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Genetic association study of a single nucleotide polymorphism of kallikrein-related peptidase 2 with male infertility

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Objective: To investigate a kallikrein-related peptidase 2 (KLK2) single nucleotide polymorphism (SNP) in relation to male infertility because of its role in semen processing. We investigated the genetic association of the KLK2+255G > A genotype with male infertility.
Methods: We genotyped the SNP site located in intron 1 (+255G > A, rs2664155) of KLK2 from 218 men with male infertility (cases) and 220 fertile males (controls). Pyrosequencing analysis was performed for the genotyping.
Results: The SNP of the KLK2 gene had a statistically significant association with male infertility ($p < 0.05$). The odds ratio for the minor allele (+255A) in the pooled sample was 0.47 (95% confidence intervals, 0.26-0.85) for rs2664155.
Conclusion: The relationship of KLK2 SNP to male infertility is statistically significant, especially within the non-azoospermia group. Further study is needed to understand the mechanisms associated with male infertility.

Keywords: Kallikrein-related Peptidase 2; Polymorphism, Single Nucleotide; Infertility, Male; Human

Introduction

Several risk factors for male infertility have recently been identified, such as chromosomal abnormalities, Y-chromosome microdeletions, translocation, cystic fibrosis transmembrane conductance regulator mutations, and other genetic factors [1]. A significant percentage of couples (30-40%) fail to achieve pregnancy despite several IUI attempts, even in cases without a clear male infertility factor, which suggests the existence of an occult cause of male infertility that has lately been linked to molecular factors and not to sperm count, motility, or morphology [2].

Semen liquefaction is achieved through a stepwise proteolytic cleavage of the gel proteins semenogelin I and II (SgI and SgII) into soluble proteins, followed by their peptidic fragmentation. Liquefaction of human semen involves proteolytic degradation of the seminal coagulum and release of motile spermatozoa. Several members of human kallikrein-related peptidases (KLKs) have been implicated in semen liquefaction [3].

Kallikreins are serine proteases that belong to the human tissue kallikrein family, which included 15 members (KLK1-KLK15) [4]. The KLK2 gene is located on chromosome 19q13.41. It consists of five exons and is 5,217 bp in length [5]. Three glandular kallikreins are encoded by the human kallikrein gene family: tissue kallikrein (KLK1), human kallikrein-related peptidase 2 (KLK2), and prostate-specific antigen (PSA or KLK3) [6]. Kallikrein is assumed to improve sperm motility due to enzyme activation [7]. Kallikrein is a glycoprotein involved in the enzymatic activation of kininogens that has been shown to have a positive effect on sperm motility *in vitro* and on the stimulation of

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sperm metabolism [4]. Since the amount of KLK2 in seminal plasma is lower than PSA (1-5%), it is a key regulatory factor for KLK protease cascade [8]. Therefore, we searched for any association of KLK2 single nucleotide polymorphism (SNP) with male infertility.

Methods

1. Patients and control

Two hundred twenty fertile men who had fathered at least one child and who lacked any history of requiring assisted reproductive technology were included as the control group. This control group was consecutively enrolled from the Division of Genome Resources, National Genome Research Institute, National Institutes of Health, and from Korea [1,9]. From January 2002 to August 2006, 709 non-obstructive infertile men were identified following physical examinations and hormone assays. Infertile men without any chromosome abnormality were selected for this association study. These non-obstructive infertile men (those with spermatogenesis problems) were further sub-divided following semen analysis performed strictly adhering to the World Health Organization guidelines [10]. The diagnoses of non-azoospermia were made on the basis of two semen analyses performed according to World Health Organization recommended procedures. Non-azoospermia (n=218) included men having at least one abnormality in sperm concentration ($<20\times10^6$), motility (<50%), or morphology (<14%). The experiments were performed at the CHA General Hospital, Seoul, Korea. Informed consent was required to participate in this study [9].

2. Pyrosequencing for genotyping

The PCR primers were designed using PRIMER3 (http://frodo.wi.mit.edu/cgi-bin/primer3/primer3_www.cgi) and Pyrosequencing Primer SNP Design ver. 1.01 software. The pyrosequencing reactions were automatically performed with a PSQ 96 system (Pyrosequencing AB) according to the manufacturer's instructions. Briefly, the biotinylated PCR product (50 μL) was purified using streptavidin-Sepharose beads. The single-strand PCR product acted as a template in the pyrosequencing reaction [1]. All reactions were constructed as recommended by the manufacturer's instructions (Pyrosequencing, Westborough, MA, USA). The pyrosequencing reaction was performed automatically using a PSQ 96 MA system (Pyrosequencing AB, Uppsala, Sweden) and also with a SNA Reagent Kit (Biotage AB, Uppsala, Sweden). The PCR conditions were 95˚C for 15 minutes, 95˚C for 30 seconds, 54˚C for 30 seconds, and 72˚C for 30 seconds for 40 cycles. The biotinylated PCR product was purified by using streptavidin-Sepharose beads. The pyrosequencing reaction was performed automatically using a PSQ 96MA system along with a SNP Reagent Kit. All reactions were performed as recommended by the manufacturer's instruction (Pyrosequencing).

3. Statistical analysis

Multiple logistic regression models were used to calculate odds ratios, 95% confidence intervals (CIs), and corresponding *p*-values. These analyses were performed using SAS statistical software (SAS Enterprise Guide, Cary, NC, USA) [11]. Student's *t*-tests (Analysis of variation, ANOVA) were used to compare the expression levels of KLK2 genotypes (GG vs. AA). Statistical significance was assumed at a *p*-value less than 0.05.

