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RESEARCH ARTICLE

C677T and A1298C polymorphisms of Methylenetetrahydrofolate Reductase Gene in Iraqi Patients with Recurrent Abortion

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Abstract

Recurrent spontaneous abortion (RSA) was significant obstetrical complications that may occur during pregnancy. Various studies in recent years have indicated that two common mutations (C677T and A1298C) of the methylenetetrahydrofolate reductase (MTHFR) gene were risk factor for RSA. This study was carried out to determine the influence of (C677T and A1298C) of the methylenetetrahydrofolate reductase (MTHFR) gene mutations in Iraqi women with RSA. A total of 80 women were included in this study: fifty women with two or more consecutive miscarriages and 30 healthy controls. Total genomic DNA was isolated from blood leukocytes and the frequency of the two common C677T and A1298C MTHFR gene mutations in the patients and controls was determined used PCR-restriction fragment length polymorphism (PCR-RFLEP).There was no significant difference in the prevalence of 677T/T genotype among women with RSA and healthy controls (P = 0.37). Also no statistically significant difference in the frequency of A1298C MTHFR gene mutation was detected between the two groups (P=0.23). In conclusion, the results indicated no significant difference in MTHFR C677T/A1298C genotype distribution among the two groups; therefore, further studies on larger population and other genetic variants to better understand the pathobiology of RSA were needed.

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INTRODUCTION

The methylenetetrahydrofolate reductase (MTHFR, EC 1.5.1.20) enzyme plays important roles in metabolism of folate, remethylation of homocysteine to methionine and reduces 5, 10-methylenetetrahydro folate to 5-methyltetrahydrofolate (Eckfeldt 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 (Frosst et al .,1995; van der et al .,1998).Another mutation is the transversion of adenosine to cytosine at nucleotide 1298 which results in the conversion of glutamate to Alanine. 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 (Frosst et al .,1995; Christensen et al .,1997; Friso et al .,2005). This function implies that gene-nutrient interactions mainly influence the pattern of DNA polymorphisms (Stern et al .,2000;Friso et al .,2002). MTHFR C677T and A1298C SNPs have been associated with human disorders cardiovascular and cerebrovascular disease (Kluijtmans et al .,1997; Nakata et al .,1998), psychiatric diseases (Bönig et al .,2003;, Bjelland et al .,2003) arteriosclerosis (van der et al .,1995; Christensen et al .,1997; Nelen et al., 1997; Sarig et al .,2002) male infertility (Nelen et al .,2002)

hyperhomocysteinemia (Frosst et al.,1995), recurrent pregnancy loss (RPL) and related complications (Naushad et al.,2010;Agodi et al .,2010). Within our population, no studies have addressed distribution of the C677T and A1298C mutations in the MTHFR gene in Iraqi women with recurrent abortion (RA) and healthy controls. Therefore, we carried out the present study to evaluate whether C677T and A1298C mutations in the MTHFR gene are associated with a predisposition for RA.

Materials and Methods

Totally, 50 cases with unexplained RA and 30 healthy controls voluntarily entered into the present study. Cases had a history of at least three consecutive fetal losses before 20 weeks of gestation from the same partner. Cases were diagnosed and sequentially selected among patients referred to the Department the Obstetrics and Gynecology at Al-yarmouk teaching hospital. The control group consisted of fertile females from the general population who had at least one uncomplicated pregnancy and no history of abortion. They were selected with regard to their past medical history and exclusion of any specific disorders such as genetic, congenital diseases and history of pregnancy loss. Patients and controls with vascular disease, obesity, and chromosomal, hormonal, immunological and anatomical abnormalities as confounding factors were excluded. All individuals (patients and controls) were matched for age, body mass index (BMI), written informed consent was obtained from patients and controls. DNA was isolated with the standard method from 3 ml EDTA-blood of samples (Miller et al .,1988). MTHFR C677T alleles and genotypes were determined by RFLP-PCR using primers 5'-CAT CCC TAT TGG CAG GTT AC-3' and 5'-GAC GGT GCG GTG AGA GTG-3'. The reaction profile was: denaturation at 94°C for 30 seconds, annealing at 59°C for 30 seconds, extension at 72°C for 30 seconds for 35 cycles and 72°C for 5 minute (Yang et al .,2007). MTHFR A1298C alleles and genotypes were determined by RFLP-PCR using primers 5'-CTTCTACCTGAAGAGCAAGTC-3' and 5'-CATGTCCACAGCATGGAG-3' and their reaction program was as follows: denaturation at 94°C for 30 seconds, annealing at 51°C for 30 seconds, extension at 72°C for 30 seconds for 35 cycles and 72°C for 5 minute (Donnelly et al .,2000). Restriction digestion with HinfI (Fermentas Life Sciences, Germany) and MboII enzymes (Fermentas Life Sciences, Germany) was used for MTHFR C677T and A1298C genotyping, respectively. Digestion of the PCR products was performed at 37°C for two hours. Separation of fragments was done by electrophoresis on 3% agarose gel containing ethidium bromide. Presence or absent of different fragments were visualized under UV transilluminator.

