Study of GT-repeat expansion in Heme oxygenase-1 gene promoter as genetic cause of male infertility

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Abstract

Purpose The length of GT-repeats polymorphic region in the promoter of human Heme oxygenase-1 gene (HO-1) alters the level of its transcriptional activity in response to oxidative stresses. Decreased level of HO-1 protein in the seminal plasma has been reported to be associated with oligospermia and azoospermia in male infertility. This is the first study to investigate the association between GT-repeats expansion in the promoter of the HO-1 gene and male infertility.

Methods The frequencies of different GT-repeats alleles in the promoter of HO-1 gene were determined in 100 cases and 100 normal controls using PCR-PAGE, ABI fragment analysis genotyping and sequencing analysis.

Results All alleles were classified into S and L alleles. S alleles were specified as number 0 to 3 with <27 GT-repeats and L alleles were specified as number 4 to 6 with >27 repeats. The L allele frequency was significantly higher among case group (54.5%) than that was obtained in the normal control group (37.5%). Statistical analysis provided a significant relationship between L allele and male infertility ($P<0.001$).

Conclusions This study shows for the first time that GT-repeats expansion in promoter of the HO-1 gene is associated with oligospermia and azoospermia among Iranian infertile cases.

Keywords Male infertility · Heme oxygenase-1 gene · Polymorphism · Oxidative stress

Capsule An association between extended GT-repeats polymorphism in the promoter of HO-1 gene and oligospermia and azoospermia in infertile men has been identified for the first time in Iranian cases ($P=0.001$).

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Introduction

Male infertility is a common reproductive disorder that has been associated with oxidative damage of sperm and leads to loss of its quality and functionality [1]. Significant levels of reactive oxygen superoxide are detectable in the semen of up to 25% of infertile men [2, 3]. Some studies have reported the effects of antioxidants on the sperm quality [4, 5]. Some investigations on the level of HO-1 gene expression in the semen in oligospermia have demonstrated oxidative damage of sperm DNA related to male infertility.

Heme oxygenase isozymes are a highly conserved family of proteins, the constitutive form referred to as HO-2 and the stress responsive form known as HO-1 [6]. Heme and other stress-related events have been shown to

chronically induce HO-1 protein and promote HO activity, while having relatively little effect on the expression of HO-2 [7]. HO-1 enzyme is a stress responsive protein which could be induced by various oxidative agents [8]. This is a rare limiting enzyme in heme degradation and plays role in cell protection and keeping cellular homeostasis [9]. It degrades heme into carbon monoxide, biliverdin/bilirubin, and free iron. The GT-repeats in heme oxygenase-1 gene promoter is highly polymorphic and modulates gene expression levels by oxidant challenge [10]. Its polymorphism has been reported to show relationship with some oxidative dependent diseases such as cardiovascular, diabetes and different kind of cancers [11–14]. Its expression is related to the number of the GT-n repeats in the promoter region of HO-1 gene [11, 12]. Longer GT-repeats resulted in lower gene expression and shorter GT-repeats resulted in higher hemeoxygenase-1 protein expression [10].

Oxidative stress is a result of the imbalance between reactive oxygen species (ROS) and antioxidants in the body which can lead to sperm damage, deformity and eventually male infertility. The small amounts of ROS are necessary for spermatozoa to acquire fertilizing capabilities [15, 16]. Low levels of seminal ROS has been reported to be essential for fertilization, acrosome reaction, hyperactivation, sperm motility and capacitation however increased level of ROS has been reported to induce sperm dysfunctions through different mechanisms such as, sperm impairment metabolism, plasma membrane lipid peroxidation, defect in sperm motility and oocyte fusion [17] and has been implicated in the pathogenesis of male infertility [18–20]. HO-1 enzyme activity in human seminal plasma is induced by ROS which is that in azoospermia and moderate in oligospermia in comparison to normal controls [21, 22].

Previous reports have explained a relationship between oxidative stress, semen parameters and male infertility. This study has evaluated the GT-repeat polymorphisms in the

Table 2 The significant relationship between L allele and male infertility obtained by Chi-square statistical analysis of S and L allele frequencies in the case and control groups

Genotypes	Samples		
	Cases	Controls	P-value
S allele	91	125	$P=0.001$
L allele	109	75	
Total	200	200	

promoter region of HO-1 and its relationship with oligospermia and azoospermia in Iranian infertile men.

