Inhibin and premature ovarian failure

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BACKGROUND: Elucidation of the causes of premature ovarian failure (POF) is difficult due to the heterogeneity of the condition. Inhibin is a potential candidate gene for POF based on its dual actions on FSH secretion by the pituitary and gametogenesis in the gonads. A missense mutation in the inhibin α subunit gene (INHA G769A) is associated with POF in several populations. However, there is phenotypic heterogeneity in INHA G769A mutation carriers.

METHODS: Relevant studies were identified by searching PubMed and mutational frequencies combined for meta-analysis.

RESULTS: Meta-analysis of published studies revealed a risk difference of 0.04 (0.030 to 0.11). The occurrence of asymptomatic carriers in populations suggests incomplete penetrance and/or a multi-genetic cause of POF. We propose that a decline in inhibin bioactivity caused by the mutation could increase FSH levels; and in a susceptible individual, the heightened sensitivity to gonadotrophins causes POF. Impaired paracrine effects of inhibin could impact folliculogenesis due to reduced antagonism of activin, bone morphogenetic protein 15 and growth differentiation factor 9. Functional studies of this mutation indicate normal production of dimeric inhibin A and B and impaired bioactivity of inhibin B.

CONCLUSIONS: The identification of an autosomal mutation in the inhibin α subunit gene that is significantly linked to POF in certain ethnic populations highlights the role of inhibin in the regulation of ovarian biology and fertility. Although the reduction of inhibin B bioactivity by the INHA G769A mutation is clearly not the only cause, evidence suggests that this change may serve as a susceptibility factor, increasing the likelihood of POF.

Key words: inhibin / premature ovarian failure / POF / inhibin A / inhibin B

Introduction

Premature ovarian failure (POF) is characterized by ovarian dysfunction leading to a menopause-like state earlier than 40 years of age. It is considered a common condition as it affects 1% of all women under the age of 40 years. In a population of women 30 years or younger, the prevalence of POF is 0.1% (Coulam et al., 1986). Women with POF may not experience a menstrual cycle at all (primary amenorrhoea) or may experience cessation of ovarian function after a period of menstrual cycling (secondary amenorrhoea).

Patients tend to have elevated serum FSH levels, and this is part of the current clinical assessment criterion for the condition of POF. Serum levels of FSH above 40 IU/l and amenorrhoea for duration of 6 months or more are the clinical parameters defining POF.
Additionally, affected women have very low levels of circulating estrogens. Clinical symptoms observed are similar to those observed with the onset of menopause, such as hot flushes, vaginal dryness, dyspareunia, insomnia, vaginitis and mood swings. In addition, psychological disturbance including depression may result from the realization that fertility is no longer possible and the perception that femininity has been lost. There are elevated risks for cardiovascular disease and low bone density (osteoporosis) due to the low levels of estrogen experienced by these women at an earlier stage in life than normal women. Additionally, women with POF have a nearly 2-fold age-specific increase in mortality rate (Kalantaridou et al., 1998).

The etiology of POF is heterogeneous with the majority being idiopathic. Known causes of POF include permanent damage to the ovaries, such as pelvic surgery, chemotherapy or radiotherapy, autoimmune conditions, exposure to environmental toxicants and genetic causes. Genetic abnormalities on the X chromosome regions Xq13.2-q21.3 (Sala et al., 1997) and Xq26–28 (Delon et al., 1997) give rise to the POF phenotype. Additionally disorders linked to the X chromosome such as Fragile X and Turner’s syndrome are associated with POF (Conway et al., 1995). POF-associated genes located on the X chromosome include DiAPH2 (Sala et al., 1997), FSHPRH1 and LRP1 (Roberts et al., 1996), BMP15 (Di Pasquale et al., 2006) and ZFX (Simpson and Rajkovic, 1999). Autosomal mutations in a number of dominantly inherited genes including GDF9, INHA, FOXL2, FSHR and others have been related to POF (Laiissue et al., 2008; Simpson, 2008).

The importance of inhibin in the regulation of the female reproductive cycle is well established. Inhibin acts principally as an endocrine modulator of pituitary FSH synthesis (Robertson et al., 1985). It is also known to act locally in the ovary, the most clearly defined paracrine function being to stimulate androgen biosynthesis in the theca cells (Hillier et al., 1991). Other paracrine roles within granulosa cells include antagonism of activin, bone morphogenetic proteins (BMPs-2,-6 and -7) (Wiater and Vale, 2003; Farnworth and Florence, 2004) and growth differentiation factor 9 (GDF9) actions (Wu et al., 2004).