The presence of T allele at nucleotide 677 of the MTHFR gene naturally produces a restriction site for the HinfI enzyme. Individuals homozygous for the T allele show two bands of 175 and 23 bp. Individuals homozygous for the C allele show a single un-cut band of 198 bp. Those heterozygous for both the C and T alleles show three bands of 198,175, and 23 bp (Yang et al .,2007).The presence of A allele at nucleotide 1298 of the MTHFR gene naturally produces a restriction site for MboII enzyme. Individuals homozygous for A allele show two bands of 204,176 bp. Individuals homozygous for C allele show a single un-cut band of 256 bp. Individuals heterozygous for C and A alleles show two bands of 204 and 176 bp (Donnelly et al .,2000). The frequencies of alleles and genotypes of MTHFR gene were determined via direct counting in the studied groups. Cases and healthy controls were tested for their fit to the Hardy-Weinberg equilibrium regarding allelic and genotypic frequencies. For every group, the expected values were calculated and then data were compared to the observed genotype frequencies. All frequencies of the MTHFR gene in cases versus healthy controls were compared using either the $\chi 2$ test or Fisher's exact test. For all statistical analysis, the $\chi 2$ and p value, odds ratio (OR) and 95% confidence interval (CI) were calculated by SPSS v.16.0 and Microsoft Excel 2003. Two-sided tests were performed and for statistical analysis, a p value less than 0.05 were considered significant.

Results

PCR amplification the region containing the C677T mutation resulted n a single specific product of 198bp without primer dimmers or any non specific product. The inclusion of a negative control that all components of the PCR except genomic DNA was used to exclude the presence of contamination by endogenous DNA .The final PCR reaction volume was 15 µl and successful amplifications was achieved with 100 ng/ml.The is sufficient for successful restriction enzyme digestion and genotype analysis. The 198 bp PCR products was digested overnight with Hinf1 and the digestion products were separated by polyacrylamide gel electrophoresis. Staining with EtBr and UV visualization resulted in the identification of the different genotypes. The absence of the C677T mutation resulted in a PCR product without a digestion site for Hinf1 and these individuals were identified as homozygote normal (figure 1).The presence of one allele with the C677T mutation and one normal allele in an individual that is heterozygote is observed following electrophoresis as two bands of 198 and 175 bp .In an individual that is

homozygote, both alleles are affected and a single band of 175 bp is observed (Figure 2). The small fragment of 23 bp that formed as a result of digestion of the 198 bp fragment is eluted together with the primers from the gel

