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GENETICS

Impact of the *FSHB* gene -211G/T polymorphism on male gonadal function

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Abstract
Purpose The *FSHB* gene -211G/T polymorphism has been reported to modulate gene expression and to cause inter-individual differences in FSH serum levels in men. This study was undertaken to assess the functional relevance of this polymorphism on gonadotropin and total testosterone serum levels and sperm parameters in men from Eastern Sicily (Italy).
Methods To accomplish this, 200 men with abnormal conventional sperm parameters or normozoospermia (according to the parameters of WHO 2010) were genotyped by TaqMan Assay.
Results The frequency of *FSHB* -211 T allele was significantly higher ($p < 0.005$) in patients with altered conventional sperm parameters (18.9% of chromosomes) compared to that observed in men with normozoospermia (10.9% of chromosomes). Decreasing serum levels of FSH and LH were observed across the three *FSHB* -211 genotype subgroups ($p < 0.001$ and $p < 0.05$, respectively). In addition, the FSHB -211G/T polymorphism showed a total testosterone downward trend that became more evident in men with the TT genotype compared to subjects with the GG genotype ($p = 0.05$). Furthermore, we found a trend towards decreased sperm concentration, total sperm count, sperm forward motility and testicular volume in men with GT and TT genotypes.
Conclusions These findings showed that the *FSHB* -211 G/T polymorphism modulates male gonadal function with a clear influence on hormonal levels and sperm parameters.
Capsule The present study was undertaken to evaluate the distribution of the *FSHB* -211 G/T in men with normal or abnormal sperm parameters from Southern Italy to assess its functional relevance on the serum levels of reproductive hormones and on sperm parameters in men.

Keywords Single nucleotide polymorphism · FSH (follicle-stimulating hormone) · Sperm count · Gonadotropins

Introduction

Follicle-stimulating hormone (FSH) is a pituitary glycoprotein hormone that plays a pivotal role in the regulation of gonadal function, pubertal maturation and reproductive processes in both sexes in mammals [1]. In men, FSH is fundamental for Sertoli cell proliferation during the fetal and neonatal development as well as for mitotic activity of spermatogonia in the pubertal phase [2]. In adult men, FSH plays a crucial role for the initiation and maintenance of spermatogenesis [3]. The effectiveness of FSH relates to the intrinsic bioactivity of the hormone, its serum concentration and the efficacy of its receptor (FSHR) signal transduction in response to hormone stimulation. FSH is a heterodimeric glycoprotein composed of an α-glycoprotein subunit (αGSU) and a β-subunit (FSHß) that ensures the binding specificity to FSHR [4]. The human *FSHB* gene (MIM 136530;11p13; genomic sequence 4.2 kb), coding for the FSH β-subunit, consists of one non-coding exon plus two translated exons that encode the 129-amino acid preprotein. The mechanisms controlling the transcription of the *FSHB* gene are critical regulatory steps in the production of the FSH hormone [5–8]. These mechanisms are

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complex, involving the actions of tissue-specific and widely expressed transcription factors [9]. The *FSHB* gene is expressed in gonadotroph cells within the anterior pituitary gland and contains a low number of polymorphisms within non-coding regions. To date, the National Center for Biotechnology Information (NCBI) SNP (Single Nucleotide Polymorphism) database (http://ncbi.nlm.nih.gov/SNP/) indicates that the *FSHB* gene links to a total of 143 SNPs. A G/T SNP at -211 relative to the transcription start site in the 5′ untranslated region of *FSHB* gene (rs 10835638) is located within an evolutionary conserved element [10], in a region of the *FSHB* promoter, which binds the LHX3 homeodomain transcription factor involved in regulating the *FSHB* gene transcription [7]. In particular, the binding of the human *FSHB* promoter carrying the -211 T allele to the LHX3 homeodomain transcription factor is reduced compared with the wild-type promoter carrying the G allele; consequently, the -211 T allele promoter in vitro relative activity is lower than 50%, compared to the wild type variant [7, 11]. Therefore, the evolutionary more recent *FSHB* -211 T allele is associated with changes in the *FSHB* transcriptional activity resulting in reduced serum FSH levels. Consistent with this functional activity, some clinical studies suggested an association between the *FSHB* -211G/T polymorphism and serum FSH and some reproductive parameters [10, 12–15]. The present study was undertaken to evaluate the distribution of the *FSHB* -211 G/T in men with normal or abnormal sperm parameters from Southern Italy to assess its functional relevance on serum levels of reproductive hormones and on sperm parameters in men.

