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Single Nucleotide Polymorphism in the Promoter Region of H1 Histone Family Member N, Testis-specific (H1FNT) and Its Association Study with Male Infertility

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Abstract

The H1 histone family, member N, testis-specific (*H1FNT*) is exclusively expressed in the testis, and had its possible role for sperm chromatin formation. The purpose of this study is to investigate any genetic association of *H1FNT* gene with male infertility, especially at the promoter region. We examined the promoter single nucleotide polymorphisms (SNP) of *H1FNT* gene which is located within transcription factor binding site for its association with male infertility. The statistical analysis showed that the −1129A>T polymorphism was present at a statistically significance in male infertility (p= 0.0059 and 0.0349 for hetero and risk type, respectively). The dual-luciferase promoter assay was performed to examine the polymorphic effect of this promoter SNP by the cloning of promoter region (1700bp fragment) into pGL3-basic vector. In our plasmid based reporter system, there is no big difference between wild and risk type. In conclusion, *H1FNT* −1129A>T promoter SNP is statistically significant with male infertility, especially with subfertile (non-azoospermia) group. Further analysis of its functional polymorphic effect *in vivo* may provide the biological significance of testis-specific histone with spermatogenesis.

Keywords: *H1FNT*, male infertility, single nucleotide polymorphism

Introduction

H1 histone family, member N, testis-specific (*H1FNT*) is exclusively expressed in the testis with a correlation of its histone like role during the spermatogenesis (Tanaka *et al*., 2006). The *H1FNT* is essential for normal spermatogenesis and male fertility (Martianov *et al*., 2005; Tanaka *et al*., 2005). The human *H1FNT* is reported to be important in the sperm chromatin formation (Tanaka *et al*., 2006). In the spermiogenesis, the physiological role of the *H1FNT* is related with the chromatin condensation (Tanaka *et al*., 2005). With the homozygous Hanp1/H1T2 (mouse *H1FNT*) knockout mice study, there was abnormal chromatin packing and nuclear formation in the sperm (Kimura *et al*., 2003). Male infertility is reported in case of the homozygous Hanp1/H1T2 mutant male mice with abnormal sperm morphology and the reduced sperm motility (Miyagawa *et al*., 2005; Tanaka *et al*., 2003). *H1FNT* gene is located on the long arm of chromosome 12 (12q13.11), and has one exon spanning a length of 1,300bp, encodes a protein of approximately 255 amino acids. Previous SNP analysis of H1FNT gene showed that five common cSNPs (coding SNP) showed no genetic association with male infertility from Japan (Tanaka *et al*., 2006).

Since H1FNT is the testis specific histone gene, so we investigated any genetic variation in promoter region of the *H1FNT* with male infertility from Korea population. We also investigated any polymorphism effect of statistically significant promoter SNP, and analyzed its clinical significance of testis specific histone.

Methods

Patients and controls

Two hundred three healthy fertile men who had at least one child and who lacked any history of requiring assisted reproduction technology were included as the nation-wide control group. Members of this group were identified at the Division of Genome Resources, National Genome Research Institute, National Institute of Health, and Seoul, Korea. Two hundred twenty-nine non-azoospermia (subfertile) patients were recruited from the CHA General Hospital, College of Medicine, CHA University from January 2000 to August 2003, (Seoul, Korea). Semen analysis was performed with infertile males further sub-divided following semen analysis performed strictly according to the World Health Organization (WHO) guidelines (WHO) (2001). The sub-division

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Table 1. Primer sequence for PCR and cloning designed based on the H1FNT sequence at NCBI

Primers	Sequence
PCR primers	
hH1FNT PCR fw	5'-AGCCTGTGACTGATGAATGTATG-3'
hH1FNT PCR rw	5'-Biotin-ATCAAGGACCTATTTTGGACACATTG-3'
hH1FNT PCR seq	5'-AAGTATTCTTAGAATAA-3'
Cloning primers	
hH1FNT cloning fw	5'-GCCGTACCAAGAACTTAGGCCTTAGG-3'
hH1FNT cloning rw	5'-CAACTGGGACACTCTGAGCA-3'
hH1FNT muta fw	5'-GGCAATTTGAAGTATTCTTAGAATTATTTAAATTATTAACAACTAAACTGTG-3'
hH1FNT muta rw	5'-CACAGTTTAGTTGGGAATAATTTAAAGAATTATTCTAAGAATACTTCAAATTGCC-3'
hH1FNT dele fw	5'-GCTTGGCAATTTGAAGTATTCTTAGCTTTAAATTATTCCCAACTAAACTG-3'
hH1FNT dele rw	5'-CAGTTTAGTTGGGAATAATTTAAAGCTAAGAATACTTCAAATTGCCAAGC-3'

