

Research Article
XRCC5 VNTR, XRCC6 -61C>G, and XRCC7 6721G>T Gene Polymorphisms Associated with Male Infertility Risk: Evidences from Case-Control and In Silico Studies

Danial Jahantigh and Abasalt Hosseinzadeh Colagar

Department of Molecular and Cell Biology, Faculty of Basic Sciences, University of Mazandaran, Babolsar, Iran

Correspondence should be addressed to Abasalt Hosseinzadeh Colagar; colagar@yahoo.com

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We evaluate the association between genetic polymorphisms of XRCC5 VNTR, XRCC6 -61C>G, and XRCC7 6721G>T with male infertility susceptibility. A total of 392 men including 178 infertile males (102 idiopathic azoospermia and 76 severe oligozoospermia) and 214 healthy controls were recruited. XRCC6 -61C>G and XRCC7 6721G>T genotyping was performed by PCR-RFLP whereas XRCC5 VNTR was performed by PCR. The 2R allele and 2R allele carriers of XRCC5 VNTR polymorphism significantly decreased risk of male infertility. The mutant GG genotypes and carriers of the CG and GG genotypes of XRCC6 -61C>G showed increased risk for the male infertility. Furthermore, the G allele of the XRCC6 -61C>G was correlated with increased susceptibility to male infertility. Likewise, the T allele of the XRCC7 6721G>T polymorphism was associated with increased susceptibility to male infertility in azoospermia. In silico analysis predicted that the presence of tandem repeats in XRCC5 gene promoter can be sequenced to bind to more nuclear factors. Also, rs2267437 (C>G) variant was located in a well-conserved region in XRCC6 promoter and this variation might lead to differential allelic expression.

The XRCC7 6721G>T gene polymorphism occurred in an acceptor-splicing site, but this polymorphism has no severe modification on XRCC7 mRNA splicing. Our results indicate the association of XRCC5 VNTR, XRCC6 -61C>G, and XRCC7 6721G>T gene polymorphisms with male infertility in Iranian men.

1. Introduction

Male infertility is responsible for 40–50% infertility problems which affects up to one in six couples worldwide [1]. However, the exact etiology and pathogenesis of approximately half of the male infertility cases remains unknown yet, and it is well known as idiopathic infertility. Some evidences declared that DNA damage in human spermatozoa is associated with poor semen quality and low fertilization rates for both in vitro and in vivo fertility, suggesting that sperm DNA damage could be used as a potential predictor of fertility [2, 3].

Defects in DNA repair pathways during spermatogenesis have negative effects on the integrity of sperm DNA; could decrease quality and quantity of sperm including morphology, movement, and number; and cause infertility [4–6]. DNA damages, single strand breaks (SSBs), and double strand breaks (DSBs) are induced by exogenous and endogenous agents such as ionizing radiation (IR), oxidative and replication stress, and naturally programmed processes including meiotic and V(D)J recombination ([7–10]; Zha et al. 2007).

The DSB is the most harmful form which should be repaired with homologous recombination (HR) or predominantly nonhomologous end joining (NHEJ) [9, 10]. NHEJ involves binding of the Ku protein, Ku70 (XRCC6)/80 (XRCC5) heterodimer, to broken DNA ends, which induces inward translocation of Ku and recruits the DNA-PK catalytic subunit (DNA-PKcs) (XRCC7), to the ends of the DSB to form DNA-dependent protein kinase (DNA-PK) [10]. All components of the DNA-PK, including Ku70, Ku80, and DNA-PKcs, were found in the radiosensitive spermatogonia, so that the Ku70 and/or Ku80-deficient testis displays
elevated levels of DSBs as well as enhanced apoptosis and reduced sperm production [11–15]. It is reported that Ku proteins are downregulated in early meiotic cells, but are again expressed in late pachytene and diplo- tene spermatocytes, and they mediate repair proficiency in somatic cells of the testis, thereby assuring the fidelity of spermatogenesis [11–13].

Strong evidences support the relationship between genetic polymorphism of genes involved in DNA repair pathways with extra sperm DNA damage and male infertility risk [16–21]. Among several genetic variants of the XRCC5, XRCC6, and XRCC7, a novel variable number of tandem repeats (VNTR) in the promoter region of XRCC5 and two single nucleotide polymorphisms (SNPs) XRCC6 -61C>G and XRCC7 6721G>T located in the promoter region of XRCC6 and intron 8 of XRCC7 genes, respectively, have been extensively studied in various disorders such as cancer [22–29] and autoimmune diseases [30, 31], but whether genetic variants in these repair pathway genes affect susceptibility to male infertility remains unknown.

Since the Ku protein, Ku70 (XRCC6)/80 (XRCC5), and DNA-PKcs (XRCC7) as critical components of NHEJ play important role in DNA integrity of spermatogenesis and can affect the offspring, we hypothesize that genetic variation of these genes may contribute to male infertility risk. Therefore, in current study, we evaluated possible relation between these common and functional polymorphisms with male infertility risk in a case-control study, and also, in silico analysis was carried out to investigate the effects of these SNPs on the interaction of several factors and motifs, involved in transcription and mRNA splicing.