Materials and methods

Samples

A total of 200 men were recruited from the Navid infertility center in Tehran-Iran that was married for more than 2 years. This study was approved by ethic committee of National Institute for Genetic Engineering and Biotechnology, Tehran, Iran. Informed consents were taken from all cases and controls participated in this study. Samples were taken from case group in two categories, azoospermia ($n=79$) with no detectable sperm and oligospermia ($n=21$) with <5 million sperm/ml. Cases were included 100 individuals of Iranian origin referred for male infertility and the normal control group consisted of 100 normospermia male with >20 million sperm/ml in the semen at the same ages with case group. A detailed medical history and physical examination were performed for the investigated cases. Infertile patients included in the study were seeking a complete andrological diagnostic work-up for couple infertility. All infertile patients were defined as ‘idiopathic’ and selected on the basis of a comprehensive andrological examination including medical history and physical examination, semen analysis, scrotal ultrasound, hormone analysis and karyotype screening. Patients with mono or bilateral cryptorchidism, varicocele, previous testis trauma, obstructive azoospermia, recurrent infections, iatrogenic infertility, hypogonadotrophic hypogonadism, karyotype anomalies were excluded.

Analysis of GT-repeats polymorphism in HO-1 gene promoter

DNA samples were extracted from blood leukocytes of infertile and fertile males using protease and phenol purification [23]. Pair of primers was obtained from

Table 1 The observed frequencies of HO-1 GT-repeat polymorphic genotypes in oligospermic and azospermic infertile men and normal control groups

Samples	Genotypes			Total
	SS	SL	LL	
Cases	30	31	39	100
	30%	31%	39%	100%
Controls	46	33	21	100
	46%	33%	21%	100%
Total	76	64	60	200

Table 3 Distribution of allele frequencies among all individuals in the case and control groups

Allele	Case group		Control group		All samples	
	frequency	percent	frequency	percent	frequency	percent
0	0	0	2	1	2	0.5
1	13	6.5	25	12.5	38	9.5
2	65	32.5	79	39.5	144	36
3	13	6.5	21	10.5	34	8.5
4	95	47.5	72	36.5	167	41.8
5	13	6.5	1	0.5	14	3.5
6	1	0.5	0	0	1	0.2
Total	200	100	200	100	400	100

SHIBAHARA *et al.* 1989 to amplify a 137 to 200 bp fragment, depending on the number of repeats, containing GT-n repeats region from promoter of HO-1 gene by PCR. The 5' end of forward primer was labeled with FAM 5'-FAMAGAGCCTGCAGCTTCTCAGA-3' for using in analysis of DNA fragmentation by ABI capillary genotyping and an unlabeled antisense primer 5'-ACAAAGTCTG GCCATAGGAC-3', which were designed on the basis of the published sequence [24]. The PCR cycle of 94°C for 45 s, 57°C for 30 s and 72°C for 30 s was carried out for a total of 32 cycles. The PCR products were subsequently run on a denaturing polyacrylamide gel (8% acrylamide:bis acrylamide 19:1) at 160–200 V for 6–8 h, followed by silver staining. Then PCR products were mixed together with a Geno Type TAMRA DNA ladder (Size range 50–500 bp; GibcoBRL) and analyzed with an automated DNA sequencer (ABI Prism 377). Each size of the GT-repeats was calculated using GeneScan Analysis software (PE Applied Biosystem). All amplified fragments were also electrophoresed on PAGE (Denaturing Polyacrilamide Gel Electrophoresis) and seven different alleles were cut from the gel, amplified by PCR and sequenced to confirm the number of GT-repeats in each allele.

Statistical analysis

Allele frequencies were calculated for each locus by allele counting. Comparisons of allele frequencies between case and control groups were determined using a Pearson χ^2 test using SPSS for windows version 16.0 (Chicago, Illinois) software. All tests were two-tailed and $p < 0.05$ was considered as significant value.

Results

All seven amplified polymorphic alleles were called alleles 0 to 6 according to their 15 to 37 GT-repeats in the promoter region of the human HO-1 gene. The distributions of the different alleles were bimodal, with one peak at allele

2 (with 22 GT-repeats) and the other at allele 4 (with 27 GT-repeats) similar to that was reported by Kaneda *et al* 2002. All alleles were classified into two major subgroups S and L according to their GT-repeats. Class S alleles was contained less than 26 GT-repeats, numbered 0 to 3 and class L alleles with more than 27 GT-repeats, numbered 4 to 6. All individuals in case and control were classified into SS, SL and LL genotypes (Table 1, 2, 3 and 4, Fig. 1).

The SS, SL and LL genotypes frequencies were 30%, 31% and 39% in the cases and 46%, 33% and 21% in the control groups respectively. The frequencies of S allele among cases and controls were 45.5% and 62.5%, whereas the L allele frequencies were 54.5% and 37.5% respectively. The statistical analysis represented significant association between male infertility and L allele ($P = 0.001$).