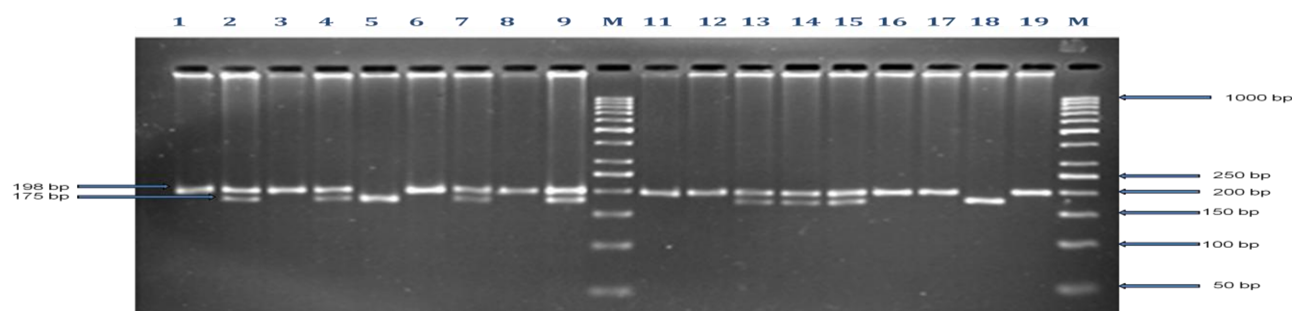

Figure 1: Eighteen samples were analyzed for MTHFR C677T mutation using HinfI based RFLP-PCR by electrophoresis on a 3% agarose gel. C/C: homozygous for wild allele; C/T: heterozygous for wild allele and mutant allele; T/T: homozygous for mutant allele). (Lane M Marker, 50 bp DNA ladder; Lanes 1-[198 bp un-cut]; Lanes 2, 4,7,9,12,13,14,15: heterozygous for mutant allele C/T genotype-(198,175-bp);Lane 5,18:T/T homozygous for mutant allele genotype-(175 bp).3,6,8 bp)3,6,8 ,11,12,16,17,19 Normal(C/C).

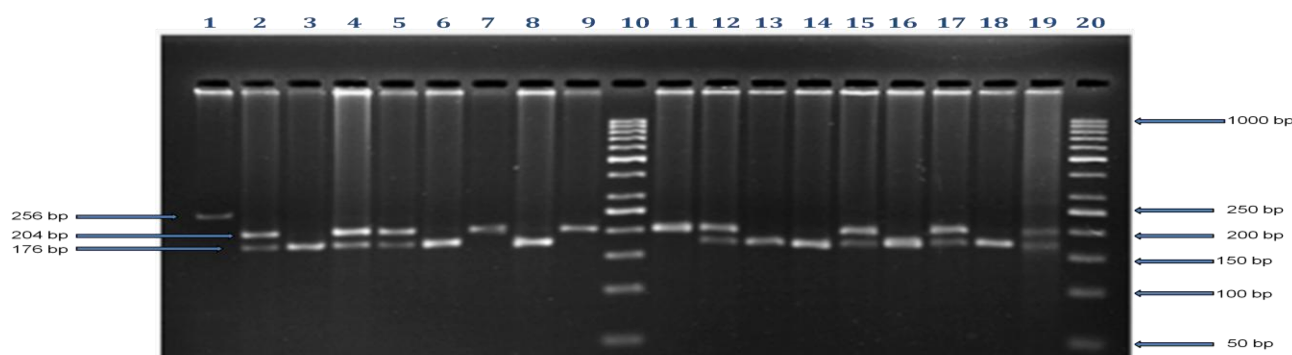

Fig 2: Eighteen samples were analyzed for MTHFR A1298Cmutation using MboII based RFLP-PCR by electrophoresis on a 3% agarose gel. (A/A: homozygous for wild allele; C/A: heterozygous for wild allele and mutant allele; C/C, homozygous for mutant allele). (Lane M: Marker, 50 bp DNA ladder; Lanes 3, 6, 8, 13,14,16,18: A/A genotype-(176-bp); Lanes 2,4,5,12,15,17,19: C/A genotype-(204,176-bp); Lane 7, 9, 11: C/C genotype-(204-bp).

Genotype Distribution of C677T and A1298C MTHFR Gene Mutations

Analyzing MTHFR gene mutations among RSA and controls are done using PCR-RFLP method and the results are summarized in table (1): The genotype distribution of each MTHFR mutations in patients and controls are shown in (Figure 3).The frequency of 677C/T genotype MTHFR gene was 36% in patients and 40% in controls while the frequency of 677T/T genotype was 10% in patients and 13.3 % in controls The frequency of 677T allele was 34 % in patients and 28.3 % in controls.

The frequencies of 1298C allele were 37% in patients and 38.3% in controls. No statistically significant difference in the frequency of A1298C MTHFR gene mutation was detected between the two groups (P>0.05). The frequencies of MTHFR 677T and MTHFR 1298C alleles were (36%, 50%) in patients and (40%, 20%) in controls, respectively (Figure 4). The total mutant allele frequencies were 27% in women experiencing RSA and 29% in fertile controls. The frequencies 677CT/1298AC combined heterozygosity in patients was 15.73% and 32% in the control group. Our findings indicated that combined MTHFR C677T/A1298C genotype distribution has no statistically significant differences. The odds ratios (ORs) of the MTHFR 677C/T (OR = 0.69; 95% confidence interval (CI) = 0.33– 1.42) and the MTHFR 1298A/C (OR = 0.50; 95% CI = 0.23–1.09).

