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Mutational analysis of the mature peptide region of inhibin genes in Indian women with ovarian failure

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BACKGROUND: Clinically, premature ovarian failure (POF) is defined as unexplained amenorrhoea (>6 months) with a high FSH level (>40 IU) before the age of 40 years. POF is a heterogeneous genetic disease with unknown aetiology. Inhibin and activin regulate the FSH level by their opposing actions and thus have been considered as strong candidate genes in the aetiology of POF. METHODS: We have screened inhibin genes in patients with POF ($n = 80$), primary amenorrhoea ($n = 33$) and secondary amenorrhoea ($n = 4$). RESULTS: *INHβB* and *INHβA* genes do not show any association with ovarian failure. We found the Ala257Thr missense mutation in *INHα* gene with high statistical significance in POF (nine out of 80, 11.2%) (Fisher's exact test, $P = 0.0005$), primary amenorrhoea (three out of 33, 9.1%) (Fisher's exact test, $P = 0.014$) and secondary amenorrhoea (two out of four, 50%) (Fisher's exact test, $P = 0.001$) with complete absence of this mutation in controls (none out of 100). CONCLUSION: The *INHα* gene is a strong candidate gene for ovarian failure. Mutations in *INHβB* and *INHβA* genes are not associated with ovarian failure.

Key words: candidate gene/inhibin/mutation analysis/POF

Introduction

The loss of functional follicles occurring in women under the age of 40 years is defined as premature ovarian failure (POF). It occurs in ~1–2% of women (Coulam *et al.*, 1986). Unexplained POF is clinically recognized as amenorrhoea (>6 months) with a low level of estrogen and raised level of FSH (>40 IU/l) before the age of 40. POF is now well documented as a heterogeneous disease, which can occur due to disturbance in any gene of the intra-ovarian as well as extra-ovarian pathways. This may lead to an elevation of the FSH level as well as poor or no response of follicles to gonadotrophins (Conway, 1997). Identification and population-based screening of candidate genes may provide a better understanding of the aetiology of POF. Abnormalities in the X chromosome have been reported in many idiopathic POF cases, suggesting the probable presence of a few candidate genes on the X chromosome, i.e. *FMR1*, *DIA*, *ZFX*, *FMR2*, the *POF1* locus, the *POF2* locus, the critical region, etc. (Zinn, 2001), but their functional role in ovarian dysfunction is not well defined. Their association studies are also not well supported in large-scale/sporadic POF cases (Mumm *et al.*, 2001; Schlessinger *et al.*, 2002).

Many association studies have been done with the key components of the hypothalamus–pituitary–ovarian axis. Very few mutations have been reported so far in gonadotrophins and the corresponding receptors in association with POF, except Ala189Val substitution in the extracellular domain of the FSH receptor (Aittomaki *et al.*, 1995) in a Finnish population. The occurrence of this mutation was not found to be associated with POF in other populations (Kohek *et al.*, 1998; Layman *et al.*, 1998). FOXL2, a member of the winged helix/forkhead transcription factor family, is expressed predominantly in the human eyelid and ovary. Crisponi *et al.* (2001) reported mutations in the *FOXL2* gene in families with the blepharophimosis/ptosis/epicanthus inversus (BPES) syndrome associated with eyelid abnormalities. In type 1 BPES, the females inherit ovarian failure in addition to the eyelid defect. In type 2 BPES, only the eyelid abnormalities are seen. Truncated FOXL2 is associated with BPES type 1, and expanded proteins with type 2 (Crisponi *et al.*, 2001). Mutations in *FOXL2* in patients with non-syndromic POF have been found (Harris *et al.*, 2002), but these are not common.

Inhibin and activin are members of the transforming growth factor-β (TGF-β) superfamily and are dimers of three gene products: the INHα subunit, the INHβA subunit and the INHβB subunit, encoded by *INHα*, *INHβA* and *INHβB* genes, respectively. Inhibin and activin are dimeric glycoprotein hormones with one INHα subunit linked to either the INHβA or INHβB subunit by disulphide bonds, resulting in inhibin-A (α–βA) and inhibin-B (α–βB) respectively. Activins are dimers of β-subunits only, forming activin A (βA–βA), activin-AB (βA–βB) and activin-B (βB–βB) (Ying, 1988).

