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Association of the Role MTHFR Infertility 677C>T and 1298A>C Polymorphisms Variants as the Risk Factor with Human Male

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ABSTRACT

Background and objectives: Spermatogenesis is a multistage process that involves various genes coordinating it. Several reports revealed that mutations in the Methylenetetrahydrofolate reductase (MTHFR) genes have been associated with infertility among males. This study aimed at investigating the relationship between male-factor infertility and MTHFR polymorphisms (677C > T; 1298A > C) in an Egyptian population.

Subjects and methods: This case-control study was carried out in Mansoura University, Egypt on 100 cases with infertility and 100 healthy controls. We used T.ARMS-PCR methods to assess the genotypes of MTHFR (677C > T; 1298A > C) gene variants and we analysed hormone levels in serum (prolactin. E2, testestrone, FSH, LH, TSH and InhibinB) by Elisa method.

Results: Genotypic and allelic frequencies of MTHFR*C677T (rs1801133) variant in infertile males were compared to control males were significant. The expected and observed findings were consistent with the Hardy-Weinberg equation (HWE) in infertile cases as comparison with control men ($p< 0.05$). Infertile patients revealed significantly increased in C/T and TT of MTHFR*C677T genotype compared with healthy controls as respectively (56.7% vs. 40% and 43% vs. 0%), the Dominant model (OR148.5, 95% CI 19.89-1108.4, $p <0.001$). The frequency of MTHFR*C677T, T allele showed a significant increase in infertile men in comparison to controls (71 % vs. 20%, OR152.1, 95% CI 9.19-2516.8, $p< 0.001$). In contrast, regarding the codominant and Recessive, heterozygote and homozygote models, infertile men indicated significantly decreased compared with control men ($p< 0.001$). The 1298A/C polymorphism was linked to male-factor infertility. The frequencies of homozygote genotype CC in fertile patients compared to healthy group was significantly increased to AA genotype ($P < 0.001$), CA to

the AA (P = 0.006), CC to the CA + AA (P < 0.001), and CC + CA to the AA (P < 0.001) genotype showed a significant difference.

Key Words:

MTHFR, (677C > T; 1298A > C) Polymorphisms, infertility.

1. INTRODUCTION

Infertility is a major worldwide public health issue because of its direct societal and economic burden. Infertility is the failure to achieve a pregnancy after 12 months of unprotected intercourse [1]. Worldwide, about 10–15% of all couples are infertile. About 50% of infertile cases are because of male factors. Male infertility heterozygous disorder affected by many mutations genetic factors [2], that due to impaired spermatogenesis [3, 4]. The polymorphic activities or mutations in genes that regulate spermatogenesis contribute to 15-30% of infertility among males [5].

Male infertility is due to impaired spermatogenesis caused by neurogenic factors, genital tumours, aplasia germ cells, impaired sperm transport, or environmental pollutants [6]. Other many factors are associated with male infertility including hormonal changes, impotence, infection, anti-sperm antibodies, exposure to chemicals and radiation, testicular cancers and varicoceles [7].

Folates are a group of co enzymes which have an important role in DNA synthesis, DNA methylation and protein synthesis. The deficiency of folates might be associated with an impairment of these metabolic pathways resulting in homocysteine accumulation, with associated increased oxidative damage and chaotic methylation reactions [8].

Previous studies reported association between folate levels, density, and normal morphological characteristics of sperms [9]. Thus, the folate pathway has a significant role in male fertility [10]. The MTHFR is a crucial enzyme in folate metabolism and it balances the storage of methyl groups between DNA synthesis and methylation [11].

DNA methylation is an epigenetic feature, which plays a crucial role in in sperm production via regulation of gene expression. The MTHFR mutations (C677T and A1298C) decrease the enzymatic activity and thus can result in male infertility [6].

