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Rs13405728 is associated with slow ovarian response in assisted reproductive technology

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Abstract. – OBJECTIVE: To explore the association between rs13405728 with slow ovarian response in assisted reproductive technology (ART).

PATIENTS AND METHODS: 236 women, aged 21 to 35 years, were enrolled and grouped according to their genotypes, The polymorphism of rs13405728 was genotyped by DNA sequencing.

RESULTS: There was no evidence of any difference in anti-Müllerian hormone (AMH), antral follicle count (AFC) among the three genotypes ($p>0.05$). The occurrence of slow response in genotype GG was lower than those in the other two genotypes ($p<0.05$). There were independent correlations between slow ovarian response with the dose of luteinizing hormone (LH) required and the genotypes of rs13405728 ($p<0.05$).

CONCLUSIONS: There were significant independent correlations between slow ovarian response with the dose of LH required and the genotypes of rs13405728. Different mechanisms may be involved in poor response and slow response.

Key Words:
rs13405728, Polymorphism, Luteinizing hormone receptor, Slow ovarian response.

Introduction

There are many indicators of ovarian response, including age, basal follicle stimulating hormone (FSH), basal FSH/basal luteinizing hormone (LH) ratio, inhibin B (INB), antral follicle count (AFC), anti-Müllerian hormone (AMH), ovarian volume, clomiphene citrate stimulation test, gonadotropin-releasing hormone-a (GnRH-a) stimulation test, etc. Despite the amount of ovarian indicators, the accurate ovarian response is not predictable. For example, in our reproductive center, a subgroup of young women with normal or good ovarian reserve function, undergoing conventional superovulation treatment using GnRH-a long protocol, did not obtain the expected results finally. We called this status (without follicle larger than 10 mm and an estradiol concentration of 180 pg/ml or lower on stimulation day 6) as "slow response". Moreover, we found a significant increase in the ovarian response with increasing doses of LH rather than with increasing doses of FSH. What were the reasons?

LH plays a key role in gonadal function. In women, LH stimulates the production of androgens serving as substrates for follicular oestradiol synthesis in the ovaries. Furthermore, LH cooperates with FSH in promoting follicular development and steroidogenesis, from the mid-follicular phase. Finally, LH is crucial for the induction of oocyte maturation, ovulation, and luteinization of the leading follicle. LH works by binding to LH receptors (LHR). If LH or LHR mutate, it may affect the growth of follicles. Alviggi et al[1] have suggested that the presence of v-βLH was more frequent in GnRH-a down-regulated women with an increased total consumption of FSH during stimulation compared with women with normal consumption. Yang et al[2] have indicated that LHR gene was a potential marker for superovulation response and could be used to predict the most appropriate dose of FSH for superovulation in Chinese Holstein cows. Recently, a genome-wide association study has been performed in a Han Chinese population[3]. Authors identified three different single nucleotide polymorphisms (SNPs) associated with polycystic ovary syndrome (PCOS) (rs13405728, rs13429458, rs2479106).

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The most significant SNP associated with PCOS in the Han Chinese GWAS at chromosome 2p16.3 was rs13405728. GTF2A1L (TFIIA-alpha and beta-like factor) and LHR genes were located nearby and in linkage disequilibrium with it.

The aim of our study was to investigate whether rs13405728 was associated with slow ovarian response in assisted reproductive technology (ART).

Patients and Methods

Patients

A total of 236 women, who underwent *In Vitro* Fertilization (IVF) or intracytoplasmic sperm injection (ICSI) treatment at the Reproductive Medicine Centre of Xuzhou Central Hospital Affiliated to Southeast University May and November 2015, were prospectively enrolled in this study and gave their informed consent to these researchers for using their venous blood.

The Xuzhou Central Hospital Experiment Research Ethics Committee approved this investigation (Certificate No: XZXY-LJ-20150504-007).