Inhibin and activin have opposing regulatory actions for modulation of the FSH level. Activin enhances FSH secretion

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while inhibin forms a negative feedback loop control of the FSH level. The presence of low serum inhibin levels in POF provides strong evidence of the involvement of inhibins and activins in the pathophysiology of POF (Petraglia *et al.*, 1998). An elevated serum FSH level and low inhibin B level in the early follicular phase has also been reported to correlate with reproductive ageing (Shahara *et al.*, 1998) and diminished ovarian reserve (MacNaughton *et al.*, 1992), and this has been included in the clinical counselling report by the American Society for Reproductive Medicine Practice Committee (2002). The first report of population screening for inhibin genes which revealed a strong association of the Ala257Thr missense mutation in the *INHα* gene with POF (Shelling *et al.*, 2000) was supported further by the study of Marozzi *et al.* (2002). Our study provides further strong support for the association of the Ala257Thr mutation in the *INHα* gene with ovarian failure.

Materials and methods

Patient information

A total of 117 non-familial cases of POF ($n = 80$), primary amenorrhoea ($n = 33$) and secondary amenorrhoea ($n = 4$) were included in the study. Patients were recruited by the Infertility Institute and Research Centre (IIRC), Hyderabad. The diagnostic criteria for POF include at least 6 months of amenorrhoea before the age of 40 years, with a high FSH serum level (>40 IU/l). Primary amenorrhoea is defined as a condition with complete absence of menses or only induced menses. Secondary amenorrhoea is defined as a cessation of menses with previous history of menses before the age of 40. All patients were assessed clinically, with complete medical and gynaecological history including history of menses, age at menopause, serum FSH level (three times at 1 month intervals) and serum LH level with no history of autoimmune disease. Karyotyping with high-resolution GTG banding was carried out for all patients for cytogenetic anomalies. Patients with chromosomal abnormalities were excluded from the study. Controls ($n = 100$) were recruited as normal females with a regular menstrual history and normal FSH level below the age of 40 years.

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 cytogenetic analysis. DNA was extracted using the Nucleon BACC2 DNA extraction kit (Amersham Pharmacia Biotech., NJ) according to the manufacturer's protocol. Chromosomal analysis was performed on phytohaemagglutinin (PHA)-stimulated peripheral lymphocyte cultures using standard cytogenetic methods (Benn and Perle, 1992; Gosden *et al.*, 1992).

PCR

Primers were synthesized for amplification of the mature peptide region of each inhibin gene, i.e. for *INHα* (nucleotides 841–1242) using INHαF and INHαR primers, *INHβB* (nucleotides 717–1061) using INHβBF and INHβBR primers, and *INHβA* (nucleotides 1167–1528) using INHβAF and INHβAR primers (Shelling *et al.*, 2000). PCR conditions for the mature peptide region of each inhibin gene were as described earlier (Shelling *et al.*, 2000; Marozzi *et al.*, 2002).

DNA sequencing

All PCR products were obtained from the above primers amplifying the mature peptide region of each inhibin gene. Sequencing was performed using the Big dye terminator sequencing protocol, supported by Applied Biosystems using an ABI prism 3700 DNA analyser.

RFLP analysis

The PCR products of the INHα1F and INHα1R primers were analysed by restriction fragment length polymorphism (RFLP) for the 769G → A mutation using *Bbv*I restriction enzyme from NEB, which is a prototype of *Bst*71I described earlier (Shelling *et al.*, 2000). This restriction digestion yields three fragments of 134, 85 and 25 bp in wild-type, four fragments of 159, 134, 85 and 25 bp in heterozygous mutants and and two fragments of 159 and 85 bp in homozygous mutant. The restriction digestion was performed using 1 × NEB2 buffer, 2 U of *Bbv*I enzyme and 10 μl of PCR product, and sterile water to make a final volume of 15 μl. All reaction mixtures were incubated at 37°C for 16 h. The RFLP products were separated by 3.5% agarose gel electrophoresis.

