

Mutation analysis of the *JUNO* gene in female infertility of unknown etiology

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Abstract

The genetic etiology of female infertility is almost completely unknown. Recently, the egg membrane protein JUNO was identified as a receptor of the sperm-specific protein IZUMO1 and their interaction functions in sperm-egg fusion in fertilization. In the present study, we examined 103 women with infertility of unknown etiology. We analyzed the *JUNO* gene in these cases by PCR and Sanger sequencing. We identified seven variants in total: four common, two synonymous, and a previously unidentified intronic mutation. However, it is not clear from these variants that JUNO has a major role, if any, in infertility. Many factors affect sterility and a larger cohort of patients will need to be screened in the future because the cause of female infertility is highly heterogeneous.

Keywords: JUNO, IZUMO1, Infertility, Sperm-egg fusion

Introduction

Approximately 10% of couples intending to have children have infertility problems, 15% of which are of unknown etiology.¹ Some failures of conventional *in vitro* fertilization (IVF) can be overcome by intracytoplasmic sperm injection (ICSI). One cause of infertility is failure of sperm-egg recognition or fusion. When a sperm reaches the ovum, it initiates an acrosome reaction to pass through the transparent zona pellucida, which is followed by fusion. However, the mechanism of sperm-egg fusion is still not fully understood. One of the molecules known to be essential for sperm-egg fusion is the sperm surface protein Izumo1.² Izumo was named after the Japanese Shrine, Izumo Taisha, known for the divine favor of matchmaking. Izumo1-deficient sperm can pass through the zona pellucida but cannot fuse with the egg.

Juno, the product of the folate receptor 4 gene (*FOLR4*), was named after the Roman goddess of marriage and women and was recently identified as the egg receptor of Izumo1.³ Dimerized Izumo1 binds tightly to Juno prior to sperm-egg fusion, after which Juno molecules are rapidly shed from the egg to protect against polyspermy.⁴ The crystal structures of human IZUMO1 and JUNO revealed the amino acids that are important for the IZUMO1-JUNO complex.⁵ Given that the Juno-deficient female mouse is infertile because of impaired sperm-egg fusion, we speculated that genetic variations in the *JUNO* gene may also be the cause of female infertility in humans.

A genome-wide association study (GWAS) may not be successful in identifying the genetic factors that underlie human female

infertility. One reason for this is that common variations found in GWAS analyses are not likely to contribute to infertility because of their reproductive fitness. Another reason is the highly heterogeneous etiology of female infertility. We, therefore, reasoned that a candidate gene approach might be successful in identifying rare variants that contribute to infertility. In this study, we sequenced the *JUNO* gene in female patients with infertility of unknown etiology to assess whether *JUNO* mutations might be associated with this phenomenon.

Methods

Cases

A cohort of 103 Japanese women with sterility of unknown cause was recruited from private fertility clinics in Nagoya, Japan. Informed consent for genomic analysis was obtained from all patients. The age range of these subjects was 24–42 years old (mean, 35 years), and the period of infertility ranged 3–144 months. The study protocol was approved by the Ethics Review Committee of Fujita Health University.⁶

DNA extraction, PCR and direct sequencing

Blood samples were collected from the study subjects and genomic DNA extracted using QuickGene 610L (Fujifilm, Tokyo, Japan). Four primer pairs were used for the amplification of *JUNO* exons, including exon-intron boundaries (Figure 1). The PCR conditions were 94° C for 2 min 30 sec, followed by 35 cycles of 94° C for 30 sec and 60° C for 60 sec, and a final incubation at 60° C for 5 min. The amplified products were purified using Exo-SAP-IT (Affymetrix, Tokyo, Japan) and used as templates for direct sequencing. Sanger sequencing was carried out using BigDye-terminator v3.1 and a Genetic Analyzer 3130 (Applied Biosystems, Tokyo, Japan) in accordance with the manufacturer's instructions.

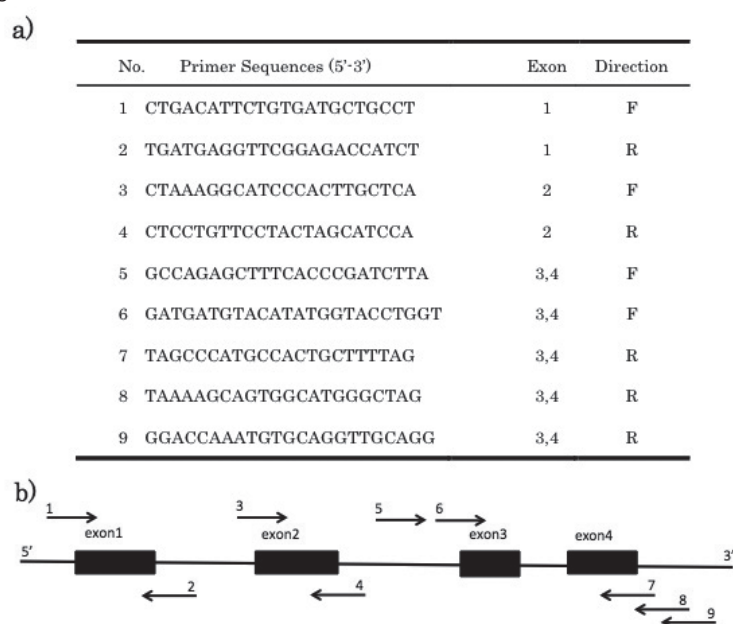
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Figure 1.