The MTHFR gene, located on the short arm of chromosome1 (1p36.3), which is comprised of 11 exons, the nucleotide of C changed to T at position MTHFR 677C>T of the gene causes the substitution of alanine by valine in the MTHFR protein and causes a decrease in enzymatic activity. The MTHFR 677C>T variant decreases the enzymatic activity by 35% in heterozygosis and by 70% in homozygosis [12]. Overall, impaired enzyme activity because of MTHFR polymorphisms can contribute to infertility.

Another common single nucleotide polymorphism (SNP) is MTHFR (A1298C). The latter causes a mutation of adenine to cytosine in exon 7 points of the gene, where glutamate position is replaced with alanine (Glu429Ala) [13]. The MTHFR A1298C polymorphism also decreases the enzymatic activity. Of note, the MTHFR A1298C polymorphism is the most controversial among various ethnicities [14].

Several studies evaluated the correlation between infertility and MTHFR polymorphisms C667T and A1298C [12]. For the C677T polymorphism, the C allele was found to be 0.94-fold more associated with azoospermia than the T allele [12].

2. STUDY AREA

Arab Republic Of Egypt.

3. Subjects AND METHODS

Patients and controls

The current study conducted on 100 volunteers aging 20–50 years attending Andrology outpatient clinic Mansoura University Hospital, patients affected by infertility with normal AZF. Other group100 apparently healthy fertile individuals

All patients included Age > 20 years (at time of AZF diagnosis by semen analysis regarding cases and at time of sampling regarding controls). Matched healthy fertile controls free from clinical manifestations and family history of infertility were included. We ruled out any patient having any AZF abnormality. All control males were non-smokers, did not have history of any disease, or were on a chronic use of any medications.

Blood samples: The levels of hormones prolactin, E2, FSH, TSH, LH, testosterone, InhibinB, were measured in serum by Elisa method, obtained from (R & D systems, Minneapolis, MN, US) according to the manufacturer's instructions.

Extraction and processing of genomic DNA:

Genomic DNA of all participated individuals underwent extraction from the whole blood utilizing GentraVR puregeneVR kit with column technique provided by (QIAGENTechnologies, Minneapolis, MN, US).

One part allowed to clot for 10–15 min then it was centrifuged.

Genotyping of MTHFR 677C > T variant (rs1801133):

These polymorphisms were analyzed by T.ARMS-PCR. The reactions of MTHFR 677C > T were performed according to Cassar, et al. [15]. primer sequences used for ARMs PCR MTHFR 677C > T genotyping are:

677-Common F: CCCAGCCACTCACTGTTTTAGTTCAGGC

677-Common R: GGTGAGAGTGGGGTGGAGGGAGCTTAT

677-C: CAAAGAAAAGCTGCGTGATGATGAAATAGG

677-T: TTGAAGGAGAAGGTGTCTGCGGGCGT

The total volume of PCR reaction mixture was 25 µl. The reaction contained 3 µl of each primer (10 pmol/µl),10 µl of Green master mix (Promega), mixed with 3 µl of DNA in tow PCR tube one for C allele and other for T allele. Cycling conditions included a denaturation cycle for 5 minutes at 95°C. It was followed by 33 Cycles, denaturation at 95°C for 25 sec, Annealing at 60°C for 30 sec and Extension 72°C for 25 C and Final extension 72°C for 10 minutes. Electrophoresis (2.5% agarose gel, stained with ethidium bromide) of PCR products was photographed under ultraviolet light. MTHFR 677C > T genotypes were appeared as CC at 273 bp, CT 273, 190, and TT at 190 bp Fig.1.

