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The Study of Genetic Variations of Human Testis-Expressed Protein 101(TEX 101) and Hormonal Levels of Fertile and Infertile (Oligospermia) Men

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ABSTRACT

Human testis-expressed protein 101(TEX 101) play an important roles in germ cell formation during its cellular localization in the gonads through gametogenesis, signal transduction and spermatogenesis. In sexually mature testes, TEX101 protein is fundamental for production of fertile spermatozoa. This study aims to estimate the genetic variations (gene polymorphism) of TEX101 gene in fertile and infertile (oligospermia) men in Baghdad. For this purpose, two groups were included; the first include 30 infertile men (oligospermia), while the other group contained 30 fertile men. Genomic DNA was extracted from each groups and PCR-sequencing was applied to detect TEX101 gene polymorphism. The fertility hormones (Luteinizing hormone (LH), follicle stimulating hormone (FSH), testosterone and prolactin) were measured for each group. Double primers were utilized to amplificate the exons 1, 2, 3, and 4 of TEX101 gene by a polymerase chain reaction (PCR) after the DNA extraction was done from blood specimens. The single nucleotide polymorphisms (SNPs) were detected through sequencing (by matching with the TEX101 gene sequencing in NCBI data bases). The results revealed five single nucleotide polymorphisms (A (516) R, T(520)W, C > S, G>T, and A>G) in chromosome 19 of TEX101 gene (exon 1,2,3, and 4) when compared with sequence ID: 83639. The variants; A (516) R and T(520)W were showed two genotypes, while other variants; C > S, G>T, and A>G showed three genotypes. In infertile (oligospermia) men, a higher significantly rise (P≤0.01) were detected in mutant genotypes (heterozygote alleles) as compared to wild genotypes (homozygote alleles) at A (516) R and T(520)W. While, in control fertile group, the wild homozygote genotypes recorded higher significantly (P≤0.01) increase in comparison with mutant homozygote genotypes. Also, the heterozygote mutant genotype (AG, TA) of variants; A (516) R and T(520)W in oligospermia infertile men presented higher significantly (P≤0.01) excess compared with mutant genotypes (heterozygote alleles) of fertile (control) men. Whereas the wild genotypes (homozygote alleles) in control appeared higher significantly (P≤0.01) raise compared with wild genotypes (AA and TT) of oligospermia infertile men. In C > S, G>T, and A>G loci, the mutant genotypes (homozygote and heterozygote alleles) recorded higher significant increment (P≤0.01) than wild homozygote genotypes in infertile (oligospermia) group. Additionally, the homozygote mutant genotypes of infertile group showed higher significant increase (P≤0.01) compared to heterozygote mutant and wild genotypes in C > S, G>T, and A>G loci. Whilst, in control fertile group, the homozygote mutant genotypes recorded higher significant rise (P≤0.01) as compared to heterozygote mutant genotypes. On the other hand, the fertile men group appeared a high significant increment (P≤0.01) in wild homozygote genotypes as compared with the wild homozygote of infertile men. In contrast, the result presented non-significant difference between fertile (control) and infertile (oligospermia) men of fertility hormones; FSH, LH, prolactin, and testosterone. As a conclusion, the gene polymorphisms in exon 1, 2, 3, and 4 influence negatively on function of TEX101 protein by reducing the signal transduction and spermatogenesis, which lead to decrease the concentration and motility of sperm.

Keywords: Men infertility, Oligospermia, TEX101 gene, TEX101 Protein and fertility hormones

