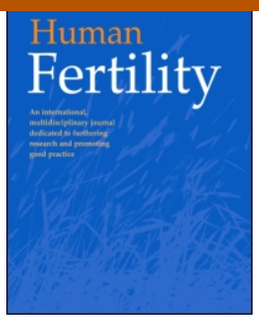

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ORIGINAL ARTICLE

Association of C3953T transition in interleukin *1β* gene with idiopathic male infertility in an Iranian population

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ABSTRACT

In this study we investigate the association of C3953T transition single nucleotide polymorphism in the fifth exon of the interleukin *1β* gene with idiopathic male infertility. In a case-control study, blood samples were collected from 230 fertile and 207 infertile men who referred to the Kashan IVF centre. Genotypes of samples at the C3953T location were determined by polymerase chain reaction-restriction fragment length polymorphism. The data showed a significant association of TT genotype (OR = 2.49, 95%CI = 1.02–6.10; $p = 0.0452$) and T allele (OR = 1.46, 95%CI = 1.07–1.99; $p = 0.0174$) with male infertility. In a subgroup analysis, we found that the TT genotype (OR = 3.28, 95%CI = 1.16–9.26; $p = 0.0249$) and T allele (OR = 1.63, 95%CI = 1.10–2.41; $p = 0.0142$) were associated with oligozoospermia. Our findings suggest that the C3953T polymorphism could be considered as a potential biomarker for a genetic diagnosis of male infertility.

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KEYWORDS
Spermatogenesis; male infertility; Interleukin1B gene; genetic polymorphism

Introduction

Infertility is an important issue affecting 15% of couples (Ji et al., 2012). Approximately 50% of infertility cases are male factor related (Tremellen, 2008), of which >70% of cases are successfully diagnosed and <30% deemed idiopathic (Huynh, Mollard, & Trounson, 2002). Physiological, environmental and genetic factors are involved in male infertility (Ferlin, Arredi, & Foresta, 2006; Oliva, Spira, & Multigner, 2001). Aberrations in expression or structure of some key genes, such as protamine (Jamali, Karimian, Nikzad, & Aftabi, 2016), folate metabolizing genes (Karimian & Colagar, 2016; Karimian & Hosseinzadeh Colagar, 2016; Karimian & Hosseinzadeh Colagar, 2017) and cytokines (Fraczek & Kurpisz, 2015) may affect male fertility. Cytokines are autocrine and paracrine growth factors involved in the normal function of the testis (Fraczek & Kurpisz, 2007). These molecules have multifaceted roles in the male reproductive system (Eggert-Kruse, Kiefer, Beck, Demirakca, & Strowitzki, 2007). For example, they are necessary for germ cell proliferation, differentiation, division and migration during spermatogenesis (Shukla et al., 2013).

Several cytokines such as IL-6, IL-8, IL-10, TNF-α, TGF-β, IL-12 and IL-1 are involved in spermatogenesis (Diemer, Hales, & Weidner, 2003; Fraczek & Kurpisz, 2007; Hedger & Meinhardt, 2003). *Interleukin-1* gene family encodes regulatory cytokines which play an important role in inflammation, maintenance of the immune environment of the testis, and other homeostatic functions (Jaiswal, Trivedi, Singh, Dada, & Singh, 2012). Members of the *IL-1*gene family such as *IL-1α*, *IL-1β* and *IL-1RA* exist in the male gonad in a homeostatic condition; their activity increases upon infection and inflammation (Busfield et al., 2000).

One of the important members of the *IL-1* gene family is *IL-1β*, located on chromosome 2 (Figure 1). In different studies, several single nucleotide polymorphisms (SNPs) have been investigated in the *IL-1β* gene (Mu et al., 2010). For example, there are two common polymorphisms in promoter region of *IL-1β* (−31C > T and −511C > T) that are associated with some cancers (Camargo et al., 2006; Chang et al., 2005; Kornman, 2006; Ruzzo et al., 2005; Zhang, Wang, Zhou, An, & Xie, 2005). In addition, there is a common SNP in exon 5 of the gene (C3953T, with ID: rs1143634) which may be associated with several disorders such as chronic periodontal disease (Nikolopoulos, Dimou, Hamodrakas, & Bagos, 2008), inflammatory bowel disease (Nemetz et al., 1999), and systemic sclerosis (Abtahi et al., 2015). As far as we are aware, there are two studies about the association of

