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Original scientific article

ASSOCIATION STUDY OF RS535296987 IN *TEX14* GENE IN AZOOSPERMIAMEN: RFLP AND DNA SEQUENCING

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Azoospermia is one of the kinds of male infertility, with clinically the most severe phenotype as the natural conception cannot occur. It has been estimated to affect 0.1 to 1% of all men and 10-15% of men in infertile couples. *TEX14* (Testis expressed 14, intercellular bridge forming factor) is a protein coding gene, which is located in human chromosome 17, (17q22). Tex14 gene appears to be crucial for perfect spermatogenesis and functional studies indicate the role of TEX14 in the intercellular bridges between developing male germ cells. The gene contains 32 exons and spans 137 kb. A heterogeneousresultis available on the association TEX14 gene and azoospermia. Therefore, it is suggested to investigate this gene in different populations. We analyzed about 200 men in two categories of azoospermia and healthy persons by RFLP as well as DNA sequencing to indicate an association between rs535296987 in *TEX14* and its adjacent nucleotides to azoospermia. We found no significant association based on RFLP data and also by clustering of case and control specimens based on DNA sequencing. In general, a low level of nucleotide variability was observed in DNA sequences. Therefore, both eternity in the studied samples and low degree of mutations in this genetic region, may be the reason for heterogeneous reports on association of *TEX14* and azoospermia.
*Keyword: a*ssociation, Azoospermia, rs535296987, *TEX14*

INTRODUCTION

Infertility is defined as inability to conceive after 1 year of unprotected intercourse. This is estimated to affect 10–15% of couples in developed and developing countries (TÜTTELMANN *et al* ., 2018).

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The clinical causes of infertility are attributed in equal parts to the male and female partners (TÜTTELMANN *et al.,* 2018). Male infertility has been divided into four categories: 1- Oligospermia, 2- asthenozoospermia, 3- teratozoospermia and 4- azoospermia. Azoospermia, is clinically the most severe phenotype of male infertility as the natural conception cannot occur. It has been estimated to affect 0.1 to 1% of all men and 10-15% of men in infertile couples (TÜTTELMANN *et al .,* 2018).

TEX14 (Testis expressed 14, intercellular bridge forming factor) is a protein coding gene, which is located in human chromosome 17, (17q22). WANG *et al.*2001, searched for genes expressed in mouse spermatogonia but not in somatic tissues, and identified 25 genes, that were expressed in only male germ cells. One of these genes, Tex14, encodes a predicted protein with 2 protein kinase domains. Tex14 shows testis-specific expression. WANG *et al.* 2001 identified an orthologous, full-length human TEX14 cDNA sequence. Latter on WU *et al.* 2003, in EST database analysis identified human TEX14, which encodes a deduced 1,451-amino acid protein that shares 64% identity with mouse Tex14. In situ hybridization of the mouse testis detected the strongest expression in pachytene, diplotene, and meiotically dividing spermatocytes.

GREENBAUM *et al.* (2006) showed that TEX14 is required for intercellular bridges in vertebrate germ cells, and these studies provide evidence that the intercellular bridge is essential for spermatogenesis and fertility.

Tex14 gene appears to be crucial for perfect spermatogenesis in the mouse and pig. The functional studies revealing the role of TEX14 in the intercellular bridges between developing male germ cells indicate the importance of this gene in sperm production across species (GREENBAUM *et al*., 2006). Latter on GERSHONI *et al.* (2017) found that TEX14 is almost exclusively expressed in testis, and in transformed fibroblasts of men and women.

WU *et al.* (2003) determined that the TEX14 gene contains 32 exons and spans 137 kb. The 5-prime end of intron 13 contains a conserved atypical splice site. By radiation hybrid analysis, WANG *et al.* (2001) mapped the human TEX14 gene to chromosome 17. The mouse Tex14 gene maps to chromosome 11. WU *et al.* 2003 stated that human TEX14 maps to chromosome 17q23.3.

MATERIAL AND METHODS

Sample size determination

We used the following formula for sample size calculations (https://www.wallstreetmojo.com/sample-size-formula).where, N = Population size, Z = Critical value of the normal distribution at the required confidence level, p = Sample proportion, e = Margin of error. Therefore, we used the following data: Population size, N = 10,000, Critical value at 95% confidence level, Z = 1.96, and Margin of error, e = 5% or 0.05. Since the current conversion rate is unknown, let us assume p = 0.5. N = (10,000 * (1.96 2) *0.05*(1-0.05)/ (0.05 2)/ (10000 – 1+((1.96 2) * 0.05*(1-0.05)/ (0.05 2)))) = 72. Therefore, a sample size of 72 customers will be adequate for deriving meaningful inference in this case. However, we used 200 samples in total, 100 for each case and control persons.

$$\text{Sample size, n} = N * \frac{\frac{Z^2 * p * (1-p)}{e^2}}{[N - 1 + \frac{Z^2 * p * (1-p)}{e^2}]}$$

These samples were collected from the Infertility Clinic of Jahad-Daneshgahi, Qom.