Table 1: MTHFR C677T and A1298C genotypes and alleles frequencies among Iraqi women with RSA compared with fertile control women

Genotype/allele	(RSA)N= 50	Control)N= 30	OR (95 CI)	X^2	P value
MTHFR C677T					
T/T	5 (10%)	4 (13.3)	0.85(0.22-3.13)	0.05425	0.82
C/T	18(36%)	12(40%)	0.87(0.4-1.82)	0.15275	0.70
C/C	27(54%)	14(46.6%)	1.22(0.56-2.52)	0.26203	0.61
T	34 (34%)	17(28.3%)	0.85(0.46-1.53)	0.25797	0.61
C	66(66%)	43(71.6%)	1.14(0.63-2.09)	0.25797	0.61
MTHFR A1298C					
C/C	9(18%)	4(13.3%)	0.95(0.33-2.69)	0.003	0.96
A/C	25(50%)	6(20%)	1.02(0.52-2.13)	0.004	0.94
A/A	16(32%)	20(66.6%)	0.96(0.46-2.14)	0.001	0.97
C	37(37%)	23(38.3%)	1(0.57-1.73)	0.0001	0.99
A	63(63%)	37(61.6%)	1(0.57-1.72)	0.0001	0.99

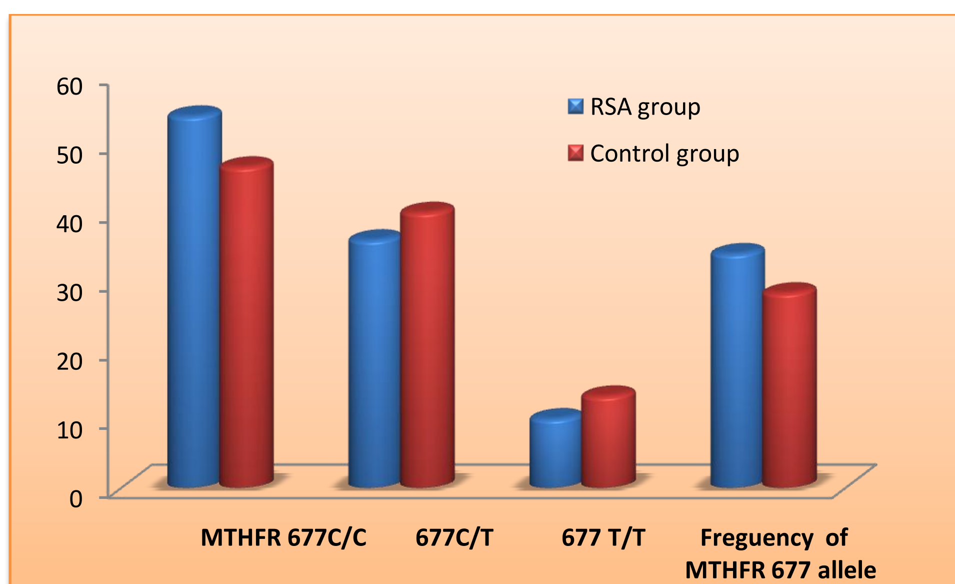

Figure 3 : Distribution of the MTHFR C677T gene mutations genotypes among Iraqi women with RSA compared with fertile control women .In each graph columns show wild type C/C, heterozygote C/T, homozygote T/T and total mutant allele frequencies respectively