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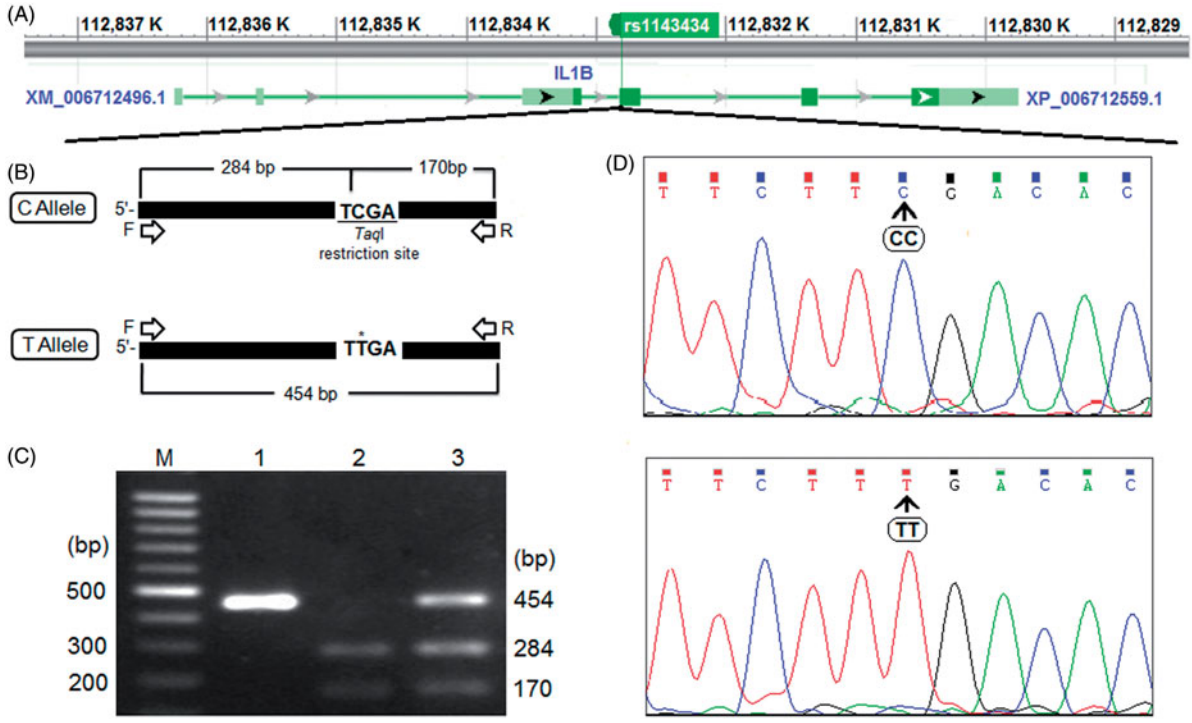

Figure 1. Polymorphism analysis of C3953T: (A) *IL-1β* gene map with7 exons; (B) Schematic of the PCR product digestion in the RFLP technique; (C) The PCR products which digested with *Taq*I restriction enzyme (lane M, DNA marker; Lane 1, TT genotype; Lane 2, CC genotype; Lane 3, CT genotype); (D) *IL-1β*sequence in sample with the genotype CC (up) and sample with genotype TT (down).

IL-1β C3953T polymorphism with male infertility (Bentz et al., 2007; Jaiswal et al., 2012). Also, there are no studies investigating the association of *IL-1β* C3953T with male infertility in the Iranian population. Therefore, in this study we investigated the association of *IL-1β* C3953T transition with idiopathic male infertility in an Iranian sub-population.

Material and methods

Subjects and inclusion criteria

In a case-control study, a total of 437 samples including 207 infertile men (32.69 ± 5.93 years of age) and 230 fertile men (33.63 ± 4.98 years of age) were selected from the Kashan Infertility Centre (Kashan City, Iran). Infertile patients were defined as idiopathic and selected based on an andrological examination including medical history, semen analysis, karyotype, and Y chromosome microdeletion screening. Patients with previous testis trauma, obstructive azoospermia, recurrent infections, chronic diseases, hypogonadotrophichypogonadism, karyotype anomalies or Y chromosome microdeletions were excluded from the study. The patients were categorized into three subgroups according to World Health Organization (WHO, 2010) 2010, criteria: (i) non-obstructive azoospermia ($n = 50$) without spermatozoa in the ejaculate; (ii) oligozoospermia ($n = 89$) with sperm concentration <15 million/ml; and (iii) asthenozoospermia ($n = 68$) with progressive motility <39%. The controls were selected from healthy fertile men with normal semen quality without a history of chronic or familial diseases and who had fathered at least one child. Finally, 2 ml of blood was collected from all males into tubes containing K3EDTA. The blood samples were stored in −20 °C for further use.