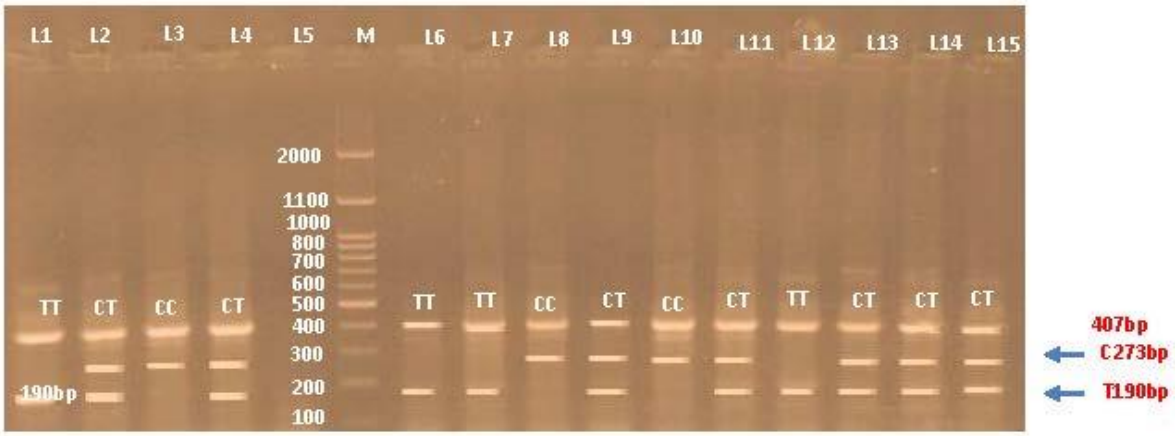

Fig 1. The electrophoresis (2.5% agarose gel, stained with ethidium bromide) of PCR products showing the MTHFR c.677C>T genotyping Polymorphism of male infirmity by T-ARMS-PCR. Lanes 1, 6, 7, 12, represent TT homozygote genotyping where band appeared at 190 bp, and internal control at 407bp, while lanes (2, 4 , 9, 11, 13, , 14 and 15) showing CT heterozygote genotyping where both C allele and T allele were present and internal control at 407bp. Lanes (M represent DNA Ladder 100bp). Lanes 3, 8, 10 represent CC homozygote genotyping where band appeared at 273 bp, and internal control at 407bp.

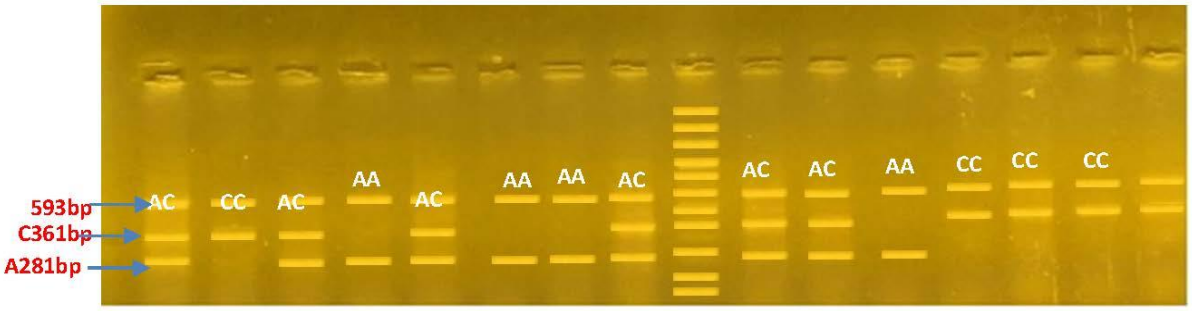

Fig. 2. PCR products of the MTHFR c.1298A>C genotyping in male infertility, whereas represent PCR Products of T-ARMS-PCR by Agarose gel Electrophoresis to detect the MTHFR A1298C Polymorphism. Lanes (1, 3, 5,8,9, 10,) showing AC heterozygote genotyping where both alleles A and C were present at 281 and 361 bp. and internal control at 593 bp, Lanes (2, 12 13, 14, 15) represent CC homozygote genotyping where band appeared at 361 bp and internal control at 593 bp. Lane (4, 6,7) represent AA homozygote genotyping where band appeared at 281 bp and internal control at 593 bp. M represent DNA Ladder 100bp.

