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RESEARCH ARTICLE

PHYSIOLOGICAL AND MOLECULAR STUDY ON POLYCYSTIC OVARY SYNDROME IN A SAMPLE OF IRAQI WOMEN

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ABSTRACT

This study investigates Poly Cystic Ovary Syndrome (PCOS) relation to infertility through a biochemical and molecular at methylene tetrahydrofolate reductase (MTHFR) gene and PCOS gene. Samples from patients suffering PCOS were collected from Kamal al-Samarrai Hospital. Twenty tissue samples were collected from Madinat al-Amamin Al-Kazimin Al-Tibbia Hospital. Fifty blood samples from healthy women served as the control. Average ages of patients and control group were 20-50 years. Subjects were divided in to three age groups (20-30), (31-40) and (41-50), years old. Serum samples for all PCOS patients were measured for fertility hormones levels like Luteinizing Hormone (LH), Follicle Stimulating Hormone FSH and Testosterone hormone. The FSH hormone recorded a significant decreased in age group (41-50) years old, while LH and Testosterone recorded a significant increase in age group (31-40) years old. The study confirmed the incidence of SNPs detected in PCOS gene. Polymerase chain reaction (PCR) was done using a specific primers set to amplify exon (2) of the methylene tetrahydrofolate reductase (MTHFR) gene. Another three primers were designed to amplify the exons (5-10) regions of (PCOS) gene. It was found after PCR product sequencing that the percentage of substitution mutation was 88%, while the deletion mutation percent was 12%.

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INTRODUCTION

Poly Cystic Ovary Syndrome (PCOS) is the most common endocrinopathy in women of reproductive age, with a prevalence of 6.5–6.7% among premenopausal women (Escobar-Morealle *et al*., 2000). PCOS was defined as the combination of chronic anovulation or oligomenorrhoea and clinical or biochemical hyperandrogenism (Zawadski and Dunaif, 1992). Consequently, PCOS has significant implications for the health and quality of life of patients. PCOS was firstly reported as Stein-Leventhal syndrome in 1935, and since then has attracted more and more attention due to its genetic heterogeneity and divers clinical manifestations. It has been used an important clinical model to investigate the relationships among endocrine functions, reproductive activity and energy metabolism (Diao *et al*., 2004). The methylenetetrahydrofolate reductase (MTHFR) enzyme plays important roles in metabolism of folates, remethylation of homocysteine to methionine and reduces 5, 10-methylenetetrahydrofolate to 5-methyltetrahydro folate (Jacques *et al.,* 1996). It has been established that MTHFR enzyme activity is associated with mutations within the MTHFR gene. The two most defined mutations of the MTHFR gene are missense mutations that include substitution of cytosine to thymine at nucleotide 677 which results in the conversion of alanine to valine. Another mutation is the transfusion of adenosine to cytosine at nucleotide 1298 which results in the conversion of glutamate to alanine (van derPut *et al.,* 1998). The influence of theses mutations varies in degree from mild to severe regarding the deficiency of MTHFR enzyme activity. Folate, as a universal methyl donor, contributes to the synthesis of nucleic acids, repair and methylation, and gene expression (Friso *et al.,* 2002). This function implies that gene-nutrient interactions mainly influence the pattern of DNA polymorphisms (Stern *et al.*, 2000).

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MATERIALS AND METHODS

The study included fifty blood samples from patients suffering Poly Cystic Ovary Syndrome (PCOS) during the period from November 2015 to January 2016, collected from Kamal al-Samarrai Hospital. Twenty tissue samples were collected during period January 2016 till February 2016 from Madinat

al-Amamin Al-Kazimin Al-Tibbia Hospital. Fifty blood samples from women were collected serving as the control group. The average ages of patients and control group were 20-50 years.

DNA Extraction: Total cellular DNA was extracted from blood and tissues samples by using the Reliaprep Blood genomic DNA MiniPrep System from Favorgene Taiwan, determination of concatenation and purity of the extracted DNA was done using nanodrop (Techne /UK).

PCR Protocols: Extracted DNA from blood, tissue samples and healthy was used in PCR for amplification of MTHFR gene (R1), and PCOS gene (R2, R3, and R4) primers table (1) Initial denaturation 94°C for 5 min., 35 cycle contain denaturation 94°C for 1 min, Annealing 61°C, 55°C, 53 ^{o}C and 52°C respectively for each primers for 30 sec, extension 72°C for 1 min., and final extension 72°C for 10 min.

DNA sequencing: The purified PCR products of the amplified MTHFR gene region and primers were sent by Macrogen company in Korea for DNA Sequencing. The obtained sequences of these samples were aligned using (Mega-6) software. 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 SNPs.

Statistical Analysis: The Statistical Analysis System- SAS (2012) program was used to effect of difference factors in study parameters. Least significant difference –LSD test (T-test) was used to significant compare between means in this study.

RESULTS AND DISCUSSION

Patients in this study were divided to three groups. For each group, fertility hormone were measured and listed in Table 2.

Table 2. Effect of age group in level of LH, FSH and Testosterone

Age group (year)	Mean ± SE		
	LH (IU/ml)	FSH (IU/ml)	Testosterone (ng/ml)
20-30	5.23 ± 0.83 b	4.97 ± 0.64 b	0.585 ± 0.09 b
31-40	5.51 ± 0.79 b	3.13 ± 0.32 b	0.696 ± 0.09 a
41-50	7.48 ± 1.40 a	2.30 ± 0.46 a	0.518 ± 0.08 b
LSD (T-test)	0.974 *	0.627 *	0.825 *
P-value	0.0392	0.0484	0.0475

* (P<0.05).

It was found that the elevation in LH hormone was more frequent in the age group (41-50) years old among the other two groups (20-30) years old and (31-40) years old groups, with a mean ±standard deviation of 7.48 ± 1.40 a, but the elevation was less in the group (20-30) years old in which the mean was5.23 ± 0.83 b. In contrast, for the hormone FSH it was noticed that this hormone decreased significantly in the age groups (41-50) years old as the difference of level of this hormone was more than the LSD value which was 0.627. Testosterone increased significantly in the age groups (31-40) years old as the difference of level of this hormone was more than the LSD value which was 0.825. Yildiz *et al*., 2012 reported that PCOS is a complex, multifactorial endocrine disorder affecting approximately 5% to 10% of all women of reproductive age. It is believed that the reason for this is due to genetic differences and geographic locations in addition to the environmental conditions and surrounded by physical and chemical effects. The etiology of PCOS is still unclear, but environmental and genetic factors may contribute to the pathogenesis of PCOS. It may be explained by the existence of a vicious perpetual circle of pathological effects, where androgen excess favoring visceral abdominal fat disposition facilitates an increased secretion of androgens by the ovaries and/or the adrenal glands (Escobar-Morreale and San Millan, 2007).