Inhibins and activins are members of the transforming growth factor β (TGFβ) superfamily encompassing a vast array of growth and differentiation factors such as TGFβs and BMPs. Inhibin was first isolated from bovine follicular fluid and was shown to suppress FSH release from cultured pituitary cells (Ling et al., 1985; Robertson et al., 1985). It is recognized that inhibins regulate FSH secretion by inhibiting the stimulatory actions of the structurally related proteins, activins (Vale et al., 1986).

Inhibins are heterodimers of an 18 kDa α-subunit disulphide-linked to one of two 14 kDa β-subunits (βA and βB), resulting in inhibin A or inhibin B, respectively. Activins are composed of two β-subunits; βA–βA (activin A), βA–βB (activin AB), βB–βB (activin B). In females, inhibin A and B are expressed in the ovary and are secreted throughout the menstrual cycle in a discordant pattern (Groome et al., 1996), with smaller follicles expressing mainly the α- and βB-subunits, whereas dominant follicles and the corpus luteum express α- and βA-subunits (Roberts et al., 1993). These individual patterns of expression raise the possibility that inhibin A and B may be functionally distinct (Burger, 1993). In early follicular phase, with rising FSH levels, there is an elevation of plasma levels of inhibins with the increase in inhibin B more pronounced than inhibin A levels (Burger et al., 1998). Inhibin B is mainly produced by the granulosa cells of the developing follicles, levels being highest in mid-follicular phase. The dominant follicle selectively produces inhibin A its levels high mid-cycle leading to the suppression of FSH. In the luteal phase, the corpus luteum maintains secretion of inhibin A (Roberts et al., 1993).

When the ovarian follicle reserve is depleted as a woman is approaching menopause, a decline in inhibin levels is correlated with increased pituitary FSH secretion (Burger, 1996; Robertson, 1996; 1997). Luteal phase inhibin A and follicular phase inhibin B are correlated inversely with age in perimenopausal women and follicular phase FSH levels (Burger, 1994; Danforth et al., 1998; Welt et al., 1999; Burger, 2000). Data from women with ovulatory or anovulatory cycles during the perimenopause also support the proposed roles of inhibin and FSH, as women with anovulatory cycles have greater FSH:inhibin ratios compared with the ovulatory group (Landgren et al., 2004).

Inhibin and FSH function in the hypothalamic–pituitary–ovarian axis has also been well established with animal models and in vitro studies. The biological role of inhibin was first demonstrated using a primary rat pituitary cell culture bioassay (Ling et al., 1985; Robertson et al., 1985; Farnworth et al., 1988; Vale et al., 1988). In this in vitro bioassay system, treatment with inhibin caused dose-dependent suppression of the FSH content of cultured pituitary cells. Evidence of the suppressive action of inhibin on FSH levels is well documented in rat (DePaolo et al., 1981; Sander et al., 1986; Fukaya et al., 1993), and sheep (Tsonis et al., 1986) endocrine systems. The regulatory role of inhibin in folliculogenesis is highlighted with experiments where ewes immunized against inhibin have increased ovulation rates (Findlay, 1993; Forage et al., 1987; Findlay et al., 2000).

Further evidence for the importance of inhibin in reproductive physiology is evident with the homozygous deletion of the inhibin α subunit gene (Inha) in mice (Matzuk et al., 1992). The Inha−/− mice develop gonadal sex cord tumours within 6 weeks, causing death in males and females in 12 and 17 weeks, respectively. These tumours develop with 100% penetrance; manifesting as Sertoli cell tumours (in males) and granulosa cell tumours (in females) that progress rapidly, resulting in haemorrhaging within the ovary. The development of cachexia, as is generally observed in cancer, occurred in these mice. Indices of cachexia included cellular necrosis and inflammation of hepatic cells, atrophy of mucosal cells in the stomach, development of anaemia and severe weight loss. These indices are thought to occur due to the lack of inhibins and the concomitant elevation of activins; with a 13- and 20-fold increase observed in the male and female, respectively. When the knockout mice were gonadectomized, life expectancy increased. Interestingly, these mice developed adrenal tumours which caused death at 36 and 33 weeks in males and females, respectively. The tissue-specific responses observed in cell growth characteristic of the adrenal and gonads highlight tissue-specific functions of inhibins and activins.

Although it is difficult to determine which factors produce the different effects observed in the Inha−/− mice, several experiments demonstrate that it is inhibin which possesses the anti-tumourigenic properties. The liver pathology and cachexia can be attributed to the over-expression of activins in the Inha−/− mouse, as this phenotype is also observed in mice given an excess of recombinant activin A (Matzuk et al., 1994). However, the additional deletion of the activin type II receptor (ActRIIA) gene in the Inha−/− mice does not prevent tumour development but does prevent cachexia. Additionally the transplant of a wild-type ovary into the Inha−/− mouse demonstrated the reduction of development of ovarian tumours, suggesting...
Furthermore, the simultaneous deletion of the inhibin might be able to diagnose the disease earlier. In addition, by to begin to understand the molecular basis of the disease, so that we be linked to idiopathic POF. Also it is becoming increasingly important if it is important to identify genes that impact ovarian function that can

... is sufficient to stop tumour development (Matzuk et al., 1996). Furthermore, the simultaneous deletion of the inhibin α and FSH β subunits in mice causes the development of ovarian tumours despite activin levels being lowered (Kumar et al., 1996).

