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Relationship between -181 (A/G) region single nucleotide polymorphisms of matrilysin gene promoter and the onset and prevalence of colorectal cancer using tetra-primer ARMS PCR and RFLP-PCR techniques

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ABSTRACT

Aim: MMP enzymes are a family of membrane proteins that are capable of digesting extracellular matrix compounds (ECM) and basement membrane. Matrilysin enzyme is the smallest member of MMP family that is encoded by MMP-7 gene (matrilysin). According to the reports, G allele of -181 A/G single nucleotide polymorphism of MMP-7 gene causes an increase in the expression of this enzyme. This study aims to investigate the effect of promoter single nucleotide polymorphism of this gene on the colorectal cancer and compare the accuracy of tetra-primer ARMS PCR technique using RFLP-PCR technique.

Methods: In this study, genomic DNA was extracted from total blood of 61 patients with colorectal cancer and 77 healthy subjects as controls. They were genotyped by tetra-primer ARMS PCR technique. A number of randomly selected samples were later genotyped by RFLP-PCR technique.

Results: : As expected, the results of RFLP-PCR technique confirmed the results of tetra-primer ARMS technique. Results of this study showed no statistical association between this polymorphism and risk of initiation and metastasis of the colorectal cancer in the population of Isfahan.

Conclusion: ATetra-primer ARMS technique can be used as an efficient clinical technique.

Key words: MMP7 gene, colorectal cancer, RFLP-PCR technique, Tetra-primer ARMS PCR technique

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Introduction

Colorectal cancer is the generation of cancer in the regions of colon, rectum (rectum), and appendices epiploicae[1]. Colorectal cancer is the third most common cancer among men (10% of all cancer cases) and second most common cancer among women (9.4% of all cancer cases) worldwide [2]. Also, colorectal cancer is considered the third most common cancer among men and the fourth most common cancer among women in Iran[3]. MMP enzymes are a family of membrane proteins that are capable of digesting extracellular matrix compounds (ECM) and basement membrane. Matrilysin enzyme is the smallest member of MMP family that is encoded by MMP-7 gene. MMP family members are structurally different in the presence or absence of different domains[4] . Expression of most of the MMP enzymes is usually low in tissues and is only inducted when ECM restructuring is required, but there is evidence which shows different mRNA stability in response to growth factors and cytokines[5]. Another regulation level of MMP enzymes is the activation of pro-enzyme form of the secretion of MMP enzymes[6] . The third level of regulation and control of the activity of these enzymes is the inhibition of their activity[7]. MMP enzymes play important roles in embryonic development and tissue morphogenesis[8] as well as release and process of biologically active molecules such as growth factors[9]. Also, these enzymes play a major role in the processes such as wound healing, bone elongation, retroversion of uterus, tissue reconstruction, and so on that need the degradation of ECM and release of growth factors. Studies have shown that unusual and excessive activity of MMP enzymes are due to a defect in any of the control levels that leads to pathological conditions such as multiple sclerosis, Alzheimer's disease, malignant glioma, arthritis, glomerulonephritis, atherosclerotic diseases, and in some cases, invasion and metastasis of cancer cells[4]. Over activation of MMP enzymes and increasing ECM degradation provide the necessary conditions for tumor growth and metastasis[10]. Overexpression of matrilysin has been observed in a variety of mesenchyme and epithelial cancers, which indicates its role in cancer. For example, its overexpression has been associated with the invasive cancers of digestive organs such as esophagus, stomach, intestine, liver, and pancreas as well as in the cancer of other organs such as the lungs, skin, breast, prostate, and head and neck. By affecting its substrates such as elastin, E cadherin, and other MMPs, matrilysin would facilitate the invasion of tumor cells[11-13]. Matrilysin inhibits apoptosis by affecting HB-EGF precursor. Also, by influencing FasL, matrilysin leads to the escape of the tumor cells from the immune system and cell resistance against apoptosis[12,14,15]. The effect of this protein on the protein inhibiting IGF (IGFBPs) and ADAM28 protein also inhibits apoptosis and stimulates tumor growth[12, 14]. MMP-7 gene is located on the long arm of the 11th chromosome. G allele of the -181 A/G single nucleotide polymorphism of matrilysin gene promoter causes its overexpression. In 2001, Jormsjo who was examining the reason of matrilysin overexpression in certain diseases found a 2 to 3 fold increase in the basic activity of -181G carrying promoters. It has been suggested that the establishment of binding sites (NGAAN) for heat shock transcription factor (HSTF) in the presence of allele G is the cause of an increase in the expression of this allele[16]. According to the physiological role of matrilysin in the body, it is thought that the overexpression of this enzyme causes cancer and metastasis. Matrilysin overexpression has been reported in colorectal tumors, colon and stomach cancers[17, 18], metastatic colorectal cancer, and colorectal cancer[11, 19]. Moreover, a report showed that matrilysin plays an important role in the development of rectal cancer[20] Also, there were reports on the association between G allele