Effect of Polycystic Ovary Syndrome on fertility hormones, Luteinizing Hormone (LH)

Figure (1) showed the mean distribution of the glycoprotein hormone concentrations Luteinizing Hormone (LH) to the females patients when compared with the mean concentration of the normal controls, a highly significant difference (P<0.01). The LH hormone level increased in PCOS patients.

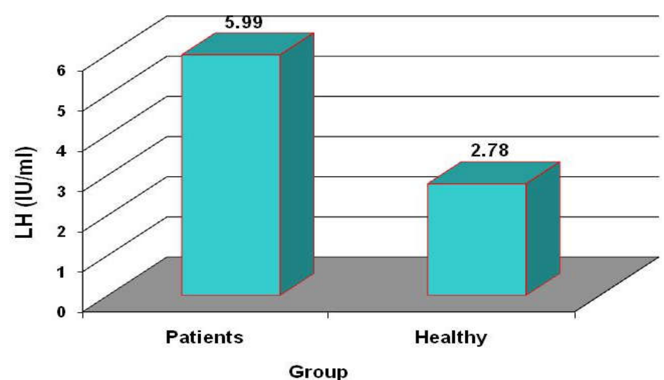

Figure 1. Comparison between patients and control in LH (IU/ml)

We found that the mean of Luteinizing Hormone (LH) hormone level in PCOS patients is 5.99 ± 0.58, while for control healthy is 2.78 ± 0.23. This higher difference is statically significant as the difference between the mean value of two groups (control and patients) is higher than the LSD value which is 1.873**.The presence of hypothalamic dysfunction is suggested by the increase in LH pulse frequency that has been described in this and numerous previous studies (Meyer *et al*., 2000) listed additional probable causes of LH hypersecretion like: Aromatization of androgens to estrogens, resulting in permanent estrogen overproduction, which favors LH hypersecretion, direct leptin-induced GnRH modulation, an insulin-mediated increase in serum LH pulse amplitude.

Follicle Stimulating Hormone (FSH)

Figure (2) showed the mean distribution of the glycoprotein hormone concentrations Follicle Stimulating Hormone (FSH) to the females patients when compared with the mean concentration of the normal controls, a highly significant difference (P<0.05).

The FSH hormone level decreased in PCOS patients. From the same table it is found that the mean of FSH hormone level in PCOS patients is 4.44 ± 0.19, while for control healthy is 5.42 ± 0.26. This higher difference is statically significant as the difference between the mean value of two groups (control and patients) is higher than the LSD value which is 0.633. The current results agreed with (Nawras, 2010) who showed that no

significant differences (p>0.05) in level of FSH. The reduction levels of FSH can be explained by the increase of the conversion of androstenedione in adipose tissue which additionally stimulates LH and inhibits FSH (Marx and Mehata, 2003). On the other hand, the FSH/LH ratio was significantly high in PCOS patients comparing to healthy group. In contrast the ratio was not significant between POCS age groups and healthy control. This is in agreement with (Nawras, 2010) who detect non significant FSH\LH ratio.

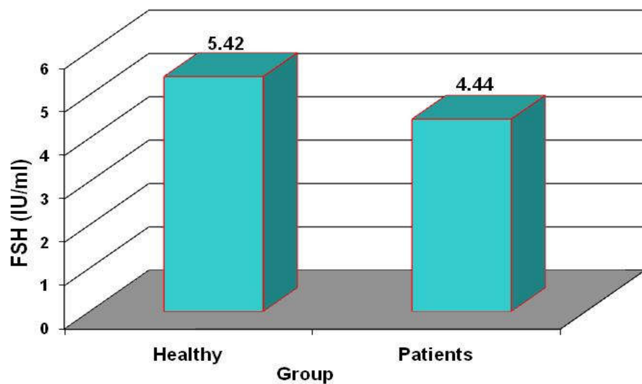

Figure 2. Comparison between patients and control in FSH (IU/ml)

Testosterone hormone

Testosterone levels were found to increase in patients with PCOS. The mean value is higher than of LSD which is 0.277 **. Figure 3 shows the comparison of testosterone levels between patients and healthy.

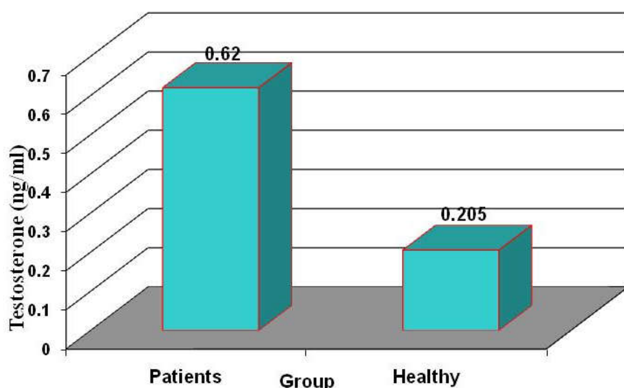

Figure 3. Comparison between patients and control in Testosterone (ng/ml)

Androgen overproduction (testosterone) often results from overproduction of LH (luteinizing hormone) which is produced by the pituitary gland. Excess of LH will stimulate ovaries to produce more testosterone when insulin level in the blood is high (Yousouf *et al.*, 2012).

Molecular detection of Polycystic ovary syndrome by PCR technique

All samples (blood, Paraffin embedded tissues (PE)) were subjected to molecular detection through PCR amplification of the MTHFR gene and PCOS using four specific primer. The first primer was used in this PCR technique (R1), the amplifies in exon (2) specific for MTHFR gene design with product lengths (198bp), The second primer used in this PCR technique (R2), amplifies exons (5 and 6) specific for PCOS gene design with product lengths (278bp), the third primer used in this PCR technique (R3), amplifies exon (7 and 8) specific for PCOS gene with product lengths (278bp), and The fourth primer amplifies in exons (9 and10) with product lengths (284bp) which is shown in Figure (4).

