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Follicle-stimulating hormone receptor polymorphism and seminal anti-Müllerian hormone in fertile and infertile men

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Summary

Follicle-stimulating hormone (FSH) is fundamental for Sertoli cell function stimulating spermatogenesis and follicular growth by a specific receptor (FSHR). This work aimed to investigate the occurrence of Asn and Ser FSHR gene variants and its relationship with seminal anti-Müllerian hormone (AMH) among normozoospermic and infertile oligoasthenozoospermic (OAT) males. Eighty-two Caucasian males grouped into normozoospermic healthy controls (n = 30) and infertile OAT males (n = 52). FSHR gene variants were determined by DNA from anti-coagulated blood and underwent polymerase chain reaction (PCR) amplification and electrophoresis in detecting amplification products. AMH in seminal plasma was determined by ELISA. The results showed that the frequency of FSHR gene variants among fertile men was 46.7% Asn/Asn (N680S), 33.3% Asn/Ser, and 20% Ser/Ser, whereas among OAT men were 34.6%, 38.5% and 26.9% respectively with nonsignificant differences. Seminal AMH was significantly higher in fertile than infertile OAT men. There was significant increase in seminal AMH with Asn/Asn variant of FSHR gene than those with Asn/Ser or Ser/Ser. It is concluded that FSH gene variants showed no difference in distribution between fertile or infertile OAT men. However, when correlated with seminal AMH values, there was an increase in Asn/Asn in men with high seminal AMH.

Introduction

Follicle-stimulating hormone (FSH) stimulates spermatogenesis and follicular growth by a specific receptor (FSHR) which is a member of the G protein-coupled receptor family (Lécureuil *et al.*, 2007; Meduri *et al.*, 2008). FSHR gene is a single-copy gene spanning a region of 54 kbp in the humans consisting of 10 exons and nine introns. The single nucleotide polymorphisms (SNP) in exon 10, influences serum FSH levels in women, but not in men. The extracellular domain of the human receptor is encoded by nine exons ranging 69–251 bp. The C-terminal part of the extra cellular domain, transmembrane and the intracellular domain is encoded by exon 10 with more than 1234 bp (Gromoll *et al.*, 1996; Simoni *et al.*, 2002). Although exon 10 is fundamental for signal transduction, it is not necessary for ligand binding. The transmembrane domain, however, might contact hormone bound to the extracellular domain (Simoni *et al.*, 1997).

The two most common SNPs in the coding region of FSHR occur at nucleotides 919 (919A>G, T307A, rs6165) and 2039 (2039A>G, N680S, rs6166) in exon 10 corresponding to amino acid positions 307 and 680 of the mature protein (Simoni *et al.*, 1999; Gromoll & Simoni, 2005). One of the polymorphisms in exon 10 is Asn/Ser 680 present in 60% of the population (Simoni *et al.*, 2002; Gromoll & Simoni, 2005) with the Ser 680 variant occurring in 40% (Gromoll & Simoni, 2001).

As regards the effects of exon 10 SNPs on testicular function, it has no effect on serum levels of FSH or other clinical parameters in men with either normal or impaired spermatogenesis (Simoni *et al.*, 1999; Asatiani *et al.*, 2002). There were no differences in FSH levels in men with different FSHR genotypes. Although the SNPs in exon 10 might have physiological effects in the testis, there is no reliable parameter to measure this. By performing variant screening, it was reported that the FSHR variants, Asn 680 and Ser 680, are similarly distributed in infertile men and fertile controls, thereby excluding the possibility that one of the two isoforms is involved in the pathogenesis of idiopathic male infertility (Simoni *et al.*, 1999; Song *et al.*, 2001). However, the two allelic variants, at position −29 and exon 10, showed a significant different distribution between controls and men with non-obstructive azoospermia (NOA) (Ahda *et al.*, 2005).

Anti-Müllerian hormone (AMH) has long been known for causing regression of the Müllerian ducts as a requirement for normal male reproductive tract development (Jost, 1947; Teixeira *et al.*, 2001). AMH is a member of the tissue growth factors superfamily of growth and differentiation factors (Massagué & Chen, 2000; Gruijters *et al.*, 2003). Visser *et al.* (1998) showed that AMH expressed by the testis during male sex differentiation is continued to be secreted into adult life. Josso *et al.* (2001) indicated that AMH is a marker of both Sertoli cell proliferation and protein synthesis activity in response to FSH before puberty and also a useful marker of FSH action in the assessment of testicular function in pre-pubertal boys.

