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Original Article

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The Effect of FSHR (G2039A) Polymorphism on Müllerian Duct Development and Hormonal Profile of Women with Primary Amenorrhea

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Abstract

Background: The function of follicle-stimulating hormone (FSH) is mediated by binding to its G-protein coupled receptor (GPCR) which is expressed on granulosa cells of the ovary. The purpose of the current study was to examine the impact of FSHR G2039A polymorphism (rs6166; $Ser^{680}Asn$) on clinical and radiology profiles of women with primary amenorrhea (PA) in Gujarat, India.

Methods: A total of 90 women (45 controls and 45 cases) were recruited for the study after obtaining informed consent. The DNA extraction was performed on the venous blood samples collected from the participants, followed by polymerase chain reaction (PCR). The presence of polymorphism was then analyzed using restriction fragment polymorphism (RFLP) with the BSeNI enzyme. The statistical analysis was conducted using an independent t-test, chi-square test, and ANOVA. Significance was determined by a $p<0.05$.

Results: Results revealed that homozygous wild type genotype was observed in 46.7% (n=21) of the control group and 11.1% (n=5) of the case group. Heterozygous genotype was observed in 33.3% (n=15) of the control group and 55.6% (n=25) of the case group ($p<0.001$). Homozygous mutant genotype was observed in 20% (n=9) of the control group and 33.3% (n=15) of the case group ($p<0.01$). The hormonal profile revealed that serum levels of FSH and luteinizing hormone (LH) were significantly higher ($p<0.05$) in the AA and AG genotypes compared to the GG genotypes.

Conclusion: The FSHR rs6166 G2039A was associated with PA in women, and the A allele could be considered a causative risk factor in developing the condition.

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Introduction

The maturation of oocytes in the ovaries is facilitated by follicle-stimulating hormone (FSH) through its binding to the FSH receptor (FSHR) (1). FSHR belongs to the G-protein-coupled receptor superfamily and requires the activation of the cAMP second messenger to assist in the signal transduction pathway (2). Human FSHR gene is mapped on chromosome 2p21 and spans a region of 54 *kbp* in size consisting of 10 exons and 9 introns (1). The initial mutation identified in Finnish women with hypergonadotropic ovarian failure involves a substitution of Alanine (Ala) to Valine (Val) at residue 189 in exon 7 of the FSHR gene (C566T) (3). Various inactivating mutations in the FSHR gene have been identified previously in women with primary or secondary amenorrhea or premature ovarian failure (POF) (2, 4–8). *In vitro* studies have established a clear link between mutations in FSHR and poor ovarian response. The receptor is target-

ed differently on the cell surface, which impedes the maturation of follicles after the primary stage (7, 9). Mutations in the FSHR lead to a loss of its ability to bind specifically to FSH. This loss of specific binding affinity occurs as a result of mutations within exon 10 of the FSHR gene. Exon 10 is responsible for encoding the C-terminal portion of the extracellular domain, as well as the transmembrane and intracellular domains, encompassing a sequence of 1251 base pairs (1). The most widely studied FSHR polymorphisms in exon 10 are A919G and G2039A (rs6166; Ser^{680} Asn). Numerous studies have sought to determine the prevalence of the G2039A polymorphism in women affected by premature ovarian failure (POF), encompassing primary and secondary amenorrhea alongside polycystic ovarian syndrome. Nevertheless, none of these studies has manifested a significant correlation with any of these conditions (10–17). In India, two studies were conducted that focused solely on women experiencing primary amenorrhea (PA) to investigate the effects of this polymorphism (18, 19). Previous research has strongly linked the distribution of polymorphism haplotypes and PA in women (18). This fact has motivated us to broaden our investigation and explore the impact of the polymorphism on the development of PA in women from other regions of western India that have not previously been studied. Moreover, the purpose of the current study was to uncover the association between the FSHR G2039A polymorphism and hormone levels and the development of Müllerian structures in women with primary amenorrhea.