Type of mutations

The First DNA sequence of the MTHFR gene located in exon (2) was taken from blood samples of patient and was compared using the NCBI nucleotide blast, as shown in the figure (5A, and B). The region of the gene of MTHFR, there are many SNPs, 3 SNPs in which are the first encode for a missense mutation (substitution) T/G in position 51 that convert A.A Cys to Pro, the other substitution A/G in position 68 that convert A.A His to Arg, also in position 111 substitution occur A/ T which convert A.A Pro to Ser. The peaks that appear the mutations are clear in Figure (5B). More confirmation was made when a tisue sample was taken for MTHFR gene amplification using the previous conditions and primer. Results are shown in the Figure (6a,and b). There are many heterozygous SNPs in patient samples 6 SNPs in this region of the gene are: in position 51 T/ G which convert A.A Val to Ala, but in position 55 the G changed to C that convert A.A Arg to Thr. In position 56-57 C / - that convert A.A His to deletion. In position 65-66 C/- that converts A.A Thr to deletion, and the other common SNP is A/T in position 66 which converts A.A Thr to Thr. The last SNP was G/C in position 87 that convert A.A Arg to Pro. The Second DNA sequence of PCOS gene located in exon (5) and(6) was taken from blood samples of patient and was compared using the NCBI nucleotide blast, as shown in the Figure (7 A, and B).

The 7 SNPs of PCOS patients are as follows: where one is substitution mutation in which G/T in position 64 that convert A.A Ala to Ser. In the same region of PCOS patients, there is a substitution mutation in position 69 G/T that convert Arg to Leu. Substitution mutation is in the position104 G/C that convert Gly toVal. Also Substitution in G/T in position 117 that convert A.A Ala to Asp.in position 119 there is substitutin G/T that convert A.A Gly to Val.another substitution mutation is in position 140 and 141CA/AC that convert A.A Gln to Thr. The Second DNA sequence of PCOS gene located in exon (5) and (6) was taken from tissue samples of patient and was compared using the NCBI nucleotide blast, as shown in the Figure (8a,and b). The region of the gene of PCOS patients, there are two SNPs, in which are G/A, in position 58 that convert A.A Gly to Arg, the other G/C in position 101that convert A.A Arg to Thr. The third DNA sequence of PCOS located in exon (7) and (8) was taken from blood samples of patient and was compared using the NCBI nucleotide blast, as shown in the figure (9A and B). The SNPs of PCOS patients are as follow: in position 88-89 deletion occur AGA/A-A that convert A.A Arg to deletion,also in position 141 substitution mutation occur CAG/CGG that convert A.A Gln to Arg,in position 157 GAG/GAT substitution convert A.A Glu to Asp, the last two mutationAGC/ACC in position 195 that convert A.A Ser to Thr, in position 217substitution convert CAG/CAC A.A Gln to His. The third DNA sequence of PCOS located in exon (7) and (8) was taken from tissue samples of patient and was compared using the NCBI nucleotide blast, as shown in the Figure (10 a and b).

Table 1. Primers used in this study

No	Name	Oligonucleotides	Product size	Sequence (5'-3')	Ref.
1-	R1-F	Forward primer	198bp	AGGACGGTGCGGTGAGAGTG	Banu *et al.,* 2010
	R1-R	Reverse primer		TGAAGGAGAAGGTGTCTGCGGGA	
2-	R2-F5	Forward primer	278bp	GGTTTTAATCCATGCCTGTT	Designed in this study*
	R2-R5	Reverse primer		AGTCTTGCTCAACAGGGTAG	
3-	R3-F	Forward primer	278bp	CATGTGTGTTTCCTTCTTTG	Designed in this study*
	R3-R	Reverse primer		TTTCCTTCCTCAATCCAGAA	
4-	R4-F	Forward primer	284bp	CTCATCACAGATGTATTATA	Designed in this study*
	R4-R	Reverse primer		GGCAGCAAGGTTAAAAATCG	

Table 2. Effect of age group in level of LH, FSH and Testosterone

Age group (year)	Mean ± SE		
	LH (IU/ml)	FSH (IU/ml)	Testosterone (ng/ml)
20-30	5.23 ± 0.83 b	4.97 ± 0.64 b	0.585 ± 0.09 b
31-40	5.51 ± 0.79 b	3.13 ± 0.32 b	0.696 ± 0.09 a
41-50	7.48 ± 1.40 a	2.30 ± 0.46 a	0.518 ± 0.08 b
LSD (T-test)	0.974 *	0.627 *	0.825 *
P-value	0.0392	0.0484	0.0475

* (P<0.05).

Table 3. Point mutations detect in patient samples

Name of Primers	Wild type	Mutant type	Change in amino acids	Site Of N.A.	Type of Mutation	Effect on translation
(R1 for blood)	TGT	CCG	Cys-Pro	51	Substitution	Missense
	CAT	CGT	His-Arg	68	Substitution	Missense
	CCA	TCA	Pro-Ser	111	Substitution	Missense
(R1 for tissue)	GTG	GGG	Val-Ala	51	Substitution	Missense
	AGG	ACG	Arg-Thr	55	Substitution	Missense
	CAT	-AT	His-Deletion	56-57	Deletion	Frame shift
	ACA	-TT	Thr-Deletion	65-66	Deletion	Frame shift
	ACA	ACT	Thr-Thr	66	Substitution	Missense
	CGT	CCT	Arg-Pro	87	Substitution	Missense
(R2 for blood)	GCT	TCT	Ala-Ser	64	Substitution	Missense
	CGT	CTT	Arg-Leu	69	Substitution	Missense
	GGG	CGG	Gly-Val	104	Substitution	Missense
	GCG	GCT	Ala-Asp	117	Substitution	Missense
	GGC	GTC	Gly-Val	119	Substitution	Missense
	CAA	ACA	Gln-Thr	140-141	Substitution	Missense
(R2 for tissue)	GGA	AGA	Gly-Arg	58	Substitution	Missense
	AGA	ACA	Arg-Thr	101	Substitution	Missense
(R3 for blood)	AGA	A-A	Arg-Deletion	88-89	deletion	Frame shift
	CAG	CGG	Gln-Arg	141	Substitutin	Missense
	GAG	GAT	Glu-Asp	157	Substitutin	Missense
	AGC	ACC	Ser-Thr	195	Substitutin	Missense
	CAG	CAC	Gln-His	217	Substitutin	Missense
(R3 for tissue)	ACA	GCC	Thr-Ala	148-150	Substitution	Missense
	CAG	CTC	Gln-Leu	152-153	Substitution	Missense
(R4 for blood)	CAT	CTC	His-Leu	246-247	Substitution	Missense
(R4 for tissue)	ATG	-AA	Met-Deletion	34-35	Deletion	Frame shift

