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Investigating the Effect of Beta-Defensin 126 Gene on Infertility Treatment by Intrauterine Insemination of Sperm through Genetic Analysis

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Abstract

This study aimed to determine the association between the mutation of Human β-defensin 126 (DEFB126) and the rate of success of intrauterine insemination (IUI) of sperm. In this study, DEFB126 gene mutation was detected in 76 Iranian males with infertility reasons for which women had positive IUI and negative IUI. After the extraction of DNA from the blood of the patients, the DNA quality extracted by the spectrophotometer was evaluated. Methods (PCR-SSCP) and sequencing were also used to confirm the results. Also, an immunocytochemistry technique was used to determine the expression of this protein on the surface of sperm cells. The results of the DNA review showed that 24.4% of the men whose fertility results were negative for their spouses had mutations, while those whose fertility results were positive did not have this mutation. Also, there was a lower protein content on the sperm level of men with a mutated allele. The results of this study indicate that having a mutant homozygote allele in the beta-daphnin 126 sequence is an effective factor in the failure of treatment with IUI.

Keywords: Beta-daphnin 126; Glycocalix; Genetic variation; intraperitoneal sperm motility.

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Introduction

One of the most serious problems that today's developed countries face is a declining birth rate. According to the World Health Organization (WHO), in 2011, infertility means non-pregnancy in couples who have had intercourse for one year without any method of prevention [1]. The cause of disbelief is unknown in approximately 20% to 30% of infertile couples [2]. One of the main treatments for intrauterine insemination (IUI: Intrauterine inseminate) is for this problem [3]. The overall success of the IUI method varies between fertility rates of 5% to 66% per cycle [4,5]. Sperm health indicators including number, morbidity, and morphology are the success factors in IUI [6]. With the apiphobia of sperm motility, several epithelium proteins, including hypnosis, cover the sperm surface to protect sperm from damage from radicals and harmful chemicals [7-9]. Daphnians are the most important antimicrobial peptides, which are the first hostile defense barrier against infections [10-12]. Definitive has a three-dimensional structure with beta b-sheet plates, and two main sub-sets are divided into 4 and 8 [11,12]. DEFB 126 is one of the sperm protein coatings that is produced in large numbers in the epididymis and is an important component of glycolic acid and plays an important role in effective sperm movement and immunological protection in the reproductive duct [13]. This protein is encoded by the beta-daphnin gene 126 located on the human chromosome 20 [14]. This protein is very similar to the ESP13 protein 2 is a secretion protein specific to the epididymis in the Cynomolgus cynomolgus monkey, and was formerly known by the same name. This small secretion protein has a stable disulfide center, which is particularly apparent in the epithelial cells that cover the tubes in Iran, the upper and lower parts of the epididymis [15,16]. The presence of beta-daphnes in coating for penetration and sperm motility in the cervix is necessary, and this seems to be due to the presence of sialic acid with a negative charge in the beta-daphnosin building [17]. DEFB 126 glycoprotein in events such as storage of sperm inside the demyelidum, sperm motility in the cervix mucus, sperm protection against the human immune system, enzymatic and microbial attacks, capacity and sperm binding to the epithelium of oviduct, and finally the creation of a reservoir of sperm is implicated in oviduct [18,19]. Studies have shown that the removal of DEFB126 by using anti-DEFB126 antibodies prevents sperm motility from entering the cervix in the laboratory and results in a significant reduction in the number of sperm that can cross the mucus of the cervix [17].