Materials and methods

Patient selection

This study included 200 men from Eastern Sicily who consulted the Division of Andrology and Endocrinology, University of Catania, for a routine diagnostic work-up for infertility or other andrological checkup. Men with known genetic causes of male infertility (karyotype anomalies, Yq chromosome microdeletions, Kallmann syndrome and *CFTR* gene mutations), cryptorchidism, testicular tumors or pituitary adenomas were excluded from this study. No patient was taking drugs capable of interfering with the hormonal levels in the last 6 months.

Serum hormone measurement

Blood sampling was performed at 8.00 am, after at least 8 h of sleep. FSH, LH and total testosterone measurements were performed by electrochemiluminescence immunoassay (ECLIA) with Cobas equipment (Roche Diagnostics GmbH, Mannheim, Germany).

Testicular volume assessment

The testicular volume was evaluated by Prader's orchidometer by the same operators (AEC and SLV) and the mean of the two evaluations was calculated and used for further analysis.

Sperm analysis

Two semen samples (2–3 weeks apart) were collected by masturbation after 4 days of sexual abstinence and sperm analysis was performed according to the World Health Organization criteria [16]. Patients were classified into two groups according to their sperm count: 90 men with alteration of conventional sperm parameters and 110 with normozoospermia according to the WHO 2010 criteria. Men with one or more conventional sperm parameters (density, total and progressive motility and normal forms) below the fifth percentile were considered as men with alteration of conventional sperm parameters.

FSHB -211G/T polymorphism analysis

Genomic DNA was isolated from peripheral lymphocytes using the High Pure polymerase chain reaction template preparation kit (Roche, Mannheim, Germany). Genotyping was performed with the StepOne Real-Time PCR System (Applied Biosystems, Carlsbad, CA, USA) for real-time PCR and TaqMan Genotyping Master Mix (Life Technologies, Pleasanton, CA, USA) and a TaqMan SNP Assay (Life Technologies, Austin, TX, USA) customized for the studied SNP (rs10835638) (C_27829553_10). The default thermal cycling conditions (10 min at 95 °C followed by 50 cycles of 15 s at 92 °C plus 1 min and 30 s at 60 °C) were applied. After each amplification, an allelic discrimination was made to determine the genotype of each subject. To find positive control DNAs representing the three genotypes (GG, GT and TT) for each genotyping reaction plate, we have previously performed direct automated DNA sequencing on AbiPrism 310 Genetic Analizer (Applied Biosystems, Carlsbad, CA, USA) of PCR products containing the specific *FSHB* -211G/T polymorphism from 60 genomic DNA samples.

Statistical analysis

The results are reported as mean ± SD and median. All analyses were performed using SPSS software (version 22) (SPSS Inc., Chicago, IL). Before statistical analysis, clinical data and hormonal and seminal variables were tested for normal distribution using the Kolmogorov-Smirnov test. Testosterone, BMI and testicular volume had normal distribution. All data were compared among different genotypes in the

whole group of subjects by one-way analysis of variance (ANOVA), for variables with normal distribution, and by non-parametric Kruskall-Wallis for non-normally distributed variables. To assess statistical differences in the variables between the carriers (GT+TT or TT homozygotes) and non-carriers (GG) of the T allele, we used one-way analysis of variance (ANOVA) and non-parametric Mann-Whitney two-tailed *U* test, as appropriate. Differences in variant tract frequencies between the group of men with alteration of conventional sperm parameters and normozoospermia were compared by the *z* test. Chi-squared analysis was used to determine whether the genotype distribution conformed to the Hardy-Weinberg equilibrium. A p value ≤0.05 was considered statistically significant and a p value 0.1 was considered suggestive.

