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Mutational analysis of the betaglycan gene-coding region in susceptibility for ovarian failure

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BACKGROUND: Elevation of FSH is frequently a consequence of impaired ovarian follicle growth. Down-regulation of the FSH levels by inhibins is mediated through its receptor betaglycan in the gonadotrophs. Understanding of germline status of the betaglycan gene (*TGFBR3*) is essential for ovarian failure pathophysiology. METHODS: Sequence analysis was performed for the coding region of *TGFBR3* gene in a cohort of 196 ovarian failure cases that include 133 premature ovarian failure (POF) cases, 63 primary amenorrhoea (PA) cases compared with 200 controls. RESULTS: Forty-six variants including six novel exonic variants and 16 novel intronic variants were revealed. Two variants were missense: (i) p.Iso184Val in a control and (ii) p.Pro775Ser in a POF case. Genotypic distribution of three variants (c.382-81C>T, c.382-77T>C and c.1200G>A) was significantly different in the patients as compared with the controls. Five variants c.382-81C>T, c.382-77T>C, c.566-216G>A, c.1200G>A and c.2022T>C were chosen for haplotyping. The CCAAT haplotype was significantly higher in the patient population as compared with the controls ($P = 0.00007$). CONCLUSION: This study establishes the first mutational report of the *TGFBR3* gene in correlation with ovarian failure. Significant diversity of genotype distribution and haplotype analysis suggested susceptibility of the *TGFBR3* gene for ovarian failure aetiology.

Key words: betaglycan/mutation/ovarian failure

Introduction

Clinically, premature ovarian failure (POF, MIM311360) is described as an unexplained amenorrhoea (>6 months) with elevated levels of gonadotrophins (FSH >40 IU/l) before the age of 40 years. It occurs in ~1% women. Various comprehensive studies have been reported at the chromosomal as well as the gene level to elucidate the aetiology of ovarian failure, but it still remains elusive (Schlessinger *et al*., 2002). Extensive germline studies have revealed very few mutations in the gonadotrophins and their corresponding receptors except noteworthy missense variant p.Ala189Val in the extracellular domain of the FSH receptor (*FSHR*) gene. This variant was strongly associated with ovarian failure in the Finnish population (Aittomaki *et al*., 1995). This variant is rare in other world populations. The truncated variants of the *FOXL2* gene were associated with familial Type 1 blepharophimosis–ptosis–epicanthus inversus syndrome (BPES) cases where females inherit ovarian failure (Crisponi *et al*., 2001). Several *FOXL2* gene mutations have been reported in the Type 1 BPES and non-syndromic POF cases but are uncommon in diverse populations (De Baere *et al*., 2005). The first germline study of the inhibin alpha (*INH*α) gene revealed a distinct missense mutation c.769G>A (p.Ala253Thr) in its mature peptide region (Shelling *et al*., 2000). Various population studies including the Indian population corroborated the significant presence of this mutation in ovarian failure cases with almost absence in the controls (Shelling *et al*., 2000; Marozzi *et al*., 2002; Dixit *et al*., 2004). Substantial significance of this mutation as a genetic marker for ovarian failure strongly urges germline studies of other key molecules networked around inhibin functions.

Inhibins are the most important factors directly involved in the down-regulation of FSH by a negative feedback mechanism. Failure to modulate FSH levels results in a diminished ovarian reserve and clinically characterized as hypergonadotrophic hypogonadism or POF (Welt *et al*., 2005). The regulation of FSH levels is maintained by opposing actions of both inhibins and activins. These growth factors are mainly produced by granulosa cells in the ovary. These factors feedback on the FSH-producing gonadotrophs in the anterior pituitary. The activins bind their Type II receptors (ActRIIA and ActRIIB) located on the gonadotroph cell surface. The activin/ActRII complex further recruits Type I receptor ALK4. This activin/ActRII/ALK4 complex in turn activates Smad2/3 signalling and results in an elevated production of FSH. Conversely,

inhibins bind to a Type III receptor betaglycan located on the gonadotrophs. This inhibin/betaglycan complex has an equal binding affinity for activin Type II receptors and therefore competes with activin-mediated signalling. Unlike activin/ActRII complex, the inhibin/betaglycan/ActRII complex fails to recruit ALK4 because of the lack of kinase activity in the cytoplasmic domain of betaglycan. This inhibin-mediated phenomenon is incompetent to initiate Smad signalling resulting in the down-regulation of FSH production (Lewis *et al*., 2000).