and risk of cancers of the esophagus, lung, cervical, ovarian, and stomach[21-23]. The association between homozygote of G allele and metastasis and involvement of lymph nodes was observed in colorectal cancer[24]. Despite the conducted studies, Koskensalo found no relationship between the overexpression and clinical signs such as tumor stage[25]. According to the authors› best knowledge, the present study was conducted for the first time in order to examine the association between allele type as a polymorphism and the risk of colorectal cancer and metastatic activity. For this purpose, tetra primer ARMS-PCR test was designed for -181 A/G polymorphism of matrilysin promoter. Furthermore, a number of samples were genotyped using RFLP-PCR and the results were compared. Evaluation of the effectiveness of tetra primer ARMS-PCR technique was among other objectives of this study.

Methods

Sample collection:

In a case-control study related to the past during 2012 to 2013, peripheral blood samples were taken from 61 patients with colorectal cancer and collected in the tubes containing anticoagulant (EDTA). Clinical and pathological data included age, sex, cancer type, grade and stage of tumor, lymph node involvement, and family background of cancer. Peripheral blood samples were collected from a control group consisting of 77 healthy individuals who had almost matching ages with the patient group.

Genomic DNA extraction and polymorphic region replication:

Genomic DNA was extracted from the leukocytes of the blood samples using centrifugation and salting out method[26]. -181 A/G single nucleotide polymorphism was first examined by tetra-primer ARMS PCR technique and, then, the validity of the results was analyzed using RFLP-PCR technique.

Designing tetra-primer ARMS PCR test:

Primer design is the first step in PCR. To design primers, first, the sequence of target fragment from the data bank should be obtained. Since the target sequence is a fragment of matrilysin gene promoter, its sequence was obtained from EPD data bank (eukaryotic promoter database). Depending on the design of PCR technique, the number of primers can be 2, 3, or 4. To genotype polymorphisms in the 4-primer method, the first and second two primers were placed on polymorphism and on both sides of the polymorphism place, respectively, in such a way that the internal primers along with external ones had products with different lengths depending on the polymorphism type. Moreover, the two external primers produced a bigger product as a result of their cooperation which was control-positive and showed the performance validity of PCR. Also, to improve the features of a pair of specific primers (internal) for the sequence of polymorphism alleles, in addition to lack of incompatibility of 3'-end with the change of the third open head of 3' internal primers, attempts were made to prevent the possible connection of the primer with the opposite allele (non-specific). After designing, the primers were analyzed using Oligo software (version 7) and NCBI site to ensure appropriate features and, most importantly, absence of secondary structures specific to place. Sequences of these primers are shown in Table 1. To examine the optimal conditions of PCR, reactions were conducted with different values of connection temperature of primers and different concentrations of substances. The mixture of PCR replication reaction in the volume of 25 µl was prepared which contained the following materials: 100-200 ng genomic min initial denaturation at 95°C followed by 35 cycles: 30 sec denaturation at 95°C, 55 sec hybridization at 58.6°C, 60 sec replication at 72°C, and finally 10 min final replication at 72°C. The replicated samples were analyzed by 1% agarose gel.

Designing RFLP-PCR test:

