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INVESTIGATION FOR VARIATION IN *MTHFR* GENE IN IRAQI ARAB FEMALE WITH PCOS

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Key words : MTHFR gene, PCOS, Polymorphism.

Abstract – In the current study, the role of MTHFR gene, especially the rs1801133, rs7533315 and rs2274976 polymorphism, has been determined in polycystic ovarian syndrome. The study involved 100 samples, 50 as polycysticovarian syndrome patients and 50 controls. After DNA extraction, samples undergo to PCR-RFLP analysis to investigate the rs1801133, rs7533315 and 2274976. Rs1801133 showed three genotypes CC,CT,TT. No statistically significant differences among the three genotypes between patients and controls were found. The three genotypes distribution agree with Hardy-Weinberg Equilibrium theory, the observed genotype frequencies had no significant differences than those predicted. Odds ratio referred that C allele could be considered as protective allele while T allele could be considered as susceptible factor for disease. While rs7533315 showed three genotypes with no significant differences among the three genotypes between patients and controls. However, AG genotype was common in both patients and controls which indicate the likelihood of undergo population of study to genetic variation that makes AG the common genotype. On other hand, the odds ratio of both A allele and G allele were close to each other's given the same susceptibility to association with disease. The further sequencing analysis for some samples revealed the association of rs2066071, rs3546336 and 17037390 with patients. This indicates to the necessity of further investigation for these SNPs with larger sample size. Moreover, some patients' samples revealed by sequencing analysis showed new insertions that may lead to frame shift reading. Finally, rs2274976 showed only GG genotype among the study samples, it seems that this region did not undergo for any genetic variation in the population of study.

INTRODUCTION

Polycystic ovary syndrome is the disorder that affects the women in reproductive age and result in subfertility and fertility. The causes of polycystic ovary syndrome is not fully understood. Even that many studies consider it as combination of genetic, environmental factors and life style lead to spectrum of endocrine, metabolic, reproductive disorders (Ozegowska, 2016).

Methylene tetrahydrofolate reductase (MTHFR), enzyme plays a vital role in folate metabolism, thus has important part for the RNA synthesis (Bai, 2009; Muslumanoglu, 2009). MTHFR catalyzes the irreversible conversion of 5,10-methylenetrahydrofolate to 5, methyltetrahydrofolate, which acts as a methyl donor in homocysteine (Hcy) to methionine conversion.Methionine plays the key role in the process of DNA methylation and nucleic acid synthesis as well as metabolism processes (Kohara, 2003)

Human MTHFR is a multi-domainprotein with 656 amino acid,the N- terminal catalytic domain is connected to C-terminal regulatory domain by a linker sequence. Serine-rich region, followed by region catalytic domain 40–337 amino acid than linker 338-362, regulatory domain involve 363–644. (Froese, 2018). The genetic polymorphisms in *MTHFR* could decrease the enzyme expression and activity. One of the most common genetic polymorphisms was observed in *MTHFR* was rs1801133 (C677T) that located at exon 5 incatalytic domain, another genetic variation candidate in this study is rs2274976 (G1793A), substitution results in replacement of arginine by glutamine at 594 amino

acid position, which located at exon12 in regulatory domain. The present study focuses on the association between rs 1801133 genetic polymorphisms and metabolic disturbances

Development of polycystic ovary syndrome, Based on this, one of the current study aims was to determine allele frequencies and genotype distributions of *MTHFR* rs1801133 polymorphisms in a sample of Iraqi female patients with polycystic ovary syndrome.

Theoretically, SNPs associated with the disease are located near the actual mutagenic disease sites in the genome. Interestingly, most of the SNPs detected by Genome-wide association study have been localized tointronregions rather than exonic or non sysnonymous sites (Li, 2012 and Welter, 2014 cited in Jo and Choi, 2015).

Thus, rs 7533315 which located at intron3 was candidate to be one of the studied SNPs in current study.

MATERIALS AND METHODS

Present study involved 100 samples, fifty were PCOS patients and fifty were controls recruited from Arab Iraqi population. Rotterdam criteria 2003 were dependent to define the PCOS.

PCOS patients with diabetes, thyroid disease, acromegaly and hyperprolactinemia were excluded from the study, control subjects were apparently healthy. Each study subject was asked to provide peripheral blood sample for DNA extraction that collected in EDTA tube. Then 200 µL of the blood was used for DNA extraction of each sample.

DNA extraction and genotyping

The blood samples for the genomic DNA which was extracted by using Quick-gDNA ™ Blood MiniPrep (Zymo/USA) in accordance with the manufacturer's instructions. The genotype was carried out using polymerase chain reaction (PCR) and RFLP. For further conformation sequencing was performed for randomly samples. Specific primers were used in the PCR showed in Table 1 were described by Ramalingam, 2015; Rosenberg *et al.*, 2002 for rs 1801133 and rs7533315 respectively. The primers, were described by Togãnel, 2007 and FARCA[a], 2010, were modified in this study after compering them with the NCBI data.

