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$C_{26232}T$ Mutation in *Nsun*7 Gene and Reduce Sperm Motility in Asthenoteratospermic Men

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Abstract
Reduced quantity and motility in sperm are primary causes of men infertility. Before researchers showed that, *Nsun*7 gene has roles in sperm motility of mice and mutation in this gene can cause defect in Nsun7 protein function and infertility. This gene in human has a hot spot exon (exon7). Our aim is study of the mutations of the exon7 in the normospermic and asthenoteratospermic men. For this, 60 semen samples including fertile and asthenoteratospermic were collected from IVF center. Semen analysis was performed according to WHO guidelines. A Phenol-chloroform method was used for total DNA extraction from sperm. The exons 7 amplified by forward primer Sun7-F: 5'-GACAAATCTCGAAGTCTTGCTG; and reverse primer Sun7-R: 5'-ACATCCTATTTTTGTGAAAAGGGT. Analyses of direct sequences of the PCR products, showed transition mutation ($C_{26232}T$) in asthenoteratospermic men. This mutation doesn't see in fertile men. Reduced semen parameters such as motility, of the asthenoteratospermic men can be close correlate with this mutation. So, analyses of the exon 7 of the *Nsun*7 gene can be candidate as a one of diagnosis genetic markers in infertile men.

Key words: *Nsun*7, Sperm motility, Transition mutation, Infertile men, Asthenoteratospermic

Introduction

Infertility is a major clinical problem that affects about 10 to 15% of couples in the world (Gnoth 2005). Men infertility included 40 to 50% of all cases of infertility (Kretser and Baker., 1999). Sperm dysfunction is one of the main causes of men infertility (Spiropoulos *et al*., 2002). Measure the number and motility of sperm in semen is used to evaluate sperm performance (Dana and Alan., 1996), especially when these parameters are extremely low, are used as the criterion for defining abnormal sperm (Dana and Alan., 1996). Evaluation of sperm motility is one of the most important semen analysis (Juhasz *et al*., 2000). Infertility is a complex disease that is caused by the interaction between genetic and environmental factors (Zhou *et al*., 2010). Defective sperm function has been identified as the greatest among the defined causes of men infertility, accounting for about 27% of all couples attending infertility clinics (Hull *et al*., 1985). Since progressive sperm motility can only pass through the cervical mucus, the assessment of sperm motility in semen analysis is necessary (Dana and Alan., 1996). If reduced sperm motility, the number of sperm that reach the female genital tract are decrease, so it is less chance of fertility (Dana and Alan., 1996). Sperm motility defect can be influenced by abnormalities of the flagellar movement (Holstein *et al*., 2003). Because loss of parts of the structure of flagella, immobilization of flagella is increases (Holstein *et al.*, 2003). Nsun7 gene has roles in sperm motility that defect in this gene can cause infertility men (Harris *et al*., 2007). Nsun7 gene being in chromosome 4 (Location: chr4 40446671-40506755 (+): 4p14. forward strand) (Fig. 1) and has 12 exons and 11 introns. *Nsun7* gene has 60085 bp length and protein coding has

3697-bp and 718 aa length. Nsun7 gene have been found in most eukaryotes and Nop2/sun domain is strongly protected and the name of this gene is taken from this domain (Fig. 2). In the present study, we investigated exon7 mutations that is hot spot and is located in protected sun domain and associated with reduced fertility and human sperm motility in idiopathic asthenoteratospermic men.

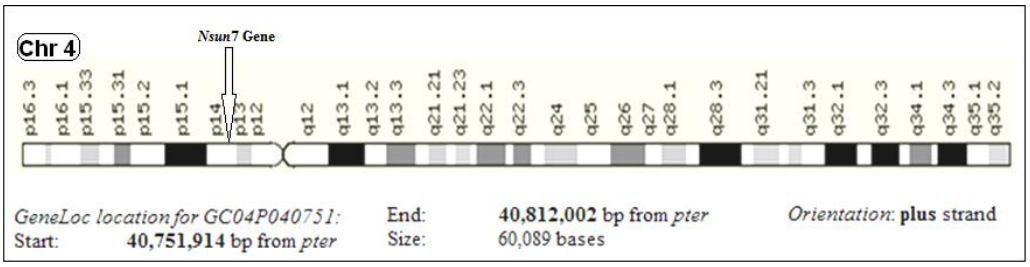

Fig. 1. Schematic drawing of the *Nsun*7 gene position.