2. Material and Methods

2.1. Patients and Control Samples. A total of 178 infertile patients, including 102 men with idiopathic nonobstructive azoospermia and 76 men with severe oligozoospermia (semen count less than $5 \times 10^6$/mL), aged 24–39 years, and mean $31.6 \pm 4.4$ years, were recruited from the Fatemeh Zahra In Vitro Fertilization (IVF) Center in Babol, Iran, between 2013 and 2015 (Table 1). Semen samples were obtained by masturbation into sterile tubes after sexual abstinence for 2 to 3 days. Before semen analysis, a questionnaire was distributed to obtain information on smoking habits, alcohol use, use or abuse of other substances and drugs, and a history of reproductive system diseases. The samples with known causes of infertility were excluded; therefore, the patients with history of cryptorchidism, orchitis, obstruction of the vas deferens, varicocele, infectious diseases, drug abuse, diabetes mellitus, abnormal hormone profile (LH, FSH, and testosterone), Y-chromosome microdeletion, and abnormal karyotype were excluded from the study. So, only men with idiopathic infertility were included in the study. All patients underwent at least two semen analyses according to World Health Organization (WHO) guidelines after 3–5 days of sexual abstinence. The control group consisted of 214 men who were proven fertile with normozoospermia aged from 23 to 48 years. These studies were approved by the Ethics Committees of University of Mazandaran

| Variable | Control (n = 214) | Total (n = 178) | Patients AS (n = 102) | Severe OS (n = 76) | Control versus total | Control versus AS | Control versus severe OS |
|----------|------------------|----------------|----------------------|-------------------|----------------------|-----------------|------------------------|
| Age (years: mean ± SD) | 31.6 ± 4.4 | 32.3 ± 2.7 | 32.05 ± 4.1 | 31.8 ± 3.6 | 0.191 | 0.540 | 0.582 |
| Body mass index (kg/m²) (%) | | | | | | | |
| ≤24.9 | 27.1 | 25.8 | 23.5 | 28.9 | | | |
| 25–34.9 | 66.3 | 64.04 | 64.7 | 63.1 | | | |
| ≥35 | 6.5 | 10.1 | 11.7 | 7.8 | 0.201 | 0.119 | 0.689 |
| Smoking (%) | | | | | | | |
| Yes | 30.8 | 34.8 | 37.2 | 31.5 | | | |
| No | 69.1 | 65.1 | 62.7 | 68.4 | 0.401 | 0.257 | 0.904 |
| Semen analysis (%) | | | | | | | |
| (1) Sperm count ≤ 20 (million/mL) | 0 | 100 | 100 | 100 | <0.001 | <0.001 | <0.001 |
| (2) Motility (grade a + b) | | | | | | | |
| ≥50 | 68.2 | 10.1 | 9.8 | 10.5 | | | |
| <50 | 31.7 | 89.8 | 90.1 | 89.4 | <0.001 | <0.001 | <0.001 |
| (3) Viscosity | | | | | | | |
| ≤30 min | 57.9 | 73.03 | 72.5 | 73.6 | | | |
| >30 min | 42.05 | 26.9 | 27.4 | 26.3 | 0.316 | 0.251 | 0.694 |
| (4) Normal morphology | | | | | | | |
| ≥30% | 34.5 | 13.4 | 11.7 | 15.78 | | | |
| <30% | 65.4 | 86.5 | 88.2 | 84.2 | 0.001 | 0.004 | 0.004 |

AS: azoospermia, OS: oligozoospermia.
and informed consent was obtained from each subject before participation.

2.2. DNA Extraction, PCR, and RFLP. Genomic DNA was extracted from blood samples, which were collected in 2 ml EDTA tubes from patients and healthy controls, by using the commercial available kit (Roche, Germany). Extracted DNA was stored at −20°C. Fragments of the XRCC5 3R/2R/1R/0R-VNTR, XRCC6 -61C>G promoter, and XRCC7 6721G>T amplified by polymerase chain reaction (PCR) using XRCC5-III-r, XRCC6-III-r, and XRCC7-III-r primers, which were designed by [26], and PCR conditions, were described by [30].

DNA fragments of the XRCC6 -61C>G and XRCC7 6721G>T were used in PCR-restriction fragment length polymorphism (PCR-RFLP) technique. Approximately 5 μl (0.1 μg) of the XRCC6 -61C>G and XRCC7 6721G>T amplified products were digested with 5 units of the BanI (with cut site: 5'-GGCGCC) and Pvu II (with cut site: 5’-CAGCCTG), respectively. Digestion reactions were performed at the total volume 10 μl with incubation at 37°C for 16h. PCR and/or digestion products were electrophoresed on a 3% agarose gel and visualized by ethidium bromide staining. All of the PCR and restriction reagents were purchased from CinnaGen Co, Iran. All of the electrophoresis materials were prepared from Merck Co, Germany.