Recent reports have shown that the removal of two nucleoside cytosine in the DEFB126 gene causes a change in the reading frame and eventually the production of a non-stop mRNA [20]. The two nucleotides of cytosine are in the position of 317 encoded sequences (CDS: Coding DNA Sequences) of the gene [21]. Research has shown that in epithelial tissue, the abnormal DEFB126 mRNAs with deldel

genotype are much less than the mRNA derived from the wild type wild-type genotype of this gene. This decrease in the amount of abnormal mRNA can be due to the mechanism of immobilized mRNA degradation in the cell (NMD: Nonsense-mediated mRNA decay and its incomplete translation [20,22-26]. The results of a study showed that 47% of European men and 45% of Chinese men carry mutants in the beta-diphenyl gene [20]. These findings highlight the important role of DEFB126 in male infertility. Considering the importance of this gene and its important role, in this study, the elimination of two nucleotides in the DEFB126 gene of men whose spouses were treated with intrauterine injections of sperm and their association with the results of treatment of infertility by intraperitoneal injection of sperm it placed.

Material and Methods

This study was performed on 76 men with unexplained infertility referred for IUI to the Royan Institute of Reproductive and Infertility Center. This case-control study was carried out and blood samples and sperm samples were collected from individuals during the period of the one year from October 2017 to October 2018 after completing the information and satisfaction form. Among those referring to the Royan Institute, the case group comprised infertile couples with infertility without explanation that their spouse was treated with intrauterine injections of sperm, which were divided into two groups.

1) Women who underwent intraperitoneal injection of sperm and were pregnant (success in making a clinical pregnancy means seeing a fetus syringe by ultrasound, positive group).

2) Women who received an intraperitoneal injection of sperm, but no pregnancy was reported (no pregnancy, negative group).

After studying the clinical records of couples treated with intrauterine injections of sperm, according to the WHO criteria, the spermicidal indices included morphology of more than 7% and total movement of more than 40% and sperm count of more than 20% was considered. Also, in these subjects, smoking, alcohol, narcotics and any other diseases that led to infertility such as varicocele, hydrocele and orchiopexy hernia were considered as criteria for the study outcomes. In women, there were also any fertility disorders Ovarian causes, endometriosis, polycystic ovarian syndrome (PCO), cervical factors, decreased ovarian reserve, ovarian failure, smoking, alcohol, and drug were excluded.

After extraction of DNA from the blood of the studied patients, the DNA quality extracted by the 2000 NanoDrop Scientific Spectrophotometer was evaluated. The 3-AAGAATGGTTGGGCAATGTGC-5 and 3-CCACCATGCTTTAATGAGTCGGG-3 were used to study the genetic changes in the beta-daphnin 126 gene in the studied groups. The PCR cycle program was as follows.

PCR products were stained with 2% agarose gels and electrophoresed with ethidium bromide (Sigma) and detected by Gel Doc XR, Gel Doc (Bio-Rad). Single-strand conformation polymorphism (SSCP) was performed as follows: DNA with bromophenol blue (Ferments) containing 95% amide form (Roche). Xylene cyanide and EDTA were mixed in half a millimeter (Merck). The mixture was incubated for 95 minutes at 95°C for 10 minutes, then cooled for 10 minutes on ice and acerbated on acrylamide gel (0.29: 0.7 mm) in a buffer of 0.05. Electrophoresis (Bi-Rad / Consort) at 5°C for 2 hours, then stained with silver nitrate method in three steps. Finally, after gel banding, the gel was scanned by a Bio-Rad scanner, with the help of Schedule 4.62 Quantity One Version, and then shot with gel using GelDoc.

The product of PCRs that exhibited similar gages in SSCP on a polyacrylamide gel was classified into distinct groups and from each group, several samples were ordered for sequencing with the company Fasbiotec in the United States by Farapage Corporation in Iran. The company performed the sequencing of the desired components using the trench sequencing method in the XLABI 3730 capillary sequencer system. The sequences were then analyzed by FinchTV software and Aligen Sequences Nucleotide BLAST. The semen was collected from the volunteers and kept at room temperature for 1 hour. The semen was washed using PBS and centrifuged at 2000 rpm. Then mixed in one ml of PBS and transferred to the slide. The slides, after drying, were washed with 4% (Sigma) phax paraformaldehyde and washed with PBS after 1 hour and dried again. The slides were then incubated with BSA 3% (Sigma) at 37°C and treated with BSA diluted 1: 50 for 1 hour after 2 hours. Then, the slides were washed 3 times with PBs for 5 minutes each time and incubated with 370°C for 1 hour with secondary antibody FITC (1: 500 dilution) (Santa Cruz Biotechnology). Finally, the specimens were washed three times in PBS. IP-AD was used to color the kernel. The cells were finally examined using a fluorescent microscope.