Genotype determination for rs 1801133 (C677T) polymorphism

MTHFR gene

The PCR cycles for 477bp segment at exon 5, the reaction began at 95 °C for 2 minutes with 1.5 µL of DNA sample was amplified for 35 cycles with denaturation at 95 °C for 45 seconds, annealing at 62 °C for 45 seconds, extention at 72 °C for 45 seconds, and final extension at 72 °C for 7 minutes. Electrophoresis on agarose gel 1.5% was used for observing PCR products. After confirmation of an amplified fragment of the expected size (477 bp) shown in Figure 1, the 5 µL PCR products were digested with 0.5 µL of restriction enzyme *Hinf1* (Biolab/newen gland) at 37 °C for 30 mints. Digested PCR products were separated by electrophoresis on 2% agarose gel at 70 volt/cm^2 for 1;30 hours, shown in Figure 4.

Genotype determination forrs 7533315 polymorphism of MTHFR gene

The PCR cycles for 893bp segment at intron3, the reaction began at 95 °C for 2 minutes,then 35 cycles of denaturation at 95 °C for 45 seconds, annealing at 68 °C for 45 seconds, extension at 72 °C for 45

Table 1. The specific primers sequences forward and reverse, ofexon 5, intron 3 and exon12 in *MTHFR* gene, the result product size is 477bp, 893bp and 310 bp using for detection of rs 1801133, rs 7533315 and rs2274976 respectively. The forward prime specific for detection rs 2274976 was modified depend on NCBI data base. The original sequence of the forward primer designed by Togãnel, 2007 and FARCA[a], 2010 was 5'-CTCTGTGTGTGTGTGCATGTGTGCC-3'shadowed base.

Primer of exon5	Sequence	Product size	Reference
1801133Exon5	F 5'- AGGCTGTGCTGTGCTGTTG- 3' R 5'- CGCTGTGCAAGTTCTGGAC - 3'	477bp	Ramalingam2015
rs7533315intron3	F 5'- GGCCTGAAGAACATCATGGCG- 3' R 5'- TCCTCTTCCCACTGGTCACC- 3'	893b	Rosenberg*et al* ,2002
rs2274976 exon12	F 5'- CTCTGTGTGTGTGTGCATGTGTGCG- 3' R 5'- GGGACAGGAGTGGCTCCAACGCAGG- 3'	310bp	Modified forward.

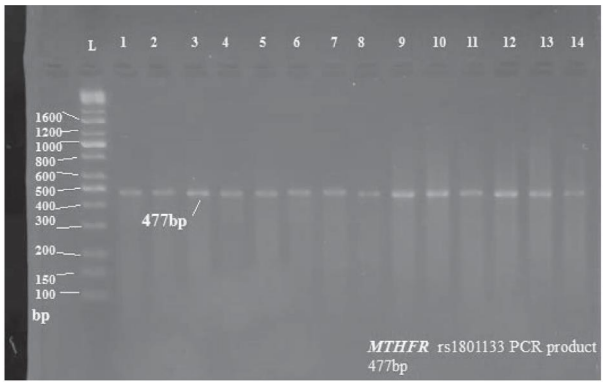

Fig. 1. PCR product the band size 477bp.The product was electrophoresis on 1.5% agarose at 70 volt/cm^2 for 1:30 hours. DNA ladder(100).

seconds, and a final extentision at 72 °C for 7 minutes. Electrophoresis on agarose gel 1.5% was used for observing PCR products showed in. After confirmation of an amplified fragment of the expected size (893 bp) shown in Figure 2, the 5 µL PCR products were digested with 0.5 µL of restriction enzyme *PeaI* (Biolab/newengland) at 37 °C for 30 mints. Digested PCR products were separated by electrophoresis on 2% agarose gel at 70 volt/cm^2 for 1:30 hours, shown in Figure 7.

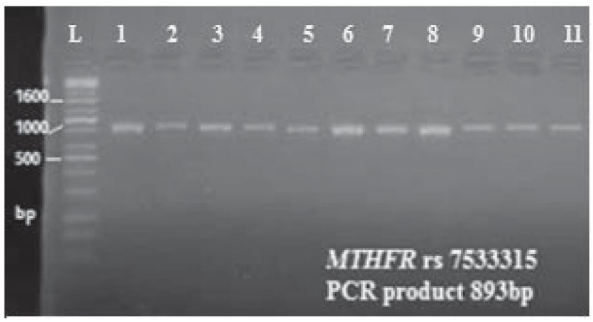

Fig. 2. PCR Product (893 bp) of the amplified region of the *MTHFR*rs 7533315 detected with 1% agarose gel for 2hr and 70 volts/cm.