2.3. In Silico Analysis. In silico analysis was performed to evaluate potential biological functions of two promoteric SNPs, rs6147172 and rs2267437, which are located in promoter region of XRCC5 and XRCC6 genes, respectively. Also, an intronic SNP, rs7003908, which is located in the intron 8 of XRCC7 gene, was subject of in silico analysis.

For detection of core promoter motifs, the DNA sequences that contain promoteric SNPs were screened by the prediction tools, EPD [32] and ElemEnt [33]. Also, SNPnexus [34] and PROMO [35] were used to find potential transcription factor binding sites in SNPs containing promoter sequences. The intronic sequence of XRCC7 gene that contains 6721G>T mutation was analyzed by Human Splicing Finder version 3, HSF3 [36], SpliceAid 2 [37], and SplicePort [38] to predict the effects of this variation on XRCC7 mRNA splicing. Moreover, the conservation of the DNA sequences containing XRCC6 -61C>G and XRCC7 6721G>T sites were also evaluated by Golden Path [39] and further illustrated by WebLogo [40].

2.4. Statistical Analysis. Hardy-Weinberg equilibrium (HWE) was calculated for both the infertile and control groups (http://www.oegge.org/software/Hardy–Weinberg.html). All data were analyzed using SPSS software version 18. Differences in frequency of alleles and genotypes were analyzed using the chi-square test or Fisher’s exact test. The odds ratio (OR) and 95% confidence intervals (CIs) were estimated. Two-tailed \( p < 0.05 \) was considered statistically significant.

According to our results, sample power was computed using STATA 10 software.

3. Results

3.1. Demographic Analysis. Demographic and clinical characteristics of infertile patients and controls have been mentioned in Table 1. The infertile men were different from fertile men in sperm count, sperm motility, and sperm morphology. However, no differences were found between the two groups in terms of semen viscosity, smoking habit, and body mass index. About 57.3% of the infertile men were azoospermic and 42.6% were severe oligospermic.

3.2. VNTR Analysis. The XRCC5 VNTR polymorphism analysis was performed for XRCC5 promoter, which contains four different alleles with three 21 bp repeats (3R), two 21 bp repeats (2R), one 21bp repeat (1R), and zero repeat (0R). Results of the PCR products by XRCC5-III/XRCC5-r primers in the agarose gel electrophoresis showed that size of fragments is 287 bp, 266 bp, 245 bp, and 224 bp for 3R, 2R, 1R, and 0R alleles, respectively (Figure 1).

The distributions of allelic and genotypic frequencies for XRCC5 VNTR polymorphism have been summarized in Table 2. In VNTR polymorphism of XRCC5 (rs6147172), among 10 probable genotypes, we observed 8 and 7 of them in control and male infertile groups, respectively. As it has been shown in Table 2, significant differences were observed between total infertile, azoospermic, severe oligozoospermic, and control groups in XRCC5 VNTR genotypes and allele frequencies.

The 2R/2R genotype has the highest frequency in total infertile, azoospermic, severe oligozoospermic, and control groups (38.78, 28.65, and 26.45 versus 31.57 percent, resp.), in which differences between total and azoospermia patients compared to controls were statistically significant \( p = 0.014, p = 0.011, \) resp.). Significant association between genotypic frequencies of 0R/2R and 1R/2R in total patients \( p = 0.039, p = 0.014, \) resp.) and also azoospermia patients \( p = 0.014, p = 0.015, \) resp.) with controls were observed. The frequency of individuals with 0R allele (0R/0R, 0R/1R, and 0R/2R genotypes) showed 1.6-fold increased risk of total infertility and was statistically different \( p = 0.039 \). Moreover, high significant differences in frequencies of the 2R allele carriers (0R/2R+ 1R/2R+ 2R/2R+ 2R/3R), between total infertile and azoospermia, together with a slight association with severe oligozoospermia compared controls were found \( p < 0.0001, \) and \( p = 0.013, \) resp.). The frequency of 2R allele of XRCC5 VNTR polymorphism was found significantly higher than 0R allele in total, azoospermic, and severe oligospermic patients compared to control group \( p = 0.0005, p = 0.001, \) and \( p = 0.01, \) resp.).

3.3. PCR-RFLP Analysis. The 320 bp PCR product contains XRCC6 -61C>G polymorphism digested by BanI (Fermentas Co., Lithuania) to 240 and 80 bp fragments for G allele and a single 320 bp, no BanI cleavage site, for C allele (Figures 2(a) and 2(a')). The wild-type G allele of XRCC7 6721 G>T polymorphism was 368 bp size and had no Pvu II cleavage site, whereas mutant allele, T allele, was digested to 274 and 94 bp fragments (Figures 2(b) and 2(b')).
The allele and genotype frequencies of XRCC6 and XRCC7 SNPs in male infertile patients and controls are summarized in Table 3. With regard to XRCC6-61C>G, the CG genotype in azoospermia was approximately 2-fold higher than controls, which was significantly different (p = 0.003). The frequencies of the GG genotypes showed approximately 2.6, 3, and 2-fold increased risk of male infertility in total infertile, azoospermic, and severe oligospermic patients compared to control group, respectively, whose differences were statistically significant (p = 0.001, p = 0.001, and p = 0.043, resp.). Furthermore, the G allele frequencies in total infertile and azoospermia were approximately 1.5- and 2-fold higher than controls, and their differences were significant as well (p = 0.001 and p = 0.0002, resp.).