Over-expression of the inhibin α subunit gene in the rat also indicates that inhibin is required for normal ovarian function (Cho et al., 2001). In rats with elevated inhibins, there was a decrease in litter size by 52% and a greater interval between pregnancies observed when compared with the wild-type animal. Although no impairment in fertility was observed in males, in the females, the reduction in fertility was caused by a decreased rate of folliculogenesis reflected by a smaller population of antral follicles, corpora lutea and a 54% decrease in the number of oocytes released/ovulated. These animal studies have confirmed the role of inhibin and FSH in the regulation of follicle growth and development, and can be used to investigate molecular interactions. However, care should be taken not to over-interpret interspecies data.

Hence, the endocrine role of inhibin on the pituitary and its paracrine roles within the ovary support the concept that inhibin plays an important role in the regulation of ovarian function and folliculogenesis, and also raise the possibility that it may be considered to be a potential candidate gene for the development of POF.

The hypothesis that in POF a decline in circulating inhibin levels could result in raised FSH concentrations, increased follicle recruitment and hence an increased rate of follicle depletion is well supported. Indeed, a decline in serum inhibin levels occurs when the stores of ovarian follicles begins to diminish prior to menopause (MacNaughton et al., 1992). This increase in FSH secretion coincides with an enhanced rate of follicular loss during the menopausal transition (Richardson et al., 1987). The hormonal patterns of POF patients also implicate inhibin as important for ovarian function, as women with idiopathic POF have low serum levels of inhibin A and inhibin B, compared with age-matched fertile women (Munz et al., 2004).

Due to the heterogeneity of the condition, diagnosis is difficult and it is important to identify genes that impact ovarian function that can be linked to idiopathic POF. Also it is becoming increasingly important to begin to understand the molecular basis of the disease, so that we might be able to be able to diagnose the disease earlier. In addition, by understanding the molecules involved in the development of the disease, we can then determine more defined avenues for improved treatment for each woman in whom a cause can be found.

**Methods**

We conducted a MEDLINE search for all reports conducting population analysis of the incidence of the inhibin α subunit mutation (INHA G769A) in women with POF. Five studies were included in the analysis of women with primary and secondary amenorrhea and controls (summarized in Fig. 1). Random effects meta-analysis to elucidate an association between the INHA G769A mutation and POF was conducted with Comprehensive Meta-Analysis Version 2 software (www.Meta-Analysis.com). The results are presented as risk difference between the two comparison groups.

**Results**

**A genetic link between inhibin and POF**

As described above, there is strong evidence that inhibin is important in regulating ovarian function. Hence it is a strong candidate gene for mutational studies in humans. A functional mutation in any one of the three inhibin genes could lead to a decrease in the amount of bioactive inhibin. This loss could remove the negative feedback on the pituitary, cause an increase in FSH levels contributing to premature depletion of follicles and hence result in POF.

The first evidence of a genetic link between inhibin and POF was established with the cytogenetic analysis of a POF patient who had a chromosomal translocation between chromosomes 2 and 15: 46,XX,t(2;15) (q32.3;q13.3) (Burton et al., 2000). As the inhibin α subunit locus is 2q33–36 (Burton et al., 2000), it was important to determine whether inhibin might play a role in the development of POF. Southern blot analysis using an inhibin α subunit gene probe did not indicate the disruption of the gene by the translocation (Shelling et al., unpublished data), however, the possibility still exists that this translocation could disrupt upstream or downstream regulatory elements (as is seen for translocations in the FOXL2 gene which causes POF).

![Figure 1](image-url)
Further investigation was aimed at mutational screening of the inhibin α subunit gene (INHA), focusing on the region encoding the inhibin mature peptide (αC), which after translation is proteolytically cleaved from the larger precursor peptide (ProαNaC). A missense mutation INHA G769A was identified causing an amino acid transition from alanine to a threonine. In a New Zealand population, a heterozygous change was identified, significantly correlated to the condition (7% in POF, n = 38 compared with 0.07% in controls, n = 150; P = 0.011) (Shelling et al., 2000). Significant mutations were not found in the inhibin βA or βB subunit genes (Shelling et al., 2000), although a silent transition 1032C>T variant was observed in the INHβA gene.

