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# Association of Hspa1b and Hspa1l Genes Polymorphisms with Risk of Severe Oligozoospermia in Sample of Iraqi Patients

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**ABSTRACT**

The present study was conducted to investigate the association between some single nucleotide polymorphisms in *hspa1b* and *hspa1l* genes with the incidence of severe oligozoospermia in Iraq. Blood samples were obtained from Kamal Al-Samraee hospital for fertility, infertility and *in vitro* fertilization –Baghdad - Iraq .Blood samples were collected from 50 of severe oligozoospermic patients and 50 apparently healthy subjects (control group). Data related with age, smoking status and semen parameters (concentration, semen volume, sperm motility and sperm abnormality) were obtained using questioner forma for each patient. DNA was extracted from all blood samples by using Promega Kit ,then the extracted DNA was used for amplification of targeted fragments genes using PCR.PCR products were incubate with the restriction enzymes used in this study(*PstI* and *NcoI* ,respectively). Then subject to electrophoresis for identification the genotypes of rs1061581 G > A SNP in *hspa1b* gene and rs2227956 C > T SNP in gene. The PCR products of positive samples were sent for sequencing to confirm the results.As related with rs1061581 G >A SNP (*hspa1b* gene),the frequency of GA genotype was significantly ($p < 0.05$) higher in control group than in severe oligozoospermic patients (48 *versus* 40% ,respectively).Whereas , the frequency of AA genotype was significantly ($p < 0.05$) higher in severe oligozoospermic patients group than in control group (38 *versus* 24% ,respectively).G allele frequency was 52 and 42% ; and A allele frequency was 48 and 58 % in control and severe oligozoospermic patients group , respectively. As related with rs2227956 C > T SNP (*hspa1l* gene) , the frequency of TT genotype was significantly($p<0.05$) higher in severe oligozoospermic patients group than in control group( 42 *versus* 34 % ,respectively) .C allele frequency was 41 and 35% ; and T allele frequency was 59 and 65% in control and severe oligozoospermic patients group ,respectively. It can be concluded that homozygous mutants (AA genotype in rs1061581 G>A of *hspa1b* gene and TT genotype in rs2227956 C>T of *hspa1l* gene) were associated with the incidence of sever oligozoospermia in Iraqi patients.



## Introduction

The World Health Organization (WHO) defines infertility as a disease of the reproductive tract characterized by the failure to achieve pregnancy after 12 months or more of regular unprotected sexual intercourse(Evers, 2002; Smith *et al.*, 2003; Dohle *et al.* ,2004; Zegers-Hochschild *et al.*,2009). Infertility has increased over the last 30 years (WHO, 2000), WHO announced that the number of infertile couples has been increasing worldwide, up to 2 million per year. In 2003, infertility was reported as the most prevalent chronic health disorder concerning couples regardless of age.The male partner accounts for the infertility 40% of the time, 40% from the female partner as well and 20% shared by both the man and the woman (Doherty and Clark, 2006; Garner, 2012). An undeniable fact is the reproductive age, to which infertility is related. The maximal female fertility is reached at the age of 19 to 29. On a contrary, male fertility potential is not limited (Wallace *et al.*, 2010). The background of infertility is based on congenital, hormonal, morphological and immunological disorders (Doherty and Clark, 2006).

It may also be related to other diseases. Nevertheless, a specific, 100% sure, cause has never been diagnosed. In 15% of all cases, the idiopathic cause is suggested, further linked to immune infertility (Alam, 2009). In Iraq infertility is a problem among men and women and considered as an important public health and clinical problem.In Iraqi study, carried out during January 2000 to May 2001 (Razzak and Wais, 2002), studied causes of infertility for 250 couples, 193 (77.2%) of whom had primary and 57 (22.8%) secondary infertility. Male infertility was found in 36.8% of cases. These percentages give a strong indication of infertility among Iraqi couples .Oligozoospermia is a low sperm count which means that the men who have a sperm concentration of less than 20 million/ml are to be classified as oligozoospermic (WHO Laboratory Manual, 1999). It is a cause of human male infertility.

Male causes for infertility are found in about 50% of infertile couples (Pryor *et al.*, 1997; Ambasudhan *et al.*, 2013). Reduced male fertility can be a result of congenital and acquired abnormalities.