Genotype determination for rs 2274976 (G1793A) polymorphism MTHFR gene

The PCR cycles for 310bp segment at exon12, the reaction began at 95 °C for 2 minutes, then 35 cycles of denaturation at 95 °C for 45 seconds, annealing at 64 °C for 45 seconds, extension at 72°C for 45 seconds, and a final extension at 72°C for 7 minutes. Electrophoresis on agarose gel 1.5% was used for observing PCR products shown in Figure 3. After confirmation of an amplified fragment of the expected size (310 bp) Figure 3, the 5 µL PCR

Fig. 3. PCR product the band size 310bp.The product was electrophoresis on 1.5% agarose at 70 volt/cm^2 for 1:30 hours. DNA ladder(100).

products were digested with 0.5 µL of restriction enzyme *BsrbI* (Biolab /newengland) at 37^{0}C for 30 mints. Digested PCR products

Were separated by electrophoresis on 2% agarose gel at 70 volt/ cm^2 1:30 hours, shown in Figure 15.

DNA sequencing

The purified PCR products of rs 1801133, rs 7533315 and rs 2274976 which were 477bp, 893bp and 310bp respectively were sent to Macrogen Company in Korea for DNA sequencing. The obtained sequences of these samples were aligned using (Mega-6) software (Tamura *et al*., 2013). Furthermore, the nucleotide sequences were compared to the information in gene bank of the National Center for Biotechnology information (NCBI) web site databases using the BLAST search tool and examined for the presence of these SNPs and screening other variation in product segments.

Statistical analysis

WINPEPI computer program (version 11.63) was used to estimate the statistical significance of the *P*values that was calculated with Fisher's exact test as well as the ODDs Ratio that was assessed by a special ÷2 formula (Abramson, 2011). Hardy-Weinberg equilibrium was tested by chi-squared test that was done using OEGE –Online Encyclopedia for Genetic Epidemiology studies (Rodriguez *et al*., 2009).

RESULTS AND DISCUSSION

Association between C677T gene polymorphism and PCOS

The PCR product 477bp fragments were digested with *Hinf1*, result in three genotypes CC,CT and TT

showed in Fig. 4. The distribution of genotypes and alleles shown in Table 2 and 3. No significant differences were found among the genotypes distribution and alleles frequency between the PCOS patients and the control groups which indicate to negative association of C677T with PCOS orcould not influence the susceptibility to PCOS development.

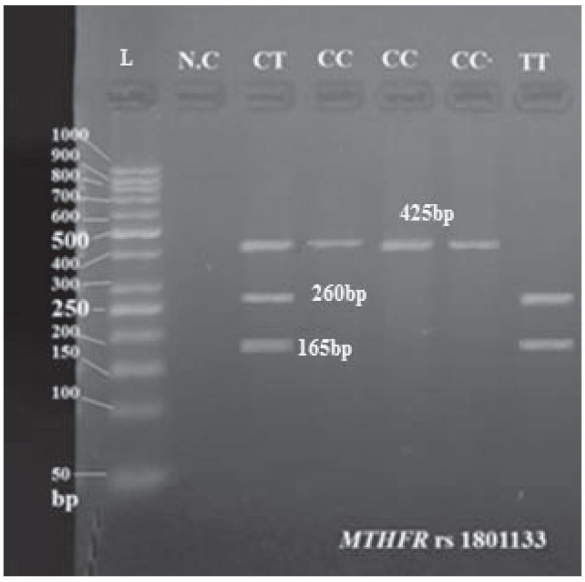

Fig. 4. PCR Product of the of the MTHFR rs 1801133 (477 bp) after digestion with *Hinf1* restriction enzyme (Two bands :52+425 bp=CC; Four band: 52+165+260+425=CT; Three band :52+165+260= TT). band 52 is not clear in the image. 2% Agarose Gel Electrophoresis, for 2hrs, 70volts/cm. L:DNA ladder (50).

In the other hand, odds ratio for the C allele was 0.77 so, negative association with the disease according to odds ratio could be considered as a protective allele. While the odds ratio for the T allele was 1.33 indicating positive association with the disease which could be considered as an etiological fraction in Iraqi patients.

The results were agree with the Hardy-Weinberg Equilibrium,the observed genetype frequencies had no significant differences than those predicted, it was 2.13 in patients and 0.08 in controls respectively Table 3. From the total observed results (patients and controls) shown in Table 3, CC genotype may be considered as the common genotype in Iraqi female population because the total observed record for this locus was highly ratio at both groups (56%+60%)=116% while the other genotypes CT and TT were 33 and 9 respectively.

Sixteen samples (11 patients and 5 control) were sent for DNA sequencing, then the resulted sequences were analyzed by NCBI blast online, these results confirmed the data obtained by the PCR-RFLP of the having the *MTHFR* rs 1801133 (C667T). Sequencing chromatogram for the three genotypes CC, CT and TT showed in Fig. 5, while

Table 2. Distribution of *MTHFR* exon5 rs 1801133 polymorphism genotypes in PCOS and controls (RFLP samples).

Groups Genotype No (%)	Study groups Patients	Control	Odds Ratio	CI 95%	Fisher's exact probability	*Attributable fraction	Prevented fraction
CC	(28) 56%	(30) 60%	0.85	0.48 to 1.49	0.571	—-	15.2%
CT	(16) 32%	(17) 34%	0.91	0.50 to 1.65	0.766	——	8.7%
TT	(6) 12%	(3) 6%	2.14	0.77 to 6.38	0.149	53.2%	—-
Total	50	50					
Alleles distribution							
C n (%)	72%	77%	0.77	0.40 to 1.46	0.424	—-	23.2%
T n (%)	28.%	23%	1.30	0.68 to 2.48	0.424	23.2%	___

Significant differences P d" 0.05*and non-significant P^ 0.05.