The betaglycan receptor is a 300-kDa protein, which is expressed in a variety of tissues including reproductive and pituitary tissues. The expression of betaglycan mRNA and receptor in gonadotrophs fluctuates sharply with menstruation cycle stages, inhibin, FSH and estradiol (E_2) levels (Chapman and Woodruff, 2003). Recent reports on the betaglycan receptor could successfully solve the mystery of inhibin action mechanism (Phillips and Woodruff, 2004). Germline mutations in the betaglycan gene (*TGFBR3*) may result in its altered mRNA expression, structure and binding affinity. These alterations would be anticipated to lead to the loss of inhibin-mediated FSH down-regulation. This study has been designed to uncover germline status of the *TGFBR3* gene in the cases of ovarian failure. Standard experiments were designed to obtain DNA sequencing data of all the exons. *In sillico* sequence analysis and various statistical parameters were designed to establish the germline status.

Materials and methods

Patient and control recruitment

One hundred and ninety-six patients with ovarian failure were recruited, which included non-familial POF cases (n = 121), familial POF cases (n = 12), non-familial PA cases (n = 56) and familial PA cases (n = 7). These patients were recruited at the Infertility Institute and Research Centre (IIRC), Hyderabad, and the Institute of Reproductive Medicine (IRM), Kolkata. Patients disclaiming any familial history of ovarian failure were categorized as non-familial/sporadic cases. Patients with the family history of ovarian failure in the same generation or parental generation were considered as familial cases. The diagnostic criteria for POF following the definition include at least 6 months of amenorrhoea before the age of 40 years, with high serum FSH levels (>40 IU/l). The criterion for PA is defined as a condition with complete absence of menses or only induced menses. All the patients were assessed clinically, with complete medical and gynaecological history, including the history of menses, age at menopause, serum FSH levels (three times at 1-month interval), LH levels, TSH levels, and with no history of any autoimmune disease. Respective consent forms from these patients were collected by the concerned clinic. Karyotyping with a high-resolution GTG banding was carried out for all the patients and controls for chromosomal anomalies. Patients and controls with chromosomal abnormalities were excluded from the study. Normal healthy females with regular menstrual history, normal FSH levels and successful pregnancies were recruited as controls (n = 200). Recruitment of the controls was entirely population-based to support the study. The Institutional Review Board of Centre for Cellular and Molecular Biology (CCMB), Hyderabad, approved the study.

DNA extraction and karyotyping

A 5-ml aliquot of peripheral blood was collected in EDTA vacutainers for genomic DNA isolation, and another 5 ml of peripheral blood was collected in heparin vacutainers for chromosomal analysis. DNA was extracted using the Nucleon BACC2 DNA extraction kit (Amersham Pharmacia Biotech, Piscataway, NJ, USA) according to the manufacturer's protocol. Chromosomal analysis was performed on phytohaemagglutinin (PHA)-stimulated peripheral lymphocyte cultures using standard conventional cytogenetic methods.

PCR

The *TGFBR3* gene is comprised of 16 exons. Primers for all the exons were designed using online Genefisher software (http://bibiserv.techfak.uni-bielefeld.de/genefisher). All the PCR conditions included initial denaturation at 94°C for 5 min, cyclic denaturation at 94°C for 45 s and extension at 72°C for 1 min with total 35 cycles. All the primer sequences, with their corresponding annealing temperatures, are summarized in Table I. The presence of all sequence variants was confirmed by performing three independent PCR reactions and subsequent DNA sequencing.

DNA sequencing and analysis

All the PCR products were obtained using the above-mentioned primers, amplifying the corresponding exon and flanking regions of the gene. Sequencing was performed using the Big Dye terminator sequencing protocol, supported by Applied Biosystems using an ABI prism 3730xl DNA analyzer. The obtained sequence data were analysed using sequence analysis and autoassembler software in MacOS. The reference *TGFBR3* sequence was downloaded from Ensembl database (www.ensembl.org/Homo_sapiens/index.html). The Human Genome Variation Society (HGVS) guidelines were followed for all the variant nomenclature and genotype representation throughout the text and tables.

Statistical analysis of results

SNPAlyzer^{TM} 5.0 software was used for performing the Hardy–Weinberg disequilibrium, Fisher's exact test, odds ratio, confidence intervals and chi-square test. Online ISTECH SNPAnalyzer software was used for haplotyping. Multinomial logistic regression analysis was performed using SPSS 11.0 software.