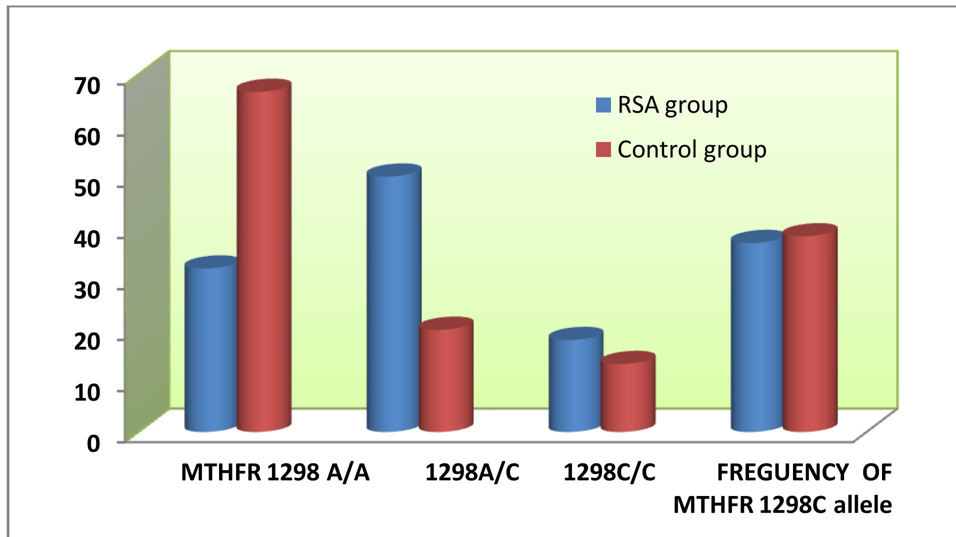

Figure 4: Distribution of the MTHFR A1298C gene mutations genotypes among Iraqi women with RSA compared with fertile control women .In each graph columns show wild type A/A, heterozygote A/C, homozygote C/C and total mutant allele frequencies respectively

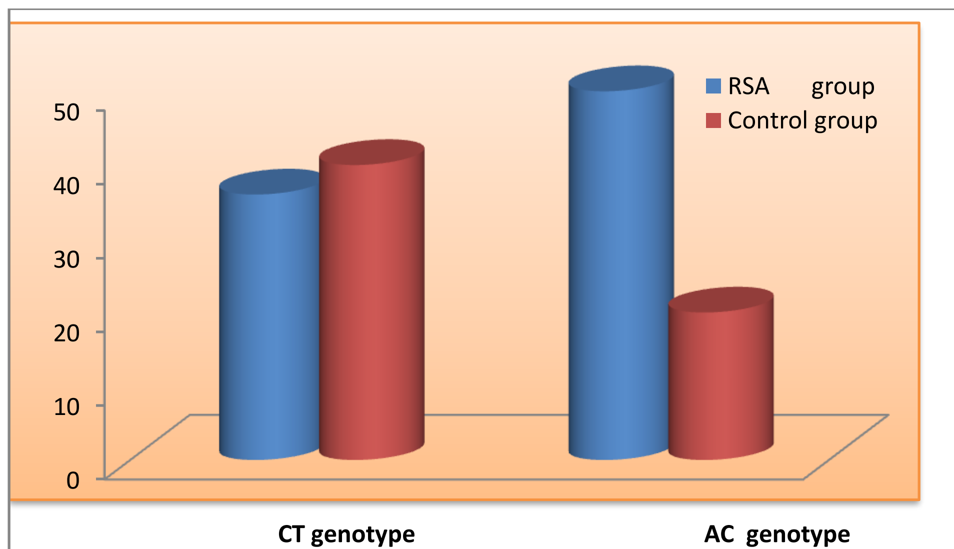

Figure 5: Frequency of heterozygous genotype for MTHFR C677T and MTHFR A1298C among Iraqi women with RSA compared with fertile control women.

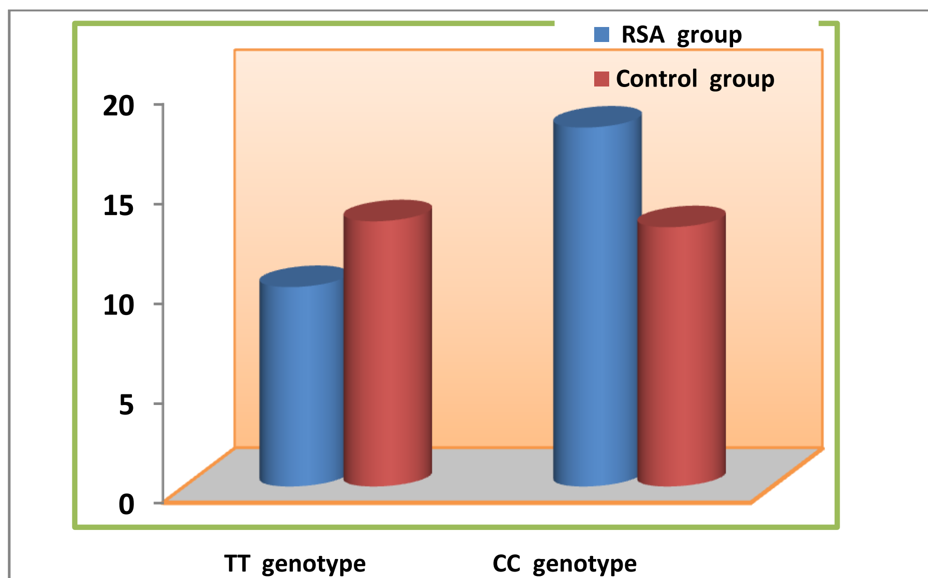

Figure 6: Frequency of homozygous genotype for MTHFR C677T and MTHFR A1298C among Iraqi women with RSA compared with fertile control women.