Written informed consent was obtained from all case and control subjects. This study had ethical approval, granted by the Medical Research Ethics Committee of Kashan University of Medical Sciences (Reference number: IR.KAUMS.REC.1394.6).

SNP genotyping

Genomic DNA was isolated from whole blood, using a DNA extraction kit (Bioneer Co., Korea) and frozen at −20 °C until required. The genotypes of *IL-1β* C3953T polymorphism were detected by the polymerase chain reaction-restriction fragment length polymorphism (PCR-RFLP) method. For this purpose, forward and reverse primers were designed for *IL-1β* gene at polymorphic position by

Oligo6 software. The forward and reverse primer sequences were: 5'-GCTAGTGTTATGACCATCACCA-3' and 5'-CAGGATGTTTCCATTTACCTTG -3', respectively. Primers were ordered from Bioneer (Daejeon, South Korea). PCR was carried out in a 20μl total volume containing 10μl pre-mix (CinnaGen, Iran), 0.5μl each of forward and reverse primers, and 3μl template DNA. PCR was performed by the following program: the first denaturation step at 94 °C for 5 min which was followed by 35 repetitive cycles including, denaturation step for 45s at 94 °C, annealing step for 1 min at 59 °C, extension step for 1 min at 72 °C, and final extension for 5 min at 72 °C. PCR products were then digested with TaqI restriction enzyme (CinnaGen, Iran). For this purpose, about 0.1μg of the PCR product was incubated with 5 units TaqI at 65 °C for 30min. The success of amplification and digestion procedures was evaluated on a 1% (v/v) agarose gel stained with DNA Green Viewer (CinnaGen, Iran). After electrophoresis and gel staining, some samples had two (284 and 170bp) fragments, and others had three (454, 284 and 170bp), while some samples had one (454bp). The samples with two, three and one fragments were identified as CC, CT and TT genotypes respectively (Figure 1). To verify PCR-RFLP results, some samples were sequenced randomly. PCR product recovery kit (Roche Applied Science, Mannheim, Germany) was used to purify the PCR product (454bp). Direct sequencing of the purified PCR product was performed with Bioneer Co. (Korea). Chromas software (version 2.33) was used to check the electropherograms sequences, and revealed that the sequence of DNA was bona fide at the C3953T position (Figure 1).

Statistical and computational analysis

The Hardy–Weinberg equilibrium for both cases and controls were tested using the Oege online server (http://www.oege.org/software/Hardy–Weinberg.html). The difference in frequencies of genotypes and alleles between the case and control groups was analyzed using the Chi-square test. Odds ratios (ORs) with a 95% confidence interval (CI) were calculated for measuring the strength of the association between C3953T transition and male infertility. A two-tailed *p*-value less than 0.05 ($p < 0.05$) was considered significant. SPSS software version 19 (SSPS Inc., IBM Corp Armonk, NY) was used for statistical analysis.

For computational analysis, the entire genomic sequences of human *IL-1β* gene was deduced from NCBI and analyzed with GeneRunner software 5.1.02 Beta. Mfold online web server (Zuker, 2003) was used to assess the impacts of C3953T on RNA structure of *IL-1β*. Finally, the network of gene-gene interaction for *IL-1β* gene was obtained from String online server (http://string-db.org/).

