

ORIGINAL

Polymorphisms of sperm protamine genes and CMA3 staining in infertile men with varicocele



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Received 26 October 2017; accepted 25 July 2018

Available online 24 November 2018

KEYWORDS

Sperm;
Protamine gene
variation;
Polymorphism;
Varicocele;
Infertility

Abstract

Objectives: The aim of this study was to evaluate polymorphisms of sperm protamine genes and their effects on the result of CMA3 staining in varicocele men.

Material and methods: In a case control study, 128 patients with male infertility due to varicocele and 128 controls were recruited. Polymorphisms of *PRM1* and *PRM2* genes in extracted DNA samples were assessed by PCR-SSCP and sequencing. Protamine deficiency was also indirectly estimated by CMA3 staining.

Result: Nine different variants including six variants in *PRM1* gene and three variants in *PRM2* gene were found among varicocele patients. The results showed that sperm count, motility and morphology were significantly different between control group without gene variations and varicocele group who had several variations in their protamine genes ($P < 0.05$).

Conclusion: Therefore, *PRM1* and *PRM2* variations in varicocele patients are associated with the production of spermatozoa with more protamine deficiency and this is one of the possible causes of infertility due to varicocele.

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PALABRAS CLAVE

Esperma;
Variación del gen de
la protamina;
Polimorfismo;
Varicocele;
Esterilidad

Polimorfismos de los genes de la protamina espermática y tinción CMA3 en varones infértiles con varicocele

Resumen

Objetivos: El objetivo de este estudio fue evaluar los polimorfismos de los genes de la protamina espermática y sus efectos sobre el resultado de la tinción CMA3 en varones con varicocele.

Material y métodos: En un estudio de casos y controles, se reclutó a 128 varones con infertilidad por varicocele y 128 controles. Los polimorfismos de los genes *PRM1* y *PRM2* en muestras de ADN extraídas se evaluaron mediante PCR-SSCP y se realizó su secuenciación. La deficiencia de protamina también se estimó indirectamente mediante tinción CMA3.

Resultado: Se encontraron 9 variantes diferentes, incluidas 6 variantes en el gen *PRM1* y 3 variantes en el gen *PRM2* entre los pacientes con varicocele. Los resultados mostraron que el recuento de espermatozoides, la movilidad y la morfología eran considerablemente diferentes entre el grupo de control sin variaciones genéticas y el grupo de varicocele que presentaba algunas variaciones en sus genes de protamina ($p < 0,05$).

Conclusión: Por tanto, las variaciones de *PRM1* y *PRM2* en los pacientes con varicocele están asociadas con la producción de espermatozoides con más deficiencia de protamina y esta es una de las posibles causas de infertilidad debida al varicocele.

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Introduction

Protamine is the most common protein in sperm nucleus and plays an important role in chromatin condensation and decondensation, paternal genome protection and hydrodynamic position of sperm nucleus.¹ Accurate histone/protamine replacement is the main step of sperm nucleus DNA packaging and it is shown that any protamine alteration may cause idiopathic male infertility.^{2,3} It is also confirmed in non-human species.

Alterations in sperm protamine-like protein in reaction to environmental stress was reported in invertebrates⁴ as well as relationship between sperm protamine content and diminished fertility in bulls.⁵ Our previous studies confirmed that the rate of spermatozoa with protamine insufficiency is significantly higher among men with varicocele,⁶ spinal cord injury⁷ and alcohol consumer animals.⁸ Protamine deficiency is also associated with abnormal sperm penetration,⁹ sperm DNA fragmentation¹⁰ and subsequent embryo development.¹¹ Furthermore, chromatin condensation and decondensation in sperm nucleus depends on protamine 1 (P1) and protamine 2 (P2) which are coded by *PRM1* and *PRM2* genes located on chromosome 16.¹ It is reported that protamine deficiency may be due to mutations and different polymorphisms of protamine genes.^{12,13}