were azoospermia group (no spermatozoa in the ejaculate) and non-azoospermia group (low quality of spermatozoa in the ejaculate). All participants gave their written informed consent for participation in the study. The non-azoospermia group (n=229) was used for this study. Exclusion criteria contains chromosome abnormality including Klinefelter syndrome (47, XXY) and Y chromosome microdeletion (Yun *et al*., 2008).

Pyrosequencing for genotyping

The PCR primers were designed using PRIMER3 site (http://frodo.wi.mit.edu/primer3/) and pyrosequencing primers were designed PSQ assay design version 1.0 software. The PCR was carried out using 30ng of genomic DNA and 10pmol each of the forward and reverse oligonucleotide PCR primers (Integrated Bioneer, Daejeon, Korea). These primers generate a 127bp fragment from nucleotide −1210 to −1083 upstream of the transcription start site. The following PCR conditions were used: predenaturation at 95°C for 5 minute, denaturation at 95°C for 1 minute, annealing at 52°C for 30 seconds, and extension at 72°C for 30 seconds for 35 cycles. The biotinylated PCR product then underwent pyrosequencing analysis for direct analysis of the −1129 polymorphism on PCR product according to the published method (Park *et al*., 2005; Yun *et al*., 2008). The sequences of all oligonucleotides in this study are listed in Table 1. The genomic location of rs2261608 was indicated at Fig. 1. rs2261608 is located at -1129 bp upstream of transcription start site of H1FNT1.

Statistical analysis

The statistical analysis used to appraisal the odds ratio and 95% confidence intervals were SAS Enterprise Guide 4. Results for the enumeration of data and comparison of percentages between groups were evaluated with a chi-squared test and Fisher's exact test.

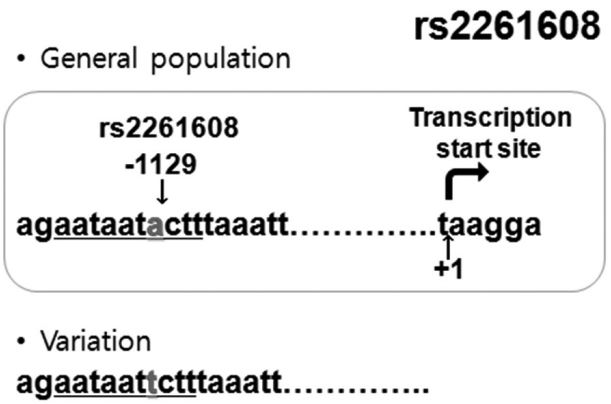

Fig. 1. −1129A/T site at the H1FNT promoter region are indicated. +1 indicated the transcription start site.

Tests for the Hardy-Weinberg equilibrium and Odd ratios (OR) were implementation by the chi-squared test. The sperm parameter statistically analyzed with the One-way ANOVA. p values < 0.05 were taken as statistically significant.

Cloning of the *H1FNT* promoter region and construction of three reporter vectors

For reporter assay, we constructed three reporters, pGL3-Wild (−1129A), pGL3-Risk (−1129T) and pGL3-Deletion (Δ−1129 to −1135) (Fig. 2A). By using the hH1FNT cloning primers, the genomic sequence from −2201 to +501 (1700 bp fragment) was amplified in the promoter region of *H1FNT*. pGL3-Wild vector was constructed by cloning the amplification product into the pGL3-Basic vector (Promega). The template DNA consisted of samples from individuals genotyped for the wild type. pGL3-Risk and pGL3-Dele (Deletion) vectors were constructed by Quik Change Site-Directed mutagenesis kit (Stratagene). The mutagenesis primers were