Results

Based on genotype frequencies (case = 7.13% and control = 1.57%) in the previous study (Bentz et al., 2007), a sample size of 437 and a significance level of 0.05, the power of our study was calculated as being greater than 80%. Based on the Hardy–Weinberg equilibrium, distribution of the *IL-1β* C3953T genotypes was evident in both the fertile and infertile groups. Alleles and genotypes frequencies of the *IL-1β* C3953T in infertile and healthy subjects are shown in Table 1. The data showed a significant association of homozygous genotype TT with male infertility (OR = 2.49, 95%CI = 1.02–6.10; $p = 0.0452$). Carriers of T (CT + TT) were at a high risk for male infertility (OR = 1.51, 95%CI = 1.03–2.21; $p = 0.0338$). Also, allelic analysis showed that the T allele is associated with infertility (OR = 1.46, 95%CI = 1.07–1.99; $p = 0.0174$). In the subgroup analysis, we found that the TT genotype is associated with oligozoospermia (OR = 3.28, 95%CI = 1.16–9.26; $p = 0.0249$). In addition, there was a significant association between carriers of T allele and oligozoospermia (OR = 1.63, 95%CI = 1.10–2.41; $p = 0.0142$). Furthermore, the T allele was significantly associated with oligozoospermia (OR = 1.63, 95%CI = 1.10–2.41; $p = 0.0142$).

In silico results

The Mfold web server was employed to evaluate the effects of C3953T on *IL-1β* RNA structure. Information extracted from the Mfold database revealed that ΔG for secondary structure of wild-type and mutant mRNA are −215.53 kcal/mol and −214.00 kcal/mol, respectively. As depicted in Figure 2, the secondary structure of mRNA showed some change at position 3953.

Data from the String database reveals that *IL-1β* interacts with 10 other molecules (Figure 3): (i) Interleukin 1 receptor accessory protein (IL1RAP); (ii) Interleukin 1 receptor, type I (IL1R1); (iii) Caspase 1, apoptosis-related cysteine peptidase (CASP1); (iv) Interleukin 1 receptor, type II (IL1R2); (v) Nuclear factor of kappa light polypeptide gene enhancer in B-cells 1(NFKB1); (vi) Interleukin 18 (IL18); (vii) Interleukin 1, alpha (IL1A); (viii) Mitogen-activated protein kinase 14 (MAPK14); (ix) Jun proto-oncogene (JUN); and (x) FBJ murine osteosarcoma viral oncogene homolog (FOS).

Table 1. Genotype and allele frequencies of C3953T in cases and controls.

Genotype/ Allele	No. and Percentage					OR (95% CI)			X^2				*p* Value			
	Control ($n = 230$)	All cases ($n = 207$)	Oligo ($n = 89$)	Asteno ($n = 68$)	NOA ($n = 50$)	Total	Oligo	Asteno	NOA	Total	Oligo	Asteno	NOA	Total	Oligo	Asteno	NOA
CC	141 (61.30%)	106 (51.21%)	43 (48.31 %)	35 (51.47%)	28 (56.00%)	–	–	–	–	–	–	–	–	–	–	–	–
TC	81 (35.22%)	86 (41.54%)	38 (42.70%)	31 (45.59%)	17 (34.00%)	1.41 (0.95-2.10)	1.54 (0.92–2.57)	1.54 (0.88–2.69)	1.06 (0.55–2.05)	2.95	2.71	2.35	0.03	0.0863	0.1011	0.1265	0.8698
TT	8 (03.48%)	15 (7.25%)	8 (8.99%)	2 (2.94%)	5 (10.00%)	2.49 (1.02-6.10)	3.28 (1.16–9.26)	1.01 (0.20–4.95)	3.15 (0.96–10.33)	4.23	5.50	0.00	3.90	**0.0452**	**0.0249**	0.9930	0.0587
TC + TT	89 (38.70%)	101 (48.79%)	46 (51.69%)	33 (48.53%)	22 (44.00%)	1.51 (1.03-2.21)	1.69 (1.03–2.78)	1.42 (0.87–2.32)	1.25 (0.67–2.31)	4.52	4.44	2.10	0.48	**0.0338**	**0.0360**	0.1487	0.4876
C	363 (78.91%)	298 (71.98%)	124 (69.66%)	101 (74.26%)	73 (73.00%)	–	–	–	–					–	–	–	–
T	97 (21.09%)	116 (28.02%)	54 (30.34%)	35 (25.74%)	27 (27.00%)	1.46 (1.07-1.99)	1.63 (1.10-2.41)	1.30 (0.83–2.02)	1.38 (0.84–2.27)	5.68	6.08	1.32	1.67	**0.0174**	**0.0142**	0.2522	0.1981

OR: odds ratio; CI: confidence interval; NOA: non-obstructive azoospermia. Significant differences ($p < 0.05$) between the case and control groups are bolded.