Results

The sequencing data collection and analysis was successfully performed for the coding regions of *TGFBR3* gene in all the cases and controls, which included non-familial POF cases (n = 121), familial POF cases (n = 12), non-familial PA cases (n = 56), familial PA cases (n = 7) and controls (n = 200). The patients as well as the control populations were in Hardy–Weinberg equilibrium for all the genetic variations. The *post hoc* power analysis confirmed the good strength of patient and control populations for this study. In the POF cases, the mean age of attaining amenorrhoea was 26 years (range 14–40 years), mean FSH level was 59.3 IU/l and mean LH level was 33.7 IU/l. Women with regular menstrual cycles, successful pregnancies and normal FSH levels (4–11 IU/l) were recruited as controls (age range 30–45 years). The control group mean FSH level was 7.3 IU/l and mean age 37 years.

This study revealed 46 sequence variants including 22 novel variants. All the sequence variants were confirmed by the repetition of three independent PCR and sequencing reactions, including sequencing in the reverse direction also. The novel variants include six exonic variants and 16 intronic variants. The details of all the variants have been summarized in Table II.

Table I. Primer details and corresponding annealing temp details for the *TGFBR3* gene

Primer	Sequence	Product size (bp)	Exon (length in bp)	Annealing temperature (45 s) (°C)
BTG01F	TAGAAAGCGCAGAGCTAGGTTTGG	173	Exon 1 (10)	57
BTG01R	GGATAATAACAGGATCTGCCCCAT			
BTG02F	GCTTTTTGGCCACTTAGTCTTGG	600	Exon 2 (231)	65
BTG02R	CAAAGTGCTGAGATTACAGGTGTG			
BTG03F	TGGCAGGGAGCTAAATCATGC	594	Exon 3 (138)	63
BTG03R	GCCTCTGCCCACAGAAGATG			
BTG04F	GACTAGGCTTGGGATTATTCCTTG	600	Exon 4 (184)	65
BTG04R	AATCCCACACAACAACCTTCTC			
BTG05F	CAGACGAGGACAGGAAGTATCAC	600	Exon 5 (169)	64
BTG05R	CATCTGATGAAGCACACCTGAAG			
BTG06F	ACAGTGGCTGCATTAAGTAACC	600	Exon 6 (148)	62
BTG06R	TGGGTCCGTCTTAATCCAAGG			
BTG07F	CGTTCTTAGCCCAAGGAAAACAG	599	Exon 7 (190)	63
BTG07R	GTCATTAGGTGTCTCGGAGAGAAG			
BTG08F	AAAGCCACTGAGCTAGTTAGAGAG	734	Exon 8 (335)	68
BTG08R	TTGACACACAAGGATCTGGAACC			
BTG09F	GTTCCTGTTGTTCTCAGTGACAG	499	Exon 9 (153)	65
BTG09R	TGGGGTGAAAACCCTCTTCATC			
BTG10 and 11F	CTTGAACCTGCATTGGGTTTCTC	709	Exons 10 and 11 (141 and159)[a]	67
BTG10 and 11R	GGAACTATAGCCCAGCACTAACC			
BTG12F	GCCTAAAGTGAAAGTGAGATGC	597	Exon 12 (300)	62
BTG12R	CCTCCATGGAAAAGCGTCTTG			
BTG13F	TTGCATCTTCAGGTACAACC	595	Exon 13 (121)	60
BTG13R	TTTGAATGCAAGGGAGAGTG			
BTG14F	AAGTGTCAGTTTCTGCTGAGAC	297	Exon 14 (42)	62
BTG14R	AGCAGGGATAAACAAAGGACAG			
BTG15F	TTGCAATGCATGATGCAGAC	390	Exon 15 (108)	60
BTG15R	AGCGGCTAAATTTCACCAAC			
BTG16F	AGAAGGTATTCCAGCCATCTAGG	390	Exon 16 (253)[b]	59
BTG16R	CACTCTGTCTTTACAAGGGAACC			

[a]Exons 10 and 11 were co-amplified because of short intron region.
[b]Full untranslated region (UTR) of exon 16 was not amplified.