On the other hand, varicocele, an abnormal dilation of the spermatic veins, which occur in 40% of subfertile men^{14,15} cause sperm DNA damage and less chromatin condensation.¹⁶ In our previous study, we showed that sperm protamine deficiency assessed by chromomycin A3 (CMA3) was significantly higher in varicocele patients than fertile men.⁶ Also, it has been reported that sperm protamine-1/2 mRNA ratio and DNA fragmentation index are associated with male infertility due to varicocele and the

post-varicocelectomy protamine-1/2 mRNA ratio and DNA fragmentation index are positively correlated to the post-varicocelectomy pregnancy rate.¹⁷

With the hypothesis that variations in *PRM1* and *PRM2* genes may cause protamine deficiency in varicocele men, we investigate the effect of protamine genes polymorphisms on protamine defects in infertile men with varicocele.

Materials and methods**Patients**

A total of 128 males with varicocele grade I, II and III attending the Research and Clinical Center for Infertility were included in this study. The varicocele diagnosis was approved by examination or through scrotal Doppler ultrasound (Ultrasonix Medical Corporation, Richmond, Canada). A population of fertile donors ($n = 128$) with normal spermogram who had fathered within the last 12 months were considered as control group. The consent form was signed by all patients.

Semen analysis

Semen samples were collected by masturbation after 2–4 days of abstinence and carried to the andrology laboratory. For each sample, routine semen analysis and Diff quick staining was performed for sperm morphology evaluation according to WHO criteria (World Health Organization, 2010).¹⁸ Azoospermic samples, patients with Diabetes and alcohol consumers were excluded from the study. All semen analyses were performed by one expert technician blinded to the study.

Sperm protamine evaluation

Chromomycin A3 (CMA3) staining is a fluorochrome specific for guanosine cytosine-rich sequence, which applied to evaluate the degree of mature spermatozoa protamination.⁶ For this purpose, smears were dried prior to fixation in Carnoy's solution (methanol/glacial acetic acid, 3:1) at 4 °C for 10 min. Each slide was then conserved with 100 µl of CMA3 (Sigma, St Louis, MO, USA) (0.25 mg ml⁻¹ in McIlvaine buffer; 7 ml citric acid, 0.1 M + 32.9 ml Na₂HPO₄·7H₂O, 0.2 M, pH 7.0 containing 10 mm MgCl₂) for 20 min. After that, the slides were washed 3 times in McIlvaine buffer and mounted with buffered glycerol (1:1). Bright yellow stained CMA3-reacted spermatozoa (CMA3+) and yellowish green stained non-reacted spermatozoa (CMA3-) were detected under axiplane fluorescent microscope with a 460-nm filter (Zeiss Co., Jena, Germany).

Polymorphism determination

Genomic DNA was extracted from peripheral blood samples according to the Accu Prep Genomic DNA Extraction Kit (Bioneer, South Korea) according to the manufacturer's manual.

PCR reaction were done in 25 µl solution comprised 2.5 µl of 10× PCR Buffer, 0.8 µl (*PRM1*) & 0.7 µl (*PRM2*) MgCl₂, 0.8 µl (*PRM1*) & 0.7 µl (*PRM2*) of dNTPs, 0.6 µl (*PRM1*) & 0.5 µl (*PRM2*) of each forward and reverse primer, 0.1 U of Taq polymerase (Cinnagene, Co., Iran) and 1.5 µl (*PRM1*) & 1 µl (*PRM2*) of template DNA.