Methods

The present study was conducted at the Department of Zoology and Biomedical Technology of Gujarat University, Ahmedabad, India where 90 women were recruited, of which 45 women had a chief complaint of primary amenorrhea (PA) and were categorized as "case group" and the rest 45 were "control group" women who had regular menstrual cycle with no other complaints regarding menstruation. The study was approved by Institutional Ethics Committee of Gujarat University (No.: GU/IEC/02/2018). Women between the ages of 14 and 35 were eligible to participate in the study. Informed consent was obtained from each individual, and in the case of minors, permission was obtained from one of the family members.

For each individual, a comprehensive proforma was completed, which included informed consent and essential information. The proforma involved a detailed pedigree spanning at least three generations, as well as a clinical profile encompassing the proband's relevant clinical data such as serum levels of FSH, LH, TSH, PRL, progesterone, estradiol, and testosterone A detailed ultrasonography (USG) profile was obtained from the clinician during recruitment.

DNA extraction, PCR, and RFLP analysis: Genomic DNA extraction was carried out by John et al.'s method (20), and the extracted DNA was subjected to polymerase chain reaction (PCR) according to the method described by Sujatha et al. (21) with slight modifications. The PCR reaction was carried out in a final volume of 25 *µl* containing 2X PCR master mix (EmeraldAmp®GT PCR Master Mix, Takara Bio Inc., Japan), 20 *pM* of each primer (forward primer: 5'-TTTGTGGTCA TCTGTGGCTGC-3' and reverse primer: 5'-CAA AGGCAAGGACTGAATTATCATT-3'sourced from Sigma–Aldrich Chemical Pvt Limited, India), 100 *ng* of the DNA template, and nuclease-free water. The PCR conditions were set as follows: initial denaturation at 94°*C* for 5 *min* followed by 35 cycles of denaturation at 94°*C* for 30 *s*, annealing at 56°*C* for 30 *s* and extension at 72°*C* for 1 *min*, and final extension at 72°*C* for 10 *min*. For restriction fragment length polymorphism (RFLP) analysis, 520 *bp* amplicon was digested with BseNI enzyme (ThermoFisher Scientific, USA). Digestion was performed in 30 *µl* of reaction volume containing 1X reaction buffer, 1 unit of restriction enzyme, and 10 *µl* of PCR product. The mixture was incubated at 65°*C* for 2 *hr*. After digestion, the product was run on 2.5% agarose gel stained with ethidium bromide (EtBr) at 110 volts for 40 *min*. Bands were visualized under a UV transilluminator. The gel electrophoresis bands for all individuals were photographed (Figure 1). The band at 413/107 shows homozygous GG genotype, the band at 520/413/107 shows heterozygous GA genotype, and the band at 520 *bp* shows homozygous mutant AA genotype against 100 *bp* ladder.

Statistical analysis: The statistical analysis was done by SPSS *vs*. 23 software (IBM Corp., USA). An independent t-test was performed to calculate the mean and standard deviation (SD), and p-value <0.05 was considered statistically significant. Chi-square (χ^2), odds ratio (OR), and 95% confidence interval (95%CI) were calculated to check the association between the groups. One-

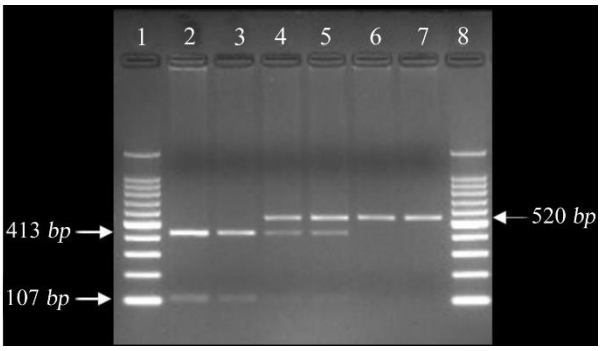

Figure 1. Gel image showing FSHR G2039A polymorphism Lane 1: 100 *bp* ladder, lanes 2 and 3: GG genotype (wild type) at 413/107 *bp*, lanes 4 and 5: GA genotype (heterozygous) at 520/413/107 *bp*, lanes 6 and 7: AA genotype (mutant) at 520 *bp*, and lane 8: 100 *bp* ladder

way analysis of variance (ANOVA) and Fishers Least Significant Difference (LSD) were employed to analyze the differences in serum levels of hormones and Müllerian duct development within the groups.

