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Relationship Between Genotype Variants Follicle-stimulating Hormone Receptor Gene Polymorphisms (FSHR) and Morphology of Oocytes Prior to ICSI Procedures

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ABSTRACT

Introduction: This study investigated association of Asn680Ser FSHR polymorphism with the ovarian response in 104 women of Albanian ethnic population enrolled in ICSI program. The reason of infertility in all cases has been identified as male factor. **Methods**: Analysis of the Asn680Ser polymorphism was performed using TaqMan® SNP Genotyping Assay. Clinical and endocrinologic parameters were analyzed based on the genotype, age, BMI, oocyte yield, number of transferred embryos and pregnancy rate. **Results.** The frequencies of the Asn680Ser genotype variants were as follows: Asn/Asn 22.1%, Asn/Ser 47.1%, and Ser/Ser 30.8%, respectively. BMI was significantly higher in the Ser/Ser group as compared to those from the Asn/Ser or the Asn/Asn group (p= 0.0010). The genotype variants Ser/Ser indicates a higher rate of oocyte retrieval (25.9%) in the immature form, metaphase I (MI) as opposed to the other two groups (Asn/Asn 23.7 % vs. Asn/Ser 21.9%), which was statistically significant (p = 0.3020). **Conclusions:** FSH receptor polymorphism is associated with different ovarian response to controlled ovarian stimulation (COS), but is not an important factor in increasing the degree of pregnancy. Polymorphisms of the FSH receptor is associated with normal morphology and genetic maturation (metaphase II) oocytes in dependence of genotypic variation polymorphisms.

Key words: FSH, FSHR, Asn680Ser, ICSI, SNP, AFC.

1. INTRODUCTION

Despite attempts to standardize controlled ovarian stimulation (COH) regimens for women undergoing assisted reproduction programs (ART), they commonly experience either poor ovarian responses or ovarian hyperstimulation syndrome (OHSS) (1). The patient response is individualized and the ovarian response to intense gonadotropin stimulation is difficult to predict even in those with similar endocrine profiles. Therefore, determining the dose of exogenous gonadotropin to attain optimum patient response thus avoiding a serious and potentially life-threatening complication of OHSS or insufficient stimulation and cycle cancellation in poor responders is one of the ongoing challenges in the field of infertility management (1). The hormone FSH (a key marker of ovarian reserve and the best-known predictor of COH response) plays a pivotal role in ovarian function, where its main effects are related to granulosa cell proliferation, oocyte maturation and estrogen synthesis via activation of the aromatase gene (1). Since the secretion of FSH is in

a negative feedback loop with the action of FSHR, the basal day 3 serum FSH levels (bFSH, one of the best predictive markers of ovarian reserve) are often indicative of the function of its receptor and could vary depending on the patient FSHR genotype background. This has led to the investigation of their potential value as predictors of ovarian response to an exogenous stimulation. Two very common SNPs present at coding position p.Thr307Ala (rs6165) and p.Asn680Ser (rs6166) in the exon 10 are currently most extensively studied to assess the response of the FSHR receptor to FSH stimulation (1, 2, 3, 4). Several reports have shown that these two SNPs are associated with ovarian response in IVF but the findings are conflicting. Some authors have shown predictability of ovarian response to FSH stimulation in patients with different alleles, while others have refuted this finding (1, 4, 5, 6). In order to verify this, in the present study we examined, for the first time, the prevalence of the Asn680Ser genotype variants in Albanian women population from Kosovo Dukagjin region who participated in an IVF/ICSI program. Furthermore, we investigated the relationship between genotype variants FSHR gene polymorphism and morphology of oocytes prior to ICSI procedures.

2. MATERIALS AND METHODS

Subjects

The present study encompass 104 prospectively recruited female patients of Albanian ethnic population from Kosovo Dukagjin region enrolled in ICSI procedures at Polyclinic - IVF Centre, Peje, Republic of Kosovo in the period from January 2014 to February 2015. The reason of infertility in all cases has been identified as male factor. Informed consent was obtained from all the participants and the study was approved by the local Medical Ethics Review Committee at Faculty of Medicine Prishtina, Republic of Kosovo.