For *PRM1* exon 1, bp 1–209 and Exon 2, bp 301–500, genotypes were identified by Polymerase chain reaction amplification using primers 5'CACAGCCCACAGATTCCAC3' (forward) and 5'CAACATTTATTGACAGGCGG3' (reverse) and Taq PCR Master Mix (AMPLIQON, Denmark). PCR conditions comprised an initial denaturing step at 94 °C for 5 min, followed by 30 cycles of 95 °C for 50 s denaturation, 58.3 °C for 50 s annealing and extension 72 °C for 50 s, with a final extension at 72 °C for 5 min. For *PRM2* exon 1, bp 1–381 and exon 2, bp 544–845, genotypes were determined by PCR amplification using primers 5'AGACCAGACCAACAGTAACAC3 (forward) and 5'AGCTTTATTGGGCAGGTGAC3' (reverse). Amplification condition were initially denaturation at 94 °C for 2 min, followed by 35 cycles of 95 °C for 30 s denaturation, 63.5 °C for 40 s annealing and extension 72 °C for 45 s, with a final extension at 72 °C for 5 min. Reaction products were fractioned on a 2% agarose gel and visualized after ethidium bromide staining with a digital camera system (Gel Digidoc II, Kiagen, Iran).

Single strand conformation polymorphism (SSCP) method was used to screen *PRM1* and *PRM2* variants. All PCR products from patients and controls were analyzed by SSCP polyacrylamide gel electrophoresis. Direct DNA sequencing of samples with altered band pattern in the SSCP gel was done by a commercial company (Macrogen, South Korea).

Statistical analysis

Statistical analysis was performed using SPSS v20 (IBM Corp.) software. Differences between variables with normal distribution were analyzed using Student's *t*-test and not

normally distributed data were assessed using nonparametric Mann–Whitney *U* test. A *P*-value of <0.05 was considered statistically significant.

Result

Clinical features

The mean age of infertile men with varicocele and normal subjects was 31.23 ± 3.67 and 32.65 ± 6.16 respectively (*P* = 0.281). Duration of infertility was 4.35 ± 2.62 in infertile men with varicocele. In the varicocele group, 25.4% of the patients defined as grade III, 74.6% presented with grade II.

Sperm concentration (83.18 ± 47.19 vs. 92.77 ± 81.02) and non-progressive motility (14.94 ± 4.48 vs. 13.27 ± 3.14) were not differing significantly between varicocele and control groups respectively (*P* > 0.05).

Sperm parameters containing sperm total (69 ± 11.35 vs. 58.76 ± 13.07) and rapid motility (55.72 ± 13.32 vs. 43.81 ± 14.05) as well as sperm morphology (32 ± 8.73 vs. 21.57 ± 10.85) was significantly higher in control men than varicocele patients.

CMA3 staining result

A significant difference was detected in CMA3 staining between the two groups. The rate of reacted spermatozoa to CMA3 in the normal and varicocele group was 29.8% and 59.4% respectively (*P* = 0.000) (Fig. 1).

Polymorphisms

Nine different variants including six variants in *PRM1* and three variants in *PRM2* genes were found among varicocele patients. The homozygous of g.805C>T in *PRM2* was the most frequent variation and was seen in all varicocele patients. Heterozygous of both g.IVS1+75T>C and g.151G>A in *PRM1* with the frequency of 3% was the lowest frequent (Table 1). Two substitutions were detected in the coding region of the *PRM1* gene but none of them leads to amino acid replacement. In control group, no variation was found in *PRM1* and *PRM2* genes (Fig. 2).