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They include infections of the genital tract, varicocele, developmental and anatomical abnormalities, endocrinepathies, immunological factors, environmental exposures, and genetic abnormalities. Frequently, however, male infertility is difficult to diagnose, and about 60-75% of cases remain idiopathic. These idiopathic cases present with no previous history associated with fertility problems and have normal findings on physical examination (Dohle *et al.*, 2004).Contemporarily, a considerable and substantial attention has been focused on the role of genetic factors in spermatogenesis failure. It has been estimated that over 4000 genes are involved in the genetic control of human spermatogenesis (Gianotten *et al.*, 2004). Some of them have been outlined and a number of genes related to infertility have been cloned, while other genes have been assigned to specific chromosome regions, but the majority of them remain undeciphered. Genetic anomalies causing male infertility can be roughly classified into three major groups: (1) chromosomal aneuploidies and rearrangements where batteries of genes on specific chromosomes have increased/decreased their expression dosage or changed their normal genomic environment; (2) interstitial deletions, whether microscopic or submicroscopic, where deletions or rearrangements of multiple genes mapped in a molecular neighborhood have changed their normal expression pattern, and (3) single gene defects where the expression of a single key element gene is changed or lost causing then male infertility (Vogt, 1996).

There are many processes involved in protecting the cell against the harmful effects of cellular stress one of these isthe synthesis of a protein family called heat shock protein (HSP) (Tóth *et al.*, 2015).The induction of HSPs is a critical and highly conserved cellular response, which protects cells from a range of stresses, including damage caused by normal physiological processes, extreme environmental stress or disease (Gullo e*t al.*, 2004). They are very important in all stages of cell metabolism, including growth, differentiation, division and even cell death the HSPA1 family is the most highly conserved of the many HSP families (Beere *et al.*, 2001). The present study was planned to investigate the analysis of single nucleotide polymorphism in heat shock protein genes (*hspa1b* and *hspa1l*) in Iraqi patients with severe oligozoospermia. Therefore, the aims of the present study were to determine the frequencies of studied SNPs (rs 1061581 in *hspa1b* and rs2227956 in *hspa1l* ) in Iraqi patients with severe oligozoospermia and to study the association between homozygous and heterozygous SNPs in these genes with the risk of severe oligozoospermia in Iraqi patients.

## Materials and Methods

Patients have selected according to clinical and laboratory examination. Men with severe oligozoospermia were examined and diagnosed as related with infertility totally 50 blood samples were collected from severe oligozoospermic patients and 50 apparently healthy individuals (Control) during a period from 1 November 2015 to 1 February 2016. Infertile patients with well-known pathological features, such as varicocele, leukospermia , hormonal abnormalities and/or obstrction were excluded. Also excluded were those with cryptorchidism, vasectomy, abnormal liver function, cigarette smoking and alcohol .The forma of severe oligozoospermia patients information in this study include the name, sex , age, height, weight, smoking ,years of diagnosis. Five ml of blood has been collected by vein from 50 cases (severe oligozoospermia) and Five ml of blood has been collected by vein from 50 Healthy men (control) were collected from Kamal AL.Samraee Hospital for Fertility, Infertility and *in vitro* Fertilization/ Baghdad. DNA was extracted from whole blood samples by using Wizard genomic DNA purification kits (Promega, USA).

The DNA concentration of samples were estimated by using Nanodrop by putting 1*µl* of the extracted DNA in the machine to detect concentration in *ng/µl* and the purity detected by noticing the ratio of optical density (OD) 260/280 nm to detect the contamination of samples with protein. The accepted 260/280 ratio for pure DNA was between 1.7-1.9. After genomic DNA extraction, agarose gel electrophoresis was adopted to confirm the presence and integrity of the extracted DNA.

PCR was performed using specific primers. Primer pairs were supplied by Alpha DNA, (Canada) as a lyophilized product of different *picomols* concentrations. Lyophilized primer was dissolved in a free DNase/RNase water to give a final concentration of (100 pmol/µl) (as stock solution) to prepare 10µM concentration as work primer resuspended 10 pmol/µl in 90 µl of deionized water to reach a final concentration 10µM. The sequences of these primers are listed in Table 1.