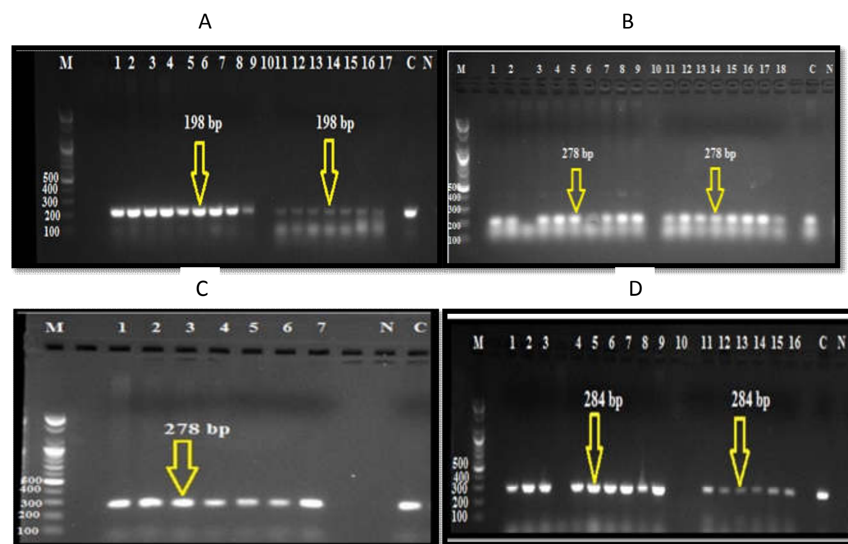

Figure 4. PCR product of (A) R1 primer, (B) R2 Primer, (C) R3 Primer, and (D) R4 primer. Agarose gel electrophoresis conditions were (2% agarose gel, 10 minutes at 100 voltage and then lowered to 70 Volts, 80minutes). Visualized under U.V light after staining with ethidium bromide Line M: 100bp marker. For (A), lane 1-9: DNA isolated from blood samples of patients, lane 11-17 DNA isolated from PE tissue, lane C: from control (healthy) and lane N: from negative control. For (B), lane 1-9: DNA isolated from blood samples of patients, lane 11-18 DNA isolated from PE tissue, lane C: from control (healthy) and lane N: from negative control. For (C), lane 1-4:DNA isolated from blood sample of patients, lane 5-7 DNA isolated from PE tissue ,lane N:from negative control and lane C: from control (healthy). Finally for (D), lane 1-9: DNA isolated from blood sample of patients, lane 11-16: DNA isolated from PE tissue, lane C: from control (healthy) and lane N: from negative control

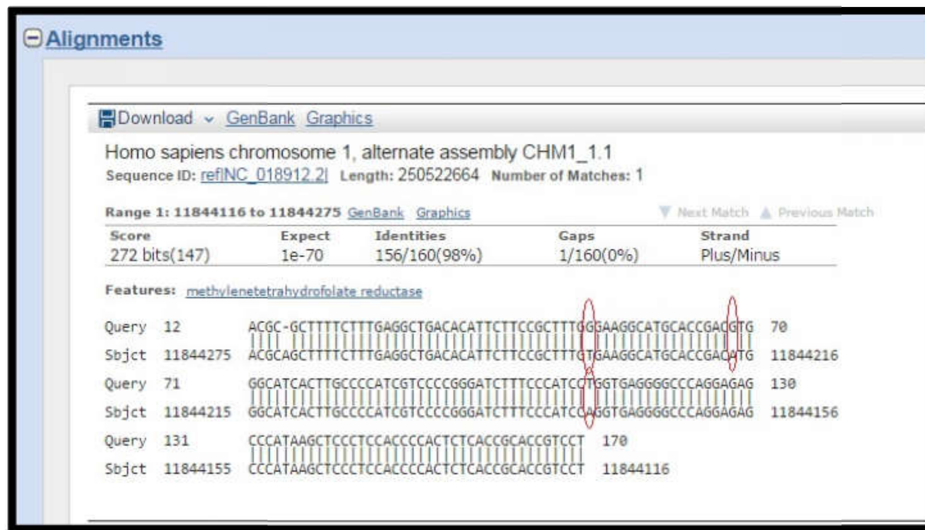

Figure 5A. blast result of the automated sequencing of MTHFR gene for DNA isolated from blood using primer R1

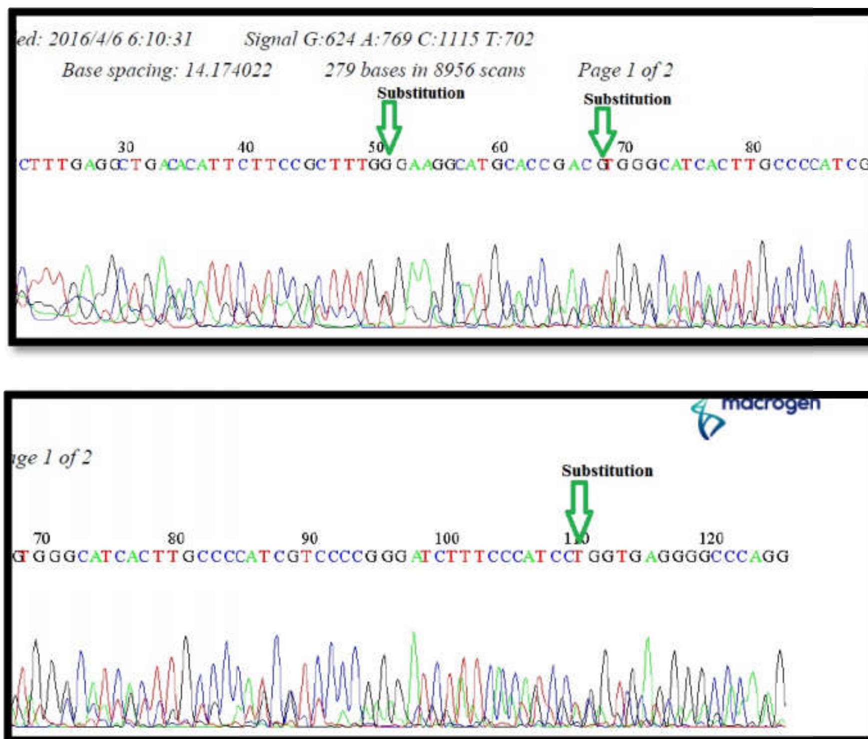

Figure 5B. A chromatogram for sample Polycystic ovary syndrome patient display a sequence and the substitution region