Incidence of the INH G769A in multi-ethnic populations

To date six studies have been performed to investigate the association of the INHA G769A mutation and POF across various ethnic populations. In this review, we combined the published studies to assess the risk difference between POF and control women carrying the INHA G769A mutation. Using random effects modelling, the combined risk difference between the two comparison groups across the studies is 0.04 (95% Confidence Interval (CI) – 0.03 to 0.11) (Fig. 1). Two populations demonstrate no association between the INHA G769A mutation and the risk of POF (Sundblad et al., 2006; Corre et al., 2009). However, in the New Zealand and Indian populations there is a positive risk difference due to the INHA G769A mutation between POF and control subjects (Fig. 1). Another meta-analysis of this data found a cumulative odds ratio of 1.38 (95% CI 0.48–3.94), and a significant odds ratio of 8.10 (95% CI 1.27–51.6) in the Asian Indian populations (Zintzaras, 2009).

There is a higher incidence of mutations in the INHA gene in the Asian Indian population (Dixit et al., 2004, 2006; Prakash et al., 2009). Dixit and co-workers reported 10.5% of the sporadic POF cases (n = 133) compared with 0.005% controls carried the INHA G769A mutation (Dixit et al., 2004, 2006). Of this population, one patient possessed a homozygous mutation. The circulating FSH levels in this patient were the highest recorded within the group studied (three separate measurements of 100, 88 and 85 IU/l). This patient reached menopause at the age of 24 years. Of the 60 women with primary amenorrhea screened separately for the inhibin α subunit mutation, six were found to be carriers (10%). Shelling et al. (2000) report one mutation carrier to have primary amenorrhea.

Another such study in the Indian population has revealed the presence of the INHA G769A and an additional three novel missense mutations (Prakash et al., 2009). The presence of the INHA G769A mutation was significantly greater in women with POF although two of the controls were also carriers (Prakash et al., 2009). The presence of the INHA G769A mutation was associated with early onset of POF in this population, similar to observations of Dixit et al. (2004, 2006) and Shelling et al. (2000).

The initial study carried out in POF women in the Italian population showed a significant correlation between the INHA G769A mutation and POF (sporadic POF 4.5%, n = 157) and primary amenorrhea (25%, n = 12) POF women (Marozzi et al., 2002). The study also demonstrated a significant link to the mutation in familial POF cases (7.7%). Recently, the study was extended to include a larger cohort of Italian and German subjects and no difference in mutation frequency was observed between in POF and control subjects (Corre et al., 2009). Interestingly, the INHA G769A mutation is rare in the Korean population where neither POF patients (n = 84) or controls (n = 100) were identified as carriers (Jeong et al., 2004).

Larger cohort studies with appropriate controls are required to further understand the relationship between the genetic variants in inhibin alpha subunit and POF. The current published studies indicate that in a susceptible population the INHA G769A mutation is associated with the early onset of POF.

Functional characterization of the INHA G769A mutation in vitro

Functional studies of this mutation include over-expression of wild-type or mutant inhibins in gonadotroph and ovarian granulosa cell lines with an activin-sensitive luciferase reporter construct (p3xGRAS), inhibin ELISA and betaglycan binding assays. We discuss these findings in the next two sections.

A gonadotroph cell line, LβT2 was co-transfected with an activin-sensitive luciferase reporter construct (pGRAS-Luc) and increasing amounts of either wild-type or mutant α-subunit. Transfection efficiency was assessed by the measurement of secreted inhibin B (LβT2 cells express inhibin βb but not βA) (Fig. 2B). The over-expression of wild-type inhibin α subunit resulted in a dose-dependent decrease in p3xGRAS-Luc activity (Fig. 2A). In contrast, activin-induced p3xGRAS-Luc activity was unaffected by transfection of increasing doses of α mutant DNA, indicating that the A257T inhibin B has compromised biological activity (Chand et al., 2007). Subsequent transfection studies in the ovarian tumour cell line COV343 have suggested that the A257T α-subunit mutation has significantly less effect on the inhibin A response. Together, these results suggest that A257T and/or juxtaposed residues (Fig. 3A) are important for inhibin A activity and highlight mechanistic differences between the inhibin isoforms.

How the INHA 769G>A mutation, which causes an amino acid substitution at position 257 (position 25 in the mature protein αC), impairs the bioactivity of inhibin B remains to be determined. In the characterization of mutant inhibin A and B in vitro, detection using a
2-site specific ELISA confirms that Thr^{257} is not crucial for the dimerization of the inhibin \(\alpha\) and \(\beta_{Ao}\) or \(\beta_{B}\) subunits. This is to be expected as Thr^{257} is located in the extended N-terminus of the \(\alpha\) subunit, well removed from the \(\alpha\beta\) dimer interface (Fig. 3B).