Results

Mutation screening analysis was performed to assess the presence of a single nucleotide polymorphism (SNP) at position 2039 within exon 10 of the FSHR gene, aiming to investigate its potential association with PA. The genotype frequencies were in agreement with the Hardy-Weinberg equilibrium. The study revealed that homozygous normal GG genotype was found in 46.7% (n=21) of control group compared to 11.1% (n=5) of case group. The heterozygous GA genotype was significantly higher in the case group, 55.6% (n=25), compared to the control group, 33.3% (n=15) ($p< 0.001$; χ^2 value=11.899; odd ratio=7.00, 95%CI= 2.180-22.478). Similarly, the homozygous mutant AA genotype was significantly higher in the case group, 33.3% (n=15), compared to the control group, 20% (n=9) ($p<0.01$; χ^2 value=9.736; odd ratio=7.00, 95% CI=1.950-25.135). The G allele distribution was higher in the control group (63.33%) than in the case group (38.89%), whereas the A allele distribution was higher in the case group (61.11%) than in the control group (36.67%) ($p<0.001$; χ^2 value=10.761; odd ratio=2.714, 95% CI=1.485-4.960) (Table 1).

Hormonal profile: All the individuals were screened for FSH, LH, TSH, prolactin, estrogen, progesterone, and testosterone serum levels. The values were represented as mean±SD in case group (Table 2A and 2B). The individuals with GA genotype exhibited non-significantly elevated levels of FSH and LH compared to those with the GG genotype (Table 2A). The mean FSH level of individuals with the AA genotype was found to be significantly higher than the normal range ($p< 0.05$), in comparison to those with the GG genotype (Table 2B). Serum levels of LH in cases with AA genotype were significantly higher ($p<0.05$) than in cases with GG genotype (Table 2B). Serum levels of TSH, estrogen, progesterone, and testosterone were within normal limits in all three genotypes whereas prolactin levels were above the normal range.

Radiology profile: Women were screened for Müllerian duct (MD) structures. As expected, all the control group showed fully developed Müllerian duct structures, whereas abnormalities were observed in the case group. ANOVA revealed a non-significant association of genotypes with the Müllerian duct anomalies. Among women with PA, 44.4% had a hypoplastic uterus and a total absence of the uterus was observed in 40% of the cases. Similarly, a higher percentage (20%) of PA women exhibited streak gonads and 22.2% lacked both ovaries. Also, a higher frequency of women with abnormal Müllerian duct development was observed in comparison to those with normal development (Table 3).

Table 1. Frequency of FSHR polymorphism in control and PA women

FSHR polymorphism	Control n=45 (%)	Cases n=45 (%)	Chi. Sq value	p-value	OR (95%CI)
GG (n=26)	21 (46.7%)	5 (11.1%)	-	-	-
GA (n=40)	15 (33.3%)	25 (55.6%)	11.899	0.00056	7.000 (2.180-22.478)
AA (n=24)	9 (20%)	15 (33.3%)	9.736	0.0018	7.000 (1.950-25.135)
G allele	57 (63.33%)	35 (38.89%)	-	-	-
A allele	33 (36.67%)	55 (61.11%)	10.761	0.0010	2.714 (1.485-4.960)

OR= odds ratio; 95%CI= 95% confidence interval

Table 2A. Association of hormonal profile with different genotypes in PA women (n=45)