Patients reported the characteristics of their menstrual cycles on a self-administered questionnaire, and all of the following criteria were met: 1) ≤35 years of age; 2) undergoing conventional IVF or ICSI treatment using the GnRH-a long protocol; 3) no history of ovarian surgery; and 4) no family history or personal history of chromosomal diseases. The infertility factors for these patients included male factor, tubal or pelvic factor, and a combination of male and female factors. Patients with a diagnosis of PCOS, hyperprolactinemia, endometriosis cysts, any functional or organic ovarian cysts or uterine myoma were excluded from the study. Poor response patients were defined according to the following criteria, meeting one or two criteria: (i) the number of oocytes retrieved ≤4, (ii) E_2 level on HCG day≤500 pg/ml.

Assisted reproduction technology (ART) protocol

The IVF or ICSI treatment regimen using a GnRH-a long protocol cycle was as followed: pituitary downregulation, accomplished by an intramuscular injection of triptorelin acetate (Diphereline; Ipsen Pharma Biotech, Paris, France), was commenced in the mid-luteal phase of the menstrual cycle before the IVF or ICSI treatment. Downregulation was then confirmed by ultrasonography and endocrinology (hormone levels): if the ovaries were inactive, the serum FSH would be <5 IU/L, LH would be <5 IU/L and E_2 levels would be <50 ng/L. Once this result was confirmed, controlled ovarian hyperstimulation (COH) was initiated using recombinant FSH (FSH; Gonal-F; Merck Serono, Geneva, Switzerland or Puregon, Organon, Barcelona, Spain), and/or highly purified human menopausal gonadotropin (HMG; Livzon, Zhuhai, China) and/or highly purified LH (LH; Luveris, Merck Serono, Geneva, Switzerland) in doses that varied from 75 to 300 IU/day from cycle day 3. Depending on age, menstrual cycles, basal hormones, AFC, follicular growth pattern, body mass index (BMI) and response to any previous COHs, the number of injections was determined. Gonadotropin dose was adjusted according to ovarian response monitored by transvaginal ultrasound assessments performed on an ultrasound machine (ALOKA 3500, Tokyo, Japan) and by measuring serum E_2 levels. Human chorionic gonadotropin (HCG, Livzon, Zhuhai, China or Ovidrel, Serono, Geneva, Switzerland) was used in doses of 5000 to 10000 IU to induce oocyte maturation; the dose depended on the mean diameter and the number of leading follicles, together with the serum E_2 level, and the administration of gonadotropins was discontinued on that day. After 36 h, aspiration of the oocytes was performed transvaginally under ultrasound guidance with a single-channel needle.

Embryo transfer was performed 2 or 3 after oocyte retrieval, with a maximum of three embryos for patients undergoing repeated cycles and two embryos for patients undergoing their first treatment cycle. A pregnancy test was performed 14 days after embryo transfer, and a primary transvaginal ultrasound examination was performed 3 weeks later to check for the number of embryonic sacs.

Luteal phase support was provided by an intramuscular injection of progesterone (60 mg/day) (Progesterone Injection, Xian Ju Pharmaceutical Co., Zhejiang, China) from the day of ovum retrieval and continuing at least until the result of the transvaginal ultrasound examination was determined.

Genotyping

The genotypes of the LHR gene were determined using DNA sequencing. All enrolled subjects were screened for LHR gene variations using genomic DNA that was isolated from blood leukocytes with the commercial kit (Omega Bio-tek, D3392-01, Norcross, GA, USA) according to the manufacturer's instructions. Genotyping was performed without knowledge of the subjects' clinical status.

Regions encompassing the polymorphism was amplified by polymerase chain reaction (PCR) and analyzed by electrophoresis in 1.5% agarose gel, which was performed in a 25 μl reaction mixture containing 5 μL buffer solution (10×), 4 ul dNTP mixed liquid (2.5 mM), 1.5 ul $MgCl_2$ (50 mM), 1 μl of each primer (5 mM), l μl of the DNA template, 0.2 μl Taq DNA polymerase, 11.3 μl sterile distilled water.