Higher frequency of L allele was observed in oligospermic infertile men (69%) and azoospermic infertile men (50.6%) compared to that in normal control group (37.5%). Statistical analysis was indicated significant differences in both oligospermia ($P = 0.00$) and azoospermia ($P = 0.01$) (Table 4) in comparison to control males. The higher frequency of L allele in oligospermic subgroup (69%) than that in azospermic subgroups (50.6%) represented a significant difference when two subgroups were compared ($P = 0.03$). It means that L allele is more common among oligospermic cases than that was observed in azospermia.

The frequencies of different genotypes related to L alleles were obtained as 4/4 (45% and 27.5%), 4/5 (5% and 1.2%), 5/5 (5% and 3.6%) and 5/6 (5% and 0%)

Table 4 Frequencies of the S and L alleles among in the oligospermia, azoospermia and control groups

Allele	Oligospermia	Azoospermia	Control
L allele	29 (69%)	80 (50.6%)	75 (37.5%)
S allele	13 (31%)	78 (49.4%)	125 (62.5%)
Total	42	158	200

Fig. 1 Distribution of different polymorphic alleles (0–6) frequencies in the cases (a, $n=100$), in the controls (b, $n=100$) and in all samples (c, $n=200$)

among oligospermic and azospermic males respectively. These results have showed that L allele is more commonly observed in oligospermic cases than that in azospermia.

Discussion

Several studies have demonstrated that the GT-repeats in the promoter region of HO-1 gene is highly polymorphic, modulated gene transcription by oxidant challenge, and associated with different diseases [1, 12, 14, 25, 26]. Recent studies indicated that oxidative stress has been implicated in the pathogenesis of male infertility [18–20, 22].

This study has reported for the first time the relationship between GT-repeats expansion in the heme oxygenase-1 gene promoter and risk of oligospermia and azospermia in Iranian infertile men ($P=0.001$). The expanded allele contained more than 27 GT-repeats, has called allele L that can reduce level of HO-1 gene expression and decrease the ability in combating with oxidative stressors [9, 10]. Therefore, male with longer L alleles may have lower level of HO-1 gene expression supported with significant P value equal to 0.001.

When cases with oligospermia were compared to azospermia, it was found that frequency of L allele was significantly higher in oligospermia compared to azospermia. This represented that L allele is more commonly found in oligospermia and may play more important role in sperm reduction. For instance, the longest L allele number 6 was only observed in cases with oligospermia and allele 5 frequency was also significantly higher compared to that observed in azospermia ($P=0.04$).

Shiraishi *et al* 2005 showed low levels of reactive oxygen such as hydrogen peroxide, a biooxidant, have been shown to be essential for fertilization, acrosome reaction, hyperactivation, motility and capacitating. Increased expression of HO-1 in response to high level of reactive oxygen preserved spermatogenesis in leydig cell. Lin *et al* 2007 demonstrated that oxidative stress is important for localization of HO-1 protein to the nucleus and activation of transcription factors [27]. The level of HO-1 enzyme activity in the seminal plasma has been shown to be related with spermatogenesis and sperm concentration ($P=0.001$) [21], a very low seminal plasma activity in azospermic, moderated in oligospermic specimens and higher activity in normospermic specimens ($P<0.01$).

Conclusion

Previous studies on the expansion of the GT repeats in promoter region of HO-1 showed that the GT-repeats is highly polymorphic and longer GT-repeats exhibits lower transcriptional activity. This study has demonstrated for the first time the significant association between expansion in GT-repeat and susceptibility to male infertility. A significant association was obtained between L allele and oligospermia and azospermia in Iranian infertile men ($P=0.001$). Long GT-repeats polymorphism might be contributed to the defect in spermatogenesis and inducing male infertility in Iranian cases. It is interesting to warrant this study by testing this polymorphism using a larger sample with a prospective design to confirm the above observation in other populations.