Results

The *FSHB* -211 G/T polymorphism was genotyped for the whole group of 200 men. The frequency distribution for the genotypes was found to be in the Hardy-Weinberg equilibrium. The overall frequency of GG, GT and TT genotypes were 72 (n = 144), 25 (n = 50) and 3% (n = 6), respectively. The frequencies for G and T alleles were 84.5 and 15.5%, respectively. Comparing groups with different sperm parameters, significant differences in -211 T allele frequencies were detected. A total of 31 men with altered sperm parameters had the -211 T allele, showing a higher frequency (18.9% or 34/180 of analyzed chromosomes) compared to that of men with normozoospermia (10.9% or 23/212 of analyzed chromosomes) (p = 0.024) (Table 1). The frequency of the other allele was 81.1% for patients with alteration of conventional sperm parameters and 89.1% for men with normozoospermia. The frequency of T-carrying genotypes (GT heterozygotes and TT homozygotes) in men with alteration of conventional sperm parameters was 34.4% (31/90), differing from that observed in men with normozoospermia (20.9% or 23/110) (p = 0.027).

Clinical characteristics, hormonal levels and sperm parameters are showed in Table 2. We observed a significant gradient of declining FSH levels across the three *FSHB* -211 G/T genotypes from the highest concentration in men with the GG genotype (6.9 ± 6.3 IU/L) to the intermediate value in men with the GT genotype (3.76 ± 2.39 IU/L) to the lowest value in men with the TT genotype (2.27 ± 0.77 IU/L) (p 0.001). Higher serum FSH levels were found in men with the GG genotype compared to the group including men with the GT genotype and TT genotype ($p < 0.001$) and to the group of men with the TT genotype (p = 0.001) (Fig. 1, upper panel).

We also found decreasing levels of LH across the *FSHB* -211 genotype subgroups: GG (4.9 ± 2.3 IU/L), GT (4.35 ± 2.14 IU/L) and TT (2.68 ± 0.8 IU/L) (p = 0.011). GG carriers had higher serum LH levels compared to both group of men with T allele-carrying genotypes, including GT heterozygotes and TT homozygotes, and group of men with the TT genotype (p = 0.031 and p = 0.007, respectively) (Fig. 1, median panel).

A suggestive downward trend in total testosterone levels was observed in men according to the different *FSHB* -211 genotypes: 5.18 ± 1.96 nmol/L in men with the GG genotype, 4.87 ± 1.6 nmol/L in men with the GT genotype and 3.6 ± 2.1 nmol/L in men with the TT genotype (p = 0.095). In particular, men with the TT genotype had lower total testosterone levels than those of men with the GG genotype (p = 0.05) (Fig. 1, lower panel).

There was a trend towards lower testicular volume, sperm concentration, total sperm count and sperm progressive motility values in TT carriers compared to GG or GT carriers, but it did not reach the statistical significance. Sperm morphology and BMI were not affected by the carrier status of the *FSHB* -211G/T polymorphism.

Discussion

The human *FSHB* promoter polymorphism -211G/T, in the evolutionary conserved element 5′ upstream of the *FSHB* gene transcription site, regulates the relative activity of the *FSHB* promoter. The in vitro activity of *FSHB* promoter carrying the

Table 1 Distribution of the FSHB -211 G/T polymorphism according to sperm parameters

FSHB -211 G/T	Patients with alteration of conventional sperm parameters (n = 180 chromosomes)	Normozoospermic men (n = 212 chromosomes)
G/G	59	87
G/T	28	22
T/T	3	1
Total T allele	34 (18.9)*	24 (10.9)

The *FSHB* -211 G/T SNP frequencies are given as numbers and percentages (in parentheses) of the total cohort

*$p \leq 0.05$ vs. group with normozoospermia

Table 2 Clinical characteristics, hormonal levels and sperm parameters of all men enrolled in the study stratified by the *FSHB* -211G/T genotype

Parameter	*FSHB* -211G/T genotype			*p* value
	GG	GT	TT	
Age (years)	32.1 ± 7.8 (31)	32.5 ± 7.2 (31.5)	27.2 ± 13.0 (22.5)	0.732
BMI (kg/m^2)	28.23 ± 4.5 (27.9)	25.13 ± 5.18 (25)	31 ± 4.5 (31)	0.324
Testicular volume (mL)	28.25 ± 9.1 (28.3)	28.7 ± 8.32 (26.6)	21.7 ± 9.5 (21.8)	0.573
FSH (IU/L)	6.9 ± 6.3 (4.6)	3.76 ± 2.39 (3)	2.27 ± 0.77 (2.15)	*<0.001*
LH (IU/L)	4.9 ± 2.3 (4.4)	4.35 ± 2.14 (3.8)	2.68 ± 0.8 (2.9)	*0.011*
Testosterone (nmol/L)	5.18 ± 1.96 (5.0)	4.87 ± 1.6 (4.9)	3.6 ± 2.1 (3.5)	**0.095**
Sperm concentration (10^6/mL)	38.9 ± 46.8 (21)	34.01 ± 43.7 (10)	18.4 ± 27.9 (6.8)	0.432
Total sperm count (×10^6/ejaculate)	116 ± 162.9 (50)	100 ± 124 (25)	60 ± 93.8 (20)	0.395
Sperm forward motility (%)	15.82 ± 11.0 (15)	14.3 ± 12.6 (10)	6.75 ± 8.95 (3.5)	0.395
Sperm with normal morphology (%)	6 ± 4 (5)	6.7 ± 6 (5)	6.2 ± 4.7 (7.5)	0.953