Results

In this study, gene variants of exon 2 from the DEFB126 gene were studied. For this study, SSCP PCR DNA extraction techniques and sequencing techniques were used. Also, the immunocytochemistry technique was used to check beta-daphnin 126 protein expressions. To check the quality of DNA used in terms of purity and concentration using Nanodrop it became clear that the quality of DNA samples from all the men was in a satisfactory condition so that about 280 to 260 between 1/8 to 1/92, which represents Non-contamination with protein and RNA. Their concentration was also between 1000 and 200 ng/ml. After applying the optimal conditions for the primer E2-Beta defensin 126, 258 pairs of games containing exon 2 of the DEFB 126 gene were amplified in 76 patients. The performance results of this primer pair are shown in (Figure 1). In all patients, the function of this primer pair has led to the proliferation of the desired part.

After performing the SSCP technique for exon 2 genomic PCR production, 126 beta-daphnin gene in 76 patients were classified into three different groups based on banding patterns (Figure 1).

In negative IUI patients, 27 patients in group 1, 7 in group 2 and eventually 11 in group 3. In IUI positive patients, according to the above figure, 16 in group 1, 15 in group 2 were divided (Figure 2).

The results of sequencing with BLAST software were fully validated by SSCP results, so that in 11 samples of patients with band 3 of SSCP,

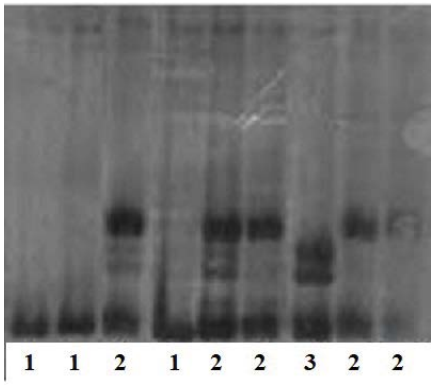

Figure 1: Denatured polyacrylamide gel after staining with sliver nitrate.

a two nucleotide change (two nucleotide deletions of cytosine) was observed in the exon 2 sequence. 55 samples from patients with groups 2 and 1 of SSCP did not show any changes in the exon 2 sequence. The wt/wt genotype is for a person who has no 2 nucleotide changes in this region (exon 2) and has all 5 cytosine (normal humosa) (Figure 3a). The genotype wt/del is also related to those who have two nucleotide changes in There is no Exon 2 region, but in FinchTV software it is observed that in the region in the nucleotide region 393 and 394 there are two couriers, one of which is the peak of the normal allele and a peak of the mutated allele (heterozygote) (Figure 3b).

The del/del genotype is for those individuals who have two nucleotide changes (removal of two nucleotides of cytosine) in the exon 2 region (mutated homozygote) (Figure 3C).

After statistical analysis of the data, there was a significant difference between the two groups in the wt/wt and del/del genotypes, which had P value of 0.001 and 0.003, respectively. These results indicate that mutated homozygote genotype can be a factor in male infertility (Figure 4, Tables 1 and 2).

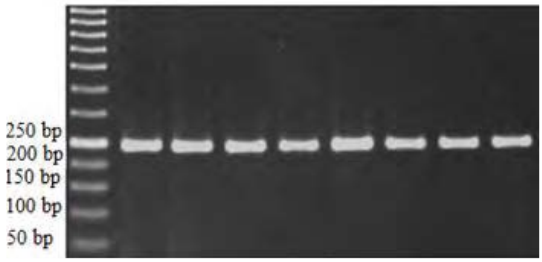

Figure 2: The image of denatured polyacrylamide gel after staining with sliver nitrate for PCR products of Exon 2 genome area of 9 persons from IUI+ and IUI- group.