Al-Qahtani *et al.* (2005) found that seminal AMH concentrations in male factor infertility were not significantly different from fertile men. Fujisawa *et al.* (2002) correlated seminal AMH significantly with sperm concentration, testicular volume, serum LH but not with serum FSH, testosterone or oestradiol. Fallat *et al.* (1996) suggested that AMH may have a function in modulating sperm motility. Seminal AMH was undetectable in patients with obstructive azoospermia (Fujisawa *et al.*, 2002; Mostafa *et al.*, 2007).

Anti-Müllerian hormone promoter activity was shown to be enhanced by the classical FSH-regulated signaling cascade involving a G protein, adenylate cyclase and PKA being capable of enhancing AMH gene transcription. The positive effect of FSH on testicular AMH production was found to be due to both the proliferation of Sertoli cells and to the increase in AMH transcriptional activity per Sertoli cell (Rey, 1998; Lukas-Croisier *et al.*, 2003). Al-Attar *et al.* (1997) demonstrated that testicular AMH output could be stimulated by FSH.

This study aimed to demonstrate the relation between the Asn and Ser FSHR gene variants and seminal AMH levels in infertile OAT males.

Materials and methods

This study included 82 Caucasian men attending the andrology outpatient clinic at the university hospital after institutional review board (IRB) approval. They were divided into group (gp)1 ($n = 30$) normozoospermic, fertile and healthy volunteers who had achieved conception within 1 year, and gp2 ($n = 52$) infertile OAT males. Normozoospermia means sperm concentration >20 million ml^{-1}, sperm motility >50% (A + B) and normal sperm morphology >30%. OAT means sperm concentration <20 million ml^{-1}, sperm motility <50% (A + B) and sperm normal morphology <30%. Exclusion criteria were varicocele, cryptorchidism, karyotype anomalies, Y chromosome microdeletions and leucocytospermia. Semen samples were obtained by masturbation after 4 days of sexual abstinence. Computer-assisted semen analyses (Autosperm) (Fertipro NV, Beerneme, Belgium) were carried out according to guidelines of the World Health Organization (1999). Sperm morphology was evaluated by phase contrast microscope and sperm Mac stain (Fertipro NV). Peroxidase-positive white blood cells were detected by Endtz stain (Shekarriz *et al.*, 1995).

Seminal plasma was obtained by centrifugation of semen samples for 15 min and the supernatant seminal plasma was analysed for alpha-glucosidase activity and AMH. Ten milliliters of overnight fasting blood samples were withdrawn from men included in the study. One ml was taken on EDTA and stored at −30 °C until the subsequent use for genomic DNA extraction. The rest of the blood sample was used to separate sera for FSH, lutenising hormone (LH), testosterone (T) and prolactin (PRL) assessment by enzyme immunosorbant (ELISA) assay.

Genotyping of FSH receptor gene using PCR

DNA was extracted from EDTA anti-coagulated blood using genomic DNA purification kit (Puregene; Gentra Systems, Minneapolis, MN, USA) for DNA purification from whole blood (Sudo *et al.*, 2002). Conventional PCR amplification was used for FSHR Gene (Asn-Ser 680) polymorphism. The region of nucleotide number 1624 to 2143 in the FSHR gene was amplified. The amplification product of 520- base pairs was indicative of the presence of gene.

Restriction analysis

Codon 680 of FSHR gene expresses asparagine (Asn) (AAT) (contain A at position 2039 nucleotide of the receptor gene). If A at position 2039 becomes G then Asn at position 680 will be Serine (Ser) (AGT). This A to G transition creates recognition site for BsrI restriction

endonuclease. The Asn 680 allele gives an undigested fragment of 520 bp, whereas the Ser 680 allele gives two fragments of 413 and 107 bp. For heterozygous (Asn/Ser), agarose gel electrophoresis allows visualisation of three bands 520 bp, 413 bp and 107 bp.