Two novel coding variants were missense, i.e. c.550 A>G (p.Iso184Val) in a control and c.2323C>T (p.Pro775Ser) in a POF case. On the basis of Fisher's exact test, allelic frequencies for three variants were almost significantly diverse in cases of ovarian failure than in controls as given below: (i) c.382-81C>T ($P = 0.0136$ for POF and $P = 0.0606$ for PA), (ii) c.382-77T>C ($P = 0.0136$ for POF, $P = 0.0606$ for PA) and (iii) c.1200G>A ($P = 0.0669$ for POF and $P = 0.0875$ for PA). The genotypic distribution for these three variants was also significantly varied (95% confidence level) in patients as compared with controls as summarized in Table III. Notably, the C allele of c.382-81C>T variant was always linked with the C allele of c.382-77T>C and vice versa. Unconditional multinomial logistic regression analysis was performed for the c.382-81C>T variant and c.1200G>A variant to estimate the relative risk for ovarian failure. These variants were chosen for regression analysis considering significant genotypic differences in the patients as compared with the controls at 95% confidence limits. The c.382-77T>C variant was not chosen for regression analysis because of its complete linkage with the c.382-81C>T variant. The multinomial regression model was significant for both the patient populations ($P < 0.00005$ for POF and $P = 0.007$ for PA) as compared with the controls with a pseudo-R^2 value (Nagelkerke) of 0.04. Both the genotypes showed almost significant regression P values as summarized in Table IV. On the basis of high frequency of minor allele (>5%) and the significant differences in genotype distribution at 80% confidence level ($P < 0.2$), five variants, namely c.382-81C>T, c.382-77T>C, c.566-216G>A, c.1200G>A and c.2022T>C, were chosen for haplotyping. The criterion for using 80% confidence limit was not to represent the association status of individual variants but rather to restrict variants for their cumulative effects. The variants other than these five were either rare or showed almost similar genotypic distribution among the patients and the controls. The details of these five variants with varied genotypic distribution in either POF cases or PA cases as compared with the controls are summarized in Table III. The significance of the haplotype analysis was represented following the Bonferroni's correction guidelines. The CCAAT haplotype was significantly higher in the patients as compared with the controls ($P = 0.00007$). The TTAGT haplotype was significantly higher in the controls than in patients ($P = 0.001$). Other less frequent haplotypes like CCGAC and TTAAT were only present in the patients ($P = 0.0096$ and 0.0003, respectively). The details of all haplotyping analysis are summarized in Table V.

Discussion

POF is largely considered a heterogeneous genetic disorder. The elevation of serum FSH levels is a prominent clinical feature of POF. Normal cyclic pulses of the serum FSH levels are regulated by opposing actions of activins and inhibins. This antagonistic activity of inhibins against activins was first

Table II. Details of variations revealed during sequence analysis of the *TGFBR3* gene