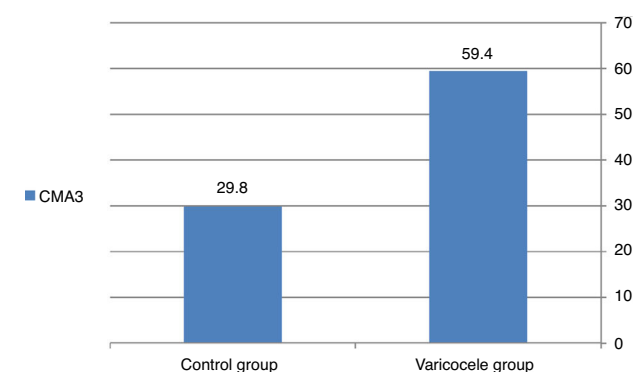
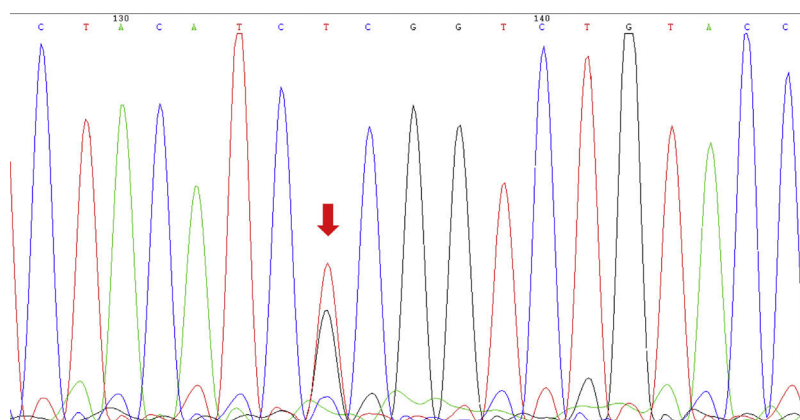


Figure 1 The percentages of samples that are above the "cut-off" value in varicocele and control group regarding to chromomycin A3.

Table 1 Identified polymorphisms in the protamine genes (I and II).

No.	Gene	Gene region	Substitution	Homo/Hetero	Percentages in varicocele men
1	PRM1	Exon 2	g.327C>A	Heterozygote	61
2	PRM1	Intron	g.IVS1+69A>C	Heterozygote	39.5
3	PRM1	Intron	g.IVS1+72A>C	Heterozygote	21.5
4	PRM1	Intron	g.IVS1+75T>C	Heterozygote	3
5	PRM1	Exon 1	g.151G>A	Heterozygote	3
6	PRM1	5'UTR	g.67C>T	Heterozygote	6
7	PRM2	Intron	g.IVS1+27G>C	Homo/Hetero	27
8	PRM2	Intron	g.IVS1+102C>A	Homo/Hetero	40
9	PRM2	5'UTR	g.805C>T	Homozygote	100

PRM1: protamine gene I; PRM2: protamine gene II; UTR: untranslated region.

**Figure 2** DNA sequencing chromatogram heterozygote g.327C>A substitution in *PRM1* gene.

The patients were categorized to 13 groups (A to M) according to the type and number of detected nucleotide substitutions in *PRM1* and *PRM2* genes. Each varicocele patient had at least two (group C & G) and maximum six substitutions (group J) in protamine genes (Table 2). Regarding CMA3 staining, significant differences for variations of *PRM1* and *PRM2* genes were observed in varicocele group compared to the controls ($P=0.0001$). Semen parameters were significantly altered in varicocele patients with variations in *PRM1* and *PRM2* genes compared to fertile men (Table 2).

Discussion

It has been considered previously that defects in sperm nuclear condensation may lead to male infertility. The normal nuclear condensation recognized by a consecutive replacement of somatic histones by proteins like transition proteins and protamines, throughout spermiogenesis.¹⁹ It is shown that defects in sperm protamination can be occurred due to some reasons and it is interesting that genetic factor has an important effect.²⁰ In the present study, we assessed the prevalence of polymorphisms of protamine genes among infertile men with varicocele. Nine different nucleotide substitutions were found in *PRM1* and *PRM2* genes among varicocele patients. Whereas, there was not any variation

in *PRM1* and *PRM2* genes in control group. According to our knowledge this is the first report on Protamine gene polymorphisms among varicocele patients. However, there are some studies assessing *PRM1* and *PRM2* gene mutations regarding abnormal semen parameters and male infertility.