Genotyping of MTHFR A1298C polymorphism variant (rs1801131):

PCR mixture of prepared in 25 µl total volume containing 2.5 µl from each primers (10 pmol/µl), 12 µl master mix, added to 3 µl DNA in a same PCR tube.

primer sequences used for ARMs PCR MTHFR A1298C genotyping are:

1298-Common F: GAAGAAGTTTGCATGCTTGTGGTTG

1298-Common R: CAGGCAAGTCACCTGGGAGAGA

1298-A: GGCAAAGAACGAAGACTTCAAAGACACATT

1298-C: GAGGAGCTGACCAGTGATGC

Cycling conditions included a denaturation cycles for 5 minutes at 95°C. It was followed by 30 cycles denaturation at 95°C for 25 sec, Annealing at 54.5.°C for 30 sec and Extension 72°C for 40 C and Final extension 72°C for 5 minutes. Electrophoresis (2.5% agarose gel, stained with ethidium bromide) of PCR products was photographed under ultraviolet light [15]. then photographed by digital camera. MTHFR

A1298C genotypes were appeared as 281 bp with A allele, AC281, 136 and 593, band at 361 bp with the C allele.

Statistical analysis:

Data were analysed by the Stata Statistical Software, Release #17 (StataCorp. 2021, College Station, TX: LLC). The G*power program v3.1.9.7 (http:// www. gpower. hhu.de/) was utilized to process the study power indicating that it could provide a power of 88% with an effect size corresponding to 0.28 for the sample size of 200 for both study groups.

The allelic/genotypic frequencies of MTHFR*(C677T and A1298C) gene variants underwent calculation by SNPstats bioinformatic tool (www. SNPst ats. net) [16, 17]. The odds ratio (OR) and the 95% confidence interval (CI) were estimated by different genetic association models [18]. The significance level was set at p-value < 0.05 [19].

4. RESULTS AND DISCUSSION

Results:

Characteristics of the studied participants

The PRL, FSH, LH levels were significantly higher among patients in comparison to control subjects (P <0.001). In contrast, T.Testosterone, Inhibin B level, and Estradiol; E2 (pg/ml) was significantly lower among infertile males than in control subjects (p<0.001 for each).

A non-significant difference existed between both groups as regards in TSH concentrations (p =0.325). in Table 1.

Table 1. The biochemical measurements of studied groups.

Variable	*Levels*	*Healthy controls* (n=100)	*AZF patients* (n=100)	*P-value*
II. Biochemical measurements				
1. Inhibin B (pg/ml)	*Median (IQR)*	55.0 (37.5-95.5)	16.0 (11.5-22.5)	< **0.001**
2. TSH (uIU/ml)	*Median (IQR)*	2.70 (1.80-3.00)	2.75 (2.00-3.30)	0.325
3. Estradiol; E2 (pg/ml)	*Median (IQR)*	29.0 (21.5-38.0)	21.0 (9.50-37.5)	< **0.001**
4. Total Testosterone (ng/ml)	*Median (IQR)*	4.00 (3.90-7.00)	2.30 (1.60-7.00)	< **0.001**
5. Prolactin; PRL (ng/ml)	*Median (IQR)*	5.40 (4.00-7.00)	19.0 (12.0-24.0)	< **0.001**
6.Luteinizing hormone; LH (uIU/ml)	*Median (IQR)*	3.00 (1.90-4.00)	10.5 (5.50-18.5)	< **0.001**
7. Follicle-stimulating hormone; FSH (uIU/ml)	*Median (IQR)*	3.40 (2.30-6.00)	14.0 (8.00-26.0)	< **0.001**
Note: Data are represented as frequencies (%) or median (IQR). Fisher's exact and two-sample Wilcoxon rank-sum tests were used.				

Relationship between MTHFR 677C > T variant (rs1801133) and male-factor infertility:

Genotypic and allelic frequencies of MTHFR*C677T (rs1801133) in the two study groups were shown in Table 2. The expected and observed findings were consistent with the HWE among infertile males compared with controls (p< 0.05). Analysis of different genetic models was processed involving allelic, dominant, recessive, heterozygote and homozygote models.