DNAextraction, PCR and RFLP

One hundred Azoospermia patients and one hundred fertile men blood samples werecollected from Jihad of Qom University; DNA was extracted by Salting out method. SNP selection was based on PHYRE2 database (http://www.sbg.bio.ic.ac.uk). Actually, Amino Acid sequence of this protein imported to PHYRE2 database and based on analysis, we selected amino acids which were high sensitive to mutation and have highly effect in protein structure if changed to another amino acid. Then these amino acids checked in NCBI database for polymorphism existence. Inthe end, we chose rs535296987 which is related to one of these important amino acids. SNP fragmentwas amplified by using forward and reverse primers which were designed by oligo7 software (primers information, Table 1).These primers produce 839 nucleotide fragment. PCR was performed in thermocycler system (BioRad, country??) with an initial denaturation at 95 °C for 5 (min), followed by 35 cycles of denaturation at 95 °C for 45 (s), annealing at 58 °C for 45 (s), extension at 72 °C for 45 (s) and Final extension was done at 72 °C for 10 (min). PCR reaction was carried out in total volume 25 µl containing 4 µl of DNA samples, 21 µl Master Mix (17.2 µl H2O, 2 µl Buffer 10×, 0.3 µl MgCl2 100 mM, 0.4 µl dNTP-Mix 40 mM, 0.8 µl Forward primer 14.72 ng/µl and 0.8 µl Reverse primer 12.77 ng/µl) and 0.5 µl Taq polymerase (5 U/µl). The PCR fragment was separated by 1.5% agarose gel electrophoresis. RFLP method done for polymorphism detection. Actually, *Bsa*WI restriction enzyme selected for rs535296987 which can cut DNA on 305th nucleotide when there is C nucleotide in this position. Digestion master mix for RFLP technique contained 10 µl PCR product, 2 µl 10× buffer, 2 µl enzyme and 18 µl water. Prepared mix incubated at 37 °C for 8h. The RFLP fragment was separated by 2% agarose gel electrophoresis.

Table 1. Information of primers

Forward Primer	CAACATCTGAGTCATGGTAAGTGC	TM= 57
Reverse Primer	CTGTGCTTGACTAGCCTGATC	TM= 56

DNA sequencing

For approving the result of RFLP technique and also for doing population genetic analysis we chose randomly30 samples of case and control for Sanger sequencing.

Data analyses

For RFLP results we did not have to perform chi-square test as all the studied samples had GG genotype. For sequencing result, we first aligned and cure DNA sequences. Then Kimura-2-parameters genetic distance was determined among the samples and use it for cluster analysis with PAST software. DCA (Dentrented correspondence analysis) was used to check if the nucleotides obtain can be used for genetic screening (PODANI, 2000).

RESULTS

RFLP

All 200 genotypes analyses had GG genotype. Therefore, the studied samples in Iran all were genotypical uniform, and that the studied SNP was present in both control as well as case samples. We therefore, conclude that, rs1294845152 of Text14 is not associated with azoospermiamen in our study.

DNA sequencing

In order to investigate and identify adjacent nucleotides/SNPs to rs535296987 in TEX14 gene, we performed DNA sequencing in 30 randomly selected individuals. DNA segment obtained after curation was 307 nucleotides in length (Fig. 1). It showed a low level of genetic diversity: pi = 0.14, with 139 polymorphic sites and 37 parsimony-informative sites. These nucleotide changes showed Tajima's D statistic:D = 3.30208e+06, p (D >= 3.30208e+06) = 0. This indicated that, nucleotide substitutions occurred randomly and mutations occurred are not in response to selection (Figure1).

Figure 1. Part of DNA sequences obtained in the studied samples after curation.

DCA plot is provided in Figure 2. It shows that several nucleotides are scattered in the plot and therefore can differentiate the studied samples.