**Is there a physiological consequence of the INHA G769A mutation?**

As the INHA G769A mutation is commonly a heterozygous mutation, it is likely that any consequence this change may have on inhibin biological function would not be a complete loss of function. It is suggested that in mutation carriers, a reduction in inhibin function by 50%, would have effects at two stages: (i) fetal gonadal development; and (ii) in the regulation of normal folliculogenesis and ovulation. A reduction in inhibin biological potency could possibly hinder the normal development of the fetal ovary, continuing to affect ovarian development and function after birth. The elevation of circulating FSH levels as a result of a reduction of the endocrine effect of inhibin may cause ovarian dysregulation, primarily at the follicular development stage. As inhibin has important paracrine effects on the action of activin, GDF9 and BMP15 within the follicles, impairment in inhibin bioactivity could contribute towards aberrant folliculogenesis, maturation and atresia.

Alignment of amino acid sequences across various mammalian species indicates that the INHA G769A mutation causes an amino acid substitution in a highly conserved region (Fig. 3A), suggesting this region may encode an important regulatory function of the protein. Currently the three dimensional structures of inhibin \(\alpha\) subunit alone and as a dimer with a \(\beta\) subunit have not been produced, however, recently extensive functional analysis has been performed by generating numerous inhibin \(\alpha\) subunit mutants. Inhibin mediates its effect principally by binding to betaglycan, this complex then associates with the activin type II receptor to prevent activin binding and suppresses the SMAD signalling pathway.

By generating mutant \(\alpha\) subunit proteins, the interaction of inhibin A with its receptor betaglycan has been characterized (Makanji et al., 2008). In this study mutations were produced in areas of \(\alpha\) subunit protein which when glycosylated reduced its affinity for betaglycan. Residues Val^{108} and Tyr^{120} were found to be crucial for betaglycan interactions and mutation of these residues to alanine reduced affinity by 6- and 8-fold, respectively (Makanji et al., 2008). Furthermore these two residues are part of the essential core residues that form the outer convex surface of the protruding, ‘finger’ motif of the protein including Arg^{109}, Thr^{110}, Thr^{111}, Ser^{112}, Ser^{117}, Phe^{118}, Lys^{119} and Tyr^{120} (Fig. 3B) (Makanji et al., 2008).
The consequence of the alanine to threonine substitution due to the INHA G769A mutation, does not include a disruption to inhibin dimer formation (Chand et al., 2007). We now know that this amino acid substitution occurs in a region that remains exposed after the dimerization of the inhibin α subunit to the inhibin/activin β subunit and does not impact betaglycan interaction (Makanji et al., 2007). This raises an interesting question on how else inhibin may be acting. Is the mutation containing region important for an interaction with an inhibin-specific receptor other than betaglycan? The three novel α subunit mutations (A245D, P252H and H259Q) are in close proximity to Ala257, highlighting the importance of this region for inhibin biological activity.

**Impact on the paracrine actions of inhibin**

The severity of the POF phenotype in mutation carriers could be compounded by the impairment of the paracrine actions of inhibin within the ovary including steroidogenesis, possibly with the dysregulation of androgen production. Within ovarian theca-interstitial cells, inhibin stimulates LH-dependent androgen production hence promoting follicular recruitment (Hsueh et al., 1994; Garrett et al., 2000). This biological effect of inhibin is mediated with its potent antagonism of activin actions, the net consequence being that inhibin is a regulating factor in ovarian steroidogenesis (Hutchinson et al., 1987; Hillier, 1991). Other paracrine roles of activin which are opposed by inhibin include granulosa cell proliferation and the potententiating effects of activin and FSH in the ovary to increase FSH receptor expression.

The action of inhibin as an activin antagonist is mediated via competition for the ActRIIs, facilitated by betaglycan (Lewis et al., 2000). Betaglycan or TGFβ type III receptor (TGFβRIII), binds to TGFβ1 and TGFβ2 via its endoglin-related domain (Lopez-Casillas et al., 1993; Ethier et al., 2002) and inhibin binds to the uromodulin-related domain with high affinity (Esparza-Lopez et al., 2001). Similar to mechanisms for activin antagonism, the betaglycan—inhibin complex binds to BMP receptor II (BMPRII) competing for BMP binding (Wiatr and Vale, 2003). In this manner inhibin could antagonize the paracrine actions of BMPs, a subgroup of growth factors and cytokines from the TGFβ superfamily.