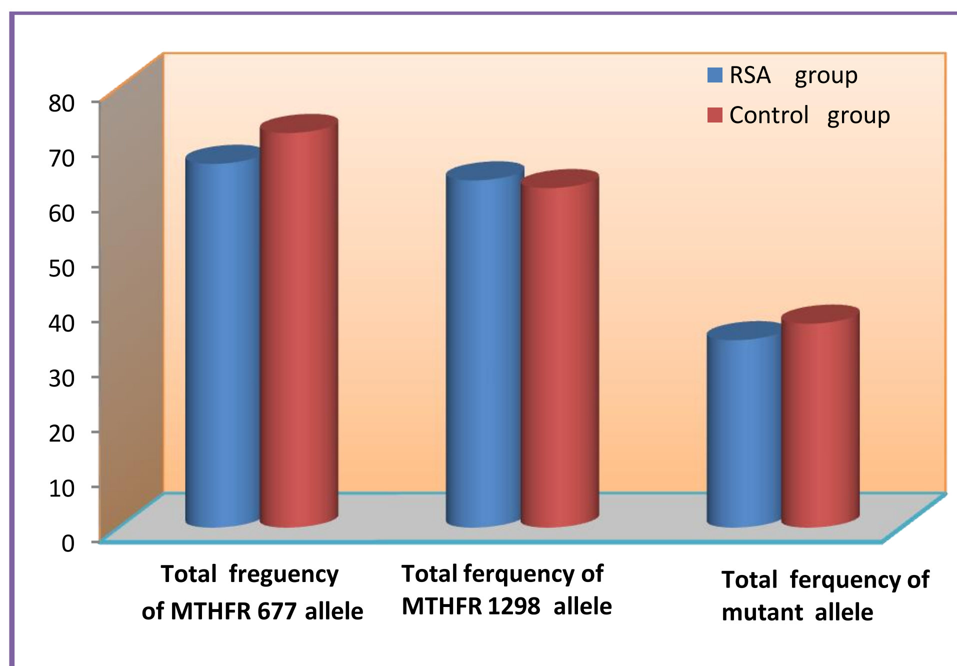

Figure 7: Frequency of mutant alleles for MTHFR (677T and 1298C) and total mutant allele among Iraqi women with RSA compared with fertile control women.

Discussion

Many pieces of literature have discussed the matter that MTHFR gene mutations might be a risk factor for recurrent spontaneous abortions (Unfried et al .,2002;Mtiraoui et al .,2006; Goodman, et al .,2006; Behjati et al .,2006 Stonek, et al 2007; Jeddi-Tehrani et al .,2011) hence, we investigated the prevalence of C677T and A1298C, two common MTHFR gene mutations in Iraqi population especially at Baghdad region to determine whether these mutations related with RSA. The genotypes distribution of C677T MTHFR gene mutation was compared in the two studied groups. It is clear from Table 1 and Figure 3 that the heterozygosity in nucleotide 677th of the MTHFR gene

has no statistically significant difference among the two groups. However, homozygosity for 677T allele of the MTHFR gene in women with RSA was higher than healthy controls, which were concordant with previous reports (Unfried et al .,2002;Mtiraoui et al .,2006). The total frequency of 677T alleles for MTHFR gene 677T was also compared between women experiencing RSA with fertile control women (34%, 28.3%), respectively. On the whole, our data has indicated no statistically significant difference in the prevalence of mutation between the two groups. These data which was observed in our study was in contrast with a previous report on the literature (Park et al., 1997;Carp et al.,2002). This difference may be explained by differences in the populations or by using low numbers of samples. On the other hand, our findings suggest that C677T mutation probably has no significant role in the etiology of first trimester RSA in Iraqi patients in Baghdad region or that other hyper coagulant gene mutations may have a role in RSA. Furthermore, the frequency of A1298C MTHFR gene mutation was also compared in patients and healthy women.