Hormonal assays

Serum levels (day 3 of the menstrual cycle) of FSH, LH, estradiol (E2), prolactin, progesterone, were measured by enzyme linked fluorescent assay (ELFA) using bioMerieux Mini Vidas Automated Immunoassay Analyzer (bioMérieux S.A. 69280 Marcy l'Etoile, France).

Treatment

In all cases, controlled ovarian stimulation was performed according to standard long protocol procedure as previously described (7).

DNA Isolation and genotyping

Analysis of the FSHR gene polymorphism at position 680 was carried out using predesigned TaqMan® SNP Genotyping Assay (rs6166; Life Technologies Corporation, Carlsbad, California, USA). Real-time PCR was performed using the TaqMan Universal master mix II and Applied Biosystems 7500 Real-Time PCR System (Life Technologies Corporation, Carlsbad, California, USA) in accordance with the manufacturer's instructions. The analysis was carried out in accordance with the instructions for the device used.

Statistical analysis

SNP	Allele frequency% (n)		Genotyps frequency% (n)		Clinical pregnancy% (n)
Ser680Asn	A (N)	45.67 (95)	AA (NN)	22.12 (23)	33.3(10)
	G (S)	54.33 (113)	AG (NS)	47.12 (49)	43.3(13)
			GG (SS)	30.77 (32)	23.3(7)

Table 1. SNP Genotyping Asn680Ser FSH gene 104 patients in the study. Asparagin-A(N), Serin-G(S), Asparagin/Asparagin AA(NN), Asparagin/Serin-AG(NS), Serin/Serin-GG(SS). BMI Body mass index , bFSH= basale Follicle-stimulating hormone, , AFC,=antral follicle count, hCG= Human Chorionic Gonadotropin, bE2=basale Estradiol

Statistical analysis was performed by applying a commercially available software package (GraphPad Prism, GraphPad Software, San Diego, CA). All statistical tests were two-sided and a conventional value of $P < 0.05$ was used to represent statistical significance.

3. RESULTS

A total of 104 patients underwent ICSI procedure and were genotyped in this study. The frequencies of the Asn680Ser genotype variants were as follows: Asn/Asn 22.1%, Asn/Ser 47.1%, and Ser/Ser 30.8%, respectively (Table 1).

Values are presented as mean ± SD if the distributed parameter, or as median and range if they are distributed non-parametric. One-way ANOVA (Tukey multiple comparative test) was used to analyze the differences in terms of age, AFC, the amount of rFSH, length of stimulation, number of leading follicles (d≥14mm) the number of oocytes retrieved and the number of transferred

Clinical and endocrinologic parameters	NN (n=23)	NS (n=49)	SS (n=32)	p
Age (years)	33.65 ± 6.278	34.06 ± 5.528	32.38 ±5.707	0.4308
BMI (kg/m^2)	22.00 (20.60-24.70)*	22.30(20.20-25.10)**	26.10 (22.88-27.70)*,**	0.0010
bFSH (mIU/ml)	10.01 (7.60-12.16)	10.20 (8.58-11.92)	10.28 (8.32-12.18)	0,8530
b Estradiol (pmol/l)	46.88 (36.75-55.09)	42.90 (34.5-61.20)	33.18 (26.25-45.53)	0.0308
AFC	7.043 ± 1.965	7.122 ± 1.822	6.625 ± 2.379	0.5474
FSH amount needed for ovulation induction (IU)	2482 ± 467.8	2441 ± 532.7	2466 ± 481.5	0.9444
Estradiol on hCG day of administration (pg / l)	1746 ± 811.0	1782 ± 681.0	1467 ± 627.3	0.1234
Number of oocytes retrieved	5.174 ±1.946	5.633 ± 1.845	4.906 ± 1.653	0.1997
Number of transferred embryos	2 (1-3)	3 (2-3)**	2 (1-2)**	0.0101
Pregnancy rate	43.48 (10/23)	26.53 (13/49)	21.875 (7/32)	0.19398