Materials and methods

Sample collection and semen analysis

We collected 60 semen samples from infertile and fertile men in the infertility clinics at Fatemeh Zahra Hospital (Babol, Iran) into sterile containers after a period for 3-4 days of abstinence. Samples were from aged 25 to 35 years. Before semen analysis, a questionnaire was distributed to get information about age and lifestyle of male.

Routine analysis of semen was carried out within 1 h according to World Health Organization guidelines (WHO 1999). Samples were then classified to fertile [including 20 "normospermic" sample] and infertile [including 40 "asthenoteratospermic" sample (with motility <50%)]. Standard semen parameters analysis according to WHO guidelines are shown in (Table 1).

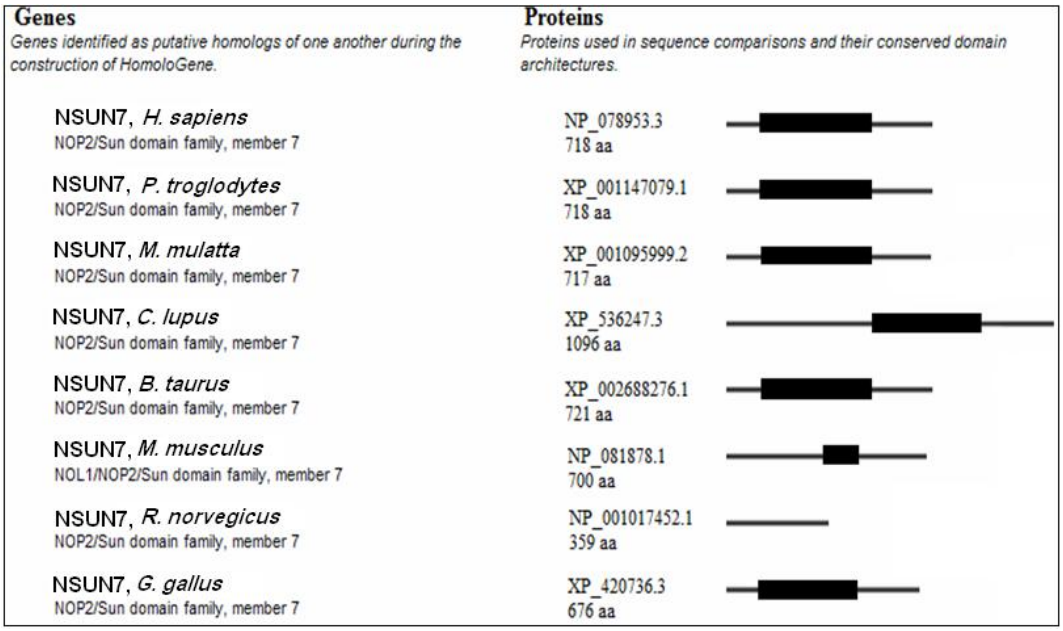

Fig. 2. Conserved domain in some of Homologous of the *Nsun*7 gene: NP_ and XP_ are protein ID number and the digits after the point, are version.

Table 1. Standard semen parameters analysis according to WHO.

Parameters	Analysis
Volume	>2.0 ml
Sperm Concentration	>20 million per ml
Total spermcount	>40% million per ejaculate
Motility	>50% with forward progression or >25% with rapid progression
Morphology	>30% (lower if strict criteria employed)

Extraction of the total sperm DNA

Total DNA of human spermatozoa was extracted according to the method of Phenol-chloroform. After centrifugation for 10 min at ×2000 g at room temperature, the supernatant was removed and the pellet of
spermatozoa was washed twice with 0.9% NaCl solution and an aliquot of 2–3 $\times 10^7$ spermatozoa was incubated at 56°C for 2 h in a lysis buffer containing 2% sodium dodecyl sulphate (SDS), 10 mM dithiothreitol, 100 µg/mL proteinase K, and 50 mM Tris–Cl (pH 8.3).
After digestion, the supernatant was extracted with phenol/chloroform (1:1, v/v). The aqueous layers were pooled and DNA was precipitated with isopropanol (1:1, v/v) and one-tenth volume of 3 M sodium acetate (pH 5.6), and incubated at -20°C overnight. After washing with 75% ethanol (v/v), the pellet was dried and resuspended in double-distilled water. They stored at –20°C until use**.**

Electrophoresis of the total sperm DNA

Extracted total DNA, were separated on a agarose gel (Fluka) at Voltage 90 V for 2 h, with 1X TBE buffer (contain: 10.8 g of Tris base. 5.5 g of boric acid. 4 ml of 0.5 M EDTA (pH 8.0)), and DNA bands were visualized by transillumination under UV light after staining with 1µg/mL ethidium bromide (Merck) at 25°C for 5 min. Agarose gel electrophoresis and gel staning performed by Green and Sambrook (2012) methods (Green and sambrook 2012).