Seminal plasma AMH was carried out using an ELISA kit (DSL, Diagnostic System Laboratories Inc., Webster, TX, USA), an enzymatically amplified two-site immunoassay. The absorbance of standards and samples in duplicates was measured by an automated, microprocessor controlled microplate reader (Sunrise absorbance reader; Tecan Austria GmbH, Grödig, Austria; Magellan software). Minimum detection limit was 3.0 pmol l^{-1} and intra- and inter-assay coefficients of variation were 2.4–4.6% & 4.8–8.0% respectively. Seminal alpha-1,4-glucosidase was estimated using double-beam spectrophotometer method (Epi screen; Fertipro, Ghent, Belgium) with reference value 0.1–14.0 mU ml^{-1} (Cooper *et al.*, 1988).

Statistical analysis

Nonparametric data were expressed as median and range. Mann-Whitney test was used as a test of significance for comparison between two groups. In addition, Bonferroni correction was applied. Spearman rank correlation coefficient was done for the relations between variables. Statistical significance was set at $P < 0.05$.

Results

Restriction fragment length polymorphism (RFLP) analysis of the Asn680Ser FSHR variant showed a 520 bp band for Asn/Asn, two bands at 413 and 107 bp for Ser/Ser, three bands at 520, 413, 107 bp for Asn/Ser. Sperm concentration, grade A motility, grade (A + B) motility, velocity, linear velocity, linearity index, normal morphology, alpha-glucosidase and seminal AMH were significantly decreased in infertile OAT men compared with fertile group. WBCs, serum FSH levels were significantly increased in infertile OAT men compared with normozoospermic group, whereas serum testosterone levels were significantly decreased (Table 1). Frequency of FSHR gene Asn/Ser 680 variants among normozoospermic and infertile OAT men showed nonsignificant difference in the distribution of these variants (Table 2).

Seminal plasma AMH showed significant increase in men with Asn/Asn variant of FSHR gene than those with Asn/Ser ($P = 0.0041$) and Ser/Ser ($P = 0.0026$). However, there was nonsignificant decrease in sperm concentration, grade A, grade A + B, velocity, linear velocity, linearity index, normal morphology and α-glucosidase in Ser/Ser and Asn/Ser allelic variants compared with the Asn/Asn one. There was significant increase in serum FSH in Ser/Ser compared with Asn/Asn ($P = 0.0008$) variant, but serum testosterone showed significant decrease in Asn/Ser and Ser/Ser variants than that of Asn/Asn ($P = 0.0023$, $P = 0.0033$ respectively) (Table 3). AMH and seminal parameters data for all groups has pooled to find the variations in these factors among different genotyping groups.

There were significant positive correlations between seminal AMH and testicular volume ($r = 0.484$, $P = 0.0001$), sperm concentration ($r = 0.807$, $P = 0.0001$), grade A sperm motility ($r = 0.747$, $P = 0.0001$), grade A + B sperm motility ($r = 0.758$, $P = 0.0001$), sperm velocity ($r = 0.653$, $P = 0.0001$), linear velocity ($r = 0.664$, $P = 0.0001$), linearity index ($r = 0.345$, $P = 0.0289$),

Table 1 Data of studied groups (median, range)

	Normozoospermia ($n = 30$)	OAT ($n = 52$)
Semen parameters		
Testicular volume (ml)	21 (18–25)	16[a] (10–22)
Semen volume (ml)	4 (2–7)	4 (1–9.2)
Sperm concentration (10^6 ml^{-1})	70.4 (44.8–96)	8.2[a] (0.85–18.8)
Grade A sperm motility (%)	54 (41–60)	4[a] (0–25)
Grade A + B sperm motility (%)	61 (51–67)	13.5[a] (2–39)
Sperm velocity (μm s^{-1})	79.3 (63.4–86.6)	36.5[a] (8.8–75.5)
Sperm linear velocity (μm s^{-1})	61 (42.9–70.7)	21[a] (5.7–52.6)
Linearity index (μm s^{-1})	82 (67.5–86.7)	60.3[a] (33.7–97.4)
Sperm normal morphology (%)	64 (58–66)	2[a] (0–18)
WBCs (million ml^{-1})	0.6 (0.4–0.6)	0.8[a] (0.3–5.4)
Alpha-glucosidase (mU ml^{-1})	69.3 (45.4–90.7)	26.4[a] (18.4–44.6)
Studied hormones		
Serum LH (mIU ml^{-1})	6.4 (4.5–9.3)	6.3 (3.0–12.4)
Serum FSH (mIU ml^{-1})	5.40 (3.8–7.1)	9.8[a] (5.2–22.0)
Serum prolactin (ng ml^{-1})	5.6 (3.5–8.6)	6.7 (2.4–12.5)
Serum testosterone (ng dl^{-1})	890 (567–1106)	560[a] (320–1200)
Seminal plasma AMH (pmol l^{-1})	118.2 (66.5–245.3)	35.8[a] (14.2–160.4)

[a]Significant.