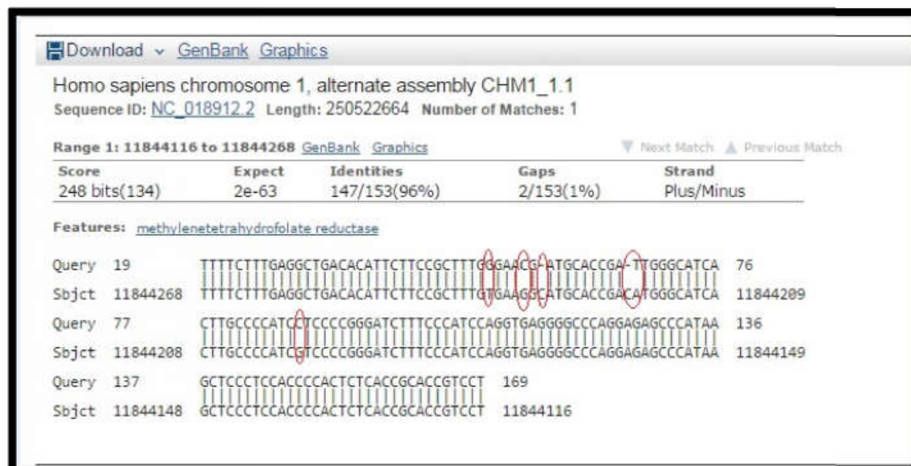

Figure 6a.The automated sequencing of MTHFR gene for DNA isolated from tissue

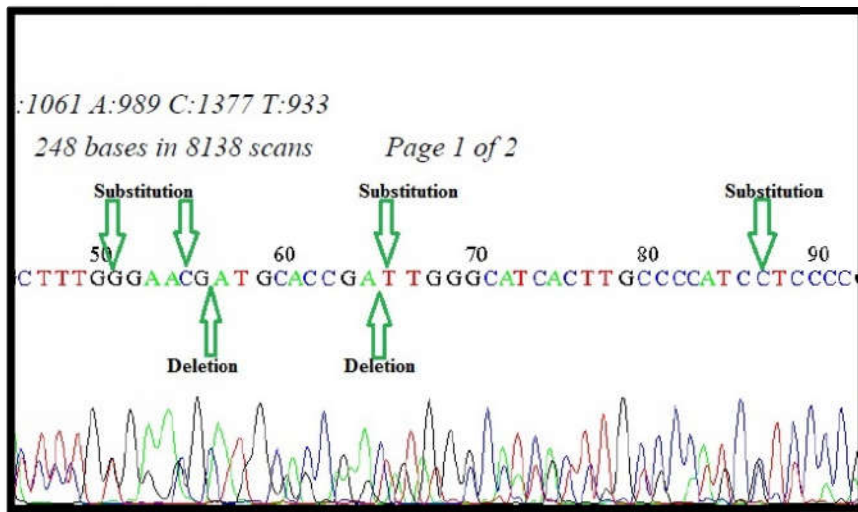

Figure 6b. A chromatogram for sample Polycystic ovary syndrome patient display a sequence and the substitution and deletion region

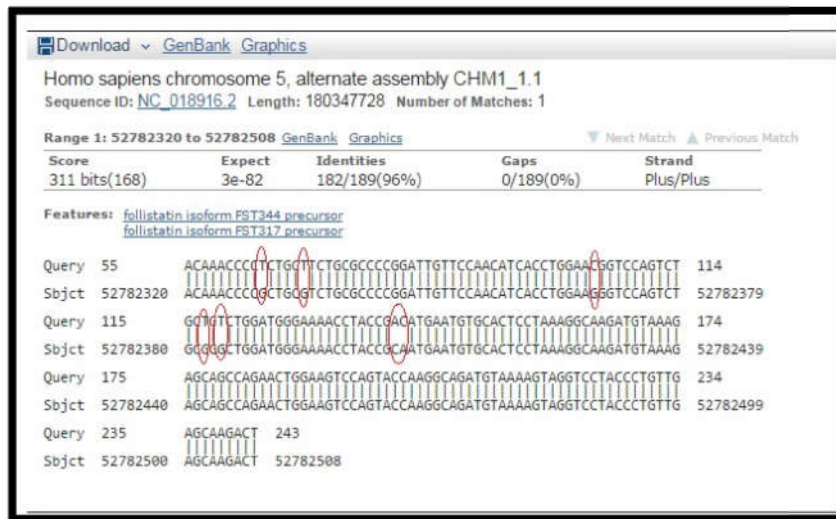

Figure 7A. The automated sequencing of PCOS for DNA isolated from blood

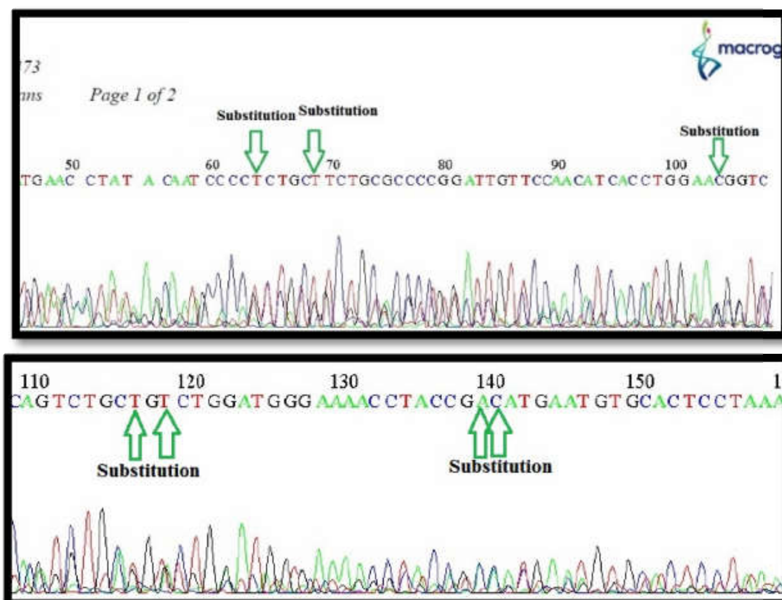

Figure 7B. A chromatogram for sample Polycystic ovary syndrome patient display a sequence and the substitution region