Synthesis of Oligonucleotide Primers for exon7

We designed primer pairs (forward and reverse) for exon7 with NCBI and Oligo7. The nucleotide sequences and sizes of the polymerase chain reaction (PCR) products amplified from each of the primer pairs are shown in Table 2.

Table 2. Oligonucleotide primers used for exon7 amplification.

Name	Sequence (5'–3')	Amplified position	Oligomer	PCR product(bp)
Sun7-F [a]	GACAAATCTCGAAGTCTTGCTG	26152 - 26173	22 mer	207
Sun7-R [b]	ACATCCTATTTTTGTGAAAAGGGT	26335 - 26358	24 mer	207

[a] Forward primer; [b] Reverse primer

Polymerase chain reaction (PCR) of exon7

After total DNA extraction from spermatozoa, we used of primer pairs sun7-F/sun7-R to amplify 207 bp fragments exon7 in all samples. The target fragments of exon7 were amplified from 20 ng of DNA in a 50 µl reaction mixture containing 5 µM buffer PCR, 1.5 µM $MgCl_2$, 1.5 µM of dNTP, 2.3 µM of each primer (forward and reverse), 5 µM of total DNA extracted, and 4 µM of *Taq* DNA polymerase. PCR was carried out for 35 cycles in a DNA thermal cycler (Eppendorf Master cycler Gradient, Germany) using the thermal profile of denaturation at 94°C for 1 min, annealing at 55°C for 1 min, and primer extension at 72°C for 1 min. PCR method done for amplify 207 bp fragments exon7 and after electrophoresis to ensure the accuracy of the PCR method, products PCR (exon7) were sent for sequencing to find mutations in them.

Electrophoresis of The PCR products

Products PCR (exon7) were separated on a agarose gel (Fluka) at 70 V for 3.30 h, with 1X TBE buffer and DNA bands were visualized by transillumination under UV light after staining with 1µg/mL ethidium bromide (Merck) at 25°C for 5 min. Agarose gel electrophoresis and gel staning performed by Green and Sambrook (2012) methods.

DNA sequencing

Direct sequencing of the purified PCR products were done with the Seq Lab Co. Gottingen, Germany. Sequence analysis was performed with the program chromas.

Results

Total subjects of 40 semen samples (asthenoteratospermic) were collected from IVF center. Analysis of standard semen parameters in normospermic and asthenospermic samples were performed according to WHO guidelines (WHO 1999). Total DNA of human spermatozoa 0was extracted according to the method of Phenol-chloroform. Then extracted total DNA, were separated on an agarose gel at Voltage 90 V for 2 h, with 1X TBE buffer. The electrophoresis results are shown in Fig. 3.

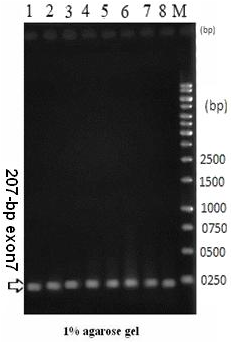

Fig. 4. Electrophoretogram of the PCR products: PCR product amplified (207-bp) from sperm total DNA for showing mutation existence in exon7, *Nsun*7 gene. Line 8, normospermic; Line 1, 2, 3, 4, 5, 6, and 7, asthenoteratospermic.

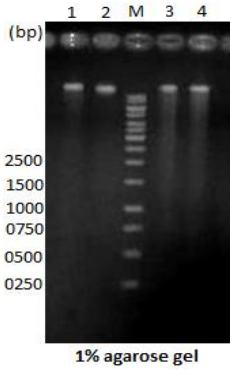

Fig. 3. Total genomic DNA of the sperm in the agarose gel electrophoresis: Line1, normospermic; Line2, 3 and 4, asthenoteratospermic.

After total DNA extraction from spermatozoa and electrophoresis, we used of primer pairs sun7-F/sun7-R to amplify 207 bp fragments exon7 in normospermic and asthenoteratospermic samples. PCR was performed in a way that was said. The PCR products of 207-bp generated from sperm total DNA by primers (sun7-F, sun7-R) respectively. Products PCR fragments (exon7) were separated on an agarose gel at 70 V for 3.30 h. The electrophoregram of the PCR products 207-bp, exon7 are shown in Fig. 4. DNA fragments of PCR products, 207-bp exon7, were Sequencing and analyzed. The Analysis results are shown in figure 5.