Table 2. Clinical and endocrine characteristics of patients with respect to the SNP Asn680Ser FSHR gene

Genotyp variants (FSHR)	Asn/Asn (n=23)	Asn/Ser (n=49)	Ser/Se (n=32)	p
Total oocyte	147	294	164	0.0001
Number of dominant follicles (d≥17mm) on the day of hCG administration	4.565 ± 1.674	4.551 ± 1.542	3.813 ±1.693	**NS
Number of oocytes retrieved (n)	5.174 ±1.946	5.633 ± 1.845	4.906 ± 1.653	**NS
Mmetaphase stage I (n)	23.7%	21.9%	25.9%	***0.3020
In vitro culture (n) (MI to MII, after 2-6 h)	8.4%	11.6%	5.2%	***0.0031
Metaphase stage II (n)	76.3% + 8.4% ——— 84.7%	78.1% + 11.6% ——— 89.7%	74.1% + 5.2% ——— 79.3%	***0.0258
Oocytes fertilized (%)	79.8%	81.8%	78.6%	NS
Pregnancy rate ,% (n)	43.48 (10/23)	26.53 (13/49)	21.875 (7/32)	**NS

Table 3. The frequency of fertilization and embryo development after ICSI in vitro matured MI and MII in three different genotype variants FSHR polymorphisms. NS= not significant (p >0.05),MI= metaphase-I, MII=metaphase-II. NS = Not significant,* Values are averages (±SD), **Chi-square test (χ2) test,***Kruskal-Wallis test

Genotyp variants (FSHR)	Asn/Asn %,(n)	Asn/Ser %.(n)	Ser/Se %,(n)	p
Normal Morphology of oocyte	67.6%(99)	70.8% (208)	65.2% (107)	0.0480*
Oocytes with abnormalities	32.4% (48)	29.2% (86)	34.8% (57)	0.0417*
Vacuolated cytoplasm	10.06%	11.20%	9.82%	NS
Dark and granular cytoplasm	8.20%	9.07%	7.70%	NS
Debris in PVS	7.24%	7.15%	7.30%	NS
Large PVS	7.20%	8.40%	7.82%	NS
Fragmented 1st PB	10.12%	12.25%	10.31%	NS
Big polar body	0.46%	0.80%	0.65%	NS
Irregular ZP	8.35%	8.21%	8.63%	
				NS
Thin zona	3.10%	3.26%	3.01%	NS
Thick zona	5.25%	4.95`%	5.62%	NS
Irregular shape	5.76%	4.35%	3.92%	NS
Giant oocyte	1.10%	1.50%	1.30%	NS
Small oocyte	4.66%	2.85%	2.62%	NS
>1 abnormalities	28.50%	17.80%	34.20%	0.0230
Total oocyte	147	294	164	

Table 4. The frequency of oocyte abnormalities observed in three different genotype variants FSHR polymorphisms. p < 0.05 significant, p >0.05 not significant,*Kruskal-Wallis test. PB- polar body, PVS–perivitilline space.ZP-zona pelucida, NS- not significant

embryos. Chi-square analysis was used to analyze pregnancy. Kruskal-Wallis and Dunn's multiple comparative test was used to analyze non-parametric variables the difference. The value of p<0.05 was considered statistically significant. Although the overall analysis of bE2 levels between the three genotype variants showed slight but statistically significant difference (p= 0.0308). No difference was also found between the genotype groups either in terms of AFC, amount of the FSH required for ovulation induction, stimulation length days, number of dominant follicles, oocyte retrieval number or endometrial thickness (Table 2). BMI was significantly higher in the Ser/Ser group as compared to those from the Asn/Ser or the Asn/Asn group (p= 0.0010) (Table 2).