Table 2 Frequency of FSHR gene Asn/Ser variants among studied groups

	Asn-Asn	Asn/Ser	Ser-Ser
Normozoospermic men ($n = 30$)	14 (46.7%)	10 (33.3%)	6 (20%)
nfertile OAT men ($n = 52$)	18 (34.6%)	20 (38.5%)	14 (26.9%)

Table 3 FSHR gene Asn/Ser680 allelic variants among studied cases (median, range)

	Asn-Asn (n = 32)	Asn/Ser (n = 30)	Ser-Ser (n = 20)
Seminal AMH (pmol l^{-1})	65.9 (43.2–245)	35.0[a] (19.5–157.0)	36.2[a] (14.2–118)
Concentration (10^6 ml^{-1})	18.8 (2.1–96)	12.0 (0.85–81.6)	8.1 (1.5–84.8)
Grade A sperm motility (%)	24.5 (0–60)	13 (0–57)	4.5 (0–59)
Grade A + B sperm motility (%)	35.5 (2–35.5)	26.0 (4–62)	11.0 (7.0–67)
Sperm velocity (μm s^{-1})	64.8 (12.8–66.6)	43.4 (14–81.9)	40.1 (8.8–82.5)
Linear velocity (μm s^{-1})	35.8 (10.1–68.4)	30.5 (9.1–70.7)	21.7 (5.7–70.7)
Linearity index	73.3 (33.7–97.4)	67.6 (38.6–86.7)	69.2 (46.3–94.7)
Normal sperm morphology (%)	16.0 (0–64)	6.0 (0–66)	2.0 (0–64)
Testicular volume (ml)	20 (10–25)	18 (11–23)	18 (10–22)
α-glucosidase (mU ml^{-1})	38.7 (18.4–89.6)	31.6 (18.4–84.2)	26.9 (20.4–90.4)
Serum LH (mIU ml^{-1})	6.4 (3.2–8.5)	7.3 (3–12.4)	5.8 (3.1–10.0)
Serum FSH (mIU ml^{-1})	6.4 (3.9–14.9)	8.8 (4.3–18.4)	9.2[a] (6.4–22.0)
Serum PRL (ng ml^{-1})	4.8 (2.4–12.5)	5.4 (3.9–11.7)	6.6 (4.0–10.0)
Serum T (ng dl^{-1})	789.5 (390–200)	567[a] (320–1106)	524[a] (301–970)

Bonferroni correction P = 0.0036.
[a]Significant comparison between Asn/Asn and other groups.

sperm normal forms (r = 0.753, P = 0.0001), seminal alpha-glucosidase (r = 0.670, P = 0.0001), serum FSH (r = 0.712, P = 0.0001) and serum T (r = 0.620, P = 0.0001). Seminal AMH demonstrated significant negative correlation with serum FSH (r = −0.712, P = 0.0001).

Discussion

Genetic abnormalities of the FSHR would be expected to affect sperm production in males. In the present study, variant screening was carried out among fertile and OAT infertile men to determine FSHR variants, Asn680 and Ser680, identified by BsrI restriction endonuclease. There was a tendency of Asn/Asn variant of FSHR gene to be higher in fertile men, while Ser/Ser variant to be higher in infertile OAT men, however, there was nonsignificant difference of these variants among groups. Furthermore, there was a tendency of semen parameters to be decreased in FSHR gene variants Asn/Ser and Ser/Ser than Asn/Asn (N680S). There was significantly increased level of serum FSH in Ser/Ser variant of FSHR gene compared with Asn/Asn variant. However, there was a significantly decreased level of serum T hormone in Asn/Ser and Ser/Ser of FSHR gene compared with Asn/Asn (N680S) variant. These data were in harmony with Song *et al.* (2001) who associated the FSHR genotypes with increased serum FSH level and decreased testicular volume. It is possible that isoforms of the FSHR may have weak and different receptor activities that may result in pathophysiological conditions such as increased serum FSH concentration or spermatogenic defects. Pengo *et al.* (2006) showed that in the Italian population, FSHR genotypes had no influence on FSH concentrations both in normal and infertile males and did not associate with spermatogenetic impairment. Tüttelmann *et al.* (2007) showed significant associations between polymorphism and male fertility only for AZF gr/gr deletions and MTHFR 677C—>T but not for POLG, DAZL, USP26 or FSHR.