Hormones studied	GG polymorphism (n=5)	GA polymorphism (n=25)
FSH (2.5-10.2 *mIU/ml*)	2.7±0.2	35.7±43.4 NS
LH (2.39-6.6 *mIU/ml*)	2.5±0.2	18.6±17.4 NS
TSH (0.4-4.2 *µIU/ml*)	3.4±1.7	2.5±1.8 NS
Prolactin (4-30 *ng/ml*)	99.4±176.3	92.1±397.3 NS
Estrogen (21-251 *pg/ml*)	14.3±3.1	57.1±65.3 NS
Progesterone (0.1-25 *ng/ml*)	0.1±0.04	1.8±2.9 NS
Testosterone (14-76 *ng/dl*)	13.8±5.1	16.1±9.5 NS

NS= not significant. An independent t-test was conducted for mean±SD calculation in GG vs GA groups. FSH= Follicle-stimulating hormone; LH= Luteinizing hormone; TSH= Thyroid-stimulating hormone. The values in parentheses represent the normal range of hormones

Table 2B. Association of hormonal profile with different genotypes in PA women (n=45)

Hormones studied	GG polymorphism (n=5)	AA polymorphism (n=15)
FSH (2.5-10.2 *mIU/ml*)	2.7±0.2	55.3±53.6 *
LH (2.39-6.6 *mIU/ml*)	2.5±0.2	24±20.3 *
TSH (0.4-4.2 *µIU/ml*)	3.4±1.7	2.2±1.1 NS
Prolactin (4-30 *ng/ml*)	99.4±176.3	41.0±106.1 NS
Estrogen (21-251 *pg/ml*)	14.3±3.1	53.3±43.3 NS
Progesterone (0.1-25 *ng/ml*)	0.1±0.04	1.6±2.5 NS
Testosterone (14-76 *ng/dl*)	13.8±5.1	19.8±13.5 NS

* $p<0.05$; values significantly different among GG and AA groups
NS= not significant. An independent t-test was conducted for mean±SD calculation in GG vs AA groups. FSH= Follicle stimulating hormone; LH= Luteinizing hormone; TSH= Thyroid stimulating hormone. The values in parentheses represent the normal range of hormones

Discussion

GnRH binding to its G-protein coupled receptor (GPCR) in the gonadotroph cells of the pituitary gland triggers the FSH release. FSH then attaches to its heptahelical GPCR, FSHR, located on the surface of ovarian granulosa cells, leading to the production of estradiol (22). Mutations in the FSHR can significantly impact glycosylation and ligand binding, decreasing or completely stopping cAMP production. A specific polymorphism known as G2039A (Ser680Asn) within the intracellular domain of the receptor plays a pivotal role in receptor trafficking and serves as a site for glycosylation and phosphorylation (23). The presence of the mutation introduces a novel phosphorylation site within the intracellular domain of the receptor, which can potentially impact the downstream mechanisms of FSHR. This alteration may contribute to the development of PA in women (19). Previously, there was uncertainty regarding the impact of the polymorphism on receptor function, particularly its potential interference with follicular growth, steroidogenesis, G-protein coupling, intracellular phosphorylation, and desensitization. This uncertainty arose due to the location of the polymorphism within the C-terminal domain of the receptor. Later, it was revealed that women with amenorrhea had higher serum FSH and required high dose of human menopausal gonadotropin (HMG) for ovulation induction (14) because G2039A polymorphism is responsible for hyporesponsiveness of ovaries for exogenous FSH (18). Several other studies have reported that two polymorphic variants (307 and 680) exhibit linkage disequilibrium; consequently, the distribution of polymorphic haplotypes is more likely to be associated with the development of amenorrhea (12, 14, 18). However, our observations suggest that the mutant allele affects folliculogenesis, receptor function, and basal FSH levels and can develop PA in West Indian women, unlike the PA women in South India (19). This provides evidence that the prevalence of the polymorphism is region-specific in India. Therefore, it raises an argument regarding the hypothesis of linkage disequilibrium and its potential impact on ovarian function. Based on our findings, it can be proposed that the functional pathway of FSHR is sig-

Table 3. Association of FSHR polymorphism with Müllerian duct development in PA women (n=45)