Infertile patients revealed significantly increased in C/T and TT of MTHFR*C677T genotype compared with healthy controls as respectively (56.7% vs. 40% and 43% vs. 0%) that was noteworthy in the Dominant model (OR148.5, 95% CI 19.89-1108.4, p <0.001). Also, the frequency of MTHFR*C677T, T allele was significantly higher among infertile men versus controls (71 % versus 20%, OR152.1, 95% CI 9.19-2516.8, p< 0.001). In contrast, as regards the codominant and Recessive, heterozygote and homozygote models, infertile men indicated significantly decreased of the MTHFR*C677T (rs1801133) variant compared with controls (p< 0.001) (Table 3).

Table 2. Genotypic/allelicfrequencies of the MTHFR* 677C > T gene variants.

Genetic polymorphisms	All participants	Healthy controls	AZF patients	*OR (95% CI)*	*P-value*
1. MTHFR*C677T gene variant					
Genotypic frequencies	**n (%) 200**	**n (%) 100**	**n (%) 100**		
C/C	61 (30.5)	60 (60.0)	1 (1.0)	1.0	
C/T	96 (48.0)	40 (40.0)	56 (56.0)	**84.0 (11.2-631.6)**	**< 0.001**
T/T	43 (21.5)	0 (0.0)	43 (43.0)	**3509 (139.6-88196.4)**	**< 0.001**
HWE	χ^2=0.21, p=0.649	χ^2=6.25, p=0.012	χ^2=12.9, p<0.001		
Allelic frequencies	**n (%) 400**	**n (%) 200**	**n (%) 200**		
C allele	218 (54.4)	160 (80.0)	58 (29.0)	1.0	
T allele	182 (45.5)	40 (20.0)	142 (71.0)	**9.79 (6.17-15.5)**	**< 0.001**

Table 3. Genetic association models of MTHFR*(C677T) gene with susceptibility for male infertility.

Model	Genotypes	Healthy controls	AZF patients	*OR (95% CI)*	*P-value*
1. MTHFR*C677T gene variant		**n (%) 100**	**n (%) 100**		
Codominant	**C/C**	60 (60.0)	1 (1.0)	1.0	**< 0.001**
	C/T	40 (40.0)	56 (56.0)	**84.0 (11.2-631.6)**	
	T/T	0 (0.0)	43 (43.0)	**3509 (139.6-88196.4)**	
Dominant	**C/C**	60 (60.0)	1 (1.0)	1.0	**< 0.001**
	C/T + T/T	40 (40.0)	99 (99.0)	**148.5 (19.89-1108.4)**	
Recessive	**C/C + C/T**	100 (100.0)	57 (57.0)	1.0	**< 0.001**
	T/T	0 (0.0)	43 (43.0)	**152.1 (9.19-2516.8)**	
Log-additive	---	---	---	**116.1 (16.03-840.8)**	**< 0.001**

Relationship between MTHFR*A1298C gene variant (rs1801131) and male-factor infertility:

The 1298A/C polymorphism has a relationship with the male-factor infertility susceptibility, the frequencies of homozygote genotype CC in fertile patients compared to healthy group was significantly increased to AA genotype (P < 0.001), CA to AA genotype (P =0.006), while CA, AA genotypes were significant decreased (P < 0.05). where Recessive model, CC to the CA + AA (P < 0.001), and Dominant CC + CA to the AA (P < 0.001) genotype showed a significant difference, Table 4.

Table 4. Genetic association models and frequency of the MTHFR A1298C gene variant with susceptibility for infertility in males.