Late studies based on larger numbers of females identified a significant correlation between the heterozygous Thr307-Asn680 (allele TN), Ala307-Ser680 (allele AS) genotype and polycystic ovary (PCO) (Sudo *et al.*, 2002). Significantly higher serum FSH levels in women with homozygous Ser at position 680 had been reported both in normal ovulatory subjects (Perez Mayorga *et al.*, 2000) as well as in anovulatory ones, suggesting that this receptor genotype might result in a mild resistance to gonadotrophin. It was indicated that FSHR gene has a role in controlled ovarian stimulation outcome. De Castro *et al.* (2003) showed that Ser680 allele might act in concert with other environmental and genetic factors that contribute to FSH efficacy.

Decreased α-glucosidase in Ser/Ser and Asn/Ser than Asn/Asn (N680S) variant could be explained by the role of FSHR in maintaining Sertoli cells to sustain normal sperm number and proper shapes of their heads and tails. In addition, Grover *et al.* (2005) observed that the shrinkage in epididymal epithelial areas may reflect direct and/or indirect changes in the functions of these cells and their role in promoting sperm motility.

The presence of an Asn residue at position 680 introduces a potential glycosylation site that might be important for post-translational receptor processing and expression at the cell surface, whereas a Ser residue could

contribute to a potential phosphorylation site involved in receptor function (Davis *et al.*, 1995). FSHR (N680S) isoforms previously described, showed similar hormone affinity and cAMP production *in vitro* and in parameters of FSH action *in vivo*, at least in adult males. These data suggest that FSHR isoforms are not functionally different in normal and infertile men. However, the possibility that different activities of the two receptor isoforms become evident in other pathophysiological conditions cannot be excluded. Depending on FSH isoform interaction, it has been speculated that FSHR can couple to different signal transduction pathways, and elicit different physiological responses (Ulloa-Aguirre *et al.*, 1995).

Kuroda *et al.* (1990) postulated FSH to be an inhibitor of AMH secretion, a hypothesis that was not supported by other investigators who found no relationship between AMH or FSH serum levels among developing boys (Rey *et al.*, 1993). However, Sertoli cells were capable of responding to FSH stimulation with an enhanced production of AMH resulting in elevated serum levels in human (Young *et al.*, 2005).

The current study showed significantly increased level of seminal AMH in fertile men compared with infertile OAT men and positive correlation with semen parameters and α-glucosidase activity. These results are in agreement with several studies performed on the rete testis fluid in mammals (Cazorla *et al.*, 1998; Vigier *et al.*, 1998) and on seminal plasma in men (Fallat *et al.*, 1996). Fénichel *et al.* (1999) and Mostafa *et al.* (2007) demonstrated that seminal AMH correlated with sperm concentration being significantly higher in fertile subjects than in oligozoospermics or in NOA. However, Fallat *et al.* (1996) demonstrated an inverse relationship between seminal AMH concentration and sperm motility index.

Significant decrease in seminal AMH concentration was demonstrated to be associated with Asn/Ser and Ser/Ser variant of FSHR (N680S) gene than with Asn/Asn, suggesting a link between AMH and spermatogenesis stimulated by FSH. The negative correlation between seminal AMH level and serum FSH, and the positive correlation with serum T confirmed these data of FSHR gene polymorphism. However, Fénichel *et al.* (1999) suggested that the decrease in seminal AMH may reflect a primary alteration in Sertoli cell function which also may lead to spermatogenic arrest.

It is concluded that FSHR (N680S) gene variants showed no difference in distribution between fertile and infertile OAT men. However, when correlated with seminal AMH values, there is an increase in Asn/Asn (N680S) in men with high seminal AMH. These data provide evidence that seminal AMH has an autocrine/paracrine effect on testicular function associated with the FSHR genotype.

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