Radiology findings		GG (n=5)	GA (n=25)	AA (n=15)
Uterus				
	Present (n=7)	0	4 (8.8%)	3 (6.6%)
	Hypoplastic (n=20)	3 (6.6%)	12 (26.6%)	5 (11.1%)
	Absent (n=18)	2 (4.4%)	9 (20%)	7 (15.5%)
Ovaries				
	Present (n=22)	3 (6.6%)	11 (24.4%)	8 (17.7%)
	Streak (n=9)	1 (2.2%)	5 (11.1%)	3 (6.6%)
	One absent (n=4)	0	2 (4.4%)	2 (4.4%)
	Both absent (n=10)	1 (2.2%)	7 (15.5%)	2 (4.4%)
Vagina				
	Present (n=28)	4 (8.8%)	14 (31.1%)	10 (22.2%)
	Small (n=5)	1 (2.2%)	4 (8.8%)	0
	Blind (n=5)	0	5 (11.1%)	0
	Absent (n=7)	0	2 (4.4%)	5 (11.1%)
Hymen				
	Intact (n=37)	5 (11.1%)	19 (42.2%)	13 (28.8%)
	Imperforated (n=8)	0	6 (13.3%)	2 (4.4%)
Kidneys				
	Present (n=38)	5 (11.1%)	21 (46.7%)	12 (26.6%)
	One absent one ectopic (n=4)	0	2 (4.4%)	2(4.4%)
	Both ectopic (n=1)	0	0	1 (2.2%)
	One absent (n=2)	0	2 (2.2%)	0
Cervix				
	Present (n=24)	2 (4.4%)	16 (35.5%)	6 (13.3%)
	Small (n=9)	2 (4.4%)	3 (6.6%)	4 (8.8%)
	Absent (n=12)	1 (2.2%)	6 (13.3%)	5 (11.1%)

A one-way ANOVA test (post hoc LSD) was conducted; a non-significant association was observed within the groups

nificantly influenced by the presence of the G2039A polymorphism, which has the potential to contribute to the development of PA in women. Also, our findings contradict the conclusion drawn by Achrekar et al. (18) and provide strong support for the notion that G2039A polymorphism in the FSHR gene is one of the causative factors in the development of PA in women. These results warrant further exploration and investigation of the role of this polymorphism.

The polymorphism frequency was earlier observed in different ethnic groups, and Indian women showed 21.2%, 27.3%, and 51.5% of GG, GA, and AA genotype frequencies, respectively (24). In contrast, various studies revealed no significant association of Ser680Asn polymorphism between control healthy women and case group women in Brazil (12), the United Kingdom (10), and Singapore (16), suggesting that the FSHR gene is highly polymorphic in Brazilian and UK population (Table 4). The difference in the association of polymorphism in different world populations might be due to heterogeneity of genetic constitution, the difference in the ethnic background, and the selection of samples (11).

Association with hormonal profile: The impact of G2039 polymorphism on the serum level of hormones was analyzed in this study. A significant

Table 4. Comparison of different studies with the present study

Study	Study population	Year	No. of cases	Homozygous mutant genotype frequency (%)	Significance
Fonte Kohek et al. (12)	Brazil	1998	15 POF	5 (33.3%)	NS
Conway et al. (10)	United Kingdom	1999	49 POF	20 (41%)	NS
Tong et al. (16)	Singapore	2001	16 POF	5 (31.2%)	NS
Sudo et al. (14)	Japan	2002	17 PA	5 (29.4%)	NS
Sundblad et al. (15)	Argentina	2004	20 POF	5 (25%)	NS
Du et al. (13)	China	2010	40 POF	16 (40%)	NS
Achrekar et al. (18)	West India	2010	48 PA	8 (16.6%)	NS
Woad et al. (17)	New Zealand	2013	80 POF	18 (22.5%)	NS
Thomas et al. (19)	South India	2014	92 PA	24 (26.1%)	NS
Cordts et al. (11)	Brazil	2015	96 POF	14 (14.5%)	NS
Present study	Gujarat (west India)	2022	45 PA	15 (33.3%)	**