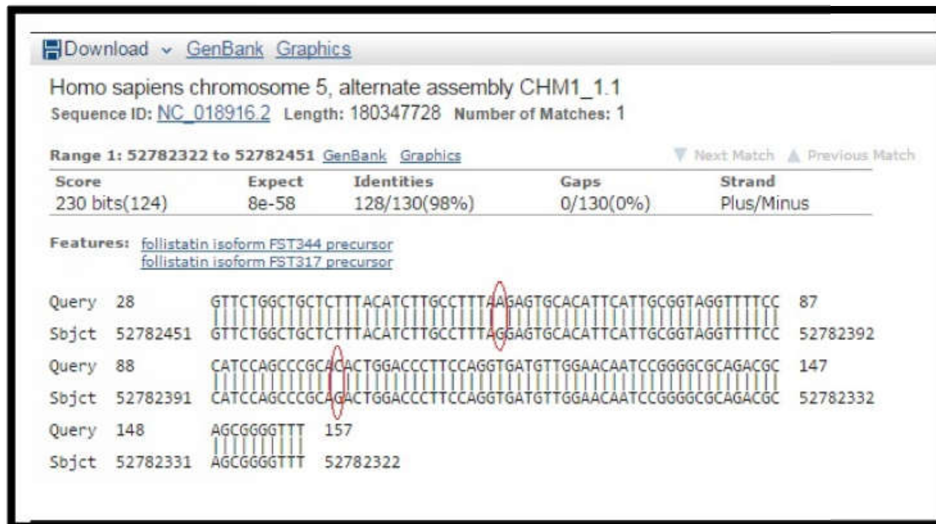

Figure 8a. The automated sequencing of PCOS for DNA isolated from tissue

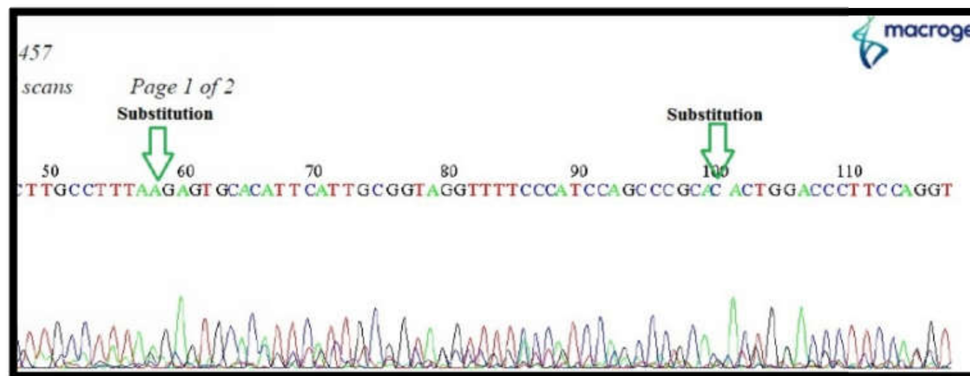

Figure 8b. A chromatogram for sample Polycystic ovary syndrome patient display a sequence and the substitution region