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INTRODUCTION

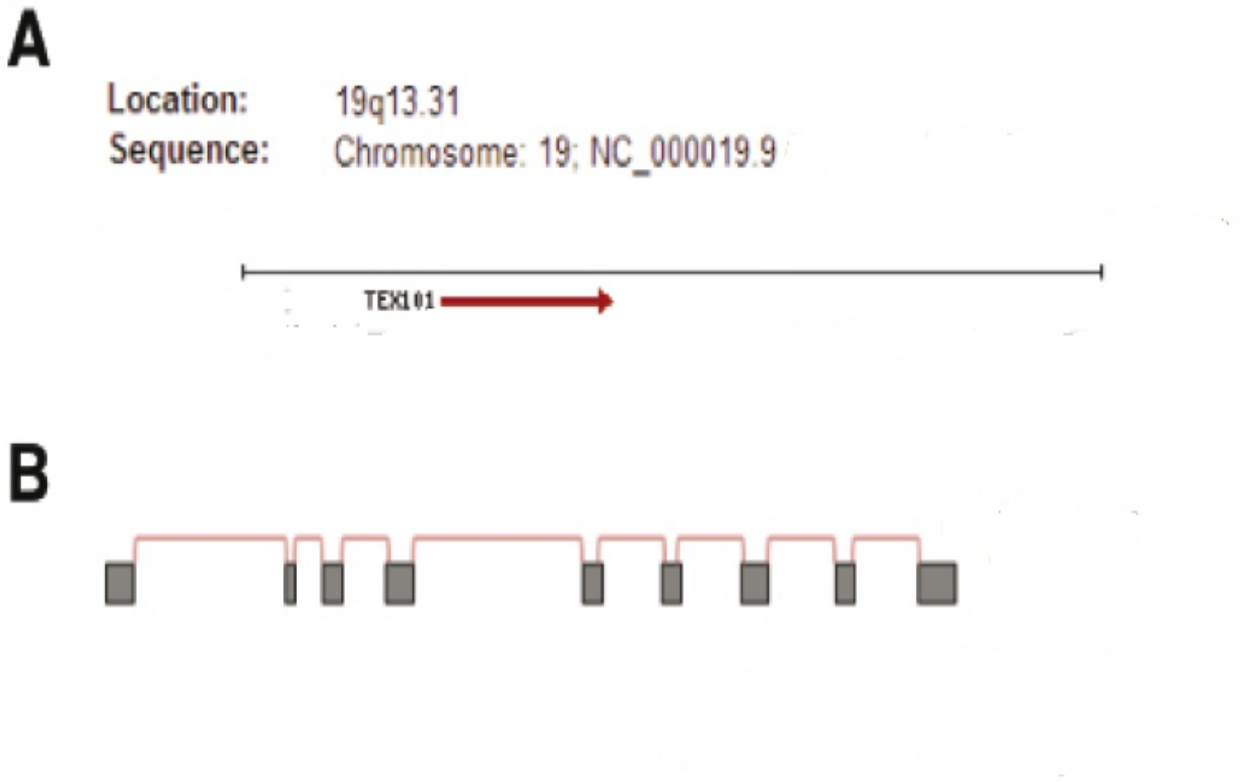
Infertility is an important health problem affecting about15% of couple. Most infertility is because of the male components of fertilization, which are fundamentally because of male factors. Infertility is described as the disability to get pregnant through twelve sequential months of unprotected copulation *(Dissanayake, 2019)*. By transporting fatherly genes to the egg, the specifically spermatozoon (sperm) nucleus, acts as a genome viaduct from one generation to another. Any changed or retrogradation in chromatin of sperm may have earnest consequences for either fertilization or the hazard for developmental upsets in the fetus *(Korbakis, 2017)*. Male infertility is due to blockages that prevent the delivery of sperm, low sperm production, or abnormal sperm function. Lifestyle choices, sickness, chronic health problems, and other agents can play a role in causing male infertility. The most common causes of male infertility are Sperm disorders Problems with making healthy sperm. Sperm may be unable to swim, immature, or abnormally shaped. In some cases, you may not have adequate sperm, or may not produce any sperm *(Abilash, V. G., 2015)*. Clinical forms of male infertility result in teratospermia (abnormal sperm morphology), oligospermia (cut-price sperm counts), asthenospermia (reduced sperm motility) and azoospermia is a complete

reduction of spermatozoa in semen. *(Bieniek, 2016, & Gudeloglu, 2013)*. Spermatogenesis takes site in the seminiferous tubules and demand normal task of both the Sertoli and the Leydig cells. Leydig cells produce testosterone, the main androgen, under the control of luteinizing hormone LH. Sertoli cells supply other testicular cells with nutrients, and also product different regulatory proteins, of which activin, inhibin, AMH Anti Mullerian Hormone and ABP androgen binding protein are the better characterized. Sertoli cell action is organized by follicle stimulating hormone FSH *(Schlatt, 2014)*. Testosterone has critical paracrine actions in the testes which are wanted for fertility and normal spermatogenesis. The whole hypothalamic pituitary testicular axis must assignment normally for spermatogenesis figure. GnRH from the hypothalamus catalyze the releasing of follicle stimulating hormone FSH and luteinizing hormone LH; its impact on luteinizing hormone releasing is more remarkable than that on follicle stimulating hormone releasing. The excretion of GnRH, and so of luteinizing hormone, happen in pulses the excretion of follicle stimulating hormone is minimal markedly pulsatile *(Walker, 2013)*. The frequency and capacity of the pulses of luteinizing hormone releasing show to be important in spend effects on testosterone produce. The excretion of luteinizing hormone is under passive feedback control from plasma (free testosterone), and the releasing of follicle stimulating hormone is prevent by inhibin and stimulated by activin, both freed by Sertoli cells. High testicular (testosterone) is guaranteed by the anatomical closeness of Sertoli, Leydig, and spermatogenic cells, and by the local releasing of ABP *(Smith, 2014, & Walker, 2013)*.

TEX101, a novel testicular germ cell-specific protein

Human TEX101 is a membrane germ cell specific glycosyl phosphatidylinositol GPI anchored protein encoded via the TEX101 gene, located in the (19q13.31) zone of chromosome19 (Figure1). According to the Human Protein Atlas, TEX101 expression is restricted to male germ cells, and testicular tissue, with no proof of expression in any other human tissue or cell kind. TEX101 is testis specific protein that exclusively expressed in germ cell of men thus that it is an important biomarker of men infertility *(Schiza, C. G., 2014 and Shen, C.C., 2014)*. Human TEX101 is not expressed in any other cell type or human tissue including Leydig and Sertoli cells of the testicular tissue. TEX101 is one of the GPI-anchored proteins which are cleaved from the sperm exterior and released into seminal plasma during epididymal maturation. It was proving that TEX101 is cleaved by a testis specific isoform of (tACE) angiotensin converting enzyme through post testicular maturation. Testicular ACE isoform is also a GPI-anchored protein and it is shed from sperm exterior in the epididymis *(Schiza, C., 2019, & Schiza, C., 2018)*. As a GPI-anchored protein, TEX101 is centralizing in specialized membrane microdomains called lipid rafts. Lipid rafts vary from plasma membranes in their lipid composition and they are-reinforced in cholesterol, sphingolipids, like sphingomyelin and GPI-anchored proteins *(Endo, S., 2016)*. TEX10 play an important roles in germ cell formation during its cellular localization in the gonads through gametogenesis, signal transduction and promote protein tyrosine phosphorylation. It also plays an important role in the acrosome reaction during fertilization *(Mobasheri, M.B., 2015)*. In sexually mature testes TEX101 is fundamental for production of fertile spermatozoa *(Tsukamoto, H., 2006, & Endo, S., 2016)*.