Genetic polymorphisms ***MTHFR*A1298C gene***	**Genotypes**	**Healthy controls n**	**patients n**	***OR (95% CI)***	***P-value***
Genotypic Model		**n (%) 100**	**n (%) 100**		
Codominant	**A/A**	22 (22.0)	3 (3.0)	1.0	**< 0.001**
	A/C	39 (39.0)	29 (29.0)	**5.45 (1.49-19.9)**	**< 0.001**
	C/C	39 (39.0)	68 (68.0)	**12.8 (3.59-45.5)**	**< 0.001**
Dominant	**A/A**	22 (22.0)	3 (3.0)	1.0	**< 0.001**
	A/C + C/C	78 (78.0)	97 (97.0)	**9.12 (2.63-31.6)**	**< 0.001**
Recessive	**A/A + A/C**	61 (61.0)	32 (32.0)	1.0	**< 0.001**
	C/C	39 (39.0)	68 (68.0)	**3.32 (1.86-5.94)**	
Log-additive	---	---	---	**2.94 (1.86-4.66)**	**< 0.001**
Genotypic frequencies	**n (%) 200**	**n (%) 100**	**n (%) 100**		
A/A	25 (12.5)	22 (22.0)	3 (3.0)	1.0	
A/C	68 (34.0)	39 (39.0)	29 (29.0)	**5.45 (1.49-19.9)**	**0.006**
C/C	107 (53.5)	39 (39.0)	68 (68.0)	**12.8 (3.59-45.5)**	**< 0.001**
Hardy–Weinberg equilibrium	χ^2*=6.67, p=0.009*	χ^2*=3.87 p=0.049*	χ^2*=0.002, p=0.965*		
Allelic frequencies	**n (%) 400**	**n (%) 200**	**n (%) 200**		
A allele	118 (29.5)	83 (41.5)	35 (17.5)	1.0	
C allele	282 (70.5)	117 (58.5)	165 (82.5)	**3.34 (2.11-5.30)**	**< 0.001**

Note: Data are represented as frequencies with percents. Chi-square test was used.
Bold values indicate significant result ($P < 0.05$).

5. DISCUSSION

DNA methylation has a crucial role in regulating gene expression during spermatogenesis. The MTHFR enzyme catalyzes the production of folate intermediates which are important for DNA synthesis and its methylation [20]. Serum folate level has a significant association with the concentration and motility of sperms [21].

Our study revealed significant higher values of FSH, LH and PRL in cases compared to controls, this results confirmed by [22-24] demonstrated that, FSH and LH values among infertile men showed a significant increase compared to fertile group. Elsaid, et al. [24] displayed that, prolactin concentrations were higher in cases. While T. Testosterone and Inhibin B level and Estradiol; E2 demonstrated significantly lower value in case group in comparison to controls this result confirmed by Elsaid, et al. [24].

Our study evaluated the likelihood of a relationship between MTHFR polymorphisms (C677T and A1298C) and male-factor infertility. Our results of infertile patients revealed significantly increased in C/T and TT of MTHFR*C677T genotype compared with healthy controls as respectively (56.7% vs. 40% and 43% vs. 0%, $p < 0.001$) confirmed by the report of Alhumaydhi, et al. [25] showed MTHFR 677 CT genotype 48.2%, and TT genotype 15.6%.

Our results consistent with a previous studies which revealed a correlation between MTHFR C677T polymorphism and male-factor infertility in Germany, Netherlands, Italy, India, South Korea and China, [26-28], this report in agreement with Zalata, et al. [29] for Egyptian cases, The Frequency of MTHR C677T genotypes and T allele showed a significant increase in cases where (CT 30.7 and TT 42.7%) this result similar to Gava, et al. [30] on Brazilian, in Indians population who found frequency of mutated allele 677T and TT genotype ($P < .001$). this reports support our results.