Table 3. Expected frequencies of *MTHFR* rs1801133 genotypes using Hardy-Weinberg Equilibrium, results showed agreement with H.W.E., (X2 <3.84) nosignificant differences between observed and expectedfrequencies for both patients and control group.

Groups		CC	CT	TT	C%	T%	X2
Patients Genotypes	Observed no.	28	16	6	72	28	2.13N.S
	Expected no.	25.92	20.16	3.92			
Control Genotypes	Observed no.	30	17	3	73	27	0.08N.S
	Expected no.	29.65	17.71	2.65			
	Total observed	58	33	9			

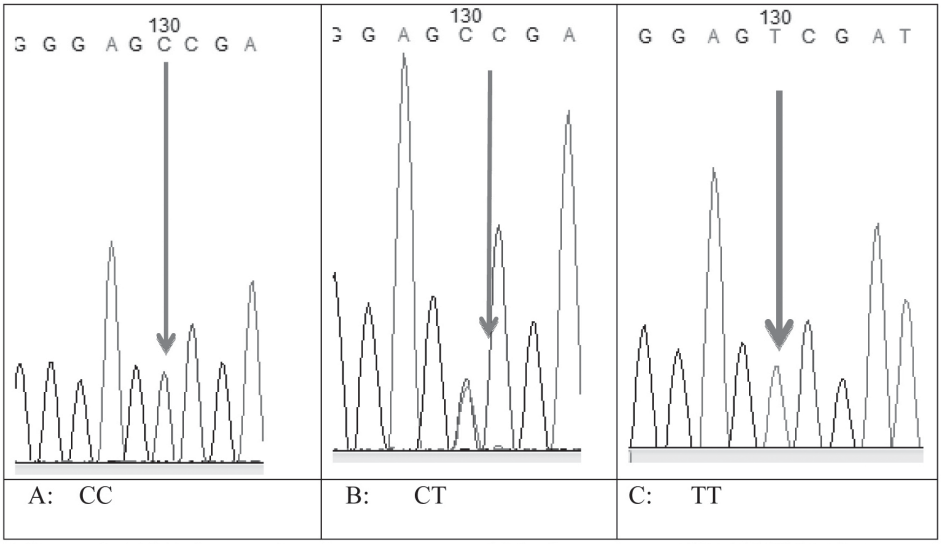

Fig. 5. DNA Sequencing chromatogram which shows the rs 1801133(C667T) that located at genomic location 14783, chromosomal location 1: 11796321. The blue arrows point at the mutation. A: the normal homozygouse CCgenotype, B: the hetorozygouse CT, C: the mutant homozygouse TT.

sequence alignment shown in Figure 6

Association between rs 7533315 gene polymorphism and PCOS

The intron3 in *MTHFR* gene spans along 839bp, but the primers which were used to detect the interesting region with rs7533315 were located in neighbor exons, that result in 893bp PCR product shown in Figure 3.

The PCR product samples were incubated with *PeaI*/ *Sph-Hi* Restriction Enzyme for 30 minutes ;and run on 2% agarose gel electrophoresis, for 2hrs, 70volts/cm. The results revealed three genotypes AA,AG and GG. Figure 7 shown the three genotypes. GG genotype was three bands: 96 + 229 +

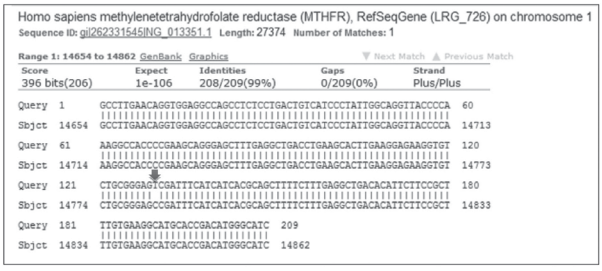

Fig. 6. Representative sequence alignment of *MTHFR* gene of exon 5 compared with standard *MTHFR* gene exon 5, obtained from Gene Bank. "Query" represents samples from this study; "Sbject" is a reference sequence from the National Center Biotechnology Information (NCBI). Red arrow indicate the position of genomic location 14783 which refers to rs 1801133 substitution C^T.

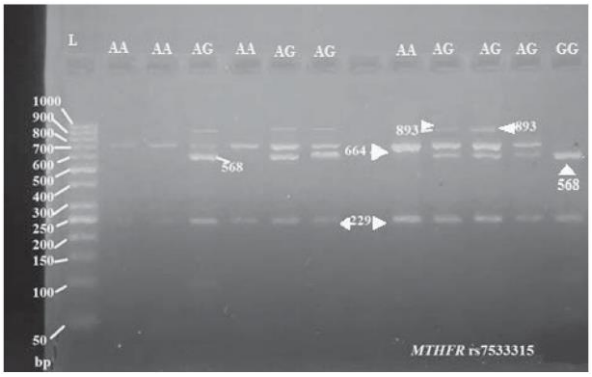

Fig. 7. PCR Product of the of the *MTHFR*rs7533315 ,the size 893bp. After digestion with PeaI/ Sph-Hi Restriction Enzyme (three bands: 96+229+568 bp=GG; five bands: 96+229+568+664+893bp = AG ; Two bands: 229+664bp = AA), 2% Agarose Gel Electrophoresis, for 2hrs, 70volts/cm. DNA ladder (50).