Positions and sequences of the PCR primers used to amplify JUNO exons.

a) The sequences of primers used for PCR are shown.

b) The exon-intron organization of *JUNO* is shown. Black boxes indicate exons. Positions and directions of the primers are depicted by arrows.

Analysis

We used sequence information from the UCSC Genome Browser⁷ as reference data and single nucleotide variation (SNV) data from the Ensembl database⁸ for population genetics analysis. The effects of amino acid substitutions were predicted using PolyPhen⁹ and SIFT¹⁰ using Ensembl data. An exome database for the Japanese population¹¹ was also used.

Results

From our sequencing analysis of the 103 study subjects, we identified seven variations in the *JUNO* gene, four of which were located within coding sequences and three in intronic regions (Table 1). c.531T>G (p.His177Gln) was observed in 10 of 103 subjects (9%) as a heterozygous variation. Although this amino acid change from histidine to glutamine was predicted as possibly damaging by PolyPhen, the histidine is not strictly conserved among vertebrates. This SNV is listed in the dbSNP database (rs76779571), and the frequency of the G/T genotype is 14%, which is similar to that of our current patient population. c.9C>G was observed in one patient (1%), and involves a coding amino acid change from cysteine to tryptophan (p.Cys3Trp). The PolyPhen and SIFT score for this variation were benign and tolerated, respectively. This SNV was also found in the dbSNP database (rs61742524). The allele frequency of this G in the Japanese population is 1%, and the frequency of the C/G genotype is 3%. It is unlikely that these changes contribute to female infertility.

The SNVs c.357G>A and c.465T>A were each found in one patient (1%). However, these are synonymous substitutions and their positions are not related to splicing function. The allele frequency of each of these SNVs (rs145715915, rs188034174, respectively) in the Asian population is 1%. c.-18G>T was detected in one of our study subjects (1%). This SNV is registered in the dbSNP database as a SNP (rs76781656). The allele frequency of this G in the Japanese population is 1%

and the frequency of the G/T genotype is 3%. IVS2-175C>T was found in three cases. One was heterozygous (C/T), and the other two were homozygous (T/T). The allele frequency (rs569781) is 44% in the global population. Based on these population data, we concluded that it is unlikely that these changes contributed to infertility in our study cohort.

Finally, an IVS1-38C>T variant was found in one patient (1%). There is no information on this substitution in either the dbSNP or exome database for the Japanese population.¹¹ This SNV thus requires further analysis to determine whether it has any effect on the splicing or transcription of *JUNO*.

Table 1. Variations found in the *JUNO* gene among the study participants

variant	substitution	count (%)	dbSNP	SIFT	PolyPhen	population genetics
c.531T>G	p.H177Q	G/T : 9 (9)	rs76779571	0.65	0.668	ALL ^a : 1%(G/T 1%) JPT ^b : 8%(G/T 14%)
c.9C>G	p.C3W	C/G : 1 (1)	rs61742524	0.19	0.001	ALL : 10%(C/G 14%) JPT : 1%(C/G 3%)
c.357G>A	p.P119P	A/G : 1 (1)	rs145715915			ALL : 0% EAS ^c : 0%(A/G 1%)
c.465T>A	p.G155G	A/T : 1 (1)	rs188034174			ALL : 0% EAS : 1%(A/T 2%)
c.-18G>T		T/G : 1 (1)	rs76781645			ALL : 10%(G/T 14%) JPT : 1%(G/T 3%)
IVS1-38C>T		T/C : 1 (1)				
IVS2-175C>T		T/C : 1 (1)	rs569781			ALL : 44%(C/T 40%) (T/T 24%) JPT : 53%(C/T 47%) (T/T 30%)
		T/T : 2 (2)				

^a ALL: All phase 3 individuals

^b JPT: Japanese residents of Tokyo

^c EAS: East Asian

Discussion

The Juno-Izumol interaction in mouse sperm-egg fusion is conserved in humans, suggesting the possibility that genetic

variations in the *JUNO* gene may also have a causal role in female infertility in humans.¹² We identified seven genetic variations in the *JUNO* gene in a cohort of infertile women. However, based on subsequent *in silico* analysis of these changes, there was no real evidence that genetic variations in *JUNO* contribute to human female infertility.

A notable limitation of our present study was its small sample size. For the genetic analysis of disorders of highly heterogeneous etiology, large numbers of cases are required. We previously examined genetic variations in the *CD9* gene, which was also a good candidate gene for infertility because of its role in sperm-egg fusion, but we did not identify any mutations.⁶ Huang and colleagues have identified mutations in the *ZPI* gene using a similar candidate approach.¹³ In their study, they selected a patient with a morphological defect of the zona pellucida. For future mutation screening of the *JUNO* gene, infertile females who conceive using ICSI after recurrent IVF failure should be selected.

In the medical treatment of infertility, several cycles of IVF followed by ICSI is a reasonable approach, regardless of etiology. However, genetic analysis for mutations related to sperm-egg recognition or fusion could help to avoid unnecessary IVF procedures and improve the efficiency of artificial reproductive technology. Furthermore, because girls conceived through ICSI might have the same mutations as the mother, leading to reproductive problems in adulthood, genetic counseling would be important in these cases.

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Conflict of interest

The authors declare no conflicts of interest in relation to this study.

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