Primers were designed and analyzed using Oligo software, version 7, and studied by NCBI to ensure they were not connected to other places in the genome. Since there is need for the enzymatic digestion of PCR products in the continuation of PCR, in designing primers, site identification of the limiting enzyme and length of the resultant fragment of the enzymatic digestion were considered. The length difference of the resultant fragment on agarose gels had to be to the extent that could be separated from each other. Due to the lack of restriction sites at the polymorphism, with the change of one nucleotide in the sequence of reverse primer, enzyme EcoRI restriction sites were created in a way that only if the polymorphism were G, restriction occurred in the PCR product. The sequence of primers listed in Table 2 was shown for determining -181 A/G genotyping polymorphism by RFLP-PCR and EcoRI enzyme cut. The mixture of PCR replication reaction was prepared similar to the 4 primer reaction with the difference that 1 µl of each of the primers was used. PCR reaction was performed at the following temperature: 4 min initial denaturation at 94°C followed by 35 cycles: 30 sec denaturation at 94°C, 30 sec hybridization at 62°C, 30 sec replication at 72°C, and finally 7 min replication at 72°C. To perform the enzymatic digestion of the microtubes containing 6 µl of PCR, 1 µl buffer x10 EcoRI, 2.5 unit of EcoRI enzyme, and 2 µl of twice distilled water were incubated at 37°C for about 16 h. Then, the digested samples were analyzed using 3% agarose gel and 10% polyacrylamide gel containing 10% acrylamide-bis-acrylamide solution, 0.01% APS, 32 µl TEMED, 7.5 ml mixture with the ratio of 1.3 TBE 10X and 2.3 Glycerol. At the final stage, the genotypes obtained from four primer method were compared with the results of RFLP-PCR.

Statistical analysis:

The relationship between -181 A/G single nucleotide polymorphism of MMP-7 (matrilysin) gene and frequency of the disease was performed by calculating Chi-square, OR, and confidence interval of 95% using SPSS software (version 16). Differences were considered significant at $p < 05$.

Result

Results of investigations by ARMS-PCR technique:

Primers were designed in such a way that the three bands were expected in the analysis of samples: the large fragment with the length of 494 base pairs was considered the control fragment (a PCR product of external primers); the medium size fragment with the

Table 1. Primer sequences of tetra primer ARMS-PCR technique (place of modified bases are specified in the internal primers)

Sequence	Primers
5 CAGAAAAAAAAATCCTTTGAAAGCCG 3´	Forward inner(G allele)
5´ TATTGGCAGGAAGCACACAATGTAGTT 3´	Reverse inner(A allele)
5´ ATTTCTTGACTTTGGTGACGGTTACAGT 3´	Forward outer
5´ TTGATTTGGTGTTTTCTGCTAGTGACTG 3´	Reverse outer

Table 2. Sequence of primers in RFLP-PCR technique (mismatch location is specified in the reverse primer)	
Sequence	Primers
Forward primer	5-ATGTCCTGAATGATACCTATGAGAGCAGTC-3
Reverse mismatch primer	5-CGTTATTGGCAGGAAGCACACAATGaATT-3

length of 219 were considered A and G alleles, respectively. Thus, in the homozygote of the G allele, replication of the control fragment of 494 bp and small fragment of 219 bp was done. In homozygote of the A allele, replication of the control fragment of 494 bp and medium fragment of 328 bp was performed. If heterozygous polymorphism is going to be done, replication of every three listed bands could be carried out. Images obtained from gel electrophoresis confirmed the fragment length (Figure 1). Figure 1.A shows the results of separation of PCR products from a heterozygous sample (AG) on 1% agarose gel. The patient samples were genotyped based on the presence or absence of bands associated with allele A (219 bp) and allele G (238 bp). The 494 bp band was located in the last column of PCR product of exterior primers and the control fragment. Figure 1-B demonstrates the results of PCR products of the samples separated by 1% agarose gel electrophoresis after optimization and setting PCR conditions. In this figure, the first and second columns of each sample show the PCR product of G and A alleles, respectively. Genotypes of people 1, 2, 3, 4, and 5 were AG, AA, GG, AG, and GG, respectively. In this step, all three genotypes were observed in the patient and control groups. Patients showed 31.2% of GG genotype, 50.8% of AG genotype, and 18% of AA genotype. The control people had 40.3% of GG genotype, 51.9% of AG genotype, and 7.8% of AA genotype. The statistical analyses showed no relationship between the studied polymorphism and colorectal cancer (P = 0.66, Table 3).

Table 3. Frequency of allele A/G in two groups of patients and controls					
OR*	P-value	Control groups	Patient group	Allele	-181 A/G
0.66	0.16	33.75%	43.5%	A	Allele
		66.25%	56.5%	G	

* In the calculation of A allele, OR is considered a reference.