Data are expressed as mean ± SD and median in parentheses. Statistically significant levels are marked in italics ($p < 0.05$) or in bold for suggestive difference ($p < 0.1$)

BMI body mass index

minor T allele is one half the activity of the wild type promoter with the G allele [7, 1]. The identification of a regulatory polymorphism in the *FSHB* promoter has paved the way to study the effect of constitutively reduced FSH levels on male fertility.

The present study was designed to investigate the impact of the *FSHB* -211 G/T on the testicular function in men from Southern Italy. We observed significant differences in the distribution of *FSHB* -211 T allele between the groups of men with abnormal sperm parameters and that with normozoospermia: T allele frequencies were 18.9 and 10.9% of analyzed chromosomes, respectively. The results reinforce data on the significance of the -211 T allele in alteration of conventional sperm parameter pathogenesis.

We observed that men with the GT genotype and TT genotype had 35 and 54% reduced FSH levels, respectively, compared to those of men with the GG genotype. The strong influence of *FSHB* -211G/T on serum FSH, seen in this study, confirms observations from previous studies reporting a significant negative association between this SNP and FSH levels, in which serum FSH levels decreased in GT group and TT group carriers of 10–27% and 24–64% respectively, compared to those of the GG group [10, 5, 7]. These results also confirm that FSH production is genetically determined by the transcription rate of the *FSHB* gene and that in turn the transcription rate is regulated by the highly conserved promoter regions of the *FSHB* gene [10] and, in particular, by an 11 bp element, encompassing the -211 nucleotide, which binds to the LHX3 homeodomain transcription factor [7].

The lower serum LH observed between the T allele-carrier men of this study may suggest additional regulatory effects of *FSHB* -211 T allele, beyond gonadal development and function, on male hormonal balance and physiology. However, it is necessary to widen the study to increase the number of TT-carrying patients to confirm these data, because previous larger studies reported that serum LH increased with the number of the T allele [5, 7]. Grigorova and colleagues detected a negative association between *FSHB* SNP and serum testosterone (GG > GT > TT) [17], not confirmed by a subsequent study [15]. In this study group, serum testosterone was lower in men with the GT genotype and further lower in men with the TT genotype compared to that observed in men with the GG genotype. These intriguing results may be explained by an indirect effect of FSH on testosterone production by Leydig cells as indicated from some studies on supernatants of Sertoli cells incubated with FSH that stimulated testosterone secretion by Leydig cells and testicular explants from rats and humans [18, 19]. Therefore, the lower testosterone levels observed in men carrying the *FSHB* -211 T allele may be explained by a compromised steroidogenic activity by Leydig cells due to an understimulation (due to low FSH levels) of the differentiation process during fetal and neonatal development.

Previous studies have shown an unclear association between *FSHB* -211 G/T and sperm parameters. In a study including Estonian men, TT homozygotes had significantly reduced sperm forward motility compared with heterozygotes GT and homozygotes GG, but an opposite trend for an increase in sperm concentration and total sperm count (GG < GT < TT) [12]. Subsequently, in two separate study groups of Baltic and German men, the authors found a decreasing trend of sperm concentration and total sperm count