568 bp; AG genotype was five bands: 96 + 229 + 568 + 664 + 893bp; AA genotype was two bands: 229 + 664 bp comparing with the DNA ladder (50). The band 96 was not clear enough in the image so we depend on the reference bands with bold font.

The results in Table 4 showed the distribution of the three genotypes.

There were no significant differences found among the genotypes distribution and allele frequency between the PCOS patients and the control groups. In the other hand, the AG genotype of rs 7533315 had the Lion's share in the population

Table 4. Distribution of MTHFR Intron3rs 7533315 polymorphism genotypes in PCOS and controls (RFLP samples)

Groups Genotype	Study groups Patients	Control	Odds Ratio	CI 95%	Fisher's exact probability	*Attributable fraction	prevented fraction
AA	(1) 2%	(3) 6%	0.32	0.04 to 1.56	0.172	——	68.0%
AG	(48) 96%	(46)92%	2.09	0.61 to 8.16	0.253	52.1%	___
GG	(1) 2%	(1) 2%	1.00	0.10 to 9.76	0.621	protective factor is the same in groups Patients and Control	
Total	50	50					
Alleles distribution							
A	0.50	0.47	1.13	0.65 to 1.97	0.674	11.3%	——
G	0.50	0.43	1.33	0.76 to 2.32	0.326	24.6%	—

Significant differences P d" 0.05*and non-significant P^ 0.05.

of the study (patients and controls). This may refer to another variation in *MTHFR* gene in Iraqi population.

Furthermore, both odds ratioof the A allele 1.13 and G allele 1.33 were close to each, so the odds ratio gave both alleles the same susceptibility to PCOS in the Iraqi population,that was confirmed by the odds ratio of heterozygote genotype and highly frequent.

The observed genotype frequencies were significantly higher than those predicted by the Hardy-Weinberg Equilibrium theory, it was 42.32 in patients and 35.53 in controls respectively (Table 5); here thepresent data departure from H.W.E, so it may be once again the evolutionary selection from inbreeding and consanguineous had a role on this locus in Iraqi population. Heterozygote genotype AG was more frequent in both groups patients and controls (48+46=96) which made it common genotype in Iraqi female population.

Ten samples (5 patients and 5 control) were sent for DNA sequencing, then the resulted sequences were analyzed by NCBI blast online, these results confirmed the results obtained by the PCR-RFLP of the having the *MTHFR* rs 7533315. The three genotypes in chromatogram sequencing shown in Figure 8, 9 and 10 respectively.

The alignment of query and sbjct shown in the figure 11, 12, 13 and 14 revealed that other SNPs, were in this interesting region of intron 3, shown frequency of (rs2066471, rs 3546336 and rs 17037390 respectively) associated with patients. Yet , it cannot be depended because of small samples size but in same time it may indicate for importance of further investigates in the future. This intron appears to be exposed to variations and needs further investigation. Figure 9 the alignment revealed insertion in intron 3 region that lead to frame shift. These variations within the intron may be represent it as an evolutionary site for *MTHFR* gene. The importance of introns associated with pathogenic mutationsas mentioned byVaz Drag 2017 which can occur deep inside the introns of more than seventy five genes whichassociated with disease. Hurtful DNA variants have been mapped more than 100 base pairs away from exon-intron junctions,they most commonly lead to pseudo-exon inclusion due to activation of non-canonical splice sites or changes in splicing regulatory elements. Additionally, deep intronic mutations can disrupt non-coding RNA genes and transcription regulatory motifs.

Results of Exon12 rs 2274976

The 310 bp PCR product were obtained by using the

Table 5. Expected Frequencies of MTHFR Intron3 rs 7533315 Genotypes Using Hardy-Weinberg Equilibrium, results showed significant differences (X2 ^3.84) between observed and expected frequencies for both patients and control group.