According to the National Center for Biotechnology Information (NCBI) data for TEX10, Official Symbol TEX101, Official Full Name: testis expressed 101, Location: 19q13.31, Exon count: 10, Organism: Homo sapiens figure (2-10 a) *(NCBI , 2020)*.



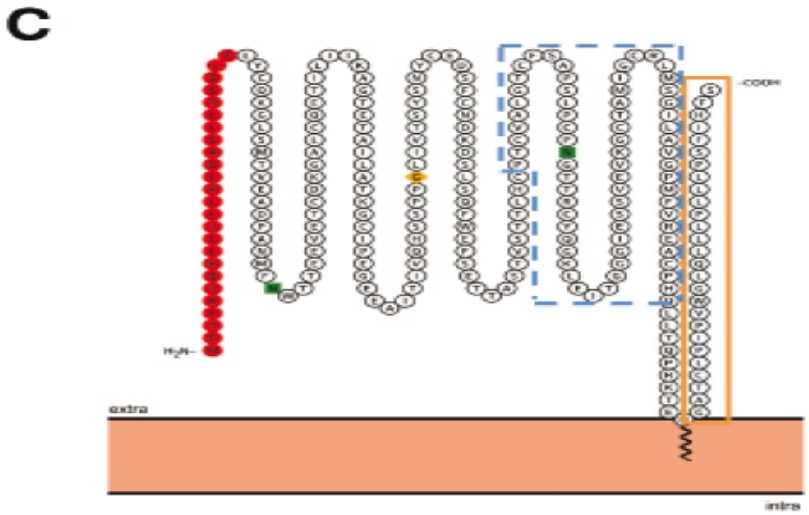


Figure 1: Genomic and proteomic organization of TEX101.

(A) Chromosomal location, orientation & position on chromosome of TEX101 gene *(NCBI, 2020)*. (B) Structure of human TEX101 gene. Exons are represented with gray boxes. (C) TEX101 protein structure *(Schiza, C., 2017).*

MATERIAL AND METHODS

Experimental work and samples collection

Five ml of blood specimens were collected randomly during June from 30 infertile men (Oligospermia) and 30 fertile men were used as a control. Average age (18-45) years in Baghdad infertility centers. One milliliter of the fresh blood sample left in the EDTA tube for TEX101 gene polymorphism. Four milliliters of blood samples were left for 20 minutes in the gel tube at room temperature. After coagulation, sera were separated by centrifugation at 2000 xg for 10 min. Sera were divided into small aliquots for measuring the level of fertility hormones in fertile and infertile men *e.g.:* (Luteinizing **hormone** (**LH**), follicle-stimulating **hormone** (FSH), prolactin and **testosterone**). Genomic DNA was isolated from blood sample according to the protocol ReliaPrep™ Blood gDNA Miniprep System. Quantus Fluorometer was used to detect the concentration of extracted DNA in order to detect the goodness of samples for downstream applications. For 1 µl of DNA, 199 µl of diluted QuantyFlour Dye was mixed. After 5min incubation at room temperature, DNA concentration values were detected. Four Primers were designed based on genomic sequences of human table 1.

Table 1. The sequences of the primers, melting temperature, Product size and its length (bp)

Primer Name	Seq.	Annealing Temp. (ºC)	Product size (bp)
TEX101_Ex1-F	5`-TAGGCTGCTGTGGGTATT-3`	60	898
TEX101_Ex1-R	5`-CCTGCGGTCCCATTTATTT-3`		
TEX101_Ex234-F	5`-CTTGCCAGGACCTAGATTTG-3`		811
TEX101_Ex234-R	5`-GGCCATACACAAGCTCTTTA-3`		

These primers were supplied by Macrogen Company in a lyophilized form. Lyophilized primers were dissolved in a nuclease free water to give a final concentration of 100pmol/µl as a stock solution. A working solution of these primers was prepared by adding 10µl of primer stock solution (stored at freezer -20 C) to 90µl of nuclease free water to obtain a working primer solution of 10pmol/µl.

Primer optimization

To examine the optimum annealing temperature of primer, the DNA template was amplified with the same primer pair, (Forward) (Reverse), at annealing temperatures of 55, 58, 60, 63 and 65°C. PCR amplifications were performed with 20µl volumes containing 10µl GoTaq Green Master Mix (2X); 1µl for each primer (10pmol); 6µl nuclease free water and 2µl of template DNA. PCR cycling was performed with PCR Express (Thermal Cycler, BioRad, USA) with the following temperature program: denatured at 94ºC for 4 min followed by 30 cycles of denaturation at 94°C for 30 sec; annealing at 55, 58, 60, 63 or 65°C for 30 sec; and extension at 72°C for 30 sec. A final extension incubation of 7 min at 72ºC was included, followed by a 10 min incubation at 4ºC to stop the reactions.