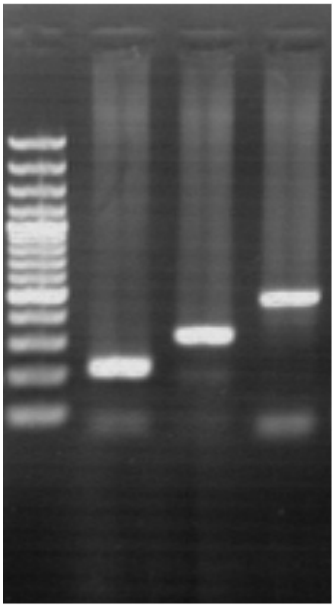

Fig. 1-A

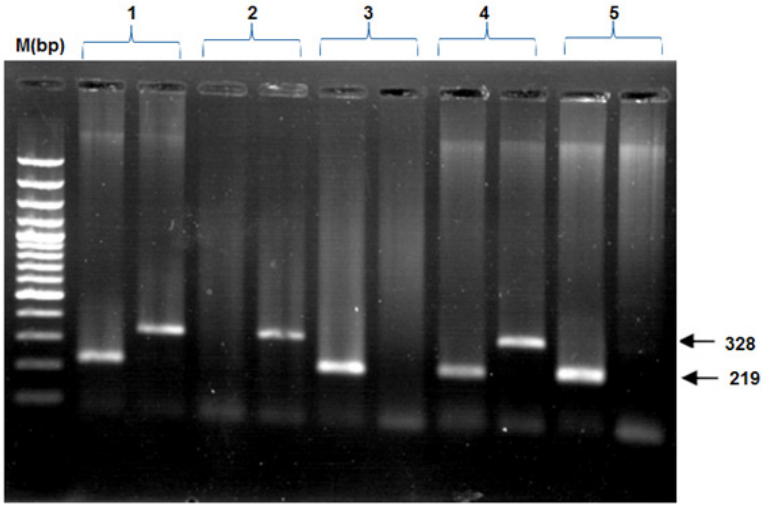

Fig. 1-B

Fig. 1. Representation of tetra primer ARMS-PCR products for -181 A/G genes of MMP7. Image 1-A shows an individual heterozygous (AG). The first column from the left shows the 100 bp marker, second column is 219 bp band of allele G, third column is 328 bp band of allele A, and the last column is 494 bp of control fragment (an external primer product). In Image 2-B, alleles G and A are genotyped by PCR products in the odd columns (first column for each sample) and even columns (second column for each sample). Genotypes of the first, second, third, fourth, and fifth subjects were as respectively follows: AG, AA, GG, AG, and GG. M is a marker of 100 bp. The samples were randomly selected from among the patient and control samples