Groups		AA	AG	GG	A	G	X2
Patients Genotypes	Observed no.	1	48	1	50	50	42.32*
	Expected no.	12.5	25	12.5	Not de tected		
Control Genotypes	Observed no.	3	46	1	54	46	35.53*
	Expected no.	13.52	24.96		11.52	Not detected	
Total observed	4	94	2				

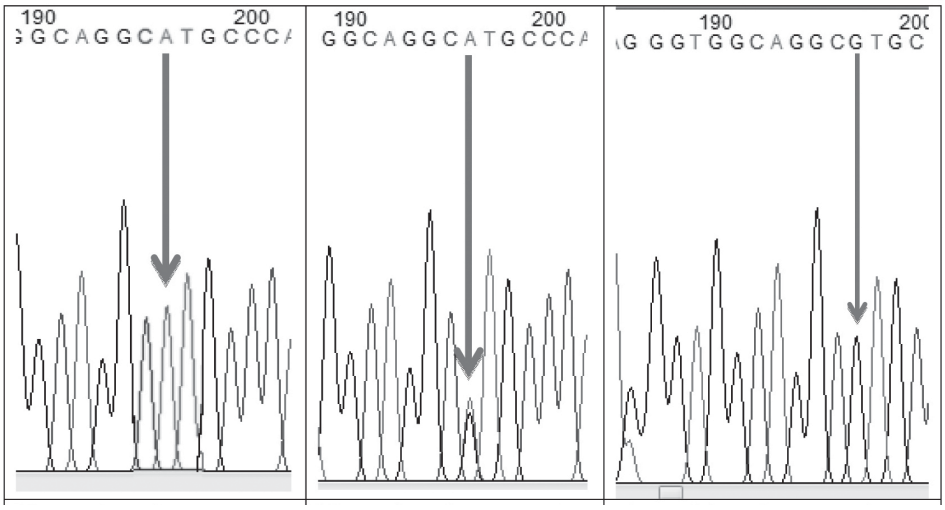

Fig. 8 Fig. 9 Fig. 10

Fig. 8. Red arrow point to AA normal homozygote rs 7533315 Genomic location 10478 Chromosomal location 1: 11800626

Fig. 9. Red arrow point to AG Mutant Heterozygote rs 7533315 Genomic location 10478 Chromosomal location 1: 11800626

Fig. 10. Red arrow point to GG mutant homozygote rs 7533315. Genomiclocation 10478 Chromosomal location 1: 11800626

specific primers pair which were designed by both Togãnel, 2007 and FARCA[a],2010. They were used after complete modification and reviewed with the NCBI in current study. Table 1shown the modified primers.

The 310 bp amplified fragment shown in Figure 5, then the fragments were digested using *BsrbI* endonuclease, resulted the same normal homozygous genotype GG in all study samples (patients and controls). This genotype is described

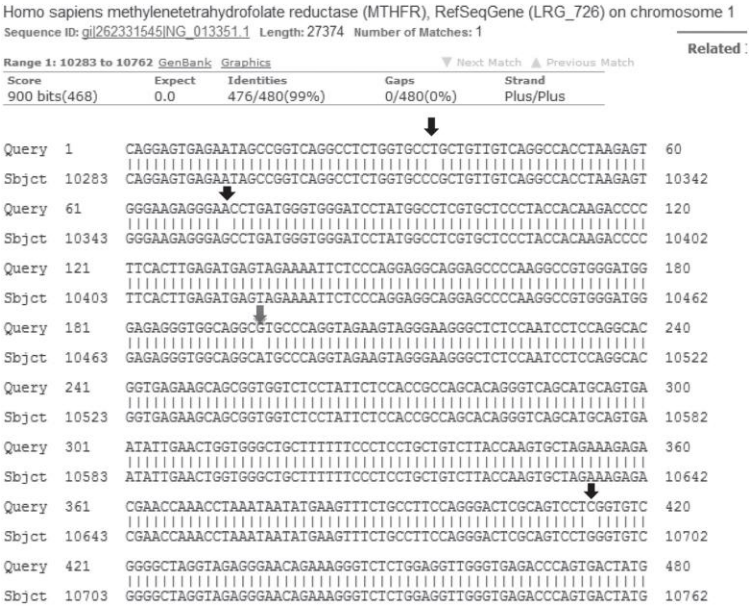

Fig. 11. Representative sequence alignment of *MTHFR* gene of intron3 compared with standard *MTHFR* gene intron3, obtained from Gene Bank. "Query" represents samples from this study; "Sbject" is a reference sequence from the National Center Biotechnology Information (NCBI). Red arrow to the rs 7533315 which located at genomic location 10478, chromosomal location 1: 11800626. Black arrows refer to other substitutions.black arrows point to rs 925478737, rs 35464336 and rs13306567 respectively.