Discussion

In this study, we analyzed the association of *IL-1β* C3953T polymorphism with male infertility in an Iranian sub-population (Kashan, Iran). Our study revealed that TT genotype and T allele were associated with male infertility. Also, subgroup analysis revealed that C3953T transition is associated with oligozoospermia. In a previous study, Bentz et al. (2007) reported that C3953T polymorphism is associated with sperm abnormalities in Caucasians. In a subgroup analysis, they showed that TT genotype was associated with oligoasthenoteratozoospermia (Bentz et al., 2007). They considered normozoospermic men as a control group and therefore they concluded that *IL-1B* C3953T polymorphism may be a risk factor for sperm pathology. In addition, Jaiswal, Trivedi, Agrawal, Singh, and Singh, (2013) showed that *IL-1B* C3953T polymorphism was associated with male infertility. They studied the correlation of C3953T with azoospermia and asthenozoospermia in an Indian population. Their results showed that there is a significant association between TT genotype and male infertility especially in the asthenozoospermia subgroup.

Different factors are needed for normal spermatogenesis. For example, cytokines are proteins that exist under normal conditions in the testis. They provide immunity for germ cells in the male gonad, protecting them against pathogens (Hales, 2002). Infection and inflammation of the male urogenital tract impacts spermatozoa, reducing their fertilizing potential (Haidl, Allam, & Schuppe, 2008). Cytokines lead to germ cells differentiation, and they affect testicular cell functions (Abu Elheija et al., 2011) and they moderate peroxidation and antioxidative activities to prevent overproduction of ROS (Fraczek & Kurpisz, 2007). The interleukin-1 family of cytokines is comprised of several members, such as IL-1β. This cytokine has been demonstrated to have an impact on spermatogenesis (Huleihel & Lunenfeld, 2004; Jaiswal et al., 2012) as well as being involved in cell survival and proliferation (Rozwadowska, Fiszer, Jedrzejczak, Kosicki, & Kurpisz, 2007). Modification of IL-1β amount together with TNFα could reduce sperm motility and therefore asthenozoospermic phenotype (Martínez, Proverbio, & Camejo, 2007). This modification results in lipid peroxidation of sperm membrane, producing excess ROS. Increased ROS causes sperm DNA damage and reduces sperm motility (Martínez et al., 2007) and can also lead to oligozoospermia (Agarwal, Virk, Ong, & duPlessis, 2014).

The C3953T polymorphism is a synonymous SNP which is located on exon 5 of *IL-1β*.