Results

1. KLK cascade for semen liquefaction

KLK2 is a highly prostate-specific serine protease (hK2), which is mainly excreted into the seminal fluid, but part of which is also secreted into circulation from prostatic tumors and benign prostatic hyperplasia tissue [12-14]. Specifically, hK2 has an 80% identity in primary structure with PSA (hK3), and both proteins have high transcripts, particularly in the prostate [15]. hK2 is responsible for the activation of PSA (hK3) by cleaving its pro-form to the enzymatically active mature form in the prostate [16] (Figure 1). In the prostate, the propeptides are removed from proPSA and prohK2, leaving the mature, catalytic forms. KLK2 might be one of the proteases responsible for these processing events. PSA and hK2 are released at high concentrations into prostatic fluid, then into seminal fluid. hK2 and PSA

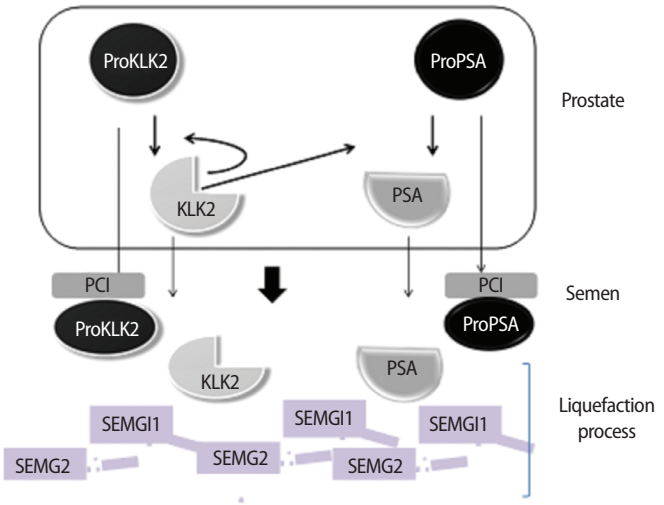

Figure 1. Kallikrein-related peptidase (KLK) cascade for semen liquefaction. Active types of prostate-specific antigen (PSA; KLK3) and KLK2 are shown as gray incomplete round forms, and inactive types as black ovals (proKLK2 and proPSA). KLK2 is responsible for the activation of PSA and itself by cleaving pro-forms to the enzymatically active mature forms inside the prostate. PCI indicates protein C inhibitor, and SEMG1-2 are semenogelin I and II.

Table 1. Association study of KLK2 SNP with male infertility

Genotype	Fertile men (n=220)	Non-azoospermia (n=218)	Odds ratio (95% confidence intervals)	*p*-value (chi-square test)
+255GG	183 (83.2)	199 (91.3)		
+255GA	36 (16.4)	19 (8.7)	0.49 (0.27-0.88)	<0.01
+255AA	1 (0.4)	0 (0)		<0.29
+255GA+AA	37 (16.8)	19 (8.7)	0.47 (0.26-0.85)	<0.01

Values are presented as number (%).
$p < 0.05$ for the statistical difference between IVS1+255 (G) and IVS1+255 (A) genotypes in fertile and non-azoospermia group.
KLK2, kallikrein-related peptidase 2; SNP, single nucleotide polymorphism.

forms in prostatic fluid are active, nicked, and complex forms with protein C inhibitor (PCI), which is a protease inhibitor [15].

2. Statistically significant association of the KLK2 gene with male infertility

Since hK2 is mostly present in semen, the genetic association study was performed the KLK2 gene with male infertility. We selected a SNP site of the KLK2 gene located in intron 1 (IVS1+255, rs2664155) because of its previously reported association with prostate cancer [5]. The association study was performed with 218 infertile men (cases) with 220 fertile male controls. Genetic analysis showed that IVS1+255 SNP was significantly associated only with the non-azoospermia group (n=218, $p < 0.05$). For IVS1+255 SNP, the allele frequency of the variant type (+255A) was 16.8% in fertile males (n=220) and 8.7% in non-azoospermia males (n=218, $p < 0.01$) (Table 1). The SNP of the KLK2 gene had a statistically significant association with male infertility ($p < 0.05$). The OR for the minor allele (+255A) in the pooled sample was 0.47 (95% CI, 0.26-0.85) for rs 2664155.

Discussion

In our study, we examined how the polymorphism of KLK2 may affect male infertility because of its role in semen processing. Genotype association analysis showed that the KLK2 intron 1 (+255G>A) SNP is associated with male infertility, especially with non-azoospermia.

Human KLKs are a family of proteases, the majority of which are found in seminal plasma. The physical properties of ejaculate (coagulation, liquefaction, volume, and viscosity) have clinical implications in male infertility with concurrent delayed liquefaction, which has also been associated with sperm parameters [17]. Kallikrein is assumed to be related to the improvement of sperm motility due to enzyme activation, in addition to semen liquefaction [18]. Kallikrein is a glycoprotein involved in the enzymatic activation of kininogens that has a positive effect on sperm motility *in vitro* and stimulates sperm metabolism. From a prostate cancer study conducted with the KLK2 gene, we know that there could be simple linkage disequilibrium with another unknown SNP involved in regulatory functioning [5]. Defining the functional significance of KLK2 SNP may be necessary for the elucidation of its biological effect with male infertility. Further study of its polymorphic effect by investigating the KLK2 intron 1 (+255G>A) SNP and other linked SNPs in the KLK2 gene is needed.

Conflict of interest

No potential conflict of interest relevant to this article was reported.

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