A BMP essential for follicle development to the secondary stages is BMP15 (also known as Growth Differentiation Factor 9B). The earliest role of BMP15 is the initiation of granulosa cell proliferation in primary follicles (Moore et al., 2003). Upon further follicular development and with rising FSH levels, the granulosa cells acquire FSHR which stimulates cumulus expansion and the formation of antral cavity (Dong et al., 1996). BMP15 also enhances estrogen and progesterone synthesis with the stimulation of aromatase expression and inhibits the premature luteinisation of large follicles (Otsuka et al., 2001). The abundance of BMP15 in large antral and Graafian follicles causes the down-regulation of FSHR expression, as a consequence, maintaining a control on progesterone production until ovulation (Otsuka et al., 2000). BMP15 also stimulates granulosa cell secretion of Kit Ligand which bind to its receptor c-kit in the oocyte. Kit Ligand actions cause negative feedback on BMP15-dependent granulosa cell proliferation (Otsuka and Shimasaki 2002).

The importance of BMP15 as a fertility regulator is evident in Inverdale and Hanna ewes (Galloway et al., 2000). Genetic mutations in the proregions (Y235C, R68W, A180T and 262insLeu) have been shown to be associated with POF in an Italian population study (DiPasquale et al., 2004). Additional genetic variations have been shown to be significantly associated with POF in the Indian population (Dixit et al., 2006), however no variations were identified in the New Zealand population cohort (Chand et al., 2006). A recent German study reported the A180T mutation in the proregion as a polymorphism and did not identify any other BMP15 genetic variations (Ledig et al., 2008).

Another TGFβ ligand that signals via BMPRII is GDF9. It is known paracrine roles within the ovarian follicle include (i) granulosa cell proliferation and differentiation (Elvin et al., 1999, Eppig, 2001, Gilchrist et al., 2006, Mottershead et al., 2008) and cumulus expansion, with the induction of hyaluronan synthase 2 (HAS2), cyclooxygenase 2 (COX2), and steroidogenic acute regulator protein (StAR) (Dong et al., 2000). Recently missense mutations have been identified in the GDF9 proregion (P103S) (Kovanci et al., 2007), mature protein (T238A) (Zhao et al., 2007) and S186Y (Laissue et al., 2006) in some populations but not others (Chand et al., 2006) indicating rare mutations in this gene are linked to POF. The oocyte derived factor, GDF9 is expressed in follicles in the primary stage and onwards, and the deletion of GDF9 renders mice infertile as folliculogenesis is blocked from the primary follicle stage onwards (Elvin et al., 2000, McPherron and Lee 1993). The phenotype of a double knockout transgenic mouse, in which the genes encoding inhibin α subunit and GDF9 have been deleted (Inha−/−GDF9−/−) (Wu et al., 2004) clearly identifies a role for inhibin in the determination of ovulation rates.

The Inha−/−GDF9−/− mouse exhibits an interesting phenotype, where the block in folliculogenesis typically observed in GDF9 knockout mice, is removed. Histological examination of the Inha−/−GDF9−/− mouse ovaries, show the presence of healthy follicles at all stages of folliculogenesis, until the late pre-antral stage, after which there is formation of granulosa cell tumours. This transgenic model demonstrates a vital role of inhibin as a potent modulator of ovarian granulosa cell proliferation. In the absence of inhibin, transgenic mice are observed to have uncontrolled granulosa cell proliferation resulting in ovarian tumours, as observed in Inha−/−GDF9−/− and Inha−/− transgenic mice. Correlating these observations to altered mechanisms in the POF ovary, it is suggested that in carriers of the INHA G769A mutation, a reduction in bioactivity of inhibins could allow uncontrolled granulosa cell proliferation leading to abnormal folliculogenesis (Fig. 3).

Thus, the effects of inhibins are numerous and current understanding is that these effects are largely mediated through its antagonistic action on activin and BMPs. However new evidence showing differences in bioactivities and betaglycan receptor affinity of inhibin A and B implies the existence of other, as yet unknown, mechanisms of inhibin function (Makanji et al., 2008). As inhibin has a potent influence on ovarian function via mechanisms described above, a decrease in inhibin biological activity, due to the INHA G769A mutation, could lead to an accelerated loss in follicle numbers, leading to POF (Fig. 4). The decrease in inhibin bioactivity could lead to more potent activin, BMP15 and GDF9 effects resulting in increased number of primordial follicles being recruited for folliculogenesis, enhanced follicular development to antral stages with increased granulosa cell proliferation, cumulus cell differentiation and expansion, and...
a dysregulation in steroid hormone production at the theca and granulosa cell level. Clearly further study is required to complete our understanding on how the paracrine actions of inhibin are facilitated within the granulosa cell and to recognize the impact of the \textit{INHA G769A} mutation within this cell type (Fig. 4).

**Figure 4** Paracrine roles of inhibin within the ovarian follicle and proposed mechanisms of POF in \textit{INHA G769A} mutation carriers.