The genotype variants Ser/ Ser indicates a higher rate of oocyte retrieval (25.9%) in the immature form, metaphase I (MI) as opposed to the other two groups (Asn/Asn 23.7% vs. Asn/Ser 21.9%), which was statistically significant (p = 0.3020). Ser/Ser groups of subjects exhibited a lower rate of oocytes which after in vitro incubation of 2-6 hours exceed the metaphase II stage (5.6%) (p =0.0031), compared to the Asn/Asn 8.4% and Asn/Ser 11.6%. Also, the trend of the difference found when comparing rates of MII oocytes between groups, Ser / Ser (78.6%) indicates a lower rate than other groups (Asn/Asn 84.7% vs. Asn/Ser 89.7%) (p = 0.0258) There was no difference between groups with respect to number of dominant follicles (d≥17mm) on the day of hCG administration and pregnancy rate (p>0.05).

Table 4 shows the morphological characteristics of the total 605 oocytes obtained by aspiration of ovarian follicles of 104 patients, of these, 414 oocytes were normal morphology and 191 abnormal morphology (p=0.0480). Asn/Asn, 71.5% had one anomaly and 28.5% more than one abnormality. Asn /Ser, 82.2%, had one abnormality and 17.80% with more than one abnormality, Ser/Ser 65.8% had one abnormality and 34.20%, with more than one abnormality (p=0.0417).

4. DISCUSSION

Pharmacogenetic studies have revealed a series of genetic markers involved in COH response, markers of ovarian reserve and reproductive lifespan (1). Among them, FSHR gene-associated SNPs, including the Asn680Ser missense variant, are the most promising genetic markers available to date. In the present study we investigated the association of Asn680Ser FSHR polymorphisms with the clinical and endocrinologic parameters of Albanian women from Kosovo Dukagjin region. Study revealed the dominant frequency distribution of the Ser/Ser genotype consistent with its highest rates found in other ethnic groups in Balkan region (2, 3, 4, 5, 6). The frequencies of the Asn680Ser genotype variants were as follows: Asn/Asn 22.1%, Asn/Ser 47.1%, and Ser/Ser 30.8% (Table 1). By all accounts, closure of the Albanian population, which is reflected low frequency of getting married with members of other ethnic groups, it could be reasonably determined by differences in the distribution of those SNP polymorphisms FSHR, compared to other ethnic groups. It seems that the social and cultural particularities preserve genetic parent "pool", which descended from the native indigenous population. Consistent with reported data present study also showed lower values for bFSH, and significantly higher AFC, peak