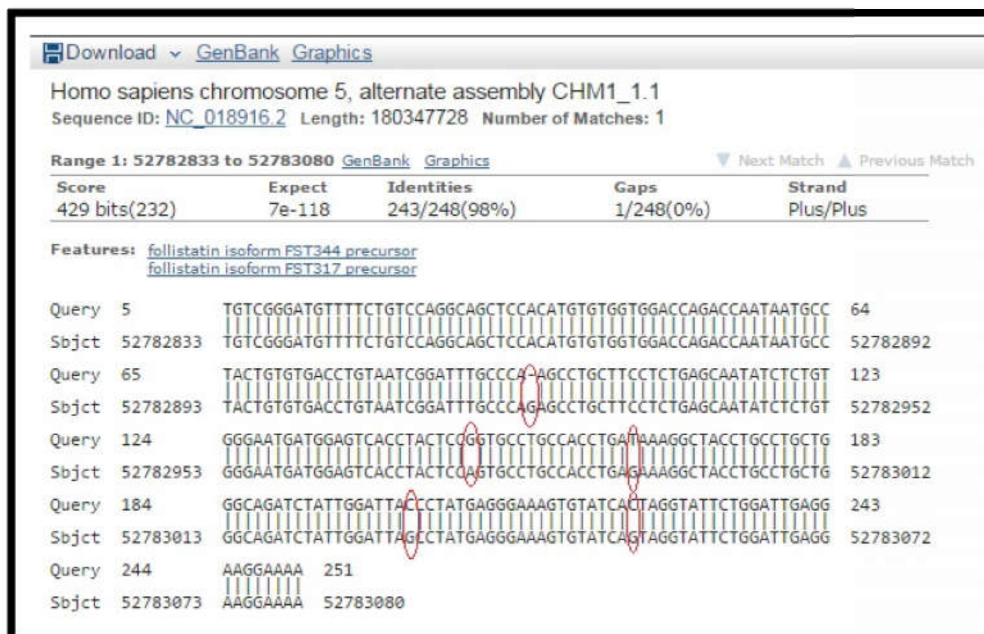

Figure 9A. The automated sequencing of PCOS for DNA isolated from blood

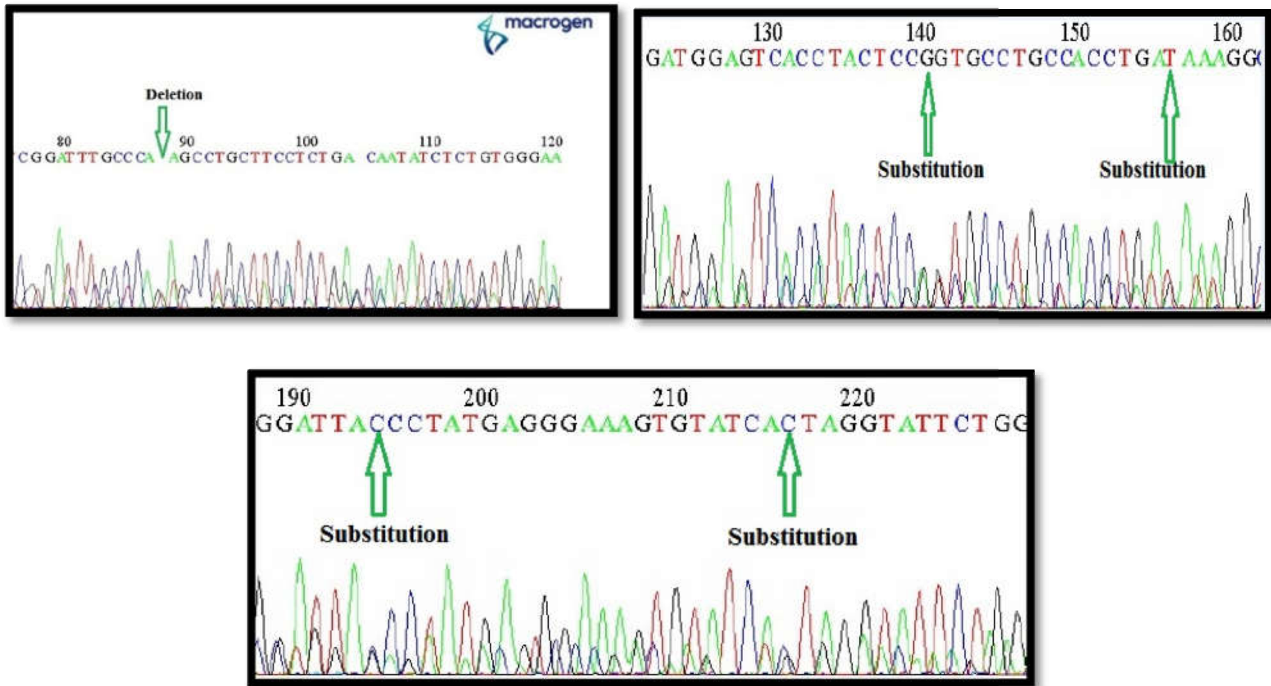

Figure 9B. A chromatogram for sample Polycystic ovary syndrome patient display a sequence and the substitution and deletion region

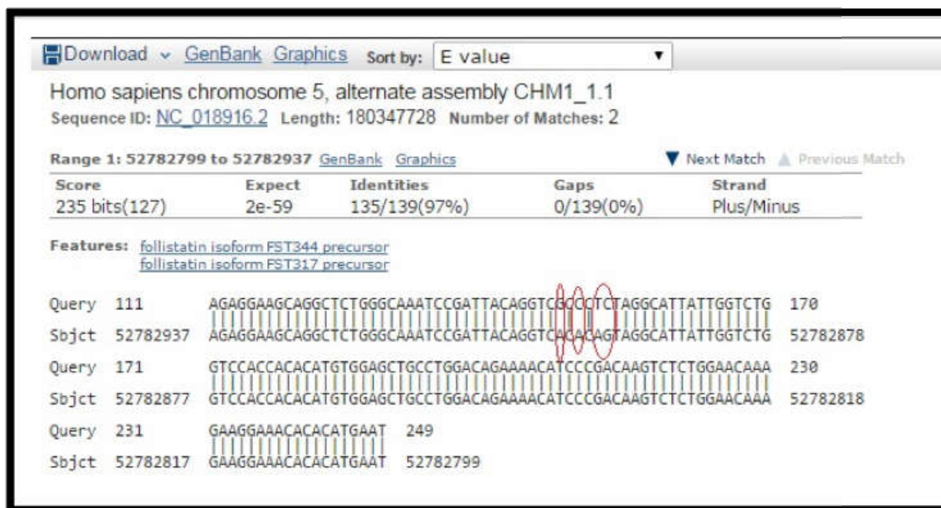

Figure 10a. The automated sequencing of PCOS for DNA isolated from tissue

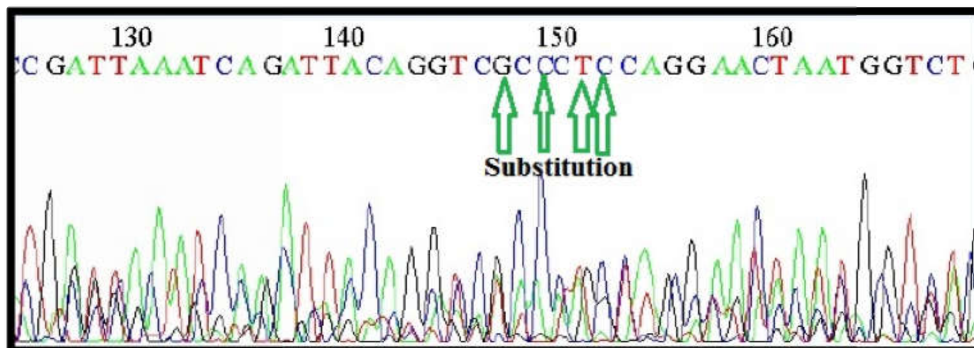

Figure 10b. A chromatogram for sample Polycystic ovary syndrome patient display a sequence and the substitution region