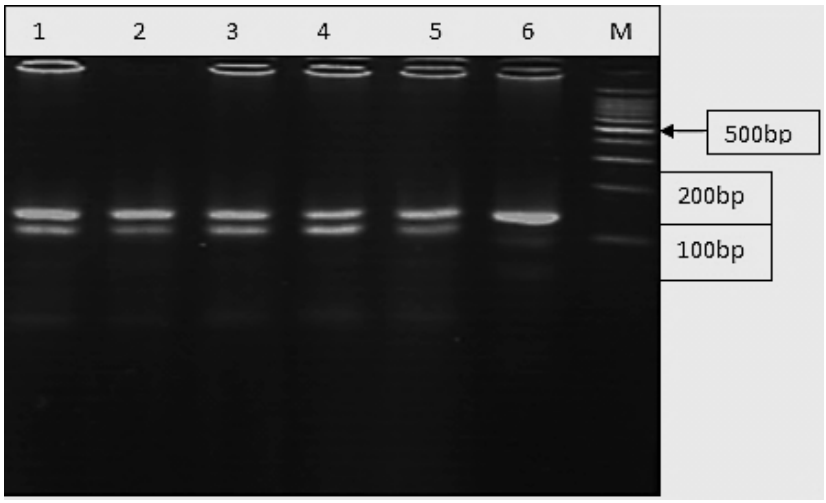

Fig. 2-A

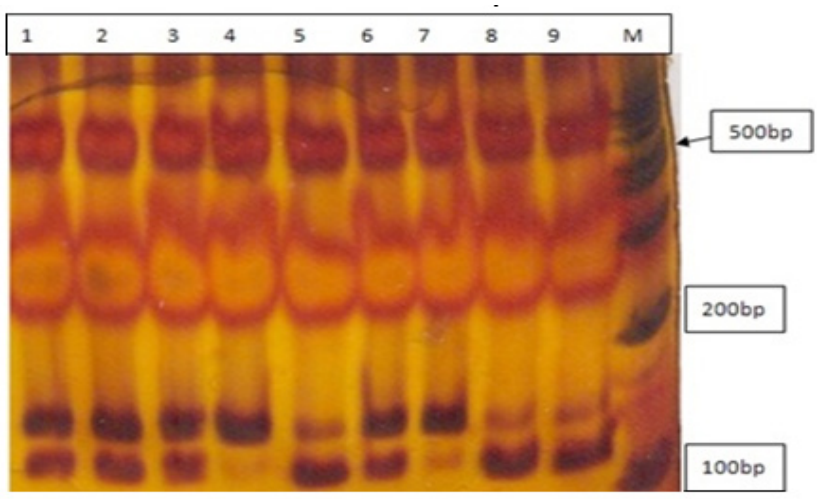

Fig. 2-B

Fig. 2. Horizontal gel electrophoresis of enzymatic digestion products (RFLP-PCR) Fig. 2-A Analysis of enzymatic digestion products is conducted by agarose gel electrophoresis (3%). Samples 1 to 5 and 6 are heterozygotes and homozygotes of A. Fig 2-B Analysis of the enzymatic digestion products is performed by polyacrylamide gel (10%). Samples 1-2-3, 6, and 5, 8, 9 are heterozygote and homozygotes of G, respectively, and 4 and 7 are heterozygote of A. M is 100 bp marker. The samples are randomly selected from the control and patient samples.

Results of investigation using RFLP-PCR technique:

Products obtained by PCR were subjected to the cutting of restriction enzymes. In the case of the presence of G allele in the polymorphism site of -181 A/G, EcoRI enzyme cuts replicated the fragment into two parts of 110 and 29 bp. According to the release of 29-bp fragment out of the gel, it was expected from the subjects to develop GG homozygote with the band of 110 bp, while it was expected from the subject of homozygous AA to develop the band of 139 bp due to the lack of cutting position. Also, it was expected from heterozygous subjects to develop both bands of 110 and 139 bp due to the presence of both alleles. Due to the slight difference in the enzymatic digestion products, 3% and 10% acrylamide agarose gel were used to separate bands (Figure 2). In Figure 2-A, the digested products were separated by agarose gel electrophoresis technique. Samples 1 to 5, due to having both 110 and 139 bp bands and sample 6 with only a 139 bp band, were considered the heterozygotes of G/A and A base pairs, respectively. For better separation, enzymatic digestion products were separated and analyzed using polyacrylamide gel electrophoresis technique in Figure 2.B. Samples 1, 2, 3, and 6 were considered heterozygotes of A/G base pair due to having both bands. Also, 4,7 and 5,8,9 samples were considered the homozygotes of A and G due to showing 139 and 110 bp bands, respectively. Then, the randomized comparison of two techniques demonstrated that the results of RFLP-PCR confirmed the results of ARMS-PCR technique; therefore, ARMS-PCR technique can be used with confidence in genotyping genes. According to previous studies on the relationship between homozygous GG, it is likely and expected that G allele in GG homozygotes and AG heterozygotes of polymorphisms causes an increase in the risk of colorectal cancer. Therefore, to investigate the association of this polymorphism with disease, GG genotype and genotype with at least one G allele were studied in comparison with genotype AA (no G allele). As can be seen in Tables 4 and 5, there was no association between these genotypes and disease ($p>0.05$). Moreover, in the present study, there was no significant correlation between AA + AG and AA genotypes and the risk of colorectal cancer at $p>0.05$ (data not shown). Also, probability and statistical correlation of allele G containing genotypes with metastatic activity of patients having colorectal cancer were studied. According to Table 6, the patients with and without metastatic activity were divided into two groups and their Chi-square test showed no difference in genotype distribution between the two groups ($p>0.05$). The association between -181A/G polymorphism and the risk of developing colorectal cancer, rectal cancer, colon cancer, and colorectal cancer was examined separately on the basis of gender groups. Statistical analyses ($p>0.05$) showed no significant correlation between the above cases (data not shown).