Finally, XRCC7 -6721G>T was evaluated in this study. Although mutant homozygotes and the T allele carriers (GT + TT) in azoospermic patients were 2.1 and 1.7-fold higher than control group, no significant differences were found (p = 0.118 and p = 0.247, resp.) (Table 2). Likewise, the frequency of allele T in azoospermia was significantly higher than controls, which was statistically different (p = 0.030).

### 3.4. Potential Biological Functions of XRCC5 VNTR, XRCC6 -61C>G, and XRCC7 6721G>T

Since the genetic variants in promoter region can affect gene transcription activity via altering the DNA-binding ability of transcription factors, we consequently evaluated the potential biological functions of XRCC5 VNTR (rs6147172) and XRCC6 -61C>G (rs2267437) using bioinformatics analysis. The XRCC5 VNTR was scanned for transcriptional start site (TSS) and core promoter elements by EPD and ElemeNT. As shown in Figure 3(a), the VNTR of XRCC5 was located in downstream of the first TATA-box, −422 to −455, in the promoter region and TSS. The various tandem repeats of XRCC5 have not shown any change in number of core promoter elements, such as TATA-box, Inr, DPE, BRE, and DCE, but insertion of these tandem repeats moved these elements to upstream or downstream. The in vitro analysis demonstrated that four different alleles of XRCC5 VNTR 0R, 1R, 2R, and 3R contain variable numbers of nuclear transcription factor Sp1 (Figure 3(b)) [27].

Additional in silico analysis of these polymorphic tandem repeat elements revealed several potential binding sites for transcription factors such as TFIH-I, E2F-1, STAT4, and NF-kappa B (Figure 4). Furthermore, the allele with more tandem repeats, 2R and 3R alleles, includes more binding site elements than the one-repeat allele, suggesting that the presence of more tandem repeats could increase the affinity of transcription factors to the region of the XRCC5 promoter (Figure 4). The prediction of core promoter elements via ElemeNT tool showed that rs2267437 (C>G) causes deletion of a BRE upstream element in the XRCC6 promoter, and also, this mutation increased the score of near BRE upstream (Figure 5(a)). The XRCC6 -61C>G variant was predicted by SNPnexus and PROMO tools to be located in the core recognition site of transcription factor E2F1 in XRCC6 gene promoter, and the transversion of allele C to allele G would lead to the loss of E2F1 binding site (Figure 5(b)). The rs189037 (C>G) was demonstrated by Golden Path tool to be located in a well-conserved region across multiple mammalian species and such conservation was further illustrated in WebLogo (Figure 5(c)).
Table 2: Genotype and allele frequencies of XRCC5 VNTR gene polymorphisms in controls and infertile patients.

| Genotype  | Control (n = 214) | Patients (n = 178) | Severe OS (n = 76) | p value [OR (95% CI)] | Study power %* |
|-----------|------------------|-------------------|-------------------|------------------------|----------------|
|           | Total (n = 214)  | Total (n = 178)   | Total (n = 76)    |                        |                |
| 0R/0R     | 4 (1.86)         | 11 (6.17)         | 4 (5.26)          | 0.510 [0.636 (0.165–2.445)] | 49.03          |
| 0R/1R     | 12 (5.60)        | 21 (11.79)        | 8 (10.52)         | 0.351 [0.705 (0.195–2.552)] | 51.85          |
| 1R/1R     | 17 (7.94)        | 33 (18.53)        | 11 (14.47)        | 0.039 [0.253 (0.068–0.937)] | 84.01          |
| 0R/2R     | 23 (10.74)       | 16 (8.98)         | 10 (13.15)        | 0.014 [0.221 (0.066–0.744)] | 38.46          |
| 1R/2R     | 64 (29.90)       | 39 (21.91)        | 17 (22.36)        | 0.014 [0.223 (0.067–0.739)] | 51.44          |
| 2R/2R     | 83 (38.78)       | 51 (28.65)        | 24 (31.57)        | 0.014 [0.223 (0.067–0.739)] | 83.11          |
| 0R/3R     | —                | —                 | —                 | —                      | —              |
| 1R/3R     | 3 (1.40)         | —                 | —                 | —                      | —              |
| 2R/3R     | 8 (3.73)         | 7 (3.93)          | 5 (4.90)          | 0.014 [0.318 (0.068–1.468)] | 3.348          |
| 3R/3R     | —                | —                 | —                 | —                      | —              |
| 0R allele | 43 (10.04)       | 59 (16.57)        | 33 (16.17)        | 0.570 [0.755 (0.286–1.991)] | 49.30          |
| 1R allele | 113 (26.40)      | 126 (35.39)       | 79 (38.72)        | 0.570 [0.755 (0.286–1.991)] | 49.30          |