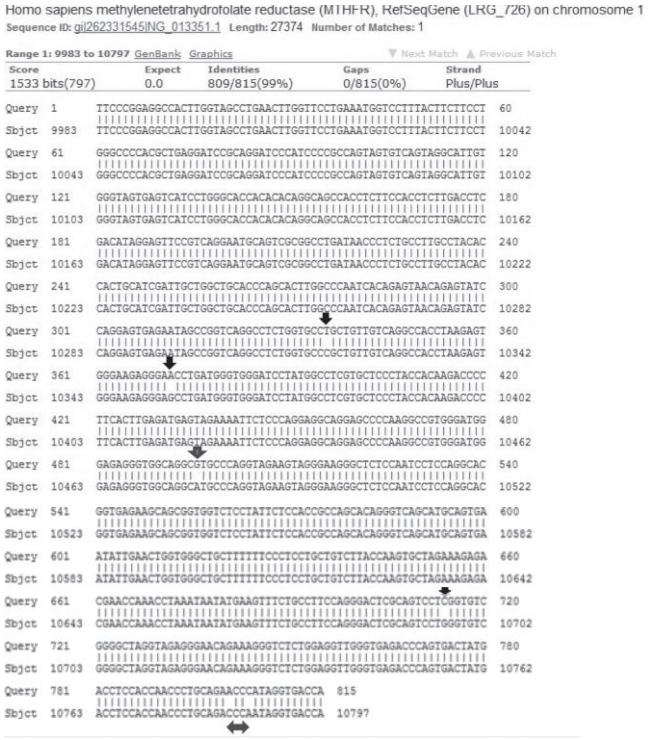

Fig. 12. Representative sequence alignment of *MTHFR* gene of intron3 compared with standard *MTHFR* gene intron3, obtained from Gene Bank. "Query" represents samples from this study; "Sbject" is a reference sequence from the National Center Biotechnology Information (NCBI); red arrow refers to the rs 7533315 which are located at genomic location 10478, chromosomal location 1: 11800626. Black arrows refer to other substitutions inrs 925478737, rs 35464336 and rs 2066471. Another variation was inversion of segment 10783-10786.

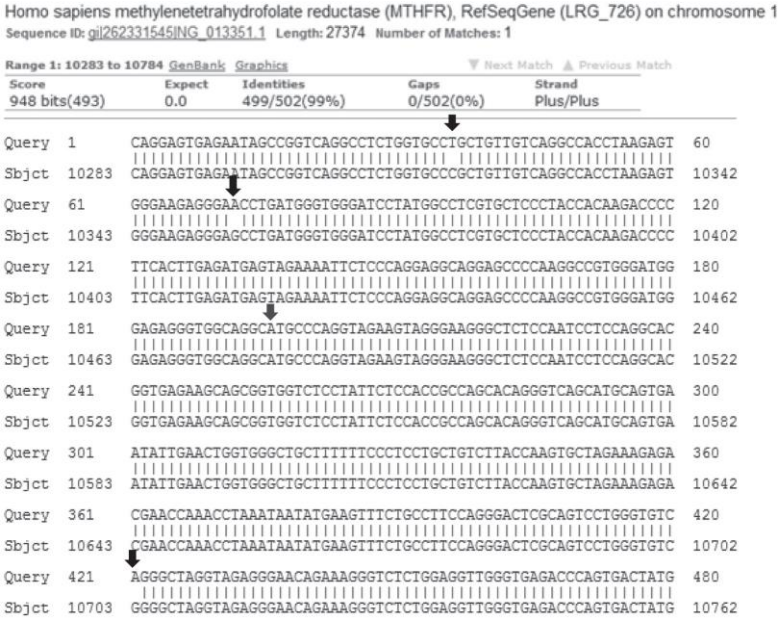

Fig. 13. Representative sequence alignment of *MTHFR* gene of intron3 compared with standard *MTHFR* gene intron3, obtained from Gene Bank. "Query" represents samples from this study; "Sbject" is a reference sequence from the National Center Biotechnology Information (NCBI). Red arrow refersthe rs 7533315 which located at genomic location 10478, chromosomal location 1: 11800626. Black arrows refer to other substitutions in rs 925478737,rs 35464336 and rs 2066471.

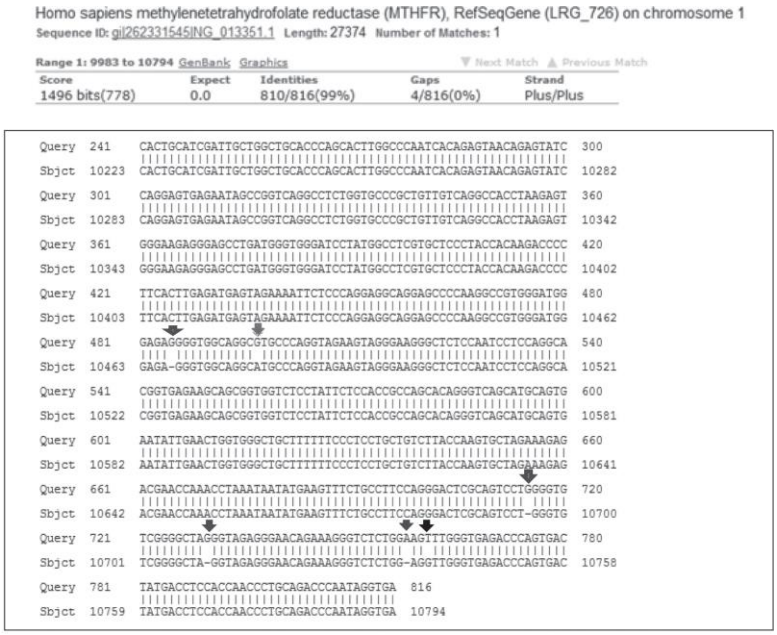

Fig. 14. Representative sequence alignment of *MTHFR* gene of intron3 compared with standard *MTHFR* gene intron3, obtained from Gene Bank. "Query" represents samples from this study; "Sbject" is a reference sequence from the National Center Biotechnology Information (NCBI). red arrow refers to the rs 7533315 which located at genomic location 10478, chromosomal location 1: 11800626. Blue arrows refer to insertion which leads to frame shift. Black arrow refer to substitution G^T at g:10739