S. No.	db SNP reference ID	Mutation	Position	AA change	POF	PA	CNT
1	rs17881268	c.11–3C>T	Intron 1	–	8/133	4/63	11/200
2	rs2810904	c.213A>G	Exon 2	p.Ala71Ala	108/133	52/63	165/200
3	rs11165441	c.244–40C>T	Intron 2	–	5/133	5/63	9/200
4	rs3738441	c.381+103G>A	Intron 3	–	46/133	20/63	64/200
5	rs284176	c.381+194C>T	Intron 3	–	133/133	63/63	198/200
6	rs111653777	c.382–118G>A	Intron 3	–	50/133	25/63	78/200
7	rs10874913	c.382–112A>G	Intron 3	–	133/133	63/63	200/200
8	Novel 01	c.382–96G>A	Intron 3	–	1/133	0/63	0/200
9	rs12124904	c.382–81C>T	Intron 3	–	19/133[a]	9/63	52/200
10	rs11165376	c.382–77T>C	Intron 3	–	133/133[a]	62/63	195/200
11	Novel 02	c.382–67C>G	Intron 3	–	0/133	0/63	1/200
12	Novel 03	c.550A>G	Exon 4	p.Iso184Val	0/133	0/63	1/200
13	Novel 04	c.565+31G>A	Intron 4	–	2/133	1/63	3/200
14	rs2306887	c.565+115A>G	Intron 4	–	30/133	10/63	45/200
15	Novel 05	c.565+137C>G	Intron 4	–	0/133	1/63	0/200
16	rs17880797	c.565+140C>A	Intron 4	–	1/133	1/63	2/200
17	rs2306886	c.565+222C>T	Intron 4	–	86/133	45/63	128/200
18	Novel 06	c.566–253A>C	Intron 4	–	133/133	63/63	200/200
19	rs10783003	c.566–216G>A	Intron 4	–	132/133	62/63	197/200
20	rs10783002	c.566–71C>T	Intron 4	–	127/133	55/63	187/200
21	Novel 07	c.734+82C>T	Intron 5	–	1/133	0/63	0/200
22	Novel 08	c.882+85T>C	Intron 6	–	2/133	1/63	0/200
23	Novel 09	c.882+150A>G	Intron 6	–	1/133	0/63	0/200
24	Novel 10	c.883–53T>A	Intron 6	–	1/133	0/63	0/200
25	rs17878768	c.1200G>A	Exon 8	p.Pro400Pro	101/133	49/63	132/200
26	Novel 11	c.1335C>T	Exon 8	p.Ser445Ser	0/133	1/63	1/200
27	Novel 12	c.1408–205T>G	Intron 8	–	1/133	0/63	0/200
28	rs2279455	c.1408–164G>A	Intron 8	–	48/133	28/63	80/200
29	Novel 13	c.1408–115G>A	Intron 8	–	5/133	4/63	9/200
30	Novel 14	c.1408–68A>G	Intron 8	–	0/133	0/63	1/200
31	Novel 15	c.1408–26_1408 –25insCCC	Intron 8	–	0/133	0/63	1/200
32	Novel 16	c.1560+27G>C	Intron 9	–	3/133	0/63	0/200
33	Novel 17	c.1923A>G	Exon 12	p.Pro641Pro	0/133	0/63	1/200
34	rs17884995	c.2022T>C	Exon 12	p.Phe674Phe	57/133	21/63	94/200
35	rs11165300	c.2160+137A>C	Intron 12	–	30/133	18/63	55/200
36	rs11466603	c.2160+147G>C	Intron 12	–	0/133	0/63	1/200
37	rs284869	c.2160+188G>T	Intron 12	–	133/133	63/63	200/200
38	rs2038931	c.2161–75C>T	Intron 12	–	13/133	7/63	23/200
39	Novel 18	c.2202G>A	Exon 13	p.Ser734Ser	1/133	0/63	0/200
40	rs284878	c.2241T>C	Exon 13	p.Thr747Thr	133/133	63/63	200/200
41	Novel 19	c.2280+56A>G	Intron 13	–	0/133	0/63	1/200
42	Novel 20	c.2323C>T	Exon 14	p.Pro775Ser	1/133	0/63	0/200
43	Novel 21	c.2430+159T>A	Intron 15	–	1/133	0/63	0/200
44	rs17879140	c.2430+204_2430 +205insTTGT	Intron 15	–	13/133	4/63	15/200
45	Novel 22	c.2567C>A	Exon 16	UTR	3/133	3/63	8/200
46	rs17878586	c.2568G>A	Exon 16	UTR	29/133	8/63	37/200

AA, amino acid; PA, primary amenorrhoea; POF, premature ovarian failure; CNT, controls.
[a]Significant fisher's exact test (95% confidence level).

explained by their direct binding competition for activin Type II receptor (Martens *et al*., 1997). The direct binding affinity of inhibins to the activin Type II receptor is ~10-fold lower than the activin-binding affinity for this receptor. Conversely, *in vivo* equimolar or even lower serum inhibin concentrations are able to antagonize activin activity with equal strength, suggesting the presence of a separate inhibin receptor (Lewis *et al*., 2000).

Lewis *et al*. (2000) reported betaglycan as a first inhibin-specific receptor that satisfies all the receptor criteria. This receptor binds inhibins with high affinity. This inhibin/betaglycan complex is equally capable of competing with activin for its binding with Type II activin receptor. This complex of inhibin/betaglycan/ActRII is unable to recruit Type I receptor ALK4 and subsequently FSH secretion (Lewis *et al*., 2000). *In situ* hybridization analyses of rat tissues confirmed localized expression of the betaglycan mRNA and protein in anterior and intermediate lobes of pituitary, oocytes, granulosa cells and theca cells. The inhibin target cells, i.e. gonadotrophs, showed 97% immunoreactivity for betaglycan co-expression (MacConell *et al*., 2002). The co-localization studies of FSHβ and betaglycan in the gonadotrophs illustrated an increased expression of betaglycan with high serum inhibin levels while being inversely correlated with serum FSH levels. In the ovary, primary follicles presented light hybridization signal for the betaglycan, whereas more moderate levels were seen in follicular granulosa, thecal cells as well as the oocytes. The ovarian interstitial cells present high levels of signal for the betaglycan (Chapman and Woodruff, 2003). The regulatory effect of FSH and E_2 on the betaglycan mRNA transcript was evaluated in cultured rat granulosa cells. Both FSH and E_2 synergistically