*International Journal of Endocrinology
| Genotype | Control (n = 214) | Patients (n = 178) | Severe OS (n = 76) | Controls versus total patients | Controls versus AS | Controls versus severe OS | Study power %a |
|----------|------------------|-------------------|-------------------|-------------------------------|----------------|--------------------------|----------------|
|          | Total n (%) | AS n (%) | Total n (%) | AS n (%) | p value [OR (95% CI)] | Controls versus total patients | Controls versus AS | Controls versus severe OS | Controls versus total patients | Controls versus AS | Controls versus severe OS | Controls versus total patients |
| 2R allele | 261 (60.98) | 87 (42.64) | 164 (46.06) | 77 (50.65) | **0.0005** [0.458 (0.295–0.710)] | **0.001** [0.434 (0.259–0.726)] | **0.010** [0.487 (0.281–0.845)] | 81.51 | 83.92 | 29.85 |
| 3R allele | 11 (2.57) | 5 (2.24) | 7 (1.96) | 2 (1.31) | 0.142 [0.463 (0.166–1.293)] | 0.372 [0.592 (0.187–1.871)] | 0.136 [0.300 (0.061–1.464)] | 4.507 | 4.188 | 2.69 |

aPower based on normal approximation with continuity correction.

*0R carriers refer to individuals with the allele 0R including 0R/0R + 0R/1R + 0R/2R + 0R/3R, **1R carriers refer to individuals with allele 1R including 0R/1R + 1R/1R + 1R/2R + 1R/3R, ***2R carriers refer to individuals with allele 2R including 0R/2R + 1R/2R + 2R/2R + 2R/3R, and ****3R carriers refer to individuals with allele 3R including 0R/3R + 1R/3R + 2R/3R + 3R/3R.

*We calculated p value for the 0R carriers and compared the allele 0R carrier’s individuals in the controls and patients against the entire individual without the allele 0R in the controls and patients, respectively; the other p value for the allele R carriers was calculated according to this formula.
The prediction of the consequence of XRCC7 -6721G>T mutation via HSF 3 tool showed that this SNP occurred in an acceptor splicing site and the transversion of allele G to allele T causes the increase of HSF score of the mutation and also increased slightly the upstream acceptor splicing site’s HSF score, but not created new cryptic splice acceptor site (Table 4(a)). The intronic variation of rs7003908 (G>T) increased branch point sequences as well (Table 4(b)). Screening of the DNA sequence flanking of rs7003908 (G>T) for enhancer motif, Exonic Splicing Enhancers (ESEs) and Intronic Splicing Enhancers (ISEs), and silencer motif, Exonic Splicing Silencers (ESSs) and Intronic Splicing Silencers (ISSs), by HSF 3 and SpliceAid 2 tools have shown almost same results. This in silico analysis revealed decrease, change, and removal of some of the ESEs such as SR (serine/arginine-rich) proteins together with slight increase in ESE motifs from HSF for mutant allele T (Tables 4(c) and 4(d); Figures 6(a) and 6(a')).

However, SpliceAid 2 has not shown any change in the silencer motifs (Figures 6(a) and 6(a')), but HSF 3 displayed an ESS site broken for wild-type allele G and production of a new silencer motif site for mutant allele T which has potential alteration of splicing (Tables 4(e), 4(f), and 4(g)). Consistent with HSF 3 prediction, subsequent analysis with SplicePort tool showed that XRCC7 -6721G>T is presented in an acceptor site and it is not only decrease score of the mutation and near acceptor sites, but also able to influence on another acceptor or even donor sites (data not shown). The conservation of the XRCC7-6721 in across multiple mammalian species was assessed by GoldenPath tool and illustrated in WebLog (Figure 6(b)).

4. Discussion

To the best of our knowledge, the present study was the first demonstration that the XRCC5 VNTR, XRCC6 -61C>G, and XRCC7 6721G>T polymorphisms are associated with susceptibility to male infertility. The results of our study showed that carriers of the 2R allele of XRCC5 VNTR were associated with a significantly decreased risk of male infertility.
### Table 3: Genotype and allele frequencies of XRCC6 and XRCC7 gene polymorphisms in controls and infertile patients.