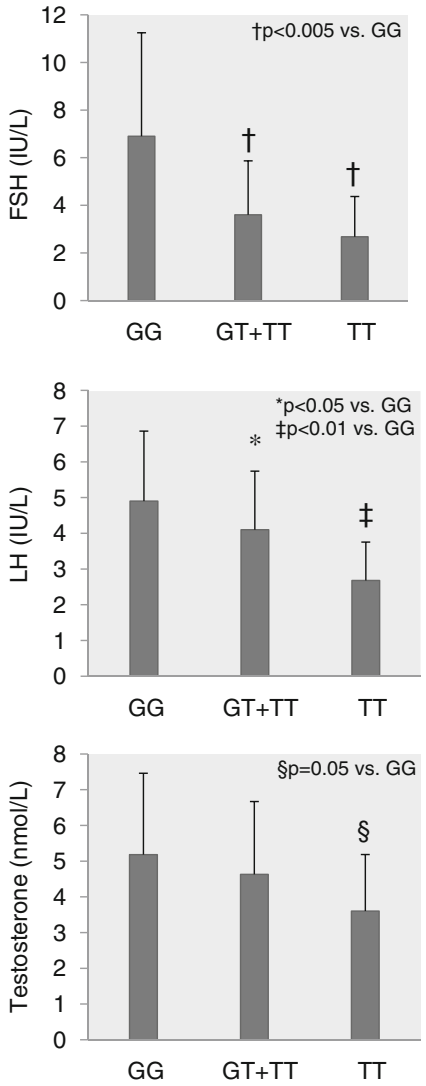

Fig. 1 Serum FSH, LH and testosterone levels in GG, GG+TT and TT carriers. Serum FSH (*upper panel*), LH (*middle panel*) and testosterone (*lower panel*) levels in men stratified according to the *FSHB* -211 GG, GT+TT and TT genotypes. *†p < 0.005*, **p < 0.05*, *‡p < 0.01* (Mann-Whitney test) and *§p = 0.05* (ANOVA) vs. the *FSHB* -211 GG genotype

(GG > GT > TT), but an increasing trend of sperm forward motility [7, 15]. A study on Italian patients revealed a trend for lower sperm concentration and sperm count, only in TT homozygotes. This study showed a decreasing trend of sperm concentration, sperm count and sperm forward motility with the highest values amongin men with the GG genotype to the lowest values in men with the TT genotype (GG > GT > TT) [14]. The significant differences in T allele frequencies between men with alterations in conventional sperm parameters and men with normozoospermia together with the alterations in sperm parameters (decreased sperm number and forward motility) reported in the -211 T allele-carrier men highlight a putative and important role of this *FSHB* SNP on spermatogenetic impairment and consequently on the male reproductive potential.

Altogether, these results, regarding the general compromised hormonal balance and the altered semen parameters reported in *FSHB* -211 T allele-carrier men, give further evidence for the crucial role of FSH on Sertoli cell proliferation, its functional significance in spermatogenesis and its contribution to male hormonal balance.

Serum FSH and reproductive parameters in men have been reported to be influenced also by the combination of *FSHB* -211G/T polymorphism with *FSHR* 2039A/G polymorphism, that influences the sensitivity of FSH receptor, and, very recently, with *FSHR* -29G/A, that modulates the transcription level [12, 15]. In particular, homozygotes *FSHR* 2039GG, with the lower sensitivity of the receptor to FSH, had significantly higher FSH levels and lower testicular volumes in the presence of *FSHB* -211 GT and TT carriers. These two *FSHR* SNPs with *FSHB* SNP explained together 2.3, 1.4, 1 and 1.1% of the measured variance in serum FSH, inhibin B, testosterone and total testes volume, respectively [12].

In conclusion, this study broadens the evidence supporting relevance of the *FSHB* -211G/T polymorphism on hormonal levels and spermatogenesis and can be considered a genetic factor contributing to the multigenic origin of male infertility. Therefore, determination of the *FSHB* promoter genotype alone, as suggested and demonstrated by a pilot study [14], or in combination with the other *FSHB*/*FSHR* genotypes could allow identification of patients that respond to FSH treatment. There is, therefore, a need for further interventional and prospective studies to ascertain with certainty whether the *FSHB* SNP alone or in combination with other SNPs is helpful for a clinically effective pharmacogenetic approach.

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Author contributions LT and SLV contributed to conception and design of the study, data acquisition, analysis and interpretation and manuscript drafting.

VT contributed to data analysis and interpretation.

RAC and LMM participated in the study design and contributed to interpretation of the data and in revising the manuscript critically for important intellectual content.

AEC contributed to conception and design of the study, data acquisition and interpretation, manuscript drafting and revising it critically for important intellectual content.

All authors have given final approval of the version to be published.

Compliance with ethical standards

Conflict of interest The authors declare that they have no conflict of interest.

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