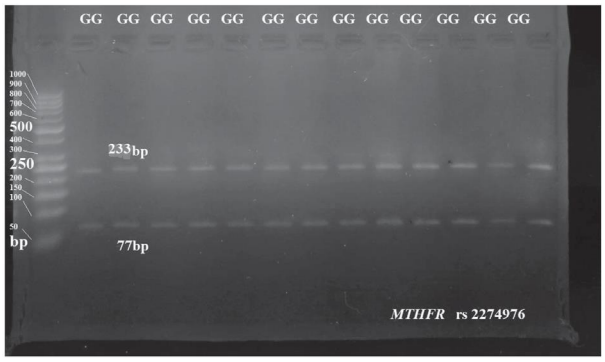

Fig. 15. PCR Product of the of the *MTHFR* rs2274976 (G1793A) 310bp after Digestion with *BsrbI* restriction enzyme(One band: 310 bp=AA; Two bands: 233 + 77bp = GG; Three bands: 310+ 233+77bp = AG), 2% Agarose Gel Electrophoresis, for 2hrs, 70volts/cm. DNA ladder (50).

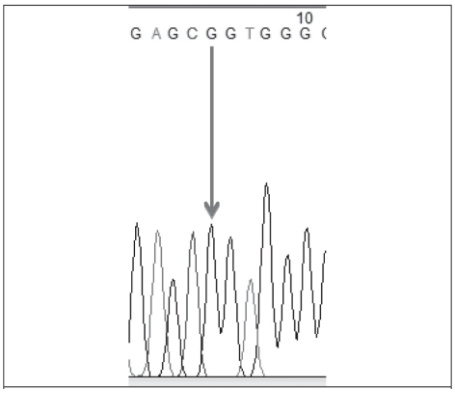

Fig. 16. Red arrow pointe to the GG peak which referred tors 2274976 that located at 20234 genomic location and 11790870 chromosomal location.

as two bands 233 and 77 bp shown in Figure-15. For more conform tenrandomly samples were sent for DNA sequencing, the results were analyzed by NCBI blast online. The sequencing results confirm the data obtained by RFLP. The GG genotype was in all the study samples. That means the G1793A is not associated with the disease, and in the other hand there were not any variation in this locus in the population of the study. Rady 2002 who first described G1793A mentioned that the polymorphic site of the G1793A allele among Americans from different races African-Americans, Hispanics and Caucasians from south Texas were analyzed then compared to those obtained from Ashkenazi Jewish individuals revealed different frequency which means this site could be affected upon different races.

Fig. 16 shows chromatogram of DNA Sequencing and Fig. 17 had shown the alignment between the query and the NCBI sbject. Thus, further

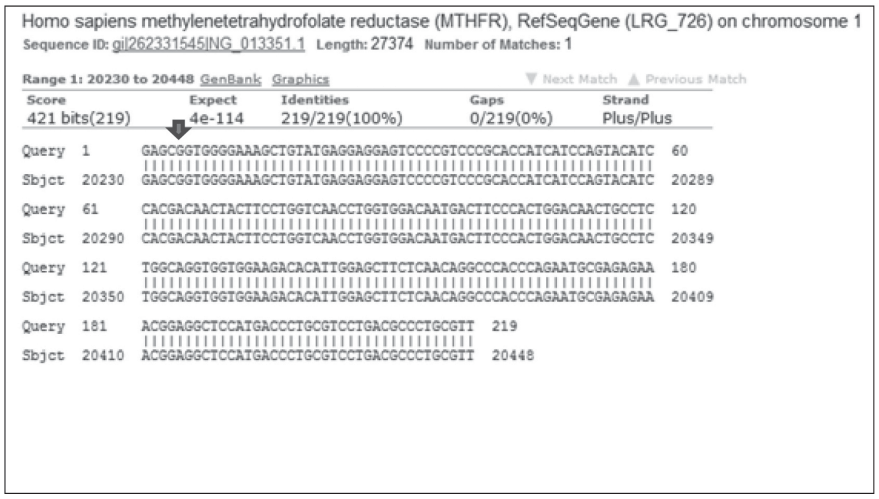

Fig. 17. Representative sequence alignment of *MTHFR* gene of exon 12compared with standard *MTHFR* gene exon 12, obtained from Gene Bank. "Query" represents samples from this study; "Sbject" is a reference sequence from the National Center Biotechnology Information (NCBI). Blue arrow indicate the position of genomic location 20234 which refers to the position of Rs 2274976(G1793A).

investigation with larger sample size may be more useful.

CONCLOSION

There were no significant diffrences among the three genotypes of rs 1801133 between patients and controls and the genotype CC was the common at this locus. While intron 3 of *MTHFR* revealed differences at the locus. Finally rs 2274976 revealed one genotype among the population of study patients and controls.

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