Primarily, Iguchi et al. (2006), evaluated the infertile men protamine genes whose sperm looked like protamine knockout mice phenotypically and found a heterozygous single nucleotide polymorphism (SNP) in the *PRM1* gene in 3 of 30 infertile men.²¹ Tanaka et al. (2003) reported three *PRM1* SNPs that did not cause amino acid substitutions. In addition, these polymorphisms were similarly prevalent in sterile Japanese male cases and proven-fertile volunteers. They claim that the preservation of protamines is critical, and even a slight change in the protamine gene may cause male infertility. Moreover they indicated that introns were highly conserved in both *PRM1* and *PRM2* genes, and deduced that they mainly effect protamine genes expression.¹¹

Furthermore, a *PRM1* polymorphism (-190C>A) was reported which is correlated to abnormal sperm head morphology and abnormal P1/P2 ratio.²² We found that the semen samples of varicoceles with SNPs have higher rates of sperm morphological abnormalities than controls. Similarly, this SNP was found in Chinese population which is associated with teratozoospermia.²³ Another study on Chinese population revealed that the *PRM1* variant rs35576928

Table 2 Semen parameters and protamine defects in varicocele patients according to the polymorphisms of protamine genes (I and II) mentioned in Table 1.

Variables	Number (%)	Concentration (10 ⁶ /mL)	Progressive motility (%)	Non-progressive motility (%)	Total motility (%)	Normal morphology (%)	CMA3+ (%)
A (1,2,3,9)	8 (6.3%)	55.59 ± 71	26.50 ± 19.77	11.00 ± 4.27	37.50 ± 24.05	11.50 ± 11.22	51.50 ± 0.53
B (1,2,5,8,9)	4 (3.1%)	13.00 ± 00	28.00 ± 00	13.00 ± 00	41.00 ± 00	18.00 ± 00	66.00 ± 00
C (7,9)	12 (9.4%)	123.66 ± 50.64	46.00 ± 7.79	13.66 ± 0.51	59.66 ± 7.50	17.66 ± 8.50	61.00 ± 13.17
D (8,9)	44 (34.4%)	73.36 ± 57	40.72 ± 16.44	15.81 ± 3.97	56.54 ± 14.66	22.90 ± 13.36	57.45 ± 13.94
E (1,2,9)	16 (12.5%)	76.25 ± 11.28	59.50 ± 6.37	11.25 ± 1.35	70.75 ± 5.59	23.00 ± 8.38	70.25 ± 12.94
F (1,6,9)	4 (3.1%)	137.00 ± 00	50.00 ± 00	18.00 ± 00	68.00 ± 00	20.00 ± 00	38.00 ± 00
G (1,9)	16 (12.5%)	89.25 ± 42.63	46.25 ± 3.49	14.25 ± 0.46	60.50 ± 3.81	20.75 ± 1.38	75.64 ± 29.15
H (1,2,8,9)	4 (3.1%)	142.00 ± 00	25.00 ± 00	17.00 ± 00	42.00 ± 00	30.00 ± 00	42.00 ± 00
I (1,2,3,7,9)	4 (3.1%)	65.00 ± 00	43.00 ± 00	20.00 ± 00	63.00 ± 00	20.00 ± 00	66.00 ± 00
J (1,2,3,4,7,9)	4 (3.1%)	38.00 ± 00	26.00 ± 00	14.00 ± 00	40.00 ± 00	20.00 ± 00	65.00 ± 00
K (1,8,9)	4 (3.1%)	126.00 ± 00	53.00 ± 00	10.00 ± 00	63.00 ± 00	38.00 ± 00	87.00 ± 00
L (1,2,3,6,9)	4 (3.1%)	73.00 ± 00	48.00 ± 00	12.00 ± 00	60.00 ± 00	42.00 ± 00	66.00 ± 00
M (1,7,9)	4 (3.1%)	96.00 ± 00	40.00 ± 00	20.00 ± 00	60.00 ± 00	8.00 ± 00	75.00 ± 00
Control group (N = 128)		92.77 ± 81.02	55.72 ± 13.32	13.27 ± 3.14	69.00 ± 11.35	32.00 ± 8.70	29.86 ± 8.60
P value*		0.000	0.000	0.000	0.000	0.000	0.000

Data are presented as mean ± standard deviation.