Our finding are in line with Xie and co-workers [31] who reported that the frequencies in the MTHFR C677T was significantly different in control males in comparison with patients where CT (39.5% versus 50%, P=0.0046) and TT (51.2% versus 7.7%). The mutant allele T was a risk factor for oligospermia (70.9% versus 32.7%). The CT genotype and the TT genotype were significantly higher in patients versus controls [59.3% versus 50% (P=0.008) and 22.2% versus 7.7% (P=4.44), respectively]. The mutant allele was a risk factor for asthenospermia (51.9% versus 32.7%, P=0.0005). The present study supported by Huang, et al. [32], suggested that T allele of MTHFR 677, was a risk factor for male-factor infertility. This study confirmed by previous researches of meta-analysis in Asian population [33, 34]. Our findings agree with that reported by Shi, et al. [35]. This study was also in line with another report revealing that 677T was a risk factor for infertility among Indian [27] and Brazilian men [36]. Inconsistent with our results, Ni and colleagues [37] found that MTHFR C677T SNP was not a risk factor for infertility among Chinese men [37, 38]. In Italy contrast to our results, [39].

Studies revealed that MTHFR polymorphisms were correlated with infertility among South East Asian, Indian, and African males. Such observation are consistent with the current study. These results were absent in the European men [27, 40, 41] and in Caucasians [42]. Study by Tavares, et al. [43] reported that genotype and allelic distribution of MTHFR polymorphisms did not show a significant association with infertility that confirmed by Balunathan, et al. [44] ($p > 0.05$). Whereas [45, 46] reported data are conflicting of MTHFR gene polymorphisms on infertility among males.

In contradiction with the study by [47, 48] demonstrated that both 677C/T polymorphisms was significantly correlated with male-factor infertility.

Our study confirmed by Aliakbari, et al. [6] were found MTHFR 677C/T variant genotypes demonstrated a significant association with infertility among males, by ethnicity analysis. This results

were supported in a meta-analysis by Nikzad and others [49], in which MTHFR 677C/T polymorphism was significantly associated with infertility in males. There results were consistent with previous meta-analysis results by [42, 50-52].

Meta-analysis by Liu and co-workers [13] indicated an association between C677T mutation and infertility in males. All of the genetic models showed a significant association with an enhanced risk of male-factor infertility with C677T mutation. In addition, the TT genotype was a risk factor among Asian men in all genetic models homozygote model ($P < 0.001$) and in American men for the homozygote comparison but a protective factor ($p = 0.033$) but in African men, it was in the dominant model and allele ($p = 0.036$). These researches are consistent with our results.

Our results were confirmed by another meta analysis which concluded 26 studies of MTHFR C677T polymorphism demonstrated a significant correlation with infertility among males [52]. Another study on 360 non obstructive infertile males found a relationship between MTHFR C677T and azoospermia [28].

Our result showed that Genotypic frequencies of MTHFR A1298C gene variant homozygote A/A, and heterozygote AC significant increase in control group than patients group (22% vs 3%),(39%vs 29%), ($p< 0.05$) while homozygote CC was significantly higher in infertile men compared with control subjects ($p<0.001$), these finding is consistent with Zalata, et al. [29] showed a significant increase in male infertility where 1298AA genotype frequency showed a significant increase among fertile group in comparison to cases group. Our result similar to Balunathan, et al. [44] conclude this variant a close association with infertility in males. Also our analysis supported by Xie, et al. [31] report in Morocco, the frequency of CC was higher among infertile males compared to fertile males ($P = 0.014$).

Contrary the present study disagreement of research by Lee and others [28] reported no relationship between SNP A1298C and infertility among Indian males, supported a previous report by Dhillon, et al. [53] and Chinese [13]. This similar to report Ren, et al. [33] concluded MTHFR A1298C were not risk factors of male infertility. Also, the frequencies of AC and CC were insignificant between fertile and normal Korean males [46]. There was also no relationship between c.1298A.C and infertility among French and Moroccan males [51].

6. CONCLUSION

In conclusion, our findings show that MTHFR gene polymorphisms (C677T and A1298C) were associated with a high risk of infertility among Egyptian males. Thus MTHFR (C677T, A1298C) variants could be considered as a diagnostic indicator of infertile cases. The confliction between our findings and other studies might probably be because of the gene-nutrient, environmental and gene racial, ethnic interactions [54].

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