PCR Program

The total PCR reaction mixture was 1.5 ul genomic DNA, 5 ul Master mix, 1 ul of
each primer, and 11.5 ul ddH2O (nuclease free water). The optimum conditions were 94°C/ 5 min (1 cycle) for initial denaturation, then, 94°C/ 45 s, 58°C/ 40 s and 72°C/ 1 min for denaturation, annealing and extension respectively (35 cycle for each phase). Lastly, the final extension temperature was 72°C/ 10 min (1 cycle).

Electrophoresis is used to detect and separate the PCR products on stained agarose gel 1% with Ethidium promide (Merck, Germany). Additionally, the DNA ladder (100 bp) (Kapa, USA) were utilized to know sizes of the products.

Sequencing and genotyping

The PCR products (amplicon) are sequenced through Macrogen foundation, Korea by using Sanger sequencing method. The homology search was conducted via utilizing online (BLAST) in NCBI website. For SNPs determination, Bioedit software was used.

Statistical analysis

In present study, the Statistical Analysis System (SAS) program was operated (SAS, 2012). For a significant compare between the means, T test is used, while Chi-square test is employed to make a significantly comparison between percentages.

RESULTS

PCR amplification, sequencing and genetic variability.

Two pairs of primers that replicate the particular regions (whole exon 1,2,3and 4) of the TEX101gene for fertile and infertile (oligospermia) men. The results of the amplification of Ex1,2,3 and 4 of TEX101 gene of human samples were fractionated on 1% agarose gel electrophoresis stained with Eth.Br. M: 100bp ladder marker showed size fragment 898bp of the PCR products (Figure 2). Whereas the target fragments which came consistent with the predicted sizes.

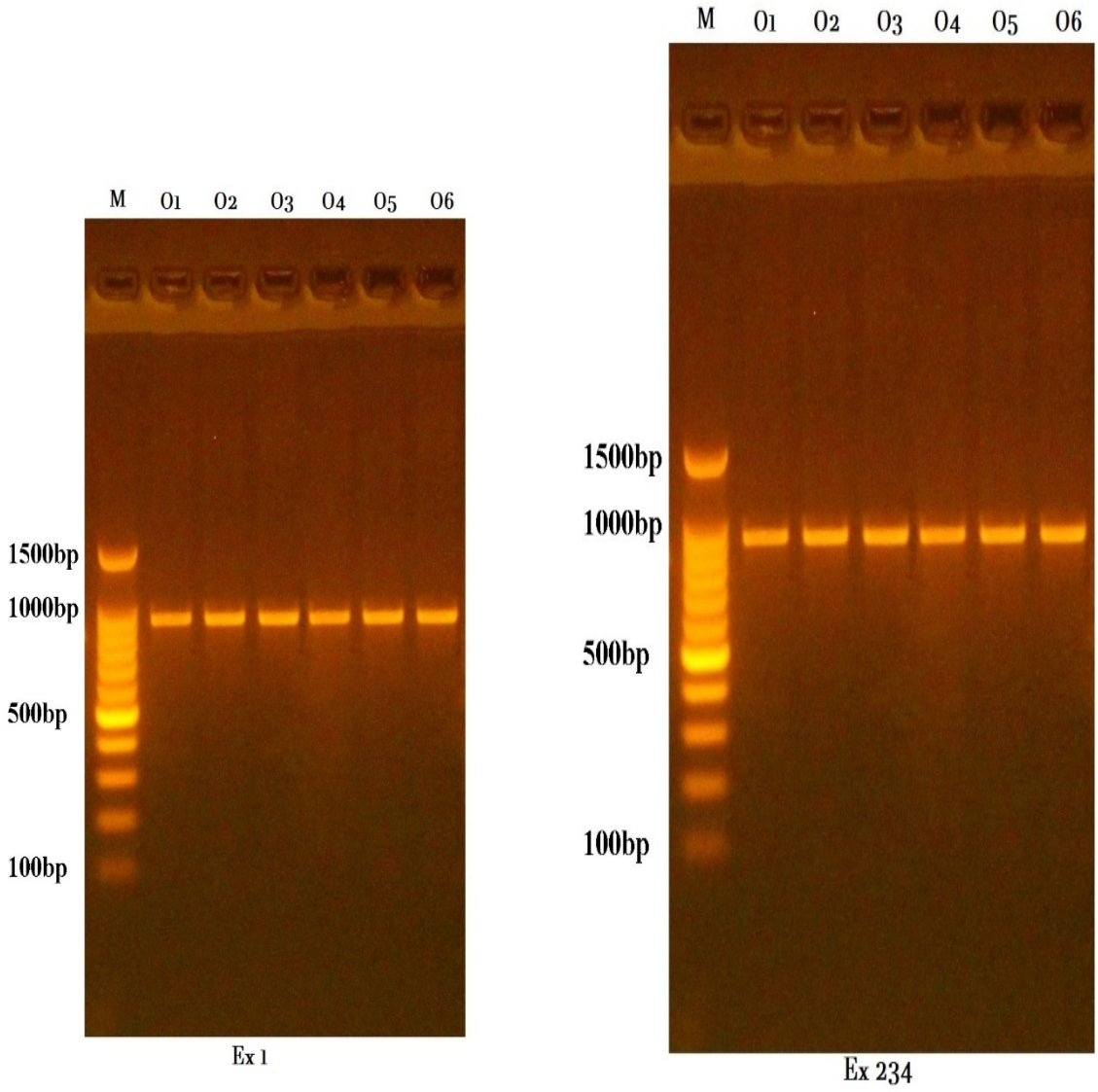


Figure 2. The PCR product of exon I,2,3 and 4 TEX101 gene. M = DNA ladder 100–10000bp