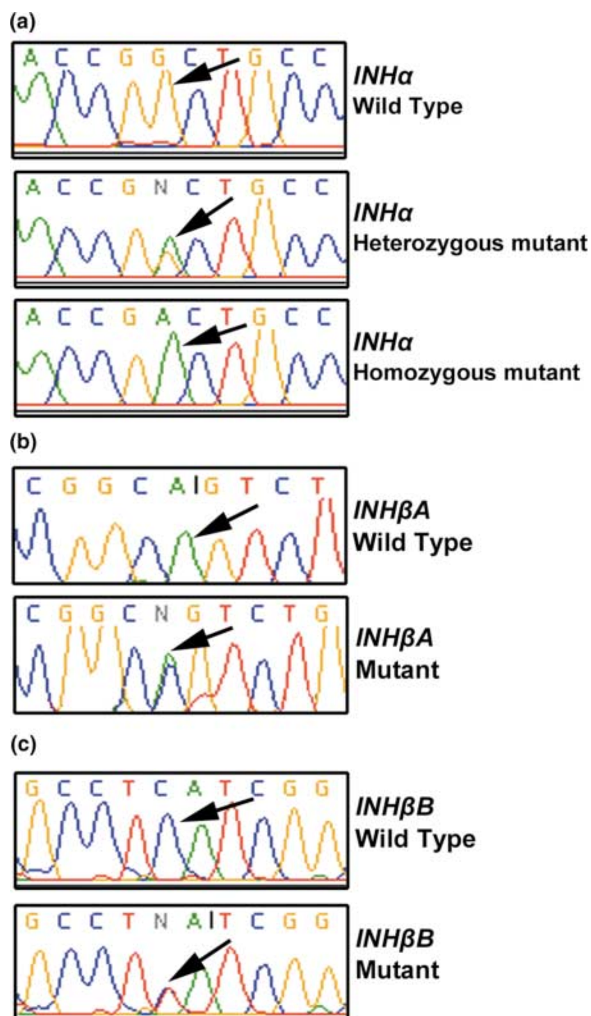

Figure 1. (**a**) Electropherogram showing heterozygous and homozygous 769G → A transitions in the *INHα* gene compared with the normal sequence. (**b**) Electropherogram showing the heterozygous 896C → A transversion in the *INHβA* gene. (**c**) Electropherogram showing the heterozygous 942C → T transition in the *INHβB* gene.

Results

In our study, we analysed POF ($n = 80$), primary amenorrhoea ($n = 33$), secondary amenorrhoea ($n = 4$) cases and controls ($n = 100$) for the mature peptide region of all three inhibin genes. The sequencing analysis of *INHα* showed a statistically significant prevalence of the 769G → A missense mutation in patients with ovarian disorder, females showed the normal sequence (Figure 1a). Eight out of 80 POF cases, three out of 33 primary amenorrhoea cases and two out of four secondary amenorrhoea cases were heterozygous for this mutation, while one POF case was homozygous. One primary amenorrhoea case showed a missense mutation 896A → C in the *INHβA* gene (Figure 1b) which leads to the Gln299Pro amino acid change. One silent mutation 942C → T was found in the *INHβB* gene in a POF case (Figure 1c). All the above mutations were confirmed by repeating the sequencing three times, including sequencing the amplification product in the reverse direction.

The presence of the 769G → A mutation was confirmed further by RFLP using the *Bbv*I restriction enzyme (Figure 2), which showed the same pattern of digestion as reported earlier (Shelling *et al.*, 2000). The mutation summary and clinical details of the patients tested positive for mutations are shown in Tables I and II, respectively.

Our sequencing results further confirm the prevalence of the 769G → A mutation in Indian women with sporadic POF (nine out of 80, 11.2%) (Fisher's exact test, $P = 0.0005$), sporadic primary amenorrhoea (three out of 33, 9.1%) (Fisher's exact test, $P = 0.014$), secondary amenorrhoea (two out of four, 50%) (Fisher's exact test, $P = 0.001$) and its absence in controls (none out of 100, 0%).

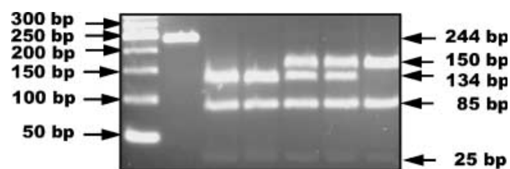

Figure 2. RFLP profile of the INHα1 product with *Bbv*I restriction enzyme digestion. Lane 1, the 50 bp marker; lane 2, undigested PCR product; lanes 3 and 4, wild-type; lanes 5 and 6, the heterozygous mutant; lane 7, the homozygous mutant (as described by Shelling *et al.*, 2000).

Table I. Summary of mutation analysis of inhibin genes

Type of mutation	Gene	Diagnosis	No. of patients with mutation	Mutation frequency
769G → A, missense	*INHα*	Sporadic POF	9/80	11.2%
		Primary amenorrhoea	3/33	9.1%
		Secondary amenorrhoea	2/4	50%
		Controls	0/100	0%
896A → C, missense	*INHβA*	Primary amenorrhoea	1	
942C → T, silent	*INHβB*	POF	1	

Discussion

Presently, POF is widely accepted as a heterogeneous disorder, which may involve a large number of genes, and, furthermore, mutation(s) in any of the genes may provide the answer to its aetiology. To date, there is no such gene known which can be accepted as a genetic marker for POF except a promising involvement of the *INHα* gene in the world population.