**Table 1. Sequences of primers used in the present study**.

| Genes | Primer * | | Band size |
|---|---|---|---|
| *hspa1b* | Forward | 5′- CATCGACTTCTACACGTCCA-3′ | 878 bp |
| | Reversed | 5′- CAAAGTCCTTGAGTCCCAAC -3′ | |
| *hspa1l* | Forward | 5′-GGACAAGTCTGAGAAGGTACAG-3′ | 1117 bp |
| | Reverse | 5′- GTAACTTAGATTCAGGTCTGG-3′ | |

- Liu *et al.* (2007).

### Genotyping

### Hspa1b gene

PCR was carried out in a total volume of 25 µl, the reaction components were as described in Table 2, and then PCR eppendroff tubes were then placed in PCR device and PCR amplification was according to the program described in Table 3.Then PCR product was detected as 1117 bp on 2% agarose gel for one hour and were visualized by ethidium bromide staining, by used 100 bp DNA ladder. Then, PCR products were digested with *PstI* enzyme for 2 hours at 37 $^{o}$C described in Table (4 ), that cut 1117 bp PCR products contained wild type( G )allele into 183 and 934 bp fragments and the A allele yielded undigested fragment (1117 bp). The digested products were separated on 2% agarose gel and visualized by ethidium bromide staining.. Both 183 and 934 bp represent GG genotype, 183, 934 and 1117 bp represent GA genotype while 1117 bp fragment only represent AA genotype.

**Table 2.PCR reaction Components for amplification of hspa1b gene.**

| Component | µl |
|---|---|
| PCR pre Mix (Ready-to-se): *Taq* DNA polymerase , dNTPs, MgCl2 and reaction buffer (PH 8.5) | 12.5 |
| Forward primer | 1 |
| Reverse primer | 1 |
| DNA template | 4 |
| D.W | 6.5 |
| Final volume | 25 |

**Table 3. PCR amplification program of *hspa1b* gene.**

| Steps | Temperature (C°) | Time | Cycles |
|---|---|---|---|
| Initial denaturation | 94 | 3 minutes | 1 |
| Denaturation | 94 | 30 seconds | 35 |
| Annealing | 58 | 30 seconds | |
| Extension | 72 | 30 seconds | |
| Final extension | 72 | 5 minutes | 1 |

**Table 4. Reaction component for *PstI* enzyme digestion.**

| Component | µl |
|---|---|
| PCR product | 10 |
| PstI Enzyme | 0.5 |
| Buffer | 2 |
| D.w | 7.5 |
| Total olume | 20 |

**hspa1l gene**

PCR was carried out in a total volume of 20 µl, the reaction components were as described in Table 5, and then PCR eppendroff tubes were then placed in PCR device and PCR amplification was according to the program described in Table 6.Then PCR product was detected as 878 bp on 2% agarose gel for one hour and were visualized by ethidium bromide staining, by used 100 bp DNA ladder. Then PCR products were digested with 0.5 U *Ncol* enzyme for 2 hours at 37 ºC as described in Table 7. The digested products were separated on 2 % agarose gel and were visualized by ethidium bromide staining. Only 878 bp represent CC genotype, 554 and 324 bp represent TT genotype, 324, 554 and 878 bp represent CT genotype.

**Table 5. PCR reaction components for amplification of hspa1l gene.**

| Component | µl |
|---|---|
| PCR pre Mix (Ready-to-se): *Taq* DNA polymerase , dNTPs, MgCl2 and reaction buffer (PH 8.5) | 12.5 |
| Forward primer | 1 |
| Reverse primer | 1 |
| DNA template | 4 |
| D.W | 6.5 |
| Final volume | 25 |

**Table 6. PCR amplification program of *hspa1l* gene.**

| Steps | Temperature (C°) | Time | Cycles |
|---|---|---|---|
| Initial denaturation | 94 | 3 minutes | 1 |
| Denaturation | 94 | 30 seconds | 30 |
| Annealing | 60 | 30 seconds | |
| Extension | 72 | 30 seconds | |
| Final extension | 72 | 5 minutes | 1 |

**Table 7. Reaction component for *Ncol* enzyme digestion.**

| Component | µl |
|---|---|
| PCR product | 10 |
| Ncol Enzyme | 0.5 |
| Buffer | 2 |
| D.W | 7.5 |
| Total volume | 20 |

**Sequencing**

PCR product of positive samples were sent for sequence analysis. Twenty µl of PCR product for each sample were sent and 25 µl (10 pmol) from the forward primer.