* The differences were statistically significant. Between varicocele and control group; CMA3+: chromomycin A3; progressive motility (%) (grade "a" + grade "b"); non progressive motility (%) (grade "c"); total motility (%) (grade "a" + grade "b" + grade "c"); A to M: patients were divided into 13 groups according to the type and number of detected mutation in PRM1 and PRM2 genes.

(p.R34S) (Arg > Ser) is correlated to severe oligozoospermia, however the dominant model of rs 35576928 has a protective effect on spermatogenesis.¹³ In general, histone H1-like plays a key role in sperm-specific chromatin compaction and comprises of higher quantities of both arginine and lysine in comparison to the somatic cells. It is assumed that phosphates were inserted between the lysine and arginine groups of histone H1 during binding to the chromatin, lead to stabilization of the protein and chromatin structures condensation.²⁴

Moreover, a novel missense mutation (c.119G>A, p.Cys40Tyr) in *PRM1* gene was detected in a man with oligoasthenozoospermia. The researchers also found the c.102G>T transversion which lead to an p.Arg34Ser amino acid change in two infertile men with oligozoospermia as well as unexplained infertility²⁵ and these mutations were not detected in control population. In contrast, Aoki et al. (2006), mentioned no association between gene variation and both protamine deficiency and male infertility. They reported 15 SNPs in *PRM1*, *PRM2* and transition protein genes which prevalence was similar between infertile males, fertile donors and protamine deficient patients.²⁶

As an altered result, Tuttelmann and colleagues (2010) described that a homozygous carrier of the common haplotype ACC showed sperm concentration twice higher than men without this haplotype. They compared Caucasian patients with oligoteratozoospermia to normal controls and determined that sperm motility and morphology were not correlated to any SNP.²⁷

Interestingly, two meta-analyses concluded that the pathogenicity of protamine gene mutations is not proven in male infertility²⁰ and only -190C>A polymorphism can be considered as a risk factor for male infertility.²⁸ In current study we found nine different variations in both *PRM1* and *PRM2* genes.

As it was specified in aforementioned studies, different polymorphisms have been detected in various populations due to male infertility. Four studies were performed in Iranian population regarding polymorphisms of protamine genes. One study showed that two previously detected SNPs in *PRM1*²¹ and *PRM2*¹¹ which correlated with male infertility, have been entirely absent in Iranian infertile men.²⁹ In consistence, Siasi et al. (2012), reported that SNPs in *PRM1* (C321A), *PRM2* (C248T) were not related to oligozoospermia and azoospermia in Iranian population.³⁰ In the third study, the c.2190 C>A transversion in promoter region of *PRM1* gene were associated significantly with increased risk of oligozoospermia.³¹ Another study showed that *PRM1* and *PRM2* mRNA copy numbers were significantly higher in normozoospermic in compared to teratozoospermic samples.³²

All the above studies suggested that polymorphisms of protamine genes were rare causes of infertility in Iranian men. But, according to our results, different polymorphisms of protamine genes and protamine deficiency are frequent in varicocele patients with abnormal semen parameters.

In conclusion, current study as the first research on polymorphisms of protamine genes may be a common cause together with the presence of alterations in CMA3 staining and they should be considered for further potential study in male infertility with varicocele.

Ethical disclosures

Protection of human and animal subjects. The authors declare that no experiments were performed on humans or animals for this study.

Confidentiality of data. The authors declare that they have followed the protocols of their work center on the publication of patient data.

Right to privacy and informed consent. The authors declare that no patient data appear in this article.

Conflict of Interest

The authors declare that they have no conflict of interest.

Acknowledgement

Current study was financially supported by Yazd Reproductive Sciences Institute, Yazd Shahid Sadoughi University of Medical Sciences. This study was approved by ethics committee of Research and Clinical Center for Infertility, Yazd Shahid Sadoughi University of Medical Sciences.

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