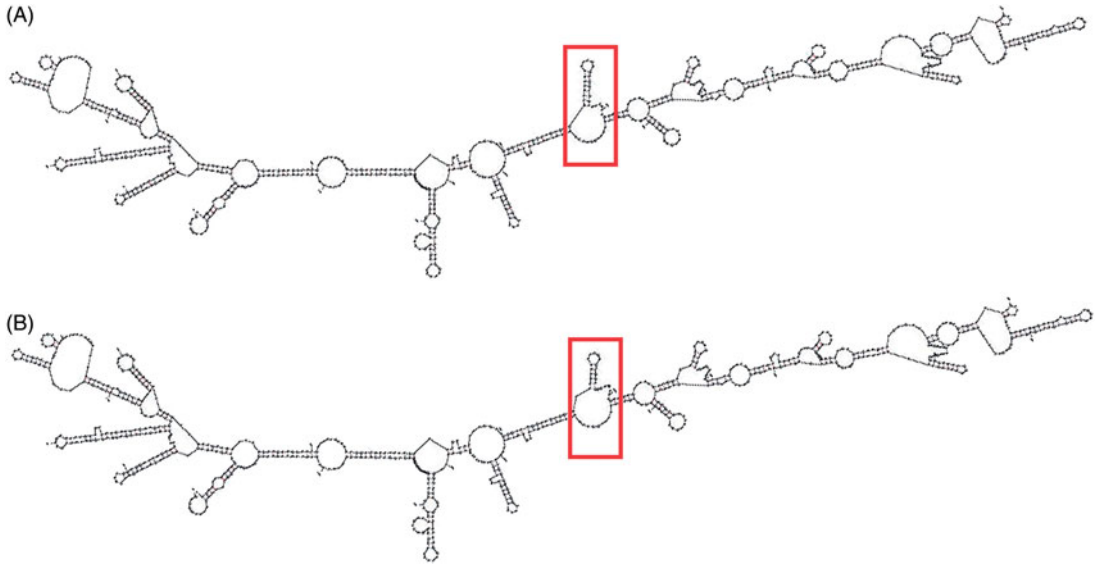

Figure 2. Secondary structure of RNA of human *IL-1β*: optimal secondary structure in the wild-type (A) and mutant (B) with minimum free energy = -215.53 kcal/mol and-214.00 kcal/mol, respectively. The altered location around 3953 position is shown with a red box.

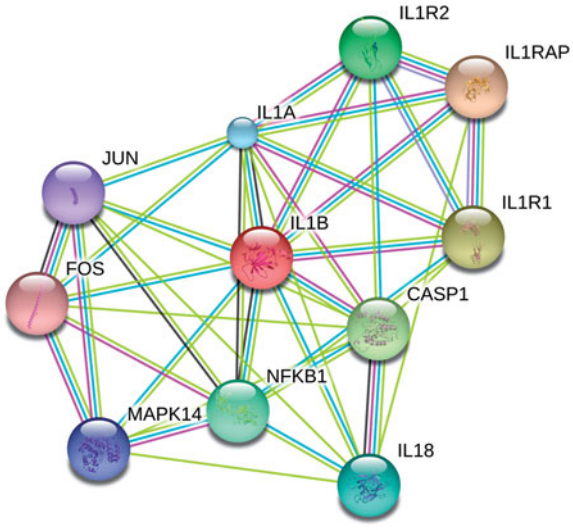

Figure 3. Human *IL-1β* interactions network obtained from String server. *IL-1β* gene interacted with 10 following genes:(1) Interleukin 1 receptor accessory protein (IL1RAP); (2) Interleukin 1 receptor, type I (IL1R1); (3) Caspase 1, apoptosis-related cysteine peptidase (CASP1); (4) Interleukin 1 receptor, type II (IL1R2); (5) Nuclear factor of kappa light polypeptide gene enhancer in B-cells 1 (NFKB1); (6) Interleukin 18 (IL18); (7) Interleukin 1, alpha (IL1A); (8) Mitogen-activated protein kinase 14 (MAPK14); (9) Jun proto-oncogene (JUN); (10) FBJ murine osteosarcoma viral oncogene homolog (FOS).

Synonymous single-nucleotide polymorphisms have been revealed to affect RNA splicing, mRNA structure, and alteration of the gene expression (Soleimani et al., 2017). Our previous studies revealed that *in silico* analysis is a helpful tool to detect the impact of SNPs on protein and mRNA structure (Nikzad, Karimian, Sareban, Khoshsokhan, & Hosseinzadeh Colagar, 2015; Raygan, Karimian, Rezaeian, Bahmani, & Behjati, 2016). In addition, our data from Mfold server revealed that C3953T could make some changes on RNA structure of IL-1β and these alterations may affect mRNA expression.

String database showed that *IL-1β* interacts with 10 other molecules. These interactions especially with the molecules involved in spermatogenesis can be very important. For example, NFKB1 which interacts with IL-1β, regulates apoptosis of male germ cell and gene expression through the spermatogenesis process (Wang, Hu, Zhen, Zhang, & Zhang, 2017). Therefore, any changes in the structure and expression of IL-1β may influence the function of NFKB1.

There are various limitations in our study that must be considered. Possible gene-environment and gene–gene interactions may affect the impact of the studied genetic variants. Interaction of these 10 genes with *IL-1β* may module the function of IL-1β. On the other hand, *IL-1* gene family contains other members, such as *IL-1α* and *IL-1RA* which should be considered in same genetic association studies. In addition, we need a larger group of case and control with various ethnic populations to obtain more accurate data. Also, *in vitro* analyses such as evaluation of *IL-1β* gene expression in case and control groups could make better the data that it suggested for further investigations.

In conclusion, our study suggests that the *IL-1β* C3953T polymorphism may be a risk factor for male infertility. But, future studies should be done in other

ethnic population with larger sample sizes, and take into consideration environmental factors to obtain more accurate data.

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Disclosure statement

The authors have no conflicts of interest.

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