The result of the sequence analysis was analyzed by BLAST in the NCBI by alignment with the refseq.

**Statistical Analysis**

The Statistical Analysis System- SAS (2012) program was used for comparing between study groups. Chi-square test was used to compare between percentages of genotypes of study groups and Odd ratio was used to identify of risk level.

**Results and Discussion**

**The heat shock 70kDa protein 1B (HSPA1B) gene**.

The *hspa1b* gene polymorphism at c.1059G>A (rs1061581) SNP, 1117 bp fragment (1086-2202; NM_005346.4) was targeted for amplification by the forward and reverse primers that used by Ciftci *et al.* (2015) using PCR (Figure 1). The rs1061581 SNP is locate within this fragment at 1269 position (G). The polymorphisms of this SNP were detected using PCR-RFLP. The conversion of G allele to A allele at this position abolish the site for *PstI* restriction enzyme ( CTGCAG to CTGCAA). Therefore, after digestion of PCR product with this enzyme, the fragments that were obtained were as follows : 183 and 934 bp for GG genotype; 183, 934 and 1117 bp for GA genotype and 1117 bp for AA genotype ( Figure 2). The other name for this SNP is c.1059G>A and ( g.31816809G>A , *NC_000006.12*). This SNP is silent because both CAG and CAA codons are code for the amino acid, glycine.

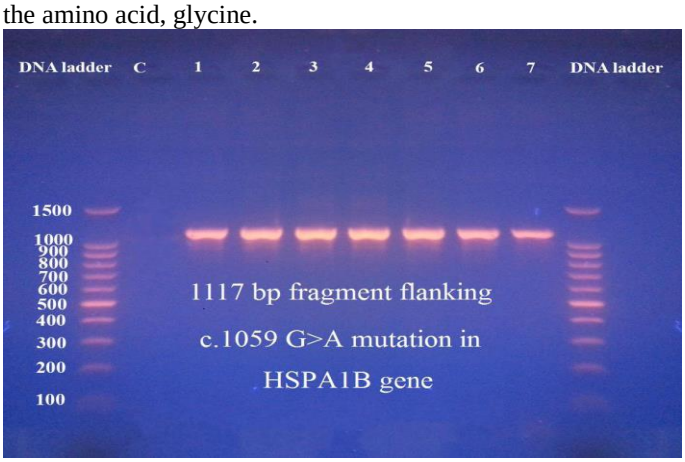


**Figure 1. PCR product (1117 bp) of targeted fragment ( HSPA1B gene) flanking the rs1061581G>A SNP (*NM_005346.4* ) visualized under UV light after staining with ethidium bromide.**

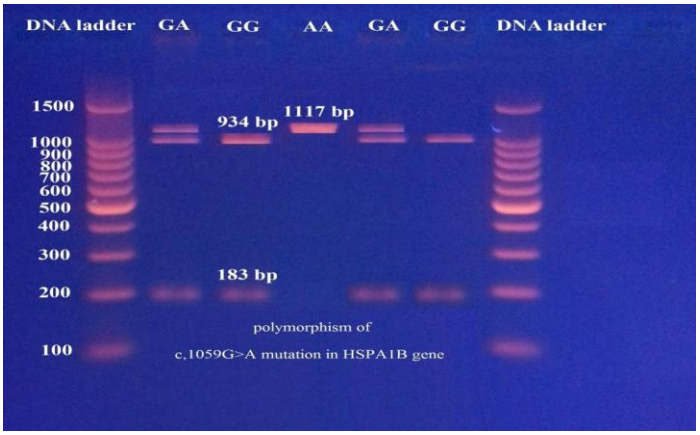


**Figure 2. PCR product (1117 bp fragment) of HSPA1B gene digested with *PstI* restriction enzyme and electrophoresed on 2% agarose. The genotypes at 1269 position (*NM_005346.4* ) are: GG (183 + 934 bp), GA (183 + 934 and 1117 bp) and AA (1117 bp).**

Table 8 show the frequency of rs1061581 SNP in severe oligozoospermic patients and apparently healthy control subjects. There was no significant difference between control subjects and severe oligozoospermic patients as related with the frequency of GG genotype. The frequency of heterozygous SNP (GA) was significantly ($p<0.05$) higher in control subjects than in patients ( 48 *versus* 40%, respectively, OR= 0.569 ; $X2$= 4.175). In contrast, the frequency of homozygous SNP (AA) was significantly ($p<0.05$) higher in patients than in

control subjects ( 38 *versus* 24%, respectively, OR= 0.598; *X2*= 5.084). The percentages of G allele were 52 and 42 % in the control subjects and patients , respectively, while, the percentages of A allele were 48 and 58 % in the control subjects and patients, respectively.