The polymorphisms of TEX101gene were detected by PCR- Sequencing. After DNA alignment with the NCBI sequences, two variants were identified at exon 2, one in intron 3 and two SNPs in 5′ prime untranslated region (5' UTR) of TEX101gene for fertile and infertile (Oligospermia) men; A (516) R, T(520)W in Exon 2, C > S (rs3810375) in intron 3 and G>T (rs11673673), A>G (rs8113556) in 5′ prime untranslated region (5' UTR) when compared with Gene ID: 83639 (Figure 3). The SNPs of exon 2 were missense mutations (not previously detected), which altered glutamine >> arginine and histidine >> glutamine respectively, while the other SNPs (C> S rs3810375) occurred in intron 3 (discovered before) and the last two SNPs (G>T rs11673673, A>G rs8113556) occurred in 5'-UTR (previously recorded) are not coding for amino acid (Table 2).

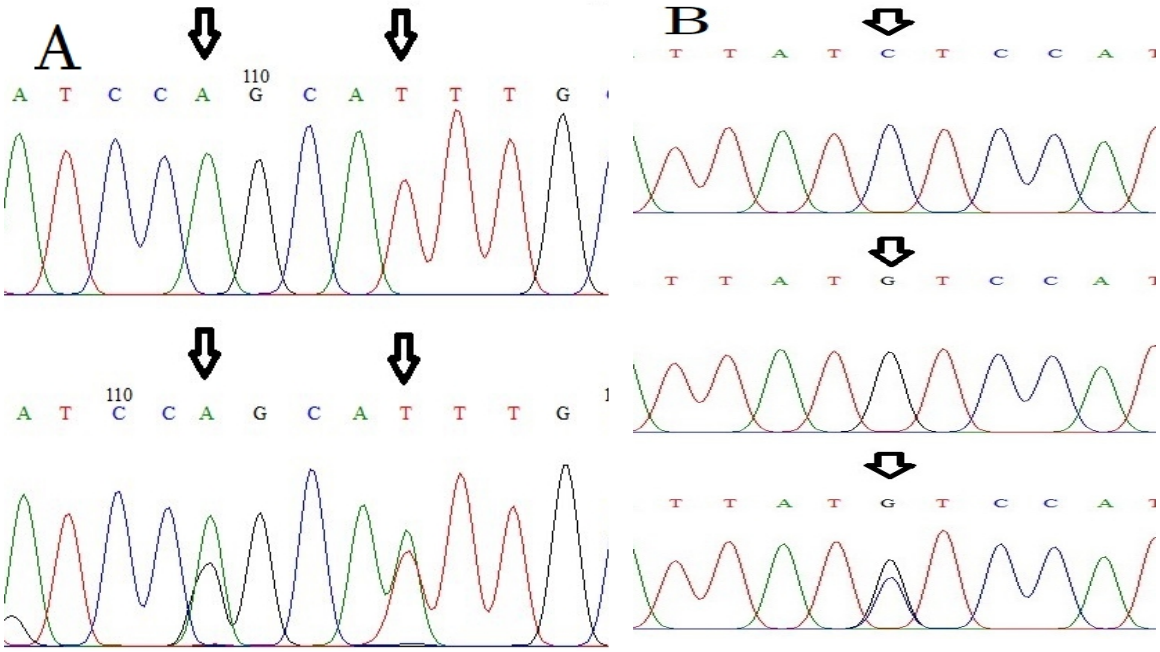


Figure 3. Wild-type and new variant; (A) A (516) R and T(520)W, (B) C>G, (C) G>T, (D) A>G of TEX101 gene.