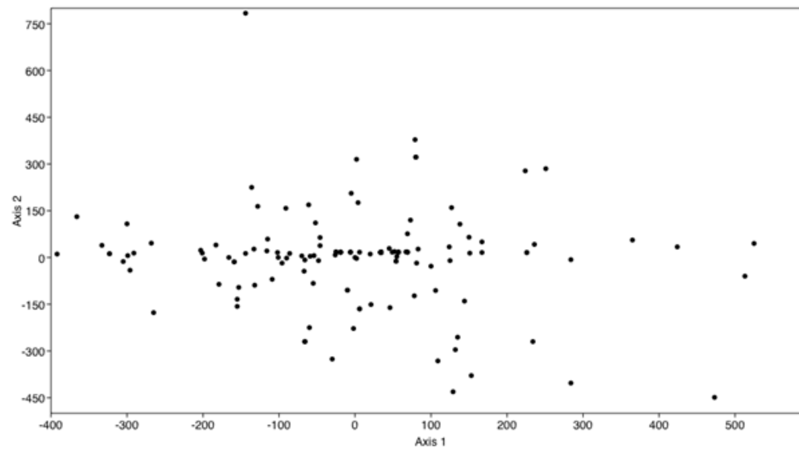

Figure 2. DCA plot od sequenced individuals, showing a good spread of many nucleotides

UPGMA clustering of DNA sequence is provided in Figure 3. It reveals that these sequences are not associated with stenospermia. Moreover, admixture of the samples from three ethnic groups of Turk, Our, and Fars in different clusters indicate that, nucleotides changes in these ethnic groups occurred at random as also evidenced by Tajima's D statistic.

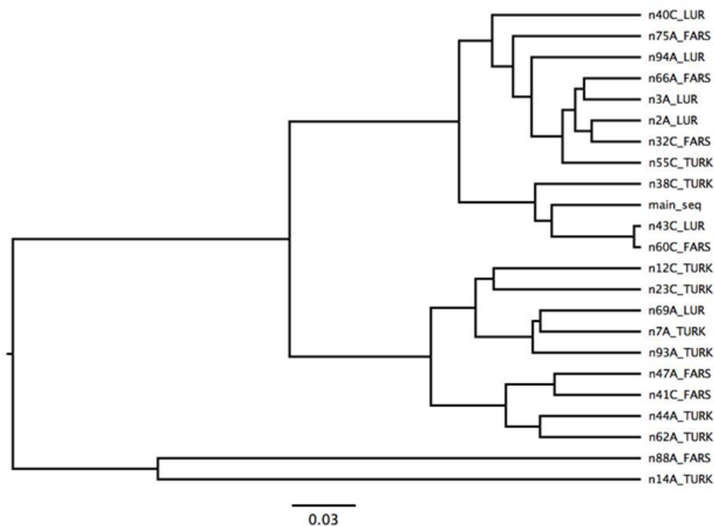

Figure 3. UPGMA dendrogram of the studied samples based on sequence data.

TCS network (Figure 4), reveals that the number of nucleotide substitutions ranges from 1-16 among the studied samples. Therefore, in general the sequence data represent an almost conserved region of the genome in our samples. TCS network of the studied samples indicate low level of genetic changes among the studied individuals and that the studied genome segment is of relatively conserved nature.

Detailed investigation of the adjacent nucleotides revealed the following known SNPs as well as variant nucleotides in our samples.

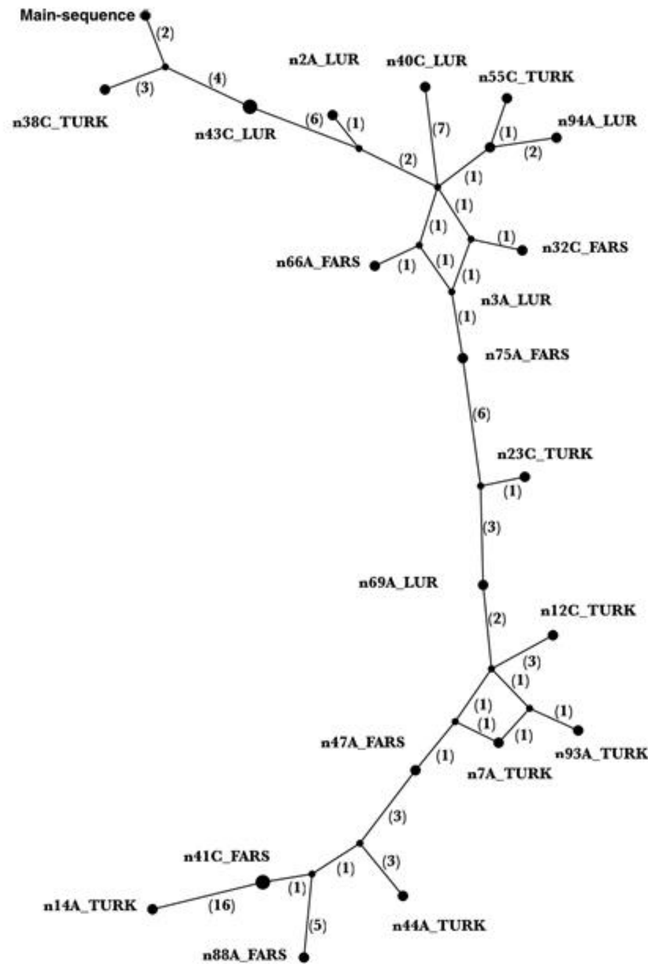

Figure 4. TCS network of the studied samples.