| Genotype | Control Total (n = 214) | Patients Total (n = 178) | Patients AS (n = 102) | Patients Severe OS (n = 76) | p value [OR (95% CI)] | Controls versus AS | Controls versus Severe OS | Study power %<sup>a</sup> |
|----------|-------------------------|--------------------------|-----------------------|-----------------------------|------------------------|-------------------|------------------------|--------------------------|
| XRCC6 -61C>G |                          |                          |                       |                             |                       |                   |                        |                          |
| CC       | 118 (55.14)             | 37 (36.27)               | 18 (23.68)            |                             | 0.348                  | 0.019             | 0.215                  | 52.31                    |
|          | (n = 102)               | (n = 76)                 |                       |                             | [1.234 (0.794–1.918)]  | [1.871 (1.107–3.160)] | [0.674 (0.361–1.257)] | 86.09                    |
| GG       | 21 (9.81)               | 16 (21.05)               |                       |                             | 0.001                  | 0.001             | 0.043                  | 82.26                    |
|          | (n = 102)               | (n = 76)                 |                       |                             | [2.631 (1.434–4.827)]  | [3.189 (1.570–6.478)] | [2.140 (1.021–4.484)] | 67.33                    |
| CG + GG  | 96 (44.85)              | 65 (63.72)               | 34 (44.73)            |                             | 0.034                  | 0.001             | 0.985                  | 52.31                    |
|          | (n = 102)               | (n = 76)                 |                       |                             | [1.540 (1.032–2.297)]  | [2.159 (1.329–3.508)] | [0.995 (0.587–1.684)] | 86.09                    |
| C        | 311 (72.66)             | 118 (57.84)              | 65 (42.15)            |                             | 0.001                  | 0.0002            | 0.194                  | 58.73                    |
|          | (n = 102)               | (n = 76)                 |                       |                             | [1.643 (1.215–2.221)]  | [1.937 (1.365–2.748)] | [1.303 (0.873–1.943)] | 70.28                    |
| G        | 117 (27.33)             | 102 (32.89)              |                       |                             | 0.720                  | 0.518             | 0.235                  | 26.71                    |
|          | (n = 102)               | (n = 76)                 |                       |                             | [0.879 (0.434–1.779)]  | [1.379 (0.519–3.664)] | [0.605 (0.264–1.386)] | 49.07                    |
| XRCC7 6721G>T |                        |                          |                       |                             | 0.599                  | 0.118             | 0.376                  | 29.26                    |
| G        | 146 (34.11)             | 55 (36.18)               |                       |                             | 0.930                  | 0.247             | 0.268                  | 26.18                    |
|          | (n = 102)               | (n = 76)                 |                       |                             | [1.030 (0.525–2.019)]  | [1.740 (0.680–4.455)] | [0.643 (0.294–1.405)] | 7.13                     |
| T        | 282 (65.88)             | 151 (74.01)              |                       |                             | 0.207                  | 0.030             | 0.719                  | 9.847                    |
|          | (n = 102)               | (n = 76)                 |                       |                             | [1.213 (0.8981–1.639)] | [1.505 (1.039–2.180)] | [0.931 (0.633–1.370)] | 7.129                    |

<sup>a</sup>Power based on normal approximation with continuity correction.
infertility. Moreover, the 2R allele of XRCC5 also significantly reduced the risk of male infertility (Table 2), suggesting that the presence of 2R allele in XRCC5 VNTR gene polymorphism may be a protective factor for male infertility. Besides, the mutant GG genotypes as well as carriers of the CG and GG genotypes showed increased risk for the male infertility. Furthermore, the G allele of the XRCC6 -61C>G variant was correlated with increased susceptibility to male infertility (Table 3). It suggests that the mutant allele G of XRCC6 -61C>G could be considered as a risk factor for male infertility susceptibility. Likewise, the T allele of the XRCC7 6721G>T polymorphism was associated with increased susceptibility to the male infertility in azoospermia (Table 3), which indicates the increased role of this polymorphism on male infertility risk.

Sperm DNA integrity is crucial for perfect transmission of genetic information; therefore, any sperm DNA damage may lead to male infertility despite the number, motility and morphology of spermatozoa, and, consequently, low fertilization rates, suggesting that it has a significant influence on the progeny [2–6]. Because of exogenous and endogenous agent’s DNA breaks, a probable case in spermatogenesis together with malfunction of DNA repair mechanisms can affect normal sperm criteria and at last result in male infertility with azoospermia or oligospermia [4–6]. It is manifested that different polymorphic variants of genes encoding the proteins responsible for DNA repair were linked with the development of sperm DNA damage and male infertility [16–21].

NHEJ is a main mechanism for the removal of broad DNA double-strand breaks (DSBs) lesions and has a critical role in maintaining normal spermatogenesis and genetic stability [13, 15]. DNA-dependent protein kinase (DNA-PK) consists of a heterodimer DNA targeting subunit Ku70/Ku80, XRCC6/XRCC5, and catalytic subunit DNA-PKcs, XRCC7, which are imperative components of NHEJ which expresses in late spermatocytes of the testis, particularly [11, 13, 14]. All components of the DNA-PK were found in the radiosensitive spermatogonia, and also, it is demonstrated that Ku ensures the fidelity of spermatogenesis so that the Ku70 and/or Ku80-deficient testis displays elevated levels of DSBs as well as enhanced apoptosis and reduced sperm production [12, 14].

The XRCC5 VNTR polymorphism, rs6147172, was located in the promoter region and could affect the transcriptional activity of this gene [27, 41]. Polymorphism of rs6147172 has been implicated in susceptibility to several cancers including bladder cancer [27], acute myeloid leukemia (AML) [26], chronic myeloid leukemia (CML) [23], and autoimmune disease such as SLE [30]. In the current study, we observed that the frequency of 2R allele and 2R allele carriers had decreased risk of male infertility which can be considered as a genetic protective factor which was consistent with those previous roles in AML [26], CML [23], and SLE [30].