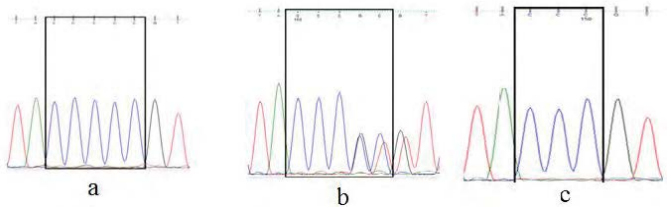

Figure 3: The sequence of different patterns of denatured polyacrylamide gel from exon 2 patients.

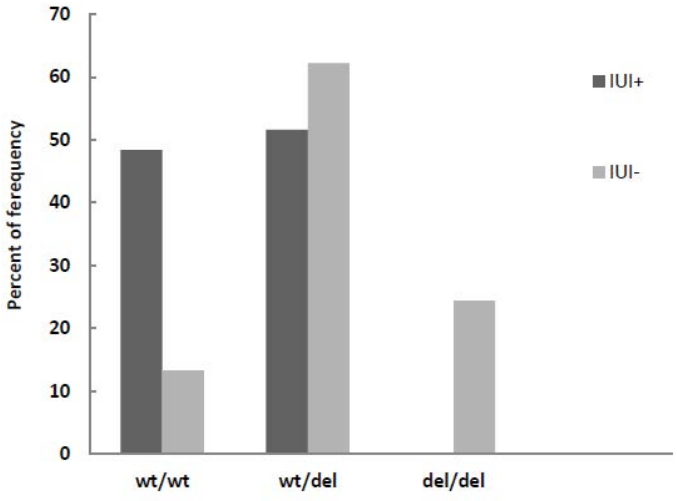

Figure 4: The percentages of different genotypes in two IUI+ and IUI- groups.

Table 1: PCR cycles program.

Repeat	Time (min)	Temperature (°C)	Performance of each stage
1 cycle	3	94	Initiation denaturation
	1	94	denaturation
30 cycles	1	58.5	Annealing
	1	72	Extension
1 cycle	7	72	Final extension

Table 2: Results of statistical analysis of data obtained from genetic study of exon Beta-deftenzen gene 129 taking into account 05> P.

	Total	Mutated homozygote	Healthy homozygote	Heterozygote
Number	45	11	6	28
Percent (%)	100	34.4	13.3	62.2
Number	31	0	15	16
Percent (%)	100	0	48.4	51.6
Number	76	11	21	44
Percent (%)	100	14.5	27.6	57.9
P.V	0.001	0.003	0.001	0.35

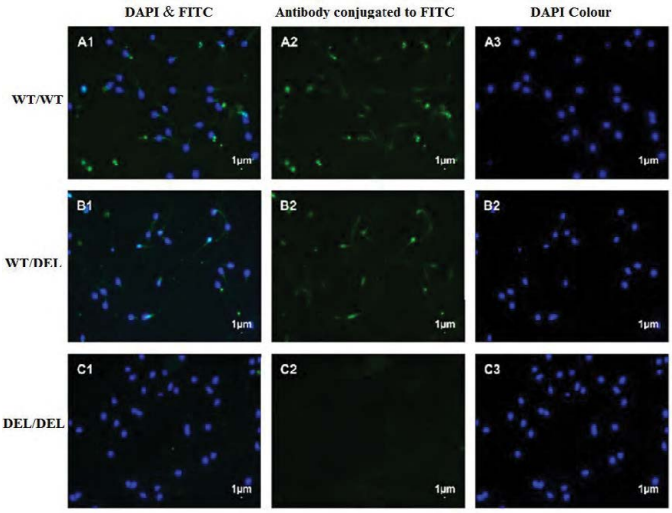

Figure 5: The results of immunogenicity staining. Column A represents the images of staining of cell nucleus with DAPI dye and cell staining with FITC-conjugated antibodies, Column B includes the images of cells stained with FITC-conjugated antibodies, and the column C includes the images of staining of cell nucleus with DAPI dye.