\textbf{(A)} The effects of GDF9, KitL, BMP15, activin and inhibins and their interactive roles are summarized in this illustration. \textbf{(B)} Reduced inhibin bioactivity, due to the \textit{INHA G769A} mutation, could lead to more potent activin, BMP15 and GDF9 effects resulting in increased number of primordial follicles being recruited for folliculogenesis, enhanced follicular development to antral stages with increased granulosa cell proliferation, cumulus cell differentiation and expansion, and a dysregulation in steroid hormone production at the theca and granulosa cell level.

**Phenotypic heterogeneity among \textit{INHA G769A} mutation carriers**

As in many other genetic diseases, the relationship between \textit{INHA G769A} and POF is not simple, and the genetic variant may be
acting more like a susceptibility allele than a disease-associated mutation with 100% penetrance. Studies have shown the occurrence of asymptomatic carriers suggesting incomplete penetrance and/or a multi-genetic cause of POF.

Examples of such cases of genetic heterogeneity are observed in a number of other similar genetic conditions such as retinitis pigmentosa (Kajiwara et al., 1994; Waseem et al., 2007) where analysis indicates up to 30 separate genes involved, with different modes of transmission. Genetic studies are difficult with infertility disorders due to the inability to ascertain large families over multiple generations, as by definition, infertility within a family will lead to small families. However, using a candidate gene approach many genes have now been identified as causes of idiopathic POF, such as FSHR, FOXL2, NOBOX and FMR1 (Fassnacht et al., 2006; Qin et al., 2007; Biesen et al., 2008; Laissire et al., 2008; Simpson, 2008).

In the New Zealand population, the clinical details of the asymptomatic carrier identified were not available as the DNA sample was provided anonymously, so the possibility that this DNA sample was from a female who experienced menopause early cannot be discounted. The second asymptomatic carrier was identified during the genetic assessment of the family of a carrier. In this family, the mother was shown to be an asymptomatic carrier, although her 16-year-old daughter who also carried the mutation was diagnosed with POF. Similarly, in the Marozzi study, the asymptomatic carrier was the sister of the POF subject with the INHA G769A change. In this particular family, the other sibling (male) also carried the inhibin mutation. From the two family histories available, the prevalence of unaffected carriers could be a relatively common occurrence in families with a history of POF.

Asymptomatic carriers of the INHA G769A mutation occur in a higher frequency in the Italian population (Corre et al., 2009) also explaining similar incidence observed in a study of control and pre-eclampsia patients (Ciarmela et al., 2005). Additionally asymptomatic mutation carriers are present in an Argentinean study (Sundblad et al., 2006).

The presence of INHA G769A mutation in the control cohort, in the New Zealand and Argentinean studies, may be due to inappropriate selection of samples. In the New Zealand study, controls included males and females whereas Sundblad et al. report six of the eight mutation carriers found in the control population were under the age of 40 years. Similarly the two asymptomatic controls in the Indian study were under the age of 40 years (Prakash et al., 2009). Population-control replaced with phenotype-specific controls where women known to have experienced menopause at the normal age of onset of ≥50 years of age may be a more appropriate control for these studies.

From all the population data available it appears that the Indian population is most profoundly affected by the INHA G769A mutation. Can this observation complement existing understanding that genetic variability is confounded by ethnic-dependent factors?

The age of onset of POF in carriers of the mutation varies amongst different population groups. This suggests that mutation carriers within some ethnicities may be at greater or lower risk of developing POF, due to other genetic or environmental influences. Furthermore the age of onset of menopause in the New Zealand and Indian population is considerably lower (13.6 years earlier) compared with that of Italian women who carried the INHA G769A mutation. The incidence of menopause before the age 25 years in the New Zealand and Indian carrier population was 100 and 90%, respectively, although in the Italian POF population, none of the carriers experienced menopause before the age of 30 years. In POF patients who were carriers of the INHA G769A mutation, approximately 30% experienced primary amenorrhea.

Can phenotypic heterogeneity among INHA G769A mutation carriers be related to ethnic-dependent factors?

Is the Indian population most profoundly affected by the INHA G769A mutation due to ethnic-dependent factors? A recent analysis of pregnancy outcomes in polycystic ovarian syndrome (PCOS) patients using IVF techniques supports variability between South Asian (Indian) and Caucasian populations. In terms of clinical pregnancy outcomes, Caucasian PCOS patients had a 2.5 times higher chance of ongoing pregnancy compared with South Asian PCOS patients (Palep-Singh et al., 2007). This discrepancy was attributed to increased sensitivity among South Asians to gonadotrophin stimulation which resulted in a greater change in FSH levels. According to this study increase in basal FSH concentration by one unit reduced the odds of pregnancy by 18.6%. Lower rates of pregnancy outcome are also observed in Asian and African American women compared with Caucasian women who undergo IVF treatment (Feinberg et al., 2006; Purcell et al., 2007).