DISCUSSION

Tex14 gene is a good candidate for association studies based on ethnic groups and geographical populations as heterogenous reports are present on its association to infertility. For example, a statistically significant difference in *Tex14* gene expression was identified in azoospermic men by OKADA *et al*. (2008).

Similarly, in a study on expression of TEX14gene and its association with impaired spermatogenesis, BOROUJENI *et al*. (2018), quantified the expression level by RT-qPCR on samples retrieved from infertile patients submitted to diagnostic testicular biopsy and reported a significant down-regulation of this gene in the case group when compared to the control group. They concluded that regular expression of TEX14 is essential for the early stages of spermatogenesis.

However, ASTON *et al*. (2010), found no significant associations between TEX14 SNPs and azoospermia in genome wide association analyses of azoospermic men. We found a low degree of nucleotide substitutions in rs535296987 and adjacent nucleotides in our samples.

We did not see any significant association between rs535296987 in TEX14 gene with azoospermia in the studied samples. Moreover, adjacent nucleotides also showed no association with the segregation of the studied cases.

However, in 2 infertile brothers with non-obstructive azoospermia (SPGF23; 617707) from a consanguineous Iraqi Jewish family, GERSHONI *et al*. (2017) performed whole-genome sequencing and identified homozygosity for a 10-bp deletion in the TEX14 gene (605792.0001) that was not found in controls. Similarly, in 2 infertile brothers with nonobstructive azoospermia from a consanguineous Jordanian family, FAKHRO *et al*. (2018) identified homozygosity for a missense mutation in the TEX14 gene (R85L; 605792.0002) that segregated with disease. In addition, 2 unrelated sporadic infertile men with azoospermia, 1 from Nepal and 1 from Tunisia, carried homozygous TEX14 mutations that were not found in ethnically matched fertile controls. Testicular histology showed maturation arrest in the Jordanian brothers and Sertoli cell-only phenotype in the other 2 men.

Therefore, the heterogenous results for TEX14 gene association with azoospermia, may be partly due to the heterogeneity of azoospermic cases and also low frequency of *Tex14* mutations (SIRONEN *et al*., 2011)

In conclusion, we may suggest that large samples based on ethnic groups within each country and geographical region must be investigated to find out association of different SNPs within TEX14 gene and azoospermia.

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ASOCIATIVNO PROUČAVANJE RS535296987 *TEX14* GENA KOD AZOOSPERMIJE: RFLP I DNA SEKVENCIRANJE

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Izvod

Azoospermija je jedna od vrsta muške neplodnosti, sa klinički najtežim fenotipom, jer se prirodno začeće ne može dogoditi. Procenjeno je da pogađa 0,1 do 1% svih muškaraca i 10-15% muškaraca u neplodnim parovima. *TEX14* je gen za kodiranje proteina, koji se nalazi u humanom hromozomu 17, (17k22). Izgleda da je gen *TEX14* presudan za savršenu spermatogenezu, a funkcionalne studije ukazuju na ulogu *TEX14* u međućelijskim mostovima prilikom razvijanja muških ćelija. Gen sadrži 32 egzona i obuhvata 137 kb. Dostupni su heterogeni rezultati koji povezuju gen *TEX14* i azoospermiju. Stoga se predlaže da se istraži ovaj gen u različitim populacijama. Analizirali smo oko 200 muškaraca u dve kategorije azoospermije i zdravih osoba pomoću RFLP-a, kao i DNK sekvenciranja kako bismo ukazali na povezanost između rs535296987 u *TEX14* i njegovih susednih nukleotida sa azoospermijom. Nismo pronašli značajnu povezanost na osnovu RFLP podataka, a takođe i grupisanjem u klastere proučavanih uzoraka i kontrola na osnovu DNK sekvenciranja. Prema tome, i starost u ispitivanim uzorcima i nizak stepen mutacija u ovom genetskom regionu mogu biti razlog za heterogene izveštaje o povezanosti *TEX14* gena i azoospermije.
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