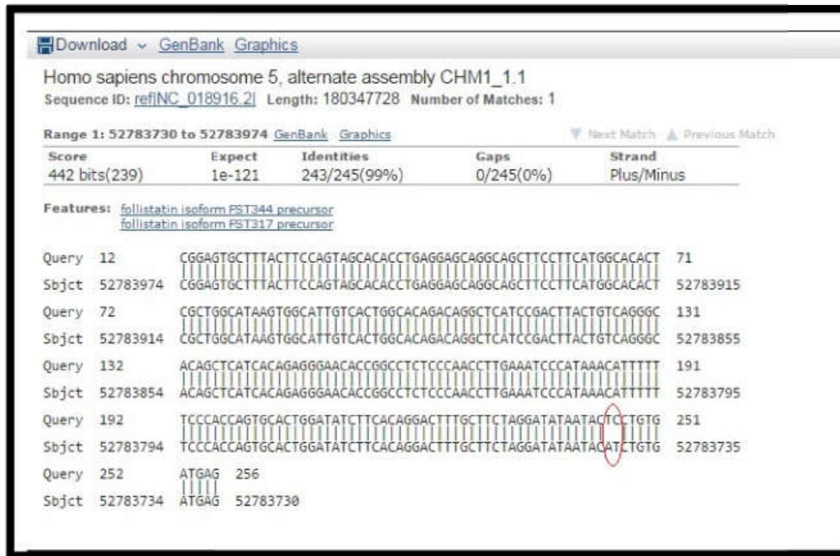

Figure 11A. The automated sequencing of PCOS for DNA isolated from blood

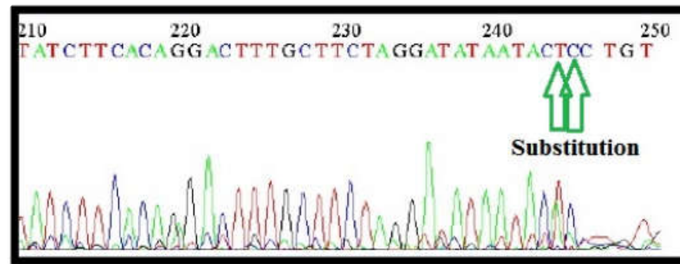

Figure 11B. A chromatogram for sample Polycystic ovary syndrome patient display a sequence and the substitution and region

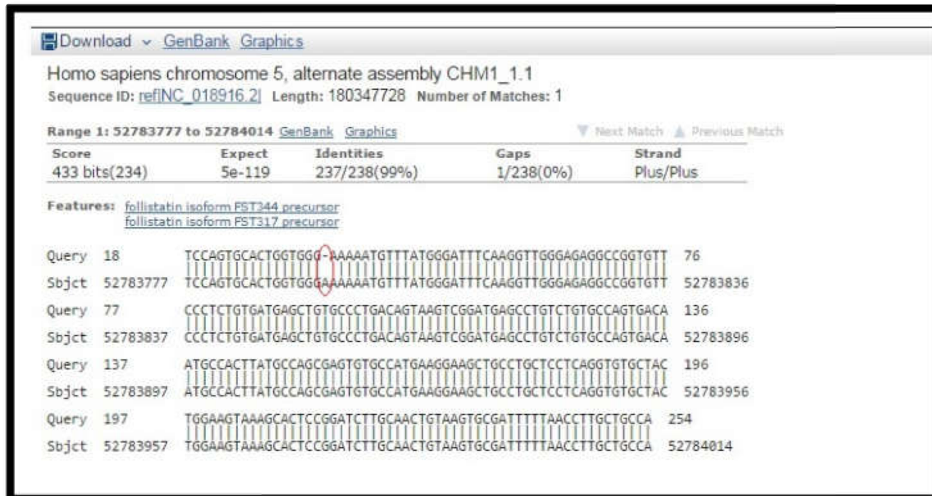

Figure 12a. The automated sequencing of PCOS for DNA isolated from tissue

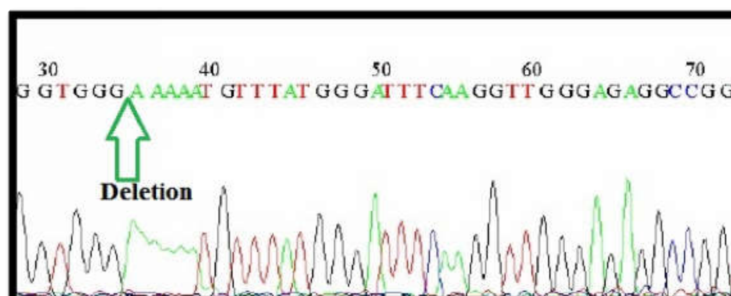

Figure 12b. A chromatogram for sample Polycystic ovary syndrome patient display a sequence and the deletion region

More than one SNPs found in this automated sequencing, the first one is substitution in position 148 and 150 ACA/GCC that convert A.A Thr to Ala, in position 152 and 153 CAG/CTC that convert A.A Gln to Leu. The fourth DNA sequence of PCOS located in exon (9) and (10) was taken from blood samples of patient and was compared using the NCBI nucleotide blast, as shown in the Figure (11 A and B). The SNPs in this region are substitution AT/TC in position 246 and 247 of PCOS patient which convert A.A His to Leu. The fourth DNA sequence of PCOS located in exon (9) and (10) was taken from tissue samples of patient and was compared using the NCBI nucleotide blast, as shown in the Figure (12 a and b). The SNPs in this region are deletion A/- in position 34-35 of PCOS patient which convert A.A Met to deletion.

Polycystic ovary syndrome (PCOS) is the most common endocrinopathy affecting women of childbearing age causing not only reproductive but also metabolic anomalies. PCOS women present with ovulatory dysfunction, abnormal hormones, hyperandrogenemia, obesity, and hyperinsulinemia (Franks *et al*., 2006). It is a heterogeneous disorder which results from interaction of multiple genes along with environmental factors (Xita *et al*., 2002). For a number of genes altered patterns of expression have been detected, suggesting that the genetic abnormality in PCOS affects signal transduction pathways controlling the expression of multiple genes rather than abnormal expression of a single gene (Jakubowski, 2005). Circulating Hcy (Homocysteine) levels could be influenced by many determinants (Refsum *et al.,*1998). Genetic factors play an important role in the metabolic pathway of Hcy synthesis. Enzymatic defects caused by genetic mutations induce a significant increase in Hcy concentrations (Jacobsen, 1998). In the general population, the most common cause of abnormal serum Hcy levels seems to be areduced efficiency of methylenetetrahydrofolate reductase (MTHFR), an enzyme involved in the folate-dependent remethylation of Hcy to methionine (Sills *et al*., 2001). The C677T mutation, *i.e*. alanine to valine substitution at nucleotide 677, in MTHFR gene causes an impairment of its enzymatic activity (McQuillan *et al*., 1999). When C677T mutation is present in a homozygous state, this polymorphism results in a variant of MTHFR enzyme, referred to as the thermolabile MTHFR (Sills *et al*., 2001), and in elevated circulating total Hcy levels (Herrmann, 2001). Different mutation of one or more than located gene region. However substitution and deletion affected the MTHFR gene and PCOS in Iraqi patients. As show in Table (3). Genetic factors are important for the disease in many samples of patients, the mutations detected in exons region of Poly Cystic Ovary Syndrome (PCOS) gene of Poly Cystic Ovary Syndrome (PCOS) patients give evidence that these mutation play a part in this syndrome.

Conclusion

Hormonal imbalance was a direct result for PCOS, and was a significant cause in infertility in women suffering from this disease. The PCOS gene was affected dramatically with genetic change, while less change was detected in MTHFR gene.

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