The PCR primer sequences for rs13405728 forward and reverse were 5′-AGAAAGACAACAAGGCTCTAAGGAA-3′ and 5′-CAGCTCCCATAACAAAGA-3′, respectively. All reactions had an initial denaturation step of 5 min at 94 °C followed by 35 cycles of denaturation at 94 °C for 30 s, annealing at 55 °C for 30 s and 72 °C for 40 s, and a final elongation step at 72 °C for 5 min on a Gene Amp PCR system 9,700 (Life Technologies Corporation, Invitrogen Trading (Shanghai) Co., Ltd).

Hormone Testing

A blood sample was obtained from each patient prior to initiating an IVF or ICSI cycle, which was, ideally, between cycle days one and five. The levels of serum sex hormones (including prolactin, LH, FSH, E_2, and AMH) were measured automatically by chemiluminescence using the Beckman Coulter UniCelDxI 800 (Beckman Coulter, Fullerton, CA, USA), Serum specimens were stored at −80°C if they were not assayed immediately.

Statistical Analysis

The data were analyzed using the statistical software SPSS version 13.0 for Windows (SPSS, Inc., Chicago, IL, USA). Kolmogorov-Smirnov test was used to test the distribution of the variables. Mean and standard deviation were used for the description of variables, which followed a normal distribution. Median and interquartile were used for the description of variables, which showed skew distribution. Categorical variables were described as a percentage. Tests were used to establish whether the allelic and genotypic frequencies of each SNP respected Hardy-Weinberg equilibrium. Statistical comparisons were performed by one-way ANOVA and Kruskal-Wallis tests, where appropriate. In the Binary Logistic analysis, the following possible confounding factors were considered: age, basal FSH, basal LH, total dose of gonadotropin and LH, FSH and LH levels on HCG day, all of which were introduced into the analysis by the Enter method. Differences with a *p*-value (two-tailed) of <0.05 were considered significant.

Results

Frequency distribution of this polymorphism

From 236 subjects, the frequency distribution of this polymorphism was 52.54% for genotype AA, 40.68% for AG, and 6.78% for GG. The genotypes frequency was in Hardy-Weinberg equilibrium.

Ovarian reserve and ovarian response of different genotypes subjects

There was no evidence of any difference in AFC or AMH among the three genotypes. The occurrence of slow response in group GG was lower than those in the other two genotypes (AA *vs.* AG *vs.* GG, 41.94% *vs.* 32.29% *vs.* 12.50%, $p=0.043$). There were no significant differences in the amount of FSH required, the number of follicles ≥14 mm on HCG day, oocytes, mature oocytes, available embryos and the clinical pregnancy rate (CPR) among the three genotypes (Table I). There were significant independent correlations between slow ovarian response with the genotypes of rs13405728, the initial dose of gonadotropins and the dose of LH required (Table II).

Discussion

Rs13405728 is located in an intron of LHR gene. Reports on this polymorphism are limited. Accordingly, there is no available data on the relationship between this polymorphism and ovarian response. However, previous studies indicated that inside of the gene LHR, rs13405728 was associated with endometrioid adenocarcinoma, PCOS, and metabolic abnormalities[3-5]. In our study, we found that there were independent correlations between slow ovarian response with the genotypes of rs13405728, the initial dose of gonadotropins and the dose of LH required. This finding suggested that rs13405728 in gene LHR might influence slow ovarian response susceptibility, intron being noncoding region. However, intronic polymorphisms were frequently implicated in increased disease susceptibility and intronic mutations have the potential to alter protein sequence dramatically[6-10]. An intronic mutation can cause the disarrangement of an existing splice site or introduce a cryptic splice site, resulting in the addition/removal of hundreds of amino acids or premature termination of translation[10-11]. However, rs13405728 being located in the middle of

Table I. Clinical and endocrinological parameters of subjects based on the genotypes of rs13405728.