Inhibin production is restricted to granulosa cells. Activins are produced mainly by granulosa cells but are also produced in a variety of tissues including the anterior pituitary (Drummond *et al.*, 1996; Findlay *et al.*, 2001). Inhibins exert their inhibitory effect either by interfering with the activin-induced signalling pathway (Lebrun and Vale, 1997) or by competition of full-length INHα precursor with FSH for binding to the FSH receptor (Schneyer *et al.*, 1991). Inhibin complexed with its co-receptor betaglycan antagonizes activin signalling by competing for the activin receptor A type-II and activin receptor B type-II with equal binding affinity. This complex does not stimulate phosphorylation of ALK4 type-I receptor and subsequent intracellular Smad-dependent signalling which is imperative for FSH production by gonadotrophins (Bernard *et al.*, 2002). Inhibin binds to betaglycan via its α-subunit (Lewis *et al.*, 2000; Esparza-Lopez *et al.*, 2001), thus a mutation in the α-chain would probably impair inhibin binding to betaglycan as well as its antagonistic role in activin signalling. A deficiency in inhibin function or secretion allows activin to elevate the FSH level, which concurs with reproductive ageing (Welt *et al.*, 1999) and rapid exhaustion of the follicular reserve during the menopausal transition (Richardson *et al.*, 1987). Increased activity of activin produced by pre-ovulatory follicles has been shown to repress the growth of neighbouring follicles (Mizunuma *et al.*, 1999).

Our study demonstrated the strong association of the 769G → A mutation in the coding region of the *INHα* gene in Indian women with sporadic POF, which agrees with two earlier studies (Shelling *et al.*, 2000; Marozzi *et al.*, 2002). Marozzi *et al.* (2002) found this mutation in 25% of patients with primary amenorrhoea (three out of 12, 25%) (Fisher's exact test, $P < 0.001$) which is a significantly higher frequency than we have found in our population (three out of 33, 9.1%) (Fisher' exact test, $P = 0.014$). The significance of this difference is unknown, but could be due to the investigation of different ethnic groups. All patients with the mutation were below the age of 25 years at the time of clinical diagnosis, except for one secondary amenorrhoea case (Figure 3). The POF patients carrying the 769G → A mutation attained menopause between the age of 18 and 24 years, with a mean age at menopause of 21 years. The POF patients without mutation presented with a wider range of age at menopause, from between the ages of 15 and 36 years, with a mean age at menopause at 27 years. Therefore, the presence of the 769G → A mutation is associated with an earlier age of menopause, as has been found previously (Shelling *et al.*, 2000).

The patient who was homozygous for the 769G → A mutation attained menopause at 23 years, with a surprisingly

Table II. Clinical details of the patients who were positive for mutations

Patient ID	Age (years)[a]	Gene	Mutation state	Phenotype	FSH level (IU/l)	LH level (IU/l)
5	20	*INHα*	+/− 769G → A	Primary amenorrhoea	9	8
32	18	*INHα*	+/− 769G → A	POF	50	30
38	16	*INHα*	+/− 769G → A	Secondary amenorrhoea	16	9
47	21	*INHα*	+/− 769G → A	POF	78	62
50	15	*INHα*	+/− 769G → A	Primary amenorrhoea, Turner's phenotype	NA	NA
56	29	*INHα*	+/− 769G → A	Secondary amenorrhoea	14	NA
64	19	*INHα*	+/− 769G → A	POF	90	42
93	23	*INHα*	−/− 769G → A	POF, prolactin: 9, thyroid-stimulating hormone: 3	100	90
101	24	*INHα*	+/− 769G → A	POF	45	26
123	22	*INHα*	+/− 769G → A	POF	59	20
131	18	*INHα*	+/− 769G → A	Primary amenorrhoea	11	NA
144	22	*INHα*	+/− 769G → A	POF	74	56
158	20	*INHα*	+/− 769G → A	POF	82	71
159	18	*INHα*	+/− 769G → A	POF	51	NA
39	18	*INHβA*	+/− 896A → C	Primary amenorrhoea, Turner phenotype, shortening of 4th metatarsal, ears low set	13	8
26	24	*INHβB*	+/− 942C → T	POF	50	13

[a]Age at menopause, except for primary amenorrhoea where it indicates age at the time of recruitment.
+/− indicates a heterozygous mutant; −/− indicates a homozygous mutant; NA = data not available.

high FSH (100, 88 and 85 IU/l) and LH (90 IU/l) levels. This is the first patient identified in the literature to be homozygous for the 769G → A mutation. It is interesting to note that she does not have a particularly severe phenotype, such as primary amenorhoea. Shelling *et al.* (2000) hypothesized that the Ala257Thr mutation is sufficient to impair the binding affinity of inhibin for its receptors, and leads to the subsequent inability to regulate the FSH level by negative feedback. The increased level of FSH leads to rapid depletion of follicles, resulting in ovarian failure. Therefore, in the homozygous patient, it is plausible that the presence of relatively higher levels of FSH and LH, as compared with patients with the heterozygous mutation, is a reflection of the increased loss of negative feedback of INHα on FSH in the homozygous state. Since a few POF patients without this mutation (8.5%) also had FSH levels (>100 IU/l) as high as the homozygous mutant for the 769G → A mutation, this is a strong indication that other candidate genes also have a role in the aetiology of ovarian failure, and these need to be identified.