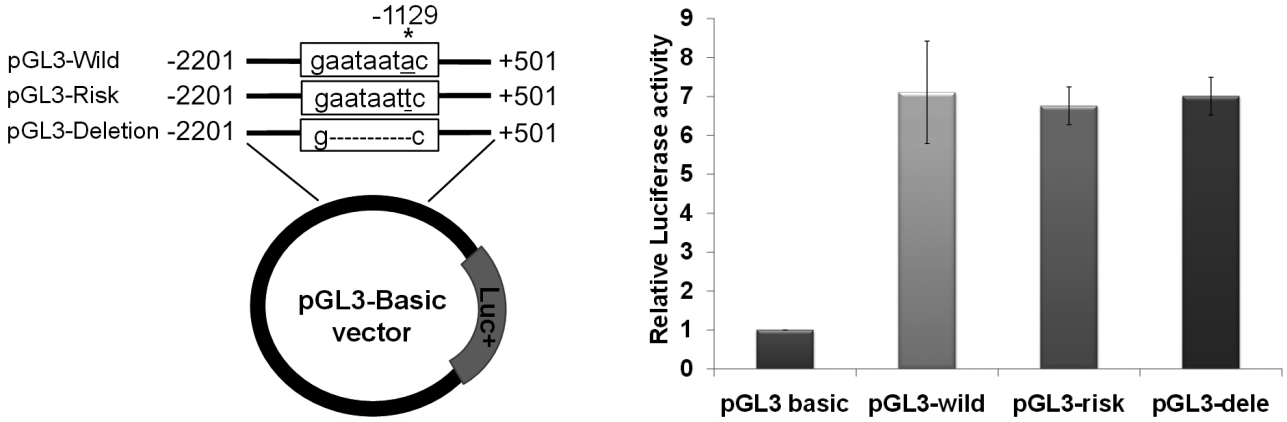

Fig. 2. The construction of three reporters for promoter assay. (A) The map of pGL3-Wild, pGL3-Risk and pGL3-Deletion. pGL3-Wild vector was constructed by the cloning of PCR fragment into the pGL3-Basic vectors, and others were constructed by *in vitro* mutagenesis based on pGL3-Wild vector. (B) A dual-luciferase assay of three reporters, pGL3-Wild, pGL3-Risk and pGL3-Deletion. The pRL-TK vector was used to correct the variation of transfection efficiency.

designed by Agilent homepage (https://www.genomics.agilent.com/). The vector sequences were confirmed by direct sequencing (SolGent. Korea).

Cell transfection and dual-luciferase assay

Human embryonic kidney HEK293T were cultured in DMEM with 10% FBS and 1% penicillin streptomycin. By using a lipofectamine 2000 (Invitrogen Life Technologies, Carlsbad, CA), we transfected the reporter vectors and pRL-TK vector (Promega). The pRL-TK vector was used to correct variation in transfection efficiency. pGL3 Basic vector was also transfected as control. After 48 hours, the cell was washed with PBS, and Dual-Luciferase Reporter Assay System (Promega) was used to measure the Firefly and Renilla luciferase activities according to the manufacturer's method.

Results

The promoter analysis H1FNT by *in silico* model

Since H1FNT is the testis specific histone gene, so we investigated any genetic variation in promoter region of the *H1FNT* from Korea population. To identify any transcription factors in the polymorphic site of the *H1FNT* promoter, we did *in silico* analysis by using the web-based bioinformatic model (http://www.cbrc.jp/research/db/TFSEARCH.html and http://www.gene- regulation.com) (Heinemeyer *et al.*, 1999; Matys *et al.*, 2003). There is only one SNP existed at the promoter region which two transcription factors can bind (Fig. 1). Our *in silico* analysis showed that HOXA7 and CDX2 can bind to the −1135～−1125 (+1 indicate the transcription start site) and −1135～−1129 region which re2261608 existed. Therefore, −1129 A to T transversion may alter the transcription factor binding affinity *in vivo*, and could affect its transcription level.

Genotyping

First, we analyzed the *H1FNT* −1129A>T SNP from non-azoospermia (n=229) and fertile (n=203) males. The SNP analysis was conducted by pyrosequencing assay for −1129A>T polymorphisms, and statistical analysis of the −1129A>T results in fertile and non-azoospermia males are shown in Table 2. The frequencies of the heterozygous −1129A>T (AT), homozygous −1129A>T (TT), −1129 (AT+TT) and T allele in the group with non-azoospermia were statistically significant ($p < 0.05$). The frequencies of heterozygous −1129A>T (AT) among the fertile and non-azoospermia were 19.7 and 30.57% ($p = 0.0059$), respectively and homozygous −1129A>T (TT) among the fertile and non-azoospermia were 0.5 and 3.05% ($p=0.0349$), respectively. The frequencies of −1129 A→T substitution in one or both alleles (AT+TT) and T allele of non-azoospermia were 33.62 and 18.34%, respectively ($p=0.0018$ and $p=0.0005$).