	AA (n=124)	AG (n=96)	GG (n=16)	*p*
Age (year)	29.55±2.97	29.68±3.42	28.92±2.78	0.522
BMI (kg/m^2)	20.50±2.30	20.24±3.74	20.03±3.01	0.402
AMH (ng/ml)	3.28±1.91	3.76±2.68	2.75±1.29	0.655
AFC	15.04±4.98	17.08±5.97	14.58±2.68	0.228
Basal FSH (IU/L)	7.45±1.71	7.45±1.93	8.31±1.33	0.411
Basal LH (IU/L)	4.19±1.69	4.89±2.68	3.79±1.23	0.281
Gn (IU)	1961.45±616.98	1900.13±717.07	1828.08±637.07	0.263
LH (IU)	392.72±335.87	395.47±321.87	468.75±725.83	0.854
≥14 mm follicles on HCG day	11.39±1.95	10.23±4.21	9.00±2.80	0.766
E_2 on HCG day (pg/mL)	3290.80±1307.41	3166.21±1370.86	3018.97±1460.02	0.526
HCG (IU)	7331.08±2218.04	7292.45±2078.82	8583.33±2108.78	0.560
Oocytes	14.04±5.36	13.79±4.76	12.08±3.99	0.818
Mature oocytes	12.30±4.94	12.13±4.53	10.75±4.37	0.608
Available embryos	7.21±4.16	8.30±4.56	7.33±3.75	0.739
Poor response (%)	4.03 (5/124)	3.13 (3/96)	0 (0/16)	0.692
Slow response (%)	41.94 (52/124)[a]	32.29 (31/96)	12.5 (2/16)[a]	0.043
CPR (%)	54.87 (62/113)	67.06 (57/85)	57.14 (8/14)	0.219

[a], A significant difference were detected between AA and GG by LSD, $p<0.05$.

an intron region, this mechanism may be unlikely, one of the reasons for the occurrence of slow ovarian response. A second mechanism by which intronic mutations might affect disease etiology may be through their effect on the miRNAs. This may be derived from the mutated intron. ~10-30% of the spliced intronic material that is exported to the cytoplasm, where there is the potential to act as a miRNA and alter protein expression[10-12]. However, the mechanisms of this finding need further studies.

Table II. The Binary Logistic regression for the relationship between genotype of rs13405728 and slow ovarian response.

	B	S.E.	*p*
Age	-0.027	0.065	0.682
BMI	0.020	0.071	0.776
AFC	0.031	0.040	0.434
BFSH	0.149	0.113	0.187
BLH	-0.044	0.093	0.634
Initial gonadotropins	-0.020	0.009	0.022
Gn	0.001	0.001	0.236
LH	0.003	0.001	0.002
FSH on HCG day	0.034	0.037	0.355
LH on HCG day	-0.038	0.185	0.839
GnRH-a	-0.501	0.609	0.411
Genotype of rs13405728	-0.736	0.357	0.039
Constant	-0.025	2.695	0.993

Note: The dependent variable is slow ovarian response or not.

Notably, a minimum proportion of subjects with slow ovarian response in GG group, but AMH, AFC and the number of retrieved oocytes were the lowest among the three genotypes. Chen et al[3] indicated that the frequency of individuals homozygous for G allele was significantly higher in the group of PCOS compared to controls. This finding suggested that different mechanisms may be involved in poor response and slow response. Our results need to be confirmed in an independent study, performed in a replicative population.

Study limitations

Selection biases may exist, since only those who are undergoing conventional IVF or ICSI treatment using the GnRH-a long protocol were selected for inclusion in the study.

Conclusions

We observed that there were significant independent correlations between slow ovarian response with the dose of LH required and the genotypes of rs13405728. Different mechanisms may be involved in poor response and slow response.

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Conflict of Interest

The Authors declare that they have no conflict of interests.

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