Discussion and conclusion:

Extensive studies have been conducted regarding the association of this polymorphism with cancer in various populations. First, Ghilardi studied this polymorphism in colorectal cancer in 2003 and found a relationship between GG genotype and risk of the disease (24, OR= 2/41). Zang in 2005 (21), Li in 2006 (12), and Sugimoto in 2008 (27) have represented that this polymorphism is associated with stomach cancer. But, in similar works, de Lima (2009, 28), Woo (2007, 29), Ohtani (2009, 30), and Fang (2010, 31) had examined the association between polymorphism and colorectal cancer and did not find any correlation between them. Two studies were conducted to examine the association of this polymorphism with metastatic

Table 4. Analyzing the association of GG genotype with risk of colorectal cancer.

P-value	OR*	Control group		Patient group		Genotype	-181 A/G
0.17	0.66	Percent	Number	Percent	Number		
		40.3	31	31.2	19	GG	Genotype
		51.9	40	50.8	31	AG	
		7.8	6	18	11	AA	

* In the calculation of OR, AA + AG genotype is considered a reference.

Table 5. Analyzing the association of GG+AG genotypes with risk of colorectal cancer.

P-value	OR *	Control group		Patient group		Genotype	-181 A/G
0.07	0.4	Percent	Number	Percent	Number		
		92.2	71	82	50	GG+AG	Genotype
		7.8	6	18	11	AA	

* In the calculation of OR, AA genotype is considered a reference.

colorectal cancer. Ghilardi (2003, 24) reported the association of GG genotype with metastasis. But, de Lima (2009) did not observe any difference between the risks of metastasis in different genotypes (28). Our results were consistent with the results by de Lima, and the relationship between genotype and risk of metastasis was rejected. However, Sugimoto (2005, 27) and Li (2006, 12) have reported the in the association of G allele carriers with the development of cancer stage in their study on the gastric cancer. The differences observed in this study from the results of Zang, Li, and Sugimoto on the gastric cancer could be due to the different expression of this enzyme in different tissues and tumors. On

Table 6. Analyzing the association between -181 A/G and risk of metastases in colorectal cancer.

OR*	P-Value	In patients without metastasis		In metastatic patients		Genotype
3.8	0.20	Percent	Number	Percent	Number	
		23.5	8	16.66	2	AA
		44.15	15	75	9	AG
		32.35	11	8.33	1	GG
		100	34	100	12	

* In the calculation of OR, AG genotype is considered a reference.

the other hand, the difference between this study and the results by Ghilardi could be attributed to differences studied genetic populations. Thus, according to Kubben›s research who stated that the presence of G allele by itself did not increase the expression of matrilysin (32), these differences in genetic diversity might have prevented the according to Kubben›s research who stated that the presence of G allele by itself did not increase the expression of matrilysin (32), these differences in genetic diversity might have prevented the risk of colorectal cancer in the studied population (gender and type of cancer did not affect the relationship). In general, the differences observed in the results of various studies together may be related to various ethnic diversities and, subsequently, the difference in the genetics of different population, effect of environmental factors, or interactions of different genes for the occurrence of disease. Despite the mismatch in the structure of the designed primers, it seems that tetra-primer Arms-PCR technique was very sensitive to PCR conditions and, if the amount of substances were regulated and adjusted in the PCR reaction, it could lead to the creation of a false band. Therefore, this technique can be questioned and considered uncertainty in terms of sensitivity and accuracy. For this reason, the samples were genotyped by this method and, then, some of them were selected randomly and genotyped by RFLP-PCR twice. Comparison of the results indicated the equal accuracy of these two techniques. Therefore, it can be stated that, if the design of primers and condition-setting of PCR were performed properly, this technique would be reliable. This method, compared with the conventional methods of genotyping like RFLP-PCR, due to the deletion of PCR stages, such as enzymatic digestion deletion, needs less time and cost and only requires agarose gel to observe and analyze the results. It should be noted that considering the lack of restriction site in sequence and limitation in the preparation of enzymes from abroad, there were some restrictions in the design of primers for RFLP-PCR technique and, subsequently, the implementation of this method. Thus, tetra-primer Arms-PCR technique restriction site in sequence and limitation in the preparation of enzymes from abroad, there were some restrictions in the design of primers for RFLP-PCR technique and, subsequently, the implementation of this method. Thus, tetra-primer Arms-PCR technique can be clinically used as an efficient technique. In short, RFLP-PCR method due to high precision and lack of sensitivity toward PCR conditions may seem as a good method. The conditions of the enzymatic digestion, especially incomplete digestion in identifying heterozygotes, are among its big disadvantage. In general, according to the above-motioned information, allele-specific PCR method seems to be a preferred alternative and reliable method.

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