As shown in Table 1 and Figure 4, the frequency of 1298A/C genotype MTHFR gene in control was more than the patients group, while the frequency of 1298C/C genotype was higher in patients with RSA compared with the control group(18% and 13.3% respectively), in contrast with the homozygote genotype. The total frequency of 1298 alleles and 677T for MTHFR gene were also compared between women experiencing RSA compared with the fertile women in the control group (34.83%, 40%),respectively. This was in accordance with earlier investigations (Goodman et al .,2006; Yenicesu et al .,2010) which reported no association between MTHFR 1298A/C and RSA. Findings of previous studies have shown that the presence of combined C677T/A1298C genotypes highly increased the risk of RSA (Zetterberg et al.,2002).Therefore, prevalence comparison of 677CT/1298AC compound heterozygosity in patients and healthy controls showed no significant difference.

In conclusion, our results showed no significant variations in MTHFR C677T and A1298C genotype distribution among patients who suffered from RSA and controls. Further studies on larger population may be needed. To better understand the pathobiology of RSA disease, we need to identify novel genetic variants and the interactive effects of these variants with each other and the environment. Due to the fact that, it is possible to detect a large number of samples and low costs, we proposed ARMS-PCR as a useful method that could be used for identification of these mutations.

References

Agodi A, Barchitta M, Cipresso R, Marzagalli R, La Rosa N, Caruso M, et al. (2010).Distribution of p53, GST, and MTHFR polymorphisms and risk of cervical intraepithelial lesions in sicily. Int J Gynecol Cancer. 20(1): 141-146.

Bönig H, Däublin G, Schwahn B, Wendel U.(2003).Psychotic symptoms in severe MTHFR deficiency and their successful treatment with betaine. Eur J Pediatr.162(3): 200-201.

Bjelland I, Tell GS, Vollset SE, Refsum H, Ueland PM.(2003).Folate, vitamin B12, homocysteine, and the MTHFR 677C->T polymorphism in anxiety and depression: the Hordal and Homocysteine Study. Arch Gen Psychiatry.60(6): 618-626.

Behjati R, Modarressi MH,Jeddi-Tehrani M, Dokoohaki P,Ghasemi J, Zarnani AH, et al. (2006).Thrombophilic mutations in Iranian patients with infertility and recurrent spontaneous abortion. Ann Hematol. 85(4): 268-271.

Carp, O. Salomon, D. Seidman, R. Dardik, N. Rosenberg, and A. Inbal,(2002).“Prevalence of genetic markers for thrombophiliain recurrent pregnancy loss,” Human Reproduction, 2002.17, pp. 1633–1637.

Christensen B, Frosst P, Lussier-Cacan S, SelhubJ, Goyette P, Rosenblatt DS, et al.(1997). Correlation of acommon mutation in the methylenetetrahydrofolate reductase gene with plasma homocysteine in patients with premature coronary artery disease. Arterioscler Thromb Vasc Biol. 17(3): 569-573

Donnelly JG.(2000). The 1298(A-->C) mutation of methylenetetrahydrofolatereductase should be designated tothe 1289 position of the gene. Am J Hum Genet. 66(2): 744-745.

Eckfeldt JH, Rosenberg IH, et al.(1996). Relation between folate status, a common mutation in methylenetetrahydrofolate reductase, and plasma homocysteine concentrations.Circulation. 93(1): 7-9.

Friso S, Girelli D, Trabetti E, Olivieri O, Guarini P, Pignatti PF, et al.(2005). The MTHFR 1298A>C polymorphism and genomic DNA methylation in human lymphocytes. Cancer Epidemiol Biomarkers Prev. 14(4):938-943.

Friso S, Choi SW. Gene-nutrient interactions and DNA methylation. J Nutr. **2002**; 132(8 Suppl): 2382S-2387S.

Frosst P, Blom HJ, Milos R, Goyette P, Sheppard CA, Matthews RG, et al.(1995). A candidate genetic risk factor for vascular disease: a common mutation in methylenetetrahydrofolate reductase. Nat Genet. 10(1): 111-113.

Goodman, C. B. Coulam, R. S. Jeyendran, V. A. Acosta,and R. Roussev,(2006).Which thrombophilic gene mutations are risk factors for recurrent pregnancy loss?” American Journal of Reproductive Immunology.56, pp. 230–236.

Jeddi-Tehrani, R. Torabi, A. H. Zarnani et al., (2011).“Analysis of plasminogen activator inhibitor-1, integrin beta3, beta fibrinogen, and methylenetetrahydrofolate reductase polymorphisms in Iranian women with recurrent pregnancy loss,” American Journal of Reproductive Immunology. 66, pp. 149–156.