PA= Primary amenorrhea; POF= Premature ovarian failure
**p<0.01; NS= Not significant

association was observed in the level of FSH and LH in PA with AA genotype. Existing literature has primarily focused on examining serum levels of FSH and LH, and no significant association has been observed thus far (18). A study by da Fonte Kohek et al. (12) and Achrekar et al. (18) found a higher FSH and LH basal level in PA and POF women. Similar findings were observed in the present study. The exact mechanism involved is still unclear, but studies have shown that the polymorphism in exon 10 is responsible for the loss of negative feedback mechanism due to the inactivation of FSHR (25), which causes FSH resistance. In women with FSH resistance, follicular maturation is impaired, and follicles are arrested at the primary or secondary stage due to reduced FSHR molecules at the membrane, thus causing an inability to proceed through the later stages of FSH-dependent follicular maturation (26). In the present study, the mean value of serum FSH and LH in GG polymorphism was within the normal range. In contrast, women in the case group with GA and AA genotypes had serum levels above the normal range. The higher levels of FSH in the mutant genotype reflect the difference between the activities of FSHR isoforms and their tuning with the feedback mechanism (14). An alternative explanation could be that the variations in responsiveness to different FSHR genotypes could influence the FSH levels observed. This could be attributed to the intraovarian modification feature of FSH function, where the presence of inhibitors/enhancers activates distinct combinations of secondary messenger systems based on the interactions between FSH isoforms and FSHR isoforms (27). The significant association of FSHR genotypes with FSH levels indicates that subtle changes in receptor will affect the hormonal regulation required for menstruation, opening a new perspective for managing the condition.

Association with radiology profile: In this study, the impact of FSHR polymorphism on the development of the Müllerian duct was assessed. A non-significant association was observed, showing that the onset of the condition is independent of abnormal Müllerian duct and genotypes of FSHR. It is known that FSHR is present on the surface of granulosa cells. Previous studies have focused on the ovarian development of PA women but found no significant relationship with the genotypes (18). It is shown that the presence of mutant genotypes in PA or POF women causes hyporesponsiveness of ovaries to exogenous FSH, eventually causing PA (14, 18, 27). The non-significant association in the present study showed that the presence of mutant or heterozygous genotypes of FSHR would not affect the Müllerian duct's development and the function of developmental genes responsible for Müllerian duct development. Another explanation could be that the structure of ovaries (normal or abnormal) is solely responsible for the onset of PA. The structure of FSHR is not affected by the presence of streak/small ovaries or vice versa. Thus, a well-designed study is required to investigate the mechanism of ovarian function loss due to FSHR polymorphism

in PA women. This will provide the etiological pathway for understanding the mechanisms at molecular levels.

Another interesting finding from the current study indicated that individuals with complete and normal development of ovaries and uterus, specifically PA women with a 46,XX karyotype (data not shown), exhibited AG and AA genotypes. This indicates that the occurrence of PA in women with normal karyotype and typical Mullerian Duct (MD) is solely due to polymorphism. Thus, in contrast to the conclusion drawn by Achrekar et al. (18), the present study strongly emphasizes the sole impact of G2039A polymorphism on the occurrence of PA in women, and the screening should be made mandatory, especially in women who show normal karyotype.

Conclusion

The present study demonstrates a significantly higher frequency of AA genotype at 2039 position of FSHR in PA, indicating that G2039A polymorphism tends to develop PA without the presence of other polymorphisms or receptor isoforms. The significantly elevated FSH levels observed in individuals with AA genotype indicate a loss of functional FSHR, leading to the disruption of the negative feedback mechanism on the hypothalamic-pituitary-ovarian (HPO) axis. Since the prevalence is significantly higher in the West Indian population, it contributes as the causative factor for the onset of the condition. Thus, a study should be designed to understand the mechanism of only the mutation and its effect on ovarian function. Also, a study regarding the mechanism associated with development of Müllerian duct and other hormones should be conducted for management and counselling purposes. This will further help in the betterment of women's life.

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Conflict of Interest

The authors declare no competing interest.

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