A similar mechanism could explain variable disease phenotypes in INHA G769A mutation carriers among the South Asian POF population. A decline in inhibin bioactivity would cause increased secretion of FSH; and in a susceptible population, a small variability in FSH levels but a heightened sensitivity to gonadotrophin stimulation would explain the earlier onset of POF observed in this population group. A subtle change in FSH levels in a hypersensitive system could account for increased follicular loss, likely through aberrant folliculogenesis and increased atresia.

The degree of variability in ovarian FSH responsiveness is observed in normal and anovulatory infertile women undergoing gonadotrophin induction of ovulation (Imani et al., 2002). Factors such as body mass index, serum leptin levels, menstrual cycle history (oligomenorrhea versus amenorrhea), ovarian response to clomiphene citrate medication, initial serum FSH levels, free IGF-I and IGFBP-1 levels are significantly correlated with individual FSH response dose. It has also been shown that follicular growth is dependent on the duration of FSH elevation above a critical threshold rather than the degree of elevation (Schipper et al., 1998). Further evidence is provided from a clinical case report where a small increase in FSH levels caused ovarian hyperstimulation, in a 37-year-old woman with an autonomous FSH-secreting pituitary adenoma. She presented with irregular cycles, and multiple ovarian cysts (Djerassi et al., 1995). Observations during IVF procedures in older women demonstrate that more follicles are recruited in the form of multiple small follicular cysts (Romeu et al., 1987) and an increased tendency for dizygotic twinning occurs in older mothers (Gillihan et al., 1996). It can be postulated that the amount of FSH required varies greatly because of individual variation in ovarian FSH sensitivity or the FSH threshold. This accounts for variability in response during gonadotrophin stimulation in IVF procedures in relation to ethnicity or ageing. These studies illustrate the importance
of FSH as a critical initiator of the progression of small responsive follicles to the antral stage and subsequent maturation towards the pre-ovulatory stages. During this time ovarian factors work in concert to establish the follicular microenvironment necessary for successful ovulation. It is suggested that in certain populations, for example, in the East Asian population who appear to respond differently to FSH than Caucasians, the presence of the inhibin mutation resulting in impaired inhibin activity may have more pronounced effects on ovarian function.

Genetic variation in INHA as a susceptibility gene for POF

During the screening of the inhibin α promoter region, a C>T polymorphism was identified, and we showed that the promoter variant c.-16C>T was significantly under-represented in POF patients, and has thus been suggested to protect women from developing POF (Harris et al., 2005). A further INHA promoter variant c.-124A>G was found to be co-ordinately inherited with a highly variable TG repeat element at −300 bp, and several haplotypes were identified that differ in repeat element length (between 76 and 94 bp) and sequence. The INHA promoter haplotype C has the shortest repeat length and was found to be in linkage disequilibrium with the -16T allele (Harris et al., 2005). In further studies, we were able to conclude that INHA promoter variants, specifically haplotype C, are associated with the development of POF (Woad et al., 2008). The C haplotype is significantly underrepresented in patients (15/118; 12.7%) compared with controls (50/238; 21%) (P = 0.037, Fisher’s exact test). It is unclear whether these polymorphisms in the INHA promoter might result in reduced inhibin expression, but this indicates that promoter polymorphisms may be another mechanism for the transcriptional regulation of the inhibin α subunit (Woad et al., 2008). Therefore, we suggest that variants within the inhibin gene cause it to act as a susceptibility allele, whereby women who carry this particular variant more likely to develop POF.

Conclusions

POF is a common condition and is of growing concern to the general population due to the increasing trend of delaying pregnancy. The use of hormonal contraceptives often conceals this condition until the woman decides to stop the contraception regime, in preparation to have a child. Diagnosis is often delayed due to an extensive variability in symptoms and no predicting factors for idiopathic POF, the largest subset of aetiology. The issues regarding personal health and quality of life of the affected women with the occurrence of menopause at a young age is also of concern. Hence there is a clear need for improved diagnostic screening before the onset of disease.

The identification of an autosomal mutation in the inhibin α subunit gene (INHA G769A) that is significantly linked to POF is of benefit in terms of improving our understanding of the role of inhibin in the regulation of ovarian biology and fertility. Although the reduction of inhibin B bioactivity by the INHA G769A mutation is clearly not the only cause for the condition, evidence suggests that this change may serve as a susceptibility factor, increasing the likelihood of POF. Other predisposing factors including other genes, ethnicity and lifestyle factors will likely play a role, but at the moment, the roles of these factors remain largely unknown. Furthermore these studies contribute to the overall understanding of oligogenetic diseases such as POF.

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