Kluijtmans LA, Kastelein JJ, Lindemans J, Boers GH, Heil SG, Bruschke AV, et al.(1997).Thermolabile methylenetetrahydrofolate reductase in coronary artery disease. Circulation. 96(8): 2573-2577.

Miller SA, Dykes DD, Polesky HF.(1998).A simple salting out procedure for extracting DNA from human nucleatedcells. Nucleic Acids Res. 16(3): 1215.

Mtiraoui N, Zammiti W, Ghazouani L, Braham NJ,Saidi S, Finan RR, et al.(2006). MTHFR C677T and A1298C polymorphism and changes in homocysteine concentrations in women with idiopathic recurrent pregnancy losses. Reproduction. 131(2): 395-401.

Nakata Y, Katsuya T, Takami S, Sato N, Fu Y,Ishikawa K, et al.(1998). Methylenetetrahydrofolate reductase gene polymorphism: relation to blood pressure and cerebrovascular disease. Am J Hypertens. 11(8Pt 1): 1019-1023.

Naushad SM, Devi AR.(2010). Role of parental folate pathway single nucleotide polymorphisms in altering the susceptibility to neural tube defects in South India. J Perinat Med. 38(1): 63-69

Nelen WL, Steegers EA, Eskes TK, Blom HJ.(1997). Genetic risk factor for unexplained recurrent early pregnancy loss. Lancet. 350(9081): 861

Nelen WL, Blom HJ, Steegers EA, den Heijer M,Eskes TK.(2000). Hyperhomocysteinemia and recurrent early pregnancy loss: a meta-analysis. Fertil Steril. 74(6): 1196-1199.

Park, V. M.(1999).“Hypercoagulable state mutation analysis in white patients with early first-trimester recurrent pregnancy loss,” Fertility and Sterility.1999. 71, pp. 1048–1053

Sarig G, Younis JS, Hoffman R, Lanir N, Blumenfeld Z, Brenner B.(2002). Thrombophilia is common in women with idiopathic pregnancy loss and is associated with late pregnancy wastage. Fertil Steril. 77(2): 342-347.

Stern LL, Mason JB, Selhub J, Choi SW.(2000). Genomic DNA hypomethylation, a characteristic of most cancers,is present in peripheral leukocytes of individuals who are homozygous for the C677T polymorphism in the methylenetetrahydrofolate reductase gene. Cancer Epidemiol Biomarkers Prev. 9(8): 849-853.

Stonek, E. Hafner, K. Philipp, L. A. Hefler, E. K.,(2007).“Methylenetetrahydrofolate reductase C677T polymorphism and pregnancy complications,” Obstetrics and Gynecology. 110, pp. 363–368.

Unfried G, Griesmacher A, Weismüller W, NageleF, Huber JC, Tempfer CB.(2002).The C677T polymorphism of the methylenetetrahydrofolate reductase gene and idiopathic recurrent miscarriage. Obstet Gynecol. 99(4): 614-619.

Van der Put NM, Gabreëls F, Stevens EM, Smeitink JA, Trijbels FJ, Eskes TK, et al.(1998). A second common mutation in the methylenetetrahydrofolate reductase gene: an additional risk factor for neural-tube defects? Am JHum Genet. 62(5): 1044-1051.

Van der Put NM, Steegers-Theunissen RP, Frosst P,Trijbels FJ, Eskes TK, van den Heuvel LP, et al. (1995).Mutated methylenetetrahydrofolate reductase as a risk factor for spina bifida. Lancet. 346(8982): 1070-1071.

Yang Y, Zhang SZ, Li N, Zhang W.(2007).Single nucleotide polymorphism C677T in the methylenetetrahydrofolatereductase gene might be a genetic risk factor for infertility for Chinese men with azoospermia or severeoligozoospermia. Asian J Androl. 9(1): 57-62.

Yenicesu, M. Cetin, O. Ozdemir et al.,(2010).“A prospective case-control study analyzes 12 thrombophilic gene mutations in Turkish couples with recurrent pregnancy loss,” American Journal of Reproductive Immunology. 63, pp. 126– 136.

Zetterberg,B.Regland,M.Palm.S,(2002).Increased frequency of combined methylenetetrahydrofolate reductase C677T and A1298C mutated alleles in spontaneously aborted embryos,” European Journal of Human Genetics. 10, pp. 113–118.