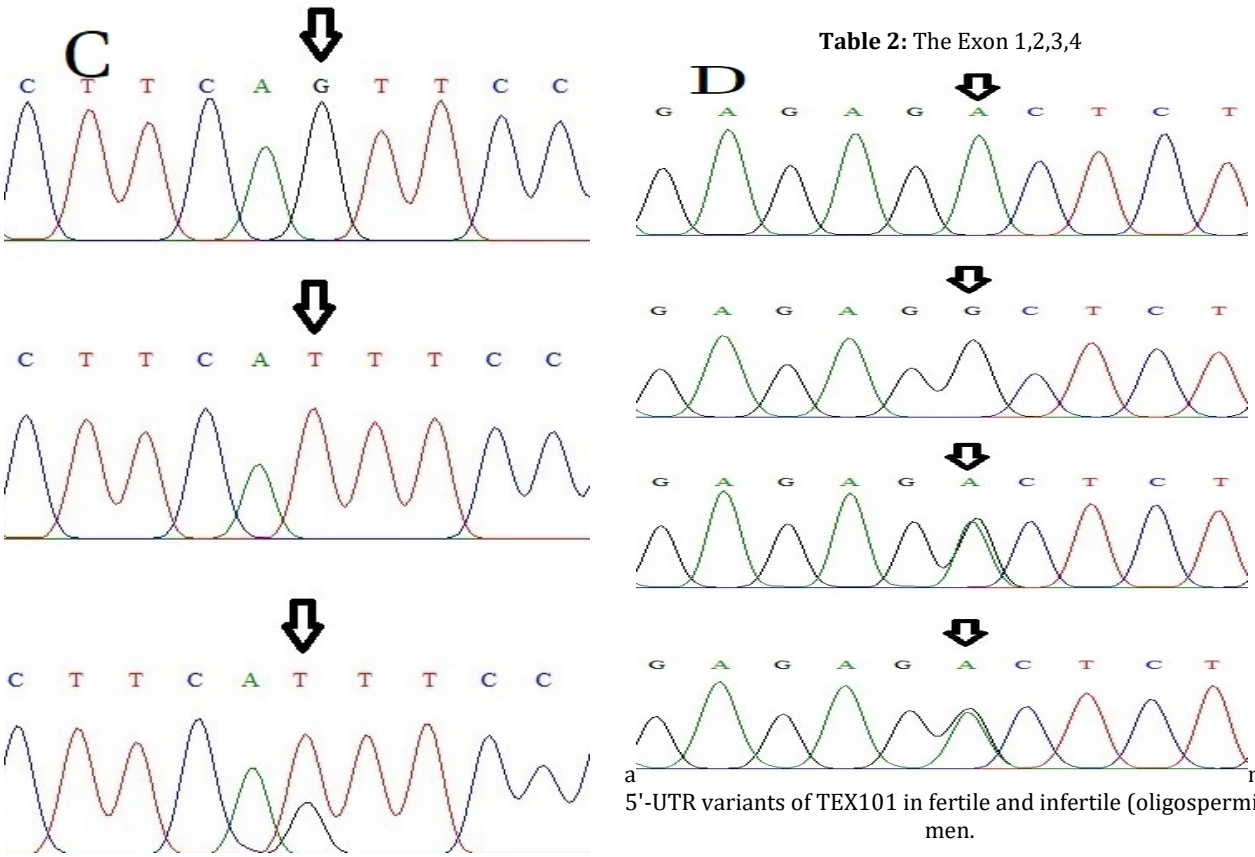


Table 2: The Exon 1,2,3,4 a nd 5'-UTR variants of TEX101 in fertile and infertile (oligospermia) men.

According to these variables, two different genotypes and alleles were observed for A (516) R, T (520) W loci and three different genotypes for C > S, G>T, A>G loci of TEX101 gene when compared between the sample sequences. The TEX101 gene frequencies of genotypes and alleles of in analyzed population showed in (Table 3).

Table 3: The Alleles and Genotypes of TEX101 in fertile and infertile(oligospermia) men (Exon 1,2,3 and 4, intron 3 and 5UTR)

	Locus	Code change	SNP Location	Amino Acid change	Type of mutation	Predicted Effect
1	A(516)R	CAG>>CRG	516 BP Exon 2	Glutamine>> Arginine (24)	Missense	Transition
2	T(520)W	CAT>>CAW	520 BP Exon 2	Histidine>> Glutamine (25)	Missense	Transversion
3	C > S rs3810375	----------	Intron Variant Intron 3	----------	----------	--------
4	G>T rs11673673	----------	5 prime UTR variant	----------	----------	Transversion
5	A>G rs8113556	---------	5 prime UTR variant	----------	----------	Transition

	Locus	Genotypes	Observed Genotypes	Genotypes Frequency	Allele Frequency		Chi- Square (χ^2)
1	A (516) R	AA	38	63.3	A	0.82	
		AG	22	36.7	G	0.18	9.06 **
2	T (520) W	TT	34	56.7	T	0.78	
		TA	26	43.3	A	0.22	11.29 **
3	C > S	CC	6	10.0	C	0.25	
	rs3810375	CG	18	30.0	G	0.75	11.86 **
		GG	36	60.0			
4	G>T	GG	22	36.7	G	0.45	
	rs11673673	TT	28	46.7	T	0.55	9.47 **
		GT	10	16.6			
5	A>G	AA	18	30.0	A	0.4	
	rs8113556	GG	30	50.0	G	0.6	9.15 **
		AG	12	20.0			
	** (P≤0.01).						

The results showed a higher significant difference ** (P≤0.01) between genotypes and alleles frequencies for each of A (516) R ,T(520)W loci in exon 2, G>T locus in intron 3 and G>T, A>G in 5′ prime untranslated region (5'-UTR) (Table 3). In infertile (oligospermia) men a higher significantly rise ** (P≤0.01) were detected in mutant genotypes (heterozygote alleles) as compared to wild genotypes (homozygote alleles) at A (516) R and T(520)W loci. While, the fertile men group, a higher significant increment ** (P≤0.01) appeared in wild homozygote genotypes as compared with the mutant heterozygote genotypes at A(516)R and T(520)W loci. On the other hand, the higher significantly rise ** (P≤0.01) detected in mutant heterozygote genotype of infertile group as compared with heterozygote allele of control group at A (516) R and T(520)W loci (**Table 4)**. Whereas the wild genotypes (homozygote alleles) in control appeared higher significantly (P≤0.01) raise_compared with wild genotypes (AA and TT) of oligospermia infertile men (**Table 4)**.