Our results in Fig. 5 showed transition mutation ($C_{26232}T$) in exon7, *Nsun*7 gene in human sperm with poor motility (asthenoteratospermic men). As a result, the incidences of mutation exon7 in asthenoteratospermic (A), groups were significantly higher than normal group. This mutation doesn't see in fertile men.

Discussion and Conclusions

Sperm motility has been established as a good indicator of semen quality and men fertility. Tredway *et al.,* 1975 announced that poor sperm motility is one of the causes of men infertility and most patients with poor sperm motility also have reduced sperm count or a greater number of abnormally shaped sperm in their semen. Kao *et al.,* 1995, expressed that poor sperm motility is one of the most significant causes of male infertility. Harris *et al*., 2007, announced the *Nsun*7 gene from mice infertile have roles in sperm motility, which this gene linked to rigidity of the midpiece and defect in this gene can be

affected in mice infertility. But we studied the mutations exon7 from the *Nsun*7 gene in infertile men that defect in this gene can cause infertility.

In the present study, we have recognized mutation in the exon7 of the *Nsun*7 gene, by using PCR. The PCR products (207-bp DNA fragments) were amplified mutation in exon7. Our PCR analysis were showed that there was a point mutation in the position of 26232 (Fig. 5C). Direct sequencing clearly was indicated transition mutation ($C_{26232}T$) in exon7, from the *Nsun*7 gene, with poor sperm motility by using the primer pair's sun7F/sun7-R (Fig. 5A/B).

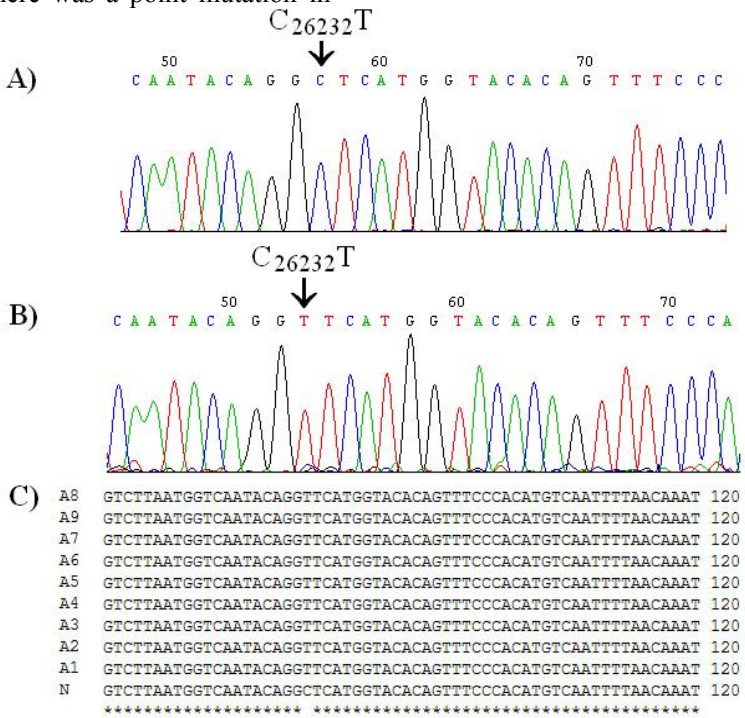

Fig. 5. Direct sequencing of exon7 of the *Nsun*7 gene in asthenoteratospermic men: (A), The wild type sequence showing 'C' at the nucleotide position 26232 in the *Nsun*7 gene; (B), Sequence of a asthenoteratospermic infertile men with poor motility showing mutant allele 'T', which was observed in a total of 20 asthenoteratospermic infertile men; (C), Blast analysis of the exon7 sequences in all A= asthenoteratospermic and N= Normospermic samples.

In conclusion, this study has demonstrated that the frequency of occurrence and proportion of transition mutation ($C_{26232}T$) in the exon7 of the *Nsun*7 gene, in poor sperm motility, asthenoteratospermic men (A) were significantly higher than in normal sperm motility, normospermic men. According to the motility of sperm is one of the most important characteristics of successful spermatozoa; so the *Nsun*7 gene products, exon7, have roles in sperm motility that creation defect in this gene can cause infertility. Probably, low semen parameters of the asthenoteratospermic men can be close correlate with mutations in the exon7 of the *Nsun*7 gene. Thus, analyses of the exon 7 direct sequence candidate as a one of diagnosis genetic markers of infertility.

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