The strong evidence introduced VNTR sequences in promoter regions as regulatory elements which can bind to nuclear factors and affect transcriptional activity [42]. The
promoter region of XRCC5 contains several copies of Sp1 recognition cis regulatory elements and its gene expression has Sp1-dependent manner (Figure 3(b)) [27, 43]. The VNTR polymorphism of XRCC5 can alter the number of Sp1 elements so that four different alleles of XRCC5 VNTR 3R, 2R, 1R, and 0R possess eight, seven, six, and five copies of Sp1 elements, respectively [41], which were able to modify the expression of XRCC5 [27, 43]. The in silico analysis of present study showed that the presence of tandem repeats in XRCC5 gene promoter can be sequenced to bind to more nuclear factors and probably affect transcriptional activity (Figure 4). This overactivity of XRCC5 leads to surplus DNA repair, which can increase the resistance of spermato- gonia to genotoxic agents and interfere with DNA damage-dependent apoptosis and, thus, increase the likelihood for the development of normal sperm during spermatogenesis.

According to results of a newly meta-analysis which was performed on the XRCC6 SNP polymorphisms and cancer risk, the rs2267437 polymorphism was found to be associated with a significant increase in risks of overall cancers, breast cancer, RCC, and HCC, and it might increase the cancer risk in Asian population [25]. The result of current study showed a high association between genetic polymorphism of XRCC6 rs2267437 with male infertility, suggesting that this SNP might be a genetic risk factor for male infertility.

The XRCC6 -61C>G polymorphism, rs2267437, was located in the promoter region and it is established that this SNP could influence the expression level and stability of the Ku70 protein in breast cancer cells and renal cell carcinoma tissues [44, 45]. Additional in silico analysis of current study to predict biological effects of rs2267437 (C>G) on XRCC6 expression showed that the XRCC6 -61C>G variant could cause deletion of a BRE upstream element in the XRCC6 promoter, firstly Figure 5(a), and the sequence region around rs2267437 (C>G) was predicated to be a DNA-binding site of
E2F1, with the allele C to allele G change leading to the loss of this site, secondly Figure 5(b). Also, it was illustrated in WebLogo that rs2267437 (C> G) lies within a high conserved region across mammalian species, which indicates potential function for this variant (Figure 5(c)). Moreover, the sequence variation in the rs2267437 may affect binding activity of the adjacent CACCC box which is extended to their 4-5 upstream nucleotides and is known binding sites for SP1 and other Kruppel-like transcription factors [44, 46]. The binding and activity of SP1 and Kruppel-like transcription factors are heavily dependent on the SP1/Kruppel-like binding sites and adjacent sequences as well [47]. It has been demonstrated that E2F1 expression was required for the development of male germ cells [48, 49]. The sequence variation in the rs2267437 may abrogate the E2F1 binding site as well as affect binding activity of the adjacent CACCC box with transcription factors, resulting in decreased Ku70 expression level, and the DSB repair activity thus was affected, finally leading to spermatogenic failure.

Genetic polymorphisms of XRCC7 6721G>T were associated with an increased risk of glioma [22], bladder cancer [28], and SLE [30], while other studies reported that there were no significant associations between this polymorphism and risk of renal cell carcinoma and differentiated thyroid cancer [24, 29]. Our result further supports that the polymorphism of the XRCC7 6721G>T may be a genetic risk factor for azoospermic male infertility but not for total infertility and severe oligozoosperma (Table 3).

Defects and alterations in pre-mRNA splicing have been revealed as a common disease-causing mechanism in several studies [50, 51]. Because the XRCC7 6721G>T polymorphism, rs7003908, was located in the intron 8 of XRCC7 gene, auxiliary in silico analysis was performed to predict the effects of this variation on XRCC7 mRNA splicing. Bioinformatics analysis showed that although this SNP occurred in an acceptor splicing site and the transversion of allele G to allele T in XRCC7 6721 causes alterations such as increase in the score of the mutation and branch point sequences and can decrease, change, and remove some of the ESEs and also able to produce a new silencer motifs site which has potential alteration of splicing (Table 4 and Figure 6(a)), this variation cannot create new cryptic splice acceptor site, so this gene polymorphism has not severe modification on XRCC7 mRNA splicing. Moreover, it was illustrated in WebLogo that rs7003908 (G>T) was mapped within a slight conserved region across mammalian species, which indicates relative function for this variant (Figure 6(b)).

However, the functional significance of XRCC7 intron G6721T polymorphism is unknown; this single-nucleotide polymorphism might affect slightly the XRCC7 mRNA splicing and, thus, decrease the level of protein expression in splicing stages, which can affect DNA repair pathway and reduce the resistance of the cells against genotoxic agents in azoospermic male infertile patients’ result. However, these possibilities should be investigated in future studies.

As an important pathway in the DNA damage repair network—NEJ—XRCC5, XRCC6, and XRCC7 play a critical role in the maintenance of genetic integrity. Thus, it would
Table 4: The prediction of the consequence of XRCC7 -6721G>T mutation via HSF 3 tool.