Table 4: Comparative between the TEX101 genotypic distribution in fertile and infertile (oligospermia) men.

	Locus	Genotypes	Fertility (control)	Chi-Square	Infertile (Oligospermia)	Chi-Square (χ^2)
1	A(516)R	AA	28 (93.3%)	14.73 **	10 (33.3 %)	9.07 **
		AG	2 (6.6%)		20 (66.6%)	
2	T(520)W	TT	26 (86.6%)	13.69 **	8 (26.6%)	12.25 **
		TA	4 (13.3%)		22 (73.3%)	
3	C > S rs3810375	CC	5 (16.6%)	11.76 **	1 (3.3%)	11.95 **
		CG	6 (20%)		12 (40%)	
		GG	19 (63.3%)		17 (56%)	
4	G>T rs11673673	GG	14 (46.6%)	10.44 **	8 (26.6%)	6.08 **
		TT	15 (50%)		13 (43%)	
		GT	1 (3.3%)		9 (30%)	
5	A>G rs8113556	AA	14 (46.6%)	9.51 **	4 (13.3%)	11.37 **
		GG	12 (40%)		18 (60%)	
		AG	4 (13.3%)		8 (26.6%)	
** (P≤0.01).						

In C > S, G>T and A>G loci, the mutant genotypes (homozygote and heterozygote alleles) recorded higher significant increment (P≤0.01) than wild homozygote genotypes in infertile (oligospermia) group.

Additionally, the homozygote mutant genotypes of infertile group showed higher significant increase (P≤0.01) compared to heterozygote mutant and wild genotypes in C > S, G>T and A>G loci. Whilst, in control fertile group, the homozygote mutant genotypes recorded higher significant rise (P≤0.01) as compared to heterozygote mutant genotypes. On the other hand, the fertile men group appeared a high significant increment ** (P≤0.01) in wild homozygote genotypes as compared with the wild homozygote of infertile men as appeared in table 4. On the other hand, the result detected non-significant difference between fertile men (control) and infertile (oligospermia) men of fertility hormones; FSH, LH, prolactin and testosterone as shown in table 5.

Table 5: Comparison between control and Oligospermia groups in hormones level

Group	Mean ± SD			
	FSH (miu/ml)	LH (miu/ml)	Prolactin (ng/ml)	Testosterone (ng/ml)
Control	5.496 ± 1.87	4.650 ± 1.50	10.95 ± 2.34	3.45 ± 0.92
Oligospermia	5.873 ± 1.96	4.703 ± 1.43	10.87 ± 1.97	3.64 ± 0.95
T-test	0.991 NS	0.760 NS	1.120 NS	0.485 NS
P-value	0.449	0.889	0.896	0.421
NS: Non-Significant.				

Most of the fertile and infertile (oligospermia) men in this study had normal levels of FSH, LH, prolactin and testosterone, as shown in the figures 4 and 5.

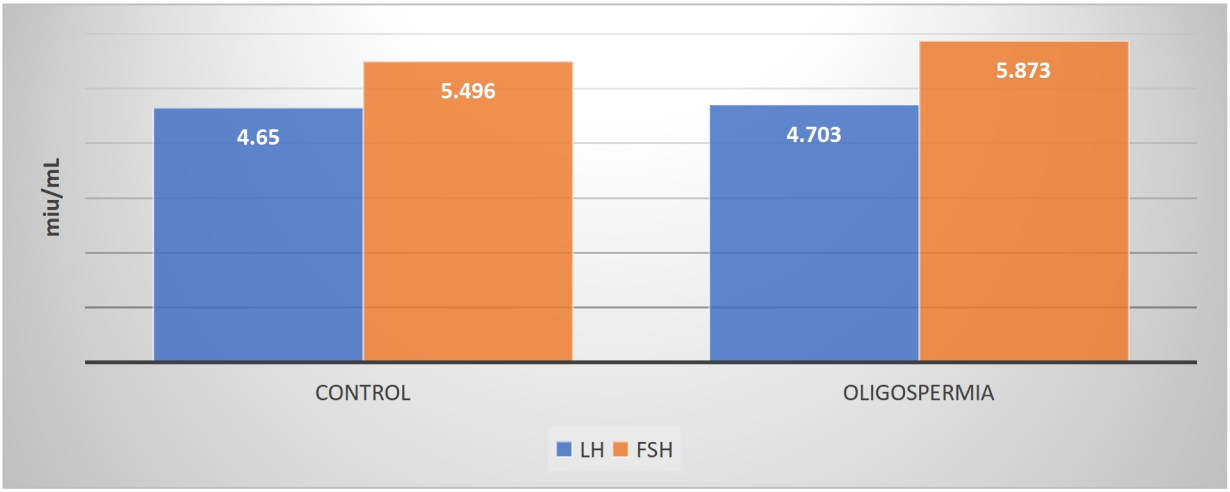


Figure 4. FSH and LH levels of fertile (control) and infertile (oligospermia) men.

