2013). There is a relationship between Hcy level and the incidence of thrombotic events in patients with systemic lupus erythematosus (Kim *et al*., 2017).

In a study conducted by (Poursadegh Zonouzi *et al.,* 2012), the C677T and A1298C mutations of MTHFR gene in women with recurrent abortions in the northwest of Iran was studied ARMS-PCR and PCR-RFLP methods were employed. Accordingly, 139 women, of whom 89 had two or more recurrent abortions, were compared with 50 healthy women. As a result, there was no significant difference in the prevalence of 677T/T genotype among women with recurrent abortion and control group. Moreover, there was no significant difference in the frequency of A1298C mutation of MTHFR gene between the two groups. The results of this study indicated that the findings from the ARMS -PCR method are completely consistent with the results of the PCR-RFLP method and, in general, there was no significant difference in the frequency of MTHFR C677T/A1298C genotype among the two groups. The frequency of MTHFR 677T allele and the MTAFR 1298C gene in the patients were 23.4% and 34%, respectively, and in the control group they were 24% and 40%, respectively. They also found that there was no significant difference between the experimental and control groups regarding the heterozygotes of 677T mutant, but the MTHFR 677T allelic homozygotes in women with recurrent abortions were greater than the control group (Poursadegh Zonouzi *et al.,* 2012).

In another study by (Khalegh Parast *et al*., 2011), it was reported that the relationship between MTHFR A1298C and MTHFR C677T polymorphisms and recurrent abortion syndrome were examined using PCR-RFLP method. From among the patients referring to Baqiyatallah Hospital and Ibn- Sina infertility center, 30 women with a history of recurrent abortion with an unspecified cause (experimental group) and 10 women without a history of abortion and having at least two successful pregnancies (control group) were studied. The findings of the study revealed a correlation between C677T and A1298C polymorphisms and 17 cases in the experimental group (56.6%) and 5 in the control group (50%) were heterozygote for C677T polymorphism and the frequency of mutants T allele in women experiencing abortion was higher than that of control group (28.4% in women experiencing abortion and 25% in control group). The frequency of A1298C polymorphism among women with recurrent abortion and women in the control group was 63.3% and 50%, respectively. They concluded that despite the greater prevalence of the C677T and A1298C polymorphysis in women with recurrent abortions than that of the control group, this difference was not statistically significant and the results

indicated that neither of the two MTHFR polymorphisms could cause recurrent abortion in women (Khaleghparast *et al*., 2011).

(Ghaffari *et al.,* 2015) conducted a study on the association between MTHFR C677T and MTHFR A1298C mutations with plasma homocysteine and venous thrombosis in pregnant women at risk of thrombosis. Accordingly, 120 pregnant women at a risk of deep venous thrombosis and 100 pregnant women without risk of deep venous thrombosis were studied using the PCR-RFLP method. Their plasma homocysteine level was measured by ELISA method. The result showed that there was no correlation between pregnant women with deep venous thrombosis and pregnant women without deep venous thrombosis with respect to the homozygous genotype of the C677T and A1298C mutations. However, for two mutations in the MTHFR gene the plasma homocysteine levels significantly increased in pregnant women with deep venous thrombosis compared to pregnant women without deep venous thrombosis (18.3 mmol/L and 8.0 mmol/L, respectively). Their results showed that there is no correlation between MTHFR C677T and MTHFR A1298C polymorphisms with plasma homocysteine levels in pregnant women with and without deep venous thrombosis. However, they concluded that plasma homocysteine levels increased significantly in pregnant women with deep venous thrombosis and the reasons for this lack of correlation are variable and multi-factorial, such as sample size, number of samples, racial diversity, diet, etc (Ghaffari *et al.,* 2015).

(Hotoleanu *et al.*, 2013) carried out a study on the importance of homozygous polymorphisms of methylenetetrahydrofolate reductase gene. They also studied the frequency of the C677T and A1298C mutations in Romanian patients with the damage of venous thromboembolism and the association between these two mutations with VTE risk. In this study, the experimental group included 90 patients diagnosed with VTE, and the control group included 75 sex- and age-matched healthy people. Using the PCR-RFLP method, these two mutations were identified and studied. The results showed that the homozygous *MTHFR* 677TT genotype was present in 18.8% of patients with VTE versus 6.6% of controls and it was significantly associated with VTE. The heterozygous *MTHFR* A1298C genotype with the highest prevalence in the VTE group (34.4%) as well as in controls (37.3%) was not associated with VTE. No associations were found for the heterozygous *MTHFR* C677T with a frequency of 32.2% in VTE and 37.3% in controls and homozygous *MTHFR* A1298C genotype with a frequency of 1.1% in VTE and 2.6% in controls. The results showed that among *MTHFR* polymorphisms, only homozygosity for *MTHFR* 677TT may be considered a risk factor for VTE and the *MTHFR* A1298C polymorphism is not significantly associated with an increased risk of VTE (Hotoleani *et al.,* 2013). While homozygosity in MTHFR677 T>C polymorphism is associated with elevated homocysteine level, it is not significantly associated with increased thrombophilic events (Nadir *et al*., 2007; Scifres and Macones 2008). The MTHFR polymorphism does not show a risk of thromboembolism in heterozygous conditions. Folic acid and vitamin B12 do not significantly reduce the risk of thrombosis (Scifres and Macones, 2008).

Conclusions

In this study, the ARMS-PCR method was used to detect two common mutations of the C677T and A1298C of methylene tetrahydrofolate reductase gene in 50 patients referring to Dr. Ashtiani's laboratory for coagulation examination in Rasht. This method has not been employed for such an examination in the north of Iran. From among these 50 cases, 13 positive cases for mutation C677T and 12 positive cases for A1298C mutations were

detected, respectively. It was concluded that the frequency of C677T mutation is greater than that of A1298C mutation. Also, the number of heterozygotes for each mutation was higher than that of homozygous. Similarly, the relative frequency of these mutations in this group of people emphasizes the need for the identification of these two mutations.

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