Table III. Genotype distribution of three variants in POF and PA cases as compared with controls

SNP and genotype	Number of subjects with genotype [*n* (%)]		
	POF	PA	Controls
c.382–81C>T	$\chi^2 = 8.410$; $P = 0.0170$[a]	$\chi^2 = 3.704$; $P = 0.1569$[b]	
CC	114/133 (85.7)	54/63 (85.7)	148/200 (74.0)
CT	19/133 (14.3)	8/63 (12.7)	47/200 (23.5)
TT	0/133 (0.0)	1/63 (1.6)	5/200 (2.5)
c.382–77T>C	$\chi^2 = 8.410$; $P = 0.0170$[a]	$\chi^2 = 3.704$; $P = 0.1569$[b]	
TT	0/133 (0.0)	1/63 (1.6)	5/200 (2.5)
TC	19/133 (14.3)	8/63 (12.7)	47/200 (23.5)
CC	114/133 (85.7)	54/63 (85.7)	148/200 (74.0)
c.566–216G>A	$\chi^2 = 3.729$; $P = 0.155$[b]	$\chi^2 = 0.354$; $P = 0.838$	
GG	1/133 (0.8)	1/63 (1.6)	3/200 (1.5)
GA	25 (18.8)	9/63 (14.3)	23/200 (11.5)
AA	107 (80.4)	53/63 (84.1)	174/200 (87)
c.1200G>A	$\chi^2 = 20.80$; $P = 0.00003$[a]	$\chi^2 = 18.68$; $P = 0.00009$[a]	
GG	44/133 (33.1)	23/63 (36.5)	25/200 (12.5)
GA	57/133 (42.8)	26/63 (41.3)	107/200 (53.5)
AA	32/133 (24.1)	14/63 (22.2)	68/200 (34.0)
c.2022T>C	$\chi^2 = 1.245$; $P = 0.536$	$\chi^2 = 3.97$; $P = 0.137$[b]	
TT	76/133 (57.1)	42/63 (66.7)	106/200 (53)
TC	49/133 (36.8)	18/63 (28.6)	85/200 (42.5)
CC	8/133 (6.0)	3/63 (4.8)	9/200 (4.5)

[a]Significant *P* value at 95% confidence interval.
[b]Significant *P* value at 80% confidence interval.

Table IV. Logistic regression analysis of the influences of different genotypes and their combination on ovarian failure

Patient group	Genotype	Odds ratio	*P* value	Pseudo-R^2
POF	Intercept		0.007[a]	
	c.382–81 = M/Hz	0.477	0.013[a]	
	c.382–81 = W			0.042[a]
	c.1200 = M/Hz	1.614	0.06	
	c.1200 = W			
PA	Intercept		<0.00005[a]	
	c.382–81 = M/Hz	0.476	0.061	
	c.382–81 = W			0.04[a]
	c.1200 = M/Hz	1.796	0.085	
	c.1200 = W			

PA, primary amenorrhoea; POF, premature ovarian failure.
[a]Significant *P* value.

Table V. Haplotype frequencies in patients groups compared with controls

Haplotype	POF	PA	Controls	χ^2	*P* value
CCAAT	0.4161	0.4533	0.2814	19.066	0.000072[a]
CCAGT	0.2989	0.2682	0.3663	6.146	0.046
CCAGC	0.0996	0.1110	0.1252	1.317	0.517
CCAAC	0.0611	0.0477	0.0541	0.312	0.855
TTGAC	0.0334	0.0183	0.0274	0.703	0.703
CCGAC	0.0255	0.0135	0.0000	9.293	0.009[a]
TTAGC	0.0185	0.0000	0.0295	4.228	0.121
TTGGT	0.0152	0.0285	0.0066	3.417	0.181
CCGGT	0.0121	0.0128	0.0301	3.968	0.137
TTAGT	0.0043	0.0081	0.0497	13.845	0.001[a]
TTAAT	0.0000	0.0244	0.0000	15.917	0.00034[a]
TTAAC	0.0000	0.0000	0.0212	7.92	0.019

PA, primary amenorrhoea; POF, premature ovarian failure.
[a]Significant *P* value after the Bonferroni's correction ($P < 0.0125$).

up-regulate its transcript levels by increasing mRNA stability (Omori *et al*., 2005). These results suggest that apart from FSH regulation in the gonadotrophs, betaglycan may also have a locally important role in ovarian follicle proliferation and oocyte maturation, but further research is required to elucidate such a role.