E2 levels, and oocyte retrieval number in the youngest age group. Most authors reported that bFSH levels vary significantly between genotype variants Asn680Ser with holders of Ser/Ser genotype has seen a slight increase bFSH and require a significantly higher dose of gonadotropins for ovulation induction (1, 2, 5, 8, 9, 10). However, the lowest values for AFC, the peak E2 levels, number of dominant follicle and oocyte retrieval number in our studied population were observed in Ser/Ser genotype group. Regarding the ovarian response, some studies report that Ser/Ser subjects in comparison to Asn/Asn or heterogeneous genotype variants have higher AFC, peak E2 levels and oocyte retrieval number and thus greater risk for OHSS (9, 11, 12, 13). Others report smaller oocyte retrieval number in Ser/Ser vs. Asn/Asn and/or Asn/Ser subjects thus suggesting quite contrary that the Ser/Ser genotype variant may be associated with a reduced ovarian response to COH (2, 10). In the present study Asn680Ser polymorphisms was significantly associated with bE2 levels in overall studied population, with the highest median level found among Asn/Asn and the lowest level in Ser/Ser group (Table 2). BMI was also significantly associated with genotype variant subgroups in the present study with the Ser/Ser group showing the highest and Asn/Asn group the lowest BMI index. Therefore, to the best of our knowledge this is the first study showing the statistically significant association of BMI and Asn680Ser FSHR polymorphism (Table 2). It is already known that increased BMI may alter hormone metabolism and clearance in several complex ways (14). Furthermore, the impact of oocyte morphology on fertility when using IVF/ICSI is a matter of debate (15). Many study data show that the quality of the egg should be considered as one of the main parameters in the environment ART (16). This paper explores the relationship between genotype polymorphism variants FSHR genes and morphology of oocytes prior to ICSI procedures. It is important to emphasize that from our research in the literature our study is the second after Mohiyiddeen,L,et. al (19). Our research results in this topic shows the opposite of Mohiyiddeen, L et which concluded the following: FSH receptor genotype does not provide metaphase II oocytes output or fertilization rates and ICSI patients. Our results show a correlation of oocytes with normal morphology and polymorphism of genetic variants of the FSH receptor. Variant genotype Ser/Ser has a poor morphology of the oocytes, our hypothesis is justified due to the age of the patient little higher in this group and a higher level of basal FSH, and most study report to the Ser/Ser genotype group has a higher basal FSH and need more doses of gonadotropins in IVF procedures/ICSI. In this study, we have shown that there is a significant difference between the patients seen at the morphological level of maturity of oocytes disease. Table 4 shows the highest percentage of mature oocytes (metaphase II) into an Asn/Asn and Asn/Ser genotype group of patients (84.7% and 89.7%) in respect of the variant polymorphism genotype Ser/Ser (79.3%) (p=0.0108). Only oocytes in metaphase II can ICSI fertilization techniques. Percentage not mature oocytes (metaphase I) between the three variants polymorphism genotype, shows significant differences. Ser/Ser group (25.9%), compared with Asn/Asn and Asn/Ser group (23.7% and 21.9%) (p = 0.0102). Immature eggs can undergo nuclear maturation and successful fertilization, is not yet completed in the cytoplasm maturation, as indicated by the ability to support the development of the embryo and successful implantation (17) It is known that a woman's age correlates well with poor quality eggs. Our data showed that the MI oocytes was higher in the group of Ser/Ser who are somewhat older. From our results we can get a very different success rates of transition from not mature oocytes (MI) in mature oocytes (MII), after 2-6 hours of in vitro maturation (Ser/Ser 5.2% compared Asn/Asn 8.4% and Asn/Ser 11.6%, p = <0 , 0001). The total population is dominated normal oocyte morphology in all three genotype polymorphism FSH variant gene. The order of the percentage of oocytes with normal morphology after the groups; Asn/Ser = 70.8%, Asn/Asn = 67.6%, and Ser/Ser = 65.2%, the results show statistical significance among groups (p = 0.0480). Ser/Ser subjects have a higher percentage of oocytes exposed to two or more abnormality (34.20%), in contrast to the Asn/Asn and Asn/Ser (28.50% and 17.80%) (p = 0.0230). The origin of the morphological abnormalities of oocytes is largely unknown, but is probably more factors. Basic factors, such as age and genetic defects or external factors, such as ovarian stimulation protocol procedures stimulation or handling immediately after aspiration have been proposed (18).

5. CONCLUSION

This work is the first study on the frequency of genotypes for the SNP Asn 680/Ser 680 in the population of Albanian women. It should be noted that there is a great heterogeneity in the results that concern the efficacy of Asn680Ser FSHR genetic marker for the prediction of the ovarian response and pregnancy outcome. FSH receptor polymorphism is associated with different ovarian response to controlled ovarian stimulation (COS), but is not an important factor in increasing the degree of pregnancy. Polymorphisms of the FSH receptor is associated with normal morphology and genetic maturation (metaphase II) oocytes in dependence of genotypic variation polymorphisms. Therefore, these findings should be confirmed in larger studies.

- Conflict of interest: none declared.

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