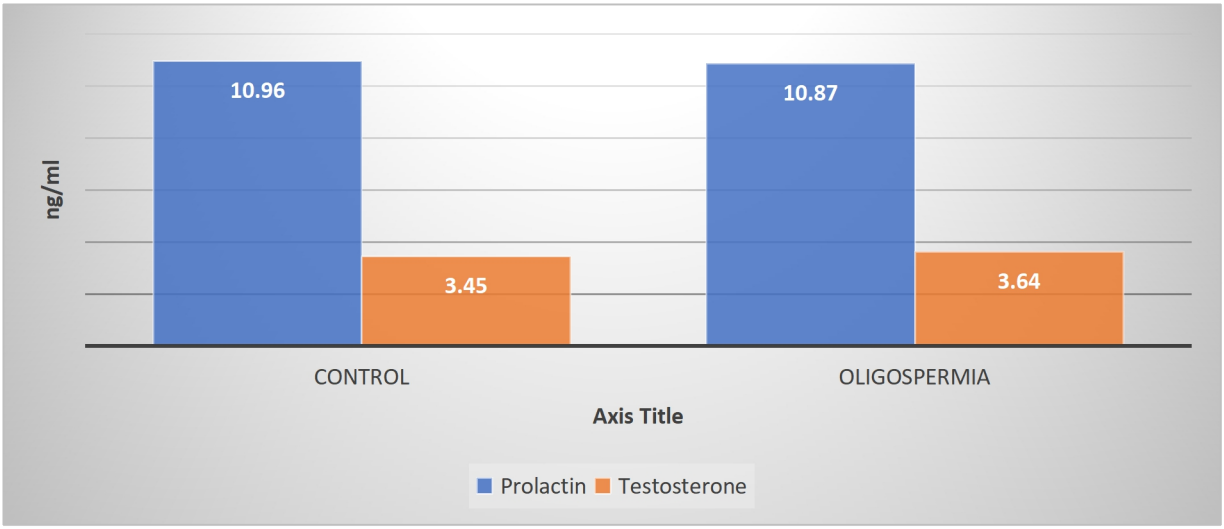


Figure 5. Prolactin and testosterone levels of fertile (control) and infertile (oligospermia) men.

DISCUSSION

The current study showed that the five variants detected in the TEX101 gene represent genetic diversity, the first

two variants were missense mutation; A (516) R changed poler, charged, weakly acid Glutamine to poler, charged, strongly basic Arginine, and T(520)W converted poler, non-charged weakly basic Histidine (with imidazole ring) to poler, charged, weakly acid Glutamine. The other three variants; C > S, G>T and A>G loci do not code amino acids (Table 2). The first two SNPs loci A(516)R and T(520)W showed two different genotypes for each, While the other SNPs loci (the last 3 SNPs) appeared three different genotypes for each (Table 3). All SNPs loci that detected in TEX101 gene of fertile and infertile (oligospermia) men showed high statistically significant differences** (P≤0.01) **(table 4)**. The first two SNPs located on the exon2; A (516)R and T(520)W were not previously detected which have effect on the transcription and translation process that lead to produce TEX101 protein with two new amino acids with new chemical properties as shown in the table 2. While the third SNP locus on the intron 3 doesn't code amino acid. On other hand the last two SNPs on the **5′** prime **untranslated region** (**5′ UTR**); G>T rs11673673 and A>G rs8113556 do not code amino acids but this region is important for regulating the translation process of TEX101 protein. The study also appeared non-significant difference between the hormonal levels; FSH, LH, prolactin and testosterone of fertile (control group) and infertile (oligospermia) men, which means they do not have hypogonadotrophic hypogonadism or hypergonadotrophic hypogonadism as shown in table 5 and figures 4,5. These findings lead to the belief that the mentioned mutations in TEX101 gene have a relative effect on TEX101 protein. The heterozygote alleles in A(516)R and T(520)W SNPs influence negatively on TEX101 protein by decreasing the expression of this protein or may be because the changes in amino acid (Glutamine to Arginine) and (Histidine to Glutamine) that made a structural change in mature protein shape and chemical characteristic alter, this effect the TEX101 protein function (signal transduction and spermatogenesis) in testes lead to reduce the concentration of sperm and may be effecting it's motility. The other SNPs: C > S rs3810375, G>T rs11673673 and A>G rs8113556 are not coding for amino acids but may have a negative influence on TEX101 protein expression, this study agreed with Endo et al (2016), Shen et al (2014) and Schiza et al (2017, 2018 and 2019).

CONCLUSION

The present study hypothesized that the exon 2 TEX101 gene polymorphisms influence negatively on signal transduction, germ cell formation and spermatogenesis in testes by changing the structure of TEX101 protein, the mutant genotypes (AG) at A (516) R and (TA) at T(520)W loci were connected with reducing the sperm concentration of infertile (oligospermia) men. On the other hand, the mutant genotypes (GT) at G>T rs11673673 and (AG) at A>G rs8113556 loci on the **5′ prime UTR** are not coding for amino acids but may have a negative influence on TEX101 protein expression. these SNPs can be considered as a genetic marker to detect the reason for reducing the sperm concentration in oligospermia infertile men.

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