**Table 8. Genotype and allele frequencies of c.1059G>A (rs1061581) SNP in the *hspa1b* gene (apparently healthy subjects1 *versus* severe oligozoospermic patients2).**

| Genotype | Control[1] n(%) | Patients[2] n(%) | OR | Chi-square $X^2$ |
|---|---|---|---|---|
| GG | 14 ( 28%) | 11 (22%) | 0.218 | 1.974NS |
| GA | 24 (48%) | 20 (40%) | 0.569 | 4.175* |
| AA | 12 (24%) | 19 (38%) | 0.598 | 5.084* |
| Allele frequency (%) | | | | |
| G | 52 | 42 | | |
| A | 48 | 58 | | |

* refer to a significant difference at 0.05 level.
NS : No significant.

These results refer to a probable negative role for the homozygous (AA) genotype of c.1059G>A SNP (*hspa1b* gene) in the spermatogenesis and this indicate that A allele may have a detrimental effects on spermatogenesis. Previous studies ( Dix *et al.*, 1996; Rockett *et al.*, 2001) showed that HSPs are effective during spermatogenesis and that degradation of the HSP70-2 (*hspa1b*) gene results in failed meiosis and male infertility. Ekambaram and Bose (2008) indicate that spermatogenesis is accompanied by the expression of different HSPs. Also, Ciftci *et al.* (2015) revealed that the participation of HSP70 is required for successful completion of meiosis during spermatogenesis. Dix *et al.* (1996) showed in a mouse model that disruption of the *hspa1b* gene results in failed meiosis, germ cell apoptosis and male infertility. In addition, Feng *et al.* (2001) reported that the HSPA1B protein is expressed in spermatocytes and spermatids in normal and maturation arrest tissues. They also found that the expression of the HSPA1B protein was low in testis with maturation arrest and no expression was found in sertoli cells. Therefore, the decreased expression of the HSPA1B protein may be associated with the pathogenesis of male infertility ( Dix *et al.* ,1996). These results indicate that the *hspa1b* gene may play a vital role in human spermatogenesis and that deficiency or dysfunction of the gene may result in male infertility. Erata *et al.* (2008) reported that the expression of HSP70 might increase the protective mechanism against apoptosis in the spermatozoa of infertile men. In contrast, Ciftci *et al.* (2015) found no significant difference between the idiopathic infertile patients and control subjects as related with the genotype distribution of the HSPA1B : c.1059G>A.

**heat shock 70kDa protein 1-like (HSPA1L) gene**

The HSPA1L gene polymorphism at c.1478C>T (rs2227956) SNP, 878 bp fragment (9238-10115; NG_011855.1 ) was targeted for amplification by the forward and reverse primers that used by Ciftci *et al.* (2015) using PCR (Figure 3). The rs2227956 SNP is locate within this fragment at 9564 position (C). The polymorphisms of this SNP were detected using PCR-RFLP. The conversion of C allele to T allele at this position create a site for *NcoI* restriction enzyme ( CCACGG to CCATGG). Therefore, after digestion of PCR product with this enzyme, the fragments that were obtained were as follows : 878 bp for CC genotype; 324, 554 and 878 bp for CT genotype and 554 and 324 bp for TT genotype ( Figure 4). This SNP is substitute the amino acid threonine with methionine.

Figure (5) show the results of sequencing and blast for checking the PCR-RFLP results of rs2227956C>T SNP.