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References

- Rao AV, Rao GL. Carotenoids and human health. *Pharmacol Res*. 2007;55:207–16.
- Iwasaki A, Gagnon C. Formation of reactive oxygen species in spermatozoa of infertile patients. *Fertile Steril*. 1992;57:409–16.
- Zini A, de Lamirande E, Gagnon C. Reactive oxygen species in semen of infertile patients: levels of superoxide dismutase and catalase-like activities in seminal plasma and spermatozoa. *Int J Androl*. 1993;16:183–8.
- Lewin A, Lavon H. The effect of coenzyme Q10 on sperm motility and function. *Mol Aspects Med*. 1997;18(suppl):213–9.
- Moncada ML, Vicari E, Cimino C, Calogero AE, Mongioi A, D'Agata R. Effect of actylcamitine in oligoasthenospermia patients. *Acta Eur Fertil*. 2002;23:221–4.
- McCoubrey KJ, Maines M. The structure, organization, and differential expression of the gene encoding rat heme oxygenase 2. *Gene*. 1994;139:155–61.
- Agarwal A, Nick HS. Renal responses to tissue injury: lessons from heme oxygenase-1. Ablation and expression. *J Am Soc Nephrol*. 2000;11:965–73.
- Lavrovsky Y, Schwartzman MC, Levere RD, Kappas A, Abraham NG. Identification of binding sites for transcription factors NF- κ B and AP-2 in the promoter region of the human heme oxygenase-1 gene. *Proc Natl Acad Sci USA*. 1994;91:5987–91.
- Deshane J, Wright M, Agarwal A. Heme oxygenase-1 expression in disease states. *Acta Biochim Pol*. 2005;52:273–84.
- Yamada N, Yamada M, Okinaga S. Microsatellite polymorphism in the heme oxygenase-1 gene promoter is associated with susceptibility to emphysema. *Am J Hum Genet*. 2000;66:187–95.
- Chen YH, Chau LY, Lin MW, Chen LC, Yo MH, Chen JW, et al. Heme oxygenase-1 gene promoter microsatellite polymorphism is associated with angiographic restenosis after coronary stenting. *Eur Heart J*. 2004;25:39–47.
- Chen YH, Chen JW, Chau LY, Lin SJ. Serum bilirubin and ferritin levels link heme oxygenase-1 gene promoter polymorphism and susceptibility to coronary artery disease in diabetic patients. *Diabetes Care*. 2008;31(8):1615–20.
- Vashist YK, Blessmann M, Trump F, Kalinin V, Kutup A, Schneider C, et al. Microsatellite GTn-repeat polymorphism in the promoter of heme oxygenase-1 gene is an independent predictor of tumor recurrence in male oral squamous cell carcinoma patients. *J Oral Pathol Med*. 2008;37:480–4.
- Wu MM, Chiou HY, Lee TC, Chen CL, Hsu L, Wang YH, et al. GT-repeat polymorphism in the heme oxygenase-1 gene promoter and the risk of carotid atherosclerosis related to arsenic exposure. *J Biomed Sci*. 2010;17(70):1–11.
- Aitken RJ. Molecular mechanisms regulating human sperm function. *Mol Hum Reprod*. 1997;3:169–73.
- Aitken RJ. The human spermatozoon a cell in crisis? *J Reprod Fertile*. 1999;115:1–7.
- Saleh RA, Agarwal A, Nada EA, El-Tonsy MH, Sharma RK, Meyer A, et al. Negative effects of increased sperm DNA damage in relation to seminal oxidative stress in men with idiopathic and male factor infertility. *Fertile Steril*. 2003;79:1597–605.
- Agarwal A, Prabakaran SA. Oxidative stress and antioxidants in male infertility: a difficult balance. *Iran J Reprod Med*. 2005;3(1):1–8.
- Agarwal A, Makker K, Sharma R. Clinical relevance of oxidative stress in male factor infertility: an update. *Am J Reprod Immunol*. 2008;59:2–11.
- Verit FF, Verit A, Ciftci H, Erel O, Celik H. Paraoxonase-1 activity in subfertile men and relationship to sperm parameters. *J Androl*. 2009;30(2):183–9.
- Abdel Aziz MT, Mostafa T, Roshdy N, Hosni H, Rashed L, Sabry D, et al. Heme oxygenase enzyme activity in human seminal plasma of fertile and infertile males. *Andrologia*. 2008;40:292–7.
- Pasqualotto FF, Sharma RK, Nelson DR, Thomas AJ, Agarwal A. Relationship between oxidative stress, semen characteristics, and clinical diagnosis in men undergoing infertility investigation. *Fertile Steril*. 2000;73(3):459–64.
- Sambrook J, Fritsch E, Maniatis T. Isolation of DNA from mammalian cells. *Molecular Cloning Cold Spring Harbour Press, Cold Spring Harbour Laboratory, New York, USA 1989, Vol 7, Chap 6*.
- Shibahara S, Sato M, Muller RM, Yoshida T. Structural organization of the human heme oxygenase gene and the function of its promoter. *Eur J Biochem*. 1989;179:557–63.
- Chen YH, Lin SJ, Lin MW, Tsai HL, Kuo SS, Chen JW, et al. Microsatellite polymorphism in promoter of heme oxygenase-1 gene associated with susceptibility to coronary artery disease in type 2 diabetic patients. *Hum Genet*. 2002;111:1–8.
- Kaneda H, Ohno M, Taguchi J, Togo M, Hashimoto H, Ogasawara K, et al. Heme oxygenase-1 gene promoter polymorphism is associated with coronary artery disease in Japanese patients with coronary risk factors. *Arterioscler Thromb Vasc Biol*. 2002;22:1680–5.
- Lin Q, Weis S, Yang G, Weng YH, Helston R, Rish K, et al. Heme oxygenase-1 protein localises to the nucleus and activates transcription factors important in oxidative stress. *J Biol Chem*. 2007;282:20621–33.