The earliest mutation study of the *TGFBR3* (betaglycan) gene reported 12 variants including one triple-nucleotide insertion (Zippert *et al*., 2000). This study was performed using mRNA and genomic DNA isolated from six healthy Caucasian individuals and 20 healthy Caucasian individuals, respectively. This study provides the first case–control study to investigate the role of *TGFBR3* gene in genetic aetiology of ovarian failure. The mutational analysis was performed for the *TGFBR3* gene-coding regions and respective flanking intronic regions that were PCR amplified in cases of ovarian failure and controls (details in Materials and methods). This study revealed 46 sequence variants including 22 novel variants. These novel variants included six exonic variants and 16 intronic variants. Of all the variants revealed in this study, only two variants c.1200G>A and c.2022T>C were similar to previously published variants by Zippert *et al*. (2000). Other reported variants were documented in the SNP database during genome sequencing projects. Two novel exonic variants were missense as follows: (i) c.550A>G (p.Iso184Val) in a control and (ii) c.2323C>T (p.Pro775Ser) in a POF case. The missense variant c.550A>G was non-conserved among all present available vertebrates sequences but seems to lack an apparent functional effect because of the substitution by the same group amino acid. The wild-type c.2323C (also p.775Pro) was found to be conserved among the present available primate sequences but not to other vertebrate sequences. This mutation results in the substitution of an imino proline to hydroxyl group containing serine which may cause some functional defects in the protein activity. The patient harbouring c.2323C>T variant was genotypically heterozygous with FSH (82 IU/l), LH (43 IU/l) and TSH (2.6 mIU/l) hormone levels. Two variants represented

significantly diverse genotypic distribution (95% confidence level) particularly in POF cases but not in PA cases than in controls as detailed: (i) c.382-81C>T ($P = 0.0170$ for POF and $P = 0.1569$ for PA) and (ii) c.382-77T>C ($P = 0.0170$ for POF and $P = 0.1569$ for PA). The c.1200G>A variant represented significantly different genotype distribution in both the patient populations (POF cases and PA cases) than in the controls ($P = 0.00003$ for POF and $P = 0.00009$ for PA). Notably, the significant diversion of this variation was not obvious at 95% confidence limits with the Fisher's exact test. The logistic regression analysis of c.382-81C>T and c.1200G>A variants was significant among cases of ovarian failure ($P < 0.00005$ for POF and $P = 0.007$ for PA) with a pseudo-R^2 value (Nagelkerke) of 0.04. Both the genotypes showed almost significant regression P values as summarized in Table IV. The haplotype analysis was performed using five variants, namely c.382-81C>T, c.382-77T>C, c.566-216G>A, c.1200G>A and c.2022T>C as detailed in the Results. The Bonferroni's correction of multiple comparison was performed for haplotype analysis to set the significant P value ($P < 0.0125$) at 95% confidence level. The CCAAT haplotype was most significantly over-represented in the patients as compared with the controls ($P = 0.00007$). The TTAGT haplotype was significantly higher in the controls than in patients ($P = 0.001$). Other less frequent haplotypes like CCGAC and TTAAT were only present in the patients ($P = 0.0096$ and 0.0003 respectively).

In conclusion, this study provides the first germline case–control status of the *TGFBR3* gene to support the aetiology of ovarian failure. This study presents a spectrum of 46 sequence variants that include six novel exonic variants and 16 novel intronic variants. So far, only two variants have been reported as a published work, whereas the remaining known variants have been documented during genome sequencing. The genotypic distributions were significantly varied for c.382-81C>T, c.382-77T>C and c.1200G>A variant, suggesting their possible importance in the genetic aetiology of ovarian failure. The haplotype analysis represented the significant association of major CCAAT haplotype with ovarian failure. In conclusion, the *TGFBR3* gene may contribute to the aetiology of ovarian failure, possibly with other genetic and environmental factors.

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