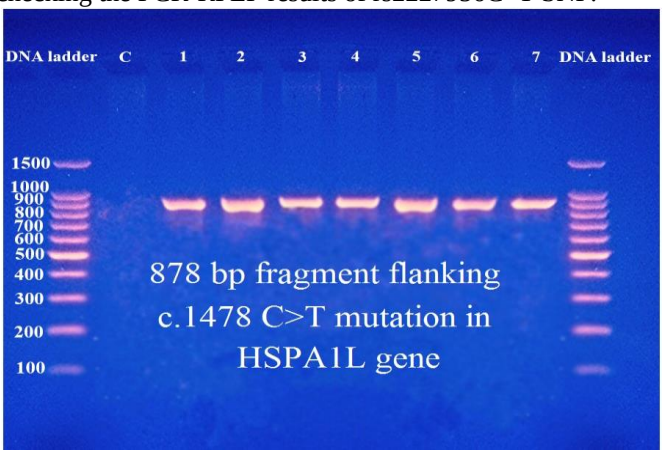


**Figure 3. PCR product (878 bp) of targeted fragment ( HSPA1L gene) flanking the rs2227956C>T SNP (*NG_011855.1* ) visualized under UV light after staining with ethidium bromide. The electrophoresis was on 2% agarose gel at 5 volt / cm for 2 hours.**

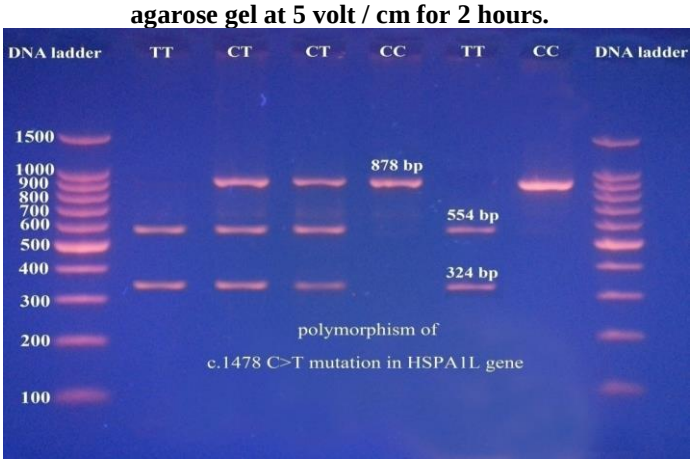


**Figure 4. PCR product (878 bp fragment) of HSPA1L gene digested with NcoI restriction enzyme and electrophoresed on 2% agarose. The genotypes at 9564 position (NG_011855.1 ) are: CC (878 bp), CT (324 + 554 + 878 bp) and TT (324 + 554 bp).**

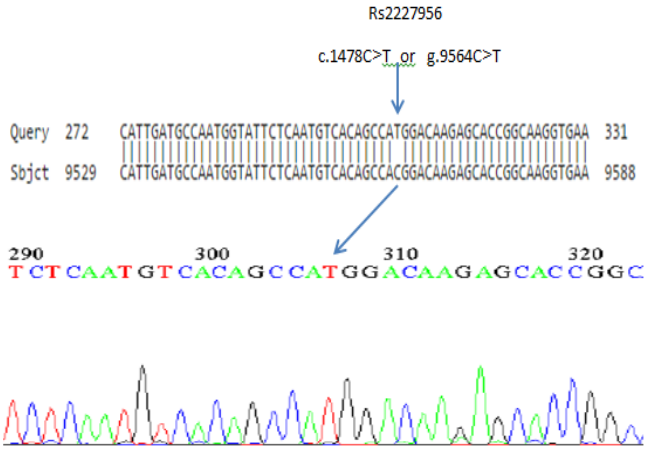


**Figure 5. Electropherogram deicting the g.9564C>T or c.1478C>T (rs2227956) SNP position and its flanks.**

Table 9 show the frequency of rs2227956 SNP in severe oligozoospermic patients and apparently healthy control subjects. There was no significant difference between control subjects and severe oligozoospermic patients as related with the frequency of CC and CT genotypeS. The frequency of homozygous SNP (TT) was significantly ($p<0.05$) higher in patients than in control subjects ( 42*versus*34%, respectively,

OR= 0.569 ; $X^2$= 4.175). The percentages of C allele were 41 and 35 % in the control subjects and patients , respectively, while, the percentages of T allele were 59 and 65 % in the control subjects and patients, respectively.