In order to confirm the existence of beta-daphnin 126 protein on the human sperm surface and the expression of this protein on the sperm level, individuals with different immunotoxicity or specific antibody staining beta-diphencin 126 were used. Immunocytochemistry results are shown in (Figure 5).

Given that the selected secondary antibody (conjugate to the FITC) is green, the level of sperm was seen in green. The blue color represents the nucleus of the cells stained with DAPI color. As shown in the figure, the amount of this protein in humans was del/del homozygote less than that of homozygous genotype wt/wt. Also, the level of this protein in the sperm level was also lower in individuals with heterozygote than in healthy homozygotes.

Discussion and Conclusion

The genetic variation studied is a two nucleotide deletion that results in the formation of a codon-free mRNA. Men who have this deletion are homozygous and produce spermatozoa that are defective

in the oligo-saccharides bound to the surface proteins and are in difficulty to penetrate the in vitro hyaluronic acid (HA) gel [27]. The present study first examined the effect of specific genotype (deldel) on the beta-daphnin gene in 126 men whose spouses were treated with intrauterine injections of sperm and their relationship with infertility with an unknown factor. The question was whether the genotype is del/del homozygote a factor in the failure to treat IUI? By analysing the data obtained from the study of the beta-defensin 126 gene in the studied patients, IUI-positive and IUI-negative groups were observed. Deletion in this gene was significantly higher in the negative IUI group than in IUI-positive. The prevalence of homozygote withdrawal in patients whose fertility was negative was 24.4% and in patients with positive fertility rates was zero ($P < 0.05$). Previously, Tollner TL, et al. (2008) examined only 638 Chinese men in the study. 29% healthy homozygote genotype, 52% heterozygote genotype and 19% mutated homozygous genotype of the subjects(19%) [20].

In the present study, the frequency of mutation subjects with negative fertility results was more than those with positive fertility. In 76 patients, IUI-positive and IUI-negative groups of people with normal homozygous genotype were 27.7% heterozygote (57%) and mutated homozygote (14.5%). After analysing the genotypes and determining the genotype of the subjects, and immunocytochemistry techniques were used for protein analysis. In this study, the sperms of the subjects were used to evaluate the quality of beta-daphnin 126 expression and its association with male infertility and the success of IUI treatment. The results showed that the expression of this protein on the sperm level was lower in patients with cytosine deletion in the beta-daphnin 126 genes than those without this deletion (deldel genotype versus wt/wt). It was also observed that the level of expression of this protein on the sperm level of those with wt/del genotype was also significantly lower than that of wt/wt genotype. These results have been shown by Rozen's study with the immunofluorescence technique that in individuals with homozygous genotype in monomeric coat, the sperm level is defective (21). Accordingly, beta-daphnin 126 is a major component of glycolic acid in the sperm surface and is effective for normal sperm function, including effective sperm motility in the reproductive duct. Since the IUI method plays an important role in sperm production in the reproductive tract, beta-daphnin 126 can be a factor in the success of treatment by intraperitoneal injection of sperm in these patients. Considering the important role of this protein in facilitating the movement of sperm in female reproductive organs, sperm protection against anti-sperm antibodies, and increasing the potential for fertilization of sperm with oocyte (20), and according to the results obtained in this study, it can be concluded that The occurrence of mutation in beta-daphnin 126 genes can affect their fertility and lead to a reduction in the success of IUI treatment.

Among the limitations of this study, the low number of infertile couples from unknown cases who are candidates for IUI action can be mentioned in the limited period of the project. If more studies are done in this field, this genotype can be used as a measure to determine and predict infertility treatment outcomes, and then doctors will be able to choose a more appropriate technique for treating infertility in these patients that health care costs will be spent.

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