(a) HSF matrices

| Sequence position | cDNA position | Splice site type | Motif               | New splice site           | Wild type | Mutant | If cryptic site use, exon length variation | Variation (%) |
|-------------------|---------------|------------------|---------------------|---------------------------|-----------|--------|-------------------------------------------|---------------|
| 83                | +83           | Acceptor         | TATGAAAAACAGCG      | TATGAAAAACAGCT            | 75.52     | 76.12  | NA                                        | +0.79         |
| 94                | +94           | Acceptor         | GCGGACTCTTAGGC      | GCTGACTCTTAGGC            | 75.48     | 78.56  | NA                                        | +4.08         |

(b) Branch points

| Sequence position | cDNA position | Branch point motif | CV for reference sequence (0–100) | CV for mutant sequence (0–100) | Variation |
|-------------------|---------------|--------------------|----------------------------------|--------------------------------|-----------|
| 93                | +93           | AGCGGAC            | 71.15                            | 86.24                          | +15.9     |

(c) ESE Finder matrices for SRp40, SC35, SF2/ASF, and SRp55 proteins

| Sequence position | cDNA position | Linked SR protein | Reference motif (value 0–100) | Linked SR protein | Mutant motif (value 0–100) | Variation |
|-------------------|---------------|-------------------|-------------------------------|-------------------|----------------------------|-----------|
| 92                | +92           | SF2/ASF (IgM-BRCA1)| CACGGGA (90.08)               | SF2/ASF (IgM-BRCA1)  | CAGCTGA (77.77)            | −13.66%   |
| 92                | +92           | SF2/ASF (IgM-BRCA1)| CACGGGA (90.08)               | SF2/ASF            | CAGCTGA (78.10)            | −13.3%    |
| 92                | +92           | SF2/ASF           | CACGGGA (89.23)               | SF2/ASF (IgM-BRCA1)  | CAGCTGA (77.77)            | −12.84%   |
| 92                | +92           | SF2/ASF           | CACGGGA (89.23)               | SF2/ASF            | CAGCTGA (78.10)            | −12.47%   |
| 93                | +93           | SRp55             | AGCGGGA (76.48)               | Site broken        | −100                      |           |
| 95                | +95           | SF2/ASF (IgM-BRCA1)| CGGACTCT (74.23)             | Site broken        | −100                      |           |
| 96                | +96           | SC35              | GGACTCTT (75.23)              | Site broken        | −100                      |           |

(d) ESE motifs from HSF

| Sequence position | cDNA position | Linked ESE protein | Reference motif (value 0–100) | Linked ESE protein | Mutant motif (value 0–100) | Variation |
|-------------------|---------------|--------------------|-------------------------------|--------------------|----------------------------|-----------|
| 94                | +94           | 9G8                | GCGGAC (77.99)                | 9G8                | GCTGAC (78.59)             | +0.77%    |

(e) Silencer motifs

| Sequence position | cDNA position | Sironi motif reference | Reference silencer (value 0–100) | Sironi mutant motif | Mutant silencer (value 0–100) | Variation |
|-------------------|---------------|------------------------|----------------------------------|--------------------|-------------------------------|-----------|
| 89                | +89           | Motif 1: CTAGAGGT      | ACAGCGGA (65.75)                | Motif 1: CTAGAGGT  | AAACAGCT (66.19)              | New site  |
| 91                | +91           | Motif 1: CTAGAGGT      | ACAGCGGA (65.75)                | Motif 1: CTAGAGGT  | AAACAGCT (66.19)              | New site  |

(f) hnRNP motifs

| Sequence position | cDNA position | Linked hnRNP protein | Reference motif (value 0–100) | Linked hnRNP protein | Mutant motif (value 0–100) | Variation |
|-------------------|---------------|----------------------|-------------------------------|----------------------|----------------------------|-----------|
| 92                | +92           | hnRNP A1             | CACGGG (66.67)                | Site broken        | −100                      |           |
| Predicted signal    | Prediction algorithm         | cDNA position | Interpretation                                      |
|--------------------|------------------------------|---------------|----------------------------------------------------|
| ESS site broken    | (1) Sironi et al., Motif 1   | a...a...a...a...g...c...t...g...a...c...t...c...t | Alteration of an intronic ESS site                  |
|                    | (2) HSF matrices-hnRNP A1    |               | Potential alteration of splicing                    |

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be supposed that these three significant SNPs that affect sperm DNA integrity could also modify male infertility risk. However, our result showed significant association between XRCC5, XRCC6, and XRCC7 gene polymorphisms and male infertility in an Iranian cohort. However, experimental studies are necessary to confirm such an assumption in the future.

In conclusion, this study provided evidences that the XRCC5 VNTR, XRCC6, and XRCC7 6721G>T gene polymorphisms were associated with male infertility risk. Large cohort and diverse ethnicity studies as well as further functional analysis are needed to elucidate the biological mechanism of these polymorphisms of XRCC5, XRCC6, and XRCC7 in male infertility.

Conflicts of Interest

The authors have declared that no conflicts of interest exist.

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