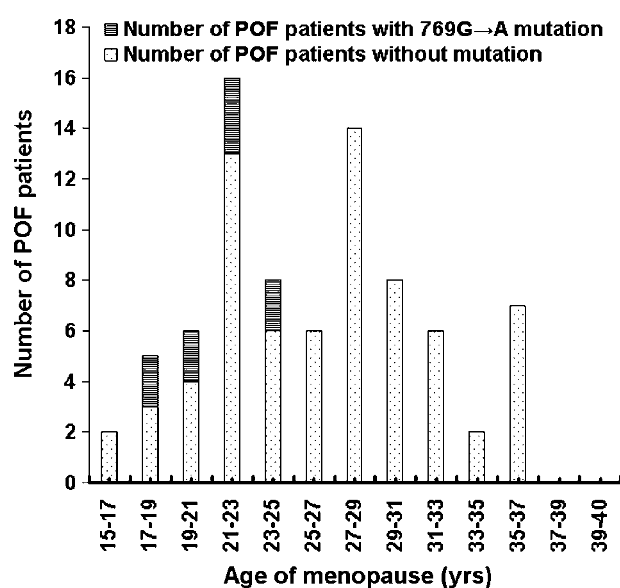

Figure 3. Graph showing the distribution of POF patients with the 769G → A mutation in the *INHα* gene and POF patients without this mutation against the age of menopause.

It is interesting to note that Matzuk *et al.* (1992) generated INHα knockout mice and showed that inhibin-deficient mice developed gonadal tumours followed by cancer cachexia such as wasting syndrome, hepatocellular necrosis around the central vein, parietal cell depletion and mucosal atrophy. Inhibin-deficient mice, if gonadectomized at an early age, do not develop a wasting syndrome but develop adrenal cortex tumours (Matzuk *et al.*, 1994). These studies indicate that inhibin acts as a gonadal and adrenal tumour suppressor. The 769G → A mutation in the *INHα* gene perhaps does not completely inactivate the functional activity of inhibin and does not show such a severe phenotypic outcome as seen in the knockout mice.

Mutations in *INHβA* and *INHβB* did not show any association with ovarian failure in our study. One patient with primary amenorrhoea was found to have an 896A → C missense mutation (Gln299Pro) in the *INHβA* gene. She had a Turner's phenotype, which included shortening of the fourth metatarsal, and her ears were low set, but she was found to have a normal karyotype. The glutamine at position 299 and its surrounding amino acids are highly conserved in cow, pig, horse, sheep, rat and mouse sequences (Figure 4). It is difficult to determine what the role of the non-conservative Gln299Pro mutation might be in ovarian failure, as it is found in the propeptide region, which would not lead to a change in the structure of the mature peptide chain.

Our study, in combination with the two earlier studies, strongly suggests that the 769G → A mutation in the *INHα* gene is a significant genetic marker for the diagnosis of POF and probably associated with early onset of POF. The presence of this mutation can be diagnosed by RFLP using

Human	EEKEQSHRPFLMLQARQSEDHPHR
Cow	EEKEQSHRPFLMLQARQSEDHPHR
Horse	EEKEQSHRPFLMLQARQSEDHPHR
Mouse	EEKEQSHRPFLMLQARQSEDHPHR
Sheep	EEKEQSHRPFLMLQARQSEDHPHR
Rat	EEKEQSHRPFLMLQARQSEDHPHR
Pig	EEKEQSHRPFLMLQARQSEEHPHR
Chicken	EEKEQSHRPFLMMLARHSEDRQHR

Figure 4. Comparison of the human INHβA protein sequence with the cow, horse, mouse, sheep, rat, pig and chicken INHβA sequences. Conservation of sequences around the glutamine (arrowhead) at position 299, which is altered to proline in one primary amenorrhoea case.

*Bst*71I or *Bbv*I restriction enzymes in clinical laboratories. This genetic test may help in the early diagnosis of POF, prior to the complete depletion of follicles, and may offer opportunities for patients to plan their families earlier.

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