Clinical significance: Sperm parameter analysis

Genetic analysis of *H1FNT* −1129A>T SNP data show that there is strong association of this SNP with non-azoospermia. Therefore, we further investigated −1129A/T genotype with sperm cell pathophysiology, and analyzed its association with sperm parameters. The sperm parameter information was available from non-azoospermia (n=144). From the analysis of sperm pa-

Table 2. The statistical analysis of the *H1FNT* −1129A/T polymorphism from fertile and non-azoospermia males

H1FNT rs2261608 (A/T)	Fertile males		Non-Azoospermia		OR (95% CI)	p
	%	n	%	n		
−1129AA	79.8	(162)	66.38	(152)	-	-
−1129AT	19.7	(40)	30.57	(70)	1.87 (1.19-2.92)	0.0059
−1129TT	0.5	(1)	3.05	(7)	7.46 (0.91-61.4)	0.0349
−1129AT+TT	20.2	(41)	33.62	(77)	2.00 (1.29-3.10)	0.0018
T allele	10.34	(42)	18.34	(84)	2.13 (1.39-3.28)	0.0005

Significance level: $p < 0.05$.

rameter with the −1129A/T genotype, the sperm count, motility, vitality and morphology were not significantly associated with −1129A/T genotype, respectively (p=0.8337, p=0.6153, p=0.5445 and p=0.5243) (Table 3).

Dual-luciferase promoter assay

We found that *H1FNT* −1129A>T polymorphism was significantly associated with non-azoospermia. Functional promoter assay was performed to investigate any polymorphic effect of −1129 A>T SNP. For reporter assay, we used three reporters, pGL3-Wild, pGL3-Risk and pGL3-Dele (Fig. 2A), The dual-luciferase assay showed that the cloning of promoter region (1700bp) of H1FNT into pGL3-basic increased the total luciferase activity, but there is no change of activity between wild (pGL3-Wild) and mutant type (pGL3-Risk) (Fig. 2B). pGL3-Dele (Deletion) vectors which deleted of transcription binding sites (Δ −1129 to −1135) has also similar activity to other reporters (Fig. 2B).

Discussion

Our study showed that the *H1FNT* −1129A>T promoter polymorphism (rs2261608) has strong genetic association with male infertility.

We analyzed the clinical significance of this promoter SNP. However, there was no direct association of sperm parameter with this −1129A>T genotype from non-azoospermia group (n=144). Interestingly, our data showed that this SNP is only associated with non-azoospermia group (low quality of spermatozoa in the ejaculate), but not with azoospermia group (no spermatozoa in the ejaculate). Therefore, our data suggested that this SNP affect total process of spermatogenesis, but may be in part. Further analysis of this genotype with sperm parameters may be required with the expanded subfertile group.

Functionally, we expected that this promoter SNP may regulate its transcriptional level. In our plasmid based reporter system, there is no polymorphic effect of this promoter SNP. Our *in silico* analysis showed two possible transcription factors can bind to the promoter of H1FNT which included this −1129A>T SNP site. The transcriptional regulation of H1FNT may be performed under very complicated condition during spermatogenesis, and so further analysis of H1FNT expression *in vivo* may be required for the analysis of this genotype. Our genetic association study of H1FNT with male infertility is the first positive genetic study with male infertility, especially with non-azoospermia in our knowledge. Further functional and clinical study of H1FNT may provide its biological significance of spermatogenesis.

Table 3. The statistical analysis of sperm count, motility, vitality and morphology according to −1129A/T genotype of non-azoospermia

Sperm parameter	Genotype	Average (SD)	p
Count (10^5/ml)	−1129A	50.47 (±47.97)	
	−1129AT	48.12 (±59.59)	0.8337
	−1129T	35.50 (±1.29)	
Motility (%)	−1129A	30.67 (±14.86)	
	−1129AT	31.24 (±17.53)	0.6153
	−1129T	38.5 (±22.96)	
Vitality (%)	−1129A	48.9 (±18.42)	
	−1129AT	45.93 (±17.97)	0.5445
	−1129T	54.5 (±28.11)	
Morphology (%)	−1129A	3.32 (±6.59)	
	−1129AT	2.22 (±2.3)	0.5243
	−1129T	4.25 (±2.99)	

Significance level: $p < 0.05$.

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