**Table 9. Genotype and allele frequencies of c.1478C>T (rs2227956) SNP in the hspa1Lgene (apparently healthy subjects1 versus severe oligozoospermic patients2).**

| Genotype | Control[1] n(%) | Patients[2] n(%) | OR | Chi-square $X^2$ |
|---|---|---|---|---|
| CC | 8 ( 16%) | 6 (12%) | 0.207 | 1.652NS |
| CT | 25 (50%) | 23 (46%) | 0.207 | 1.652NS |
| TT | 17 (34%) | 21 (42%) | 0.569 | 4.175* |
| Allele frequency (%) | | | | |
| C | 41 | 35 | | |
| T | 59 | 65 | | |

* refer to a significant difference at 0.05 level.
NS : No significant.

The results of this study are disagree with the results of Ciftci *et al.* (2015) who found , in Turkey, that infertility in males with normal sperm parameters was not significantly associated with HSPA1L: c.1478C>T (rs2227956) gene polymorphisms. Also, in Iran, Kohan and Tabiee (2016) showed that the HSPA1L rs2227956 polymorphism is associated with the idiopathic male infertility risk and that the individuals with TC and CC genotype had an increased risk of male infertility.

The Hsp70 loci within the major histocompatibility complex (MHC) class III region on chromosome 6 contains three intronless genes. The HSPA1A and HSPA1B genes are 12 kb apart and encode an identical heat-inducible protein, but have divergent 50 and 30 untranslated region (UTR) sequences. The third gene, HSPA1L is located 4 kb telomeric to HSPA1A. HSPA1L encodes a constitutively expressed protein that shares 90% identity with HSPA1A, the sequences differing most in the C-terminal 100 amino acids.

The polymorphism of *hsp70 gene*, in several genes are associated with male infertility(Rajender *et al* 2011). The *h*sp70 gene encodes a highly inducible stress protein, which is fundamentally important in protein transport and folding (Rajender *et al* 2011).Several a studies have proposed that *hsp* loci are possible susceptibility genes in the development of many diseases,such as Grave's disease, diabetes mellitus, celiac disease,systemic lupuserythematous and asthma(Vargas-Alarcón *et al* 2000).Spermatogenesis is a developmental process. However, a genetic programme intrinsic to germ cells probably controls the progressive steps through mitotic, meiotic and postmeiotic phases of development. As all these developmental stages represent situations where dramatic transformations and cellular differentiation take place, it is not surprising that spermatogenesis is accompanied by the expression of different HSP(Ekambaram *et al* 2008). The participation of HSP70 is required for successful completion of meiosis during spermatogenesis.

There are few studies of the relationship between the *h*sp70 gene polymorphism and infertility in the literature. Present studies mainly deal with *h*sp70-2, which is only constitutively expressed in experimental animals.( Dix *et al* 1996; Dix *et al* 1997). A recent study showed in a mouse model that disruption of the *h*SP70-2 gene using gene targeting results in failed meiosis, germ cell apoptosis and male infertility (Dix *et al* 1996). Another stud y of HSP70 and infertility reported that the *h*SP70-2 protein is expressed in spermatocytes and spermatids in normal and maturation arrest tissues (Feng *et al* 2001).

However, the expression the HSP70-2 protein was low in testes with maturation arrest, and no expression of the HSP70-2 protein was demonstrated in sertoli- only specimens. Therefore, the decreased expression of the HSP70-2 protein may be associated with the pathogenesis of male infertility (Dix *et al* 1996).

These results suggest that the *hsp*70-2 gene may play a specific role in human spermatogenesis and that deficiency or dysfunction of the gene may result in male infertility. HSP70 is known to be induced in several stress conditions, including heat, and has been shown to protect various human cell lines from heat-induced apoptosis ( Dix *et al* 1997).

Many studies reported that heat shock factor is activated and that consequently *hsp*70 expression is increased in spermatids exposed to heat (Zakeri *et al* 1990 ; Mori *et al* 1997). One study reported that the expression of HSP70 might increase as a protective mechanism against apoptosis in the spermatozoa of infertile men.( Erata et al 2008). Two additional unique members of the HSP70 family are expressed during spermatogenesis. Spermatocyte- specific HSP70-2 is expressed at high levels in pachytene spermatocytes during the meiotic phase of spermatogenesis (Allen *et al* 1988; Zakeri, *et al* 1990), and testis-specific HSC70t is expressed in postmeiotic spermatids (Maekawa *et al* 1989;Matsumoto and Fujimoto, 1990). The *hspa1l* gene is constitutively expressed at high levels in testis and at very low levels in other tissues (Milner *et al* 1990).

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