

Premature Ovarian Failure (POF) Syndrome: Towards the Molecular Clinical Analysis of its Genetic Complexity

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Abstract: The Premature Ovarian Failure (POF) syndrome is a very heterogeneous clinical disorder due probably to the complex genetic networks controlling human folliculogenesis. Clinical subgroups of POF patients whose aetiology of ovarian failure is based on the same genetic factors are therefore difficult to establish. Some experimental evidence suggests that these genes might be clustered on the female sex chromosome in the *POF1* and *POF2* loci. This review is aimed to present an overview of the actual structural changes of the X chromosome causing POF, and to present a number of X and autosomal female fertility genes which are probably key genes in human folliculogenesis and are therefore prominent POF candidate genes. Towards the molecular analysis of their functional contribution to the genetic aetiology of POF in the clinic, an interdisciplinary scheme for their diagnostic analysis is presented in a pilot study focussed on chromosome analyses and the expression analysis of some major POF candidate genes (*DAZL*, *DBX*, *FOXL2*, *INH*, *GDF9*, *USP9X*) in the leukocytes of 101 POF patients. It starts with a comprehensive and significantly improved clinical diagnostic program for this large and heterogenous patient group.

Keywords: POF-syndrome, Turner-syndrome, POF loci and key genes, human folliculogenesis.

1. INTRODUCTION

Premature ovarian failure (POF) is defined as a secondary amenorrhea with hypergonadotropic hormone status in women not older than 40 years [1]. It occurs in approximately 1% of women in that age group [2]. In women under the age of 45 years, the rate of hypergonadotropic amenorrhea is as high as 5% [3]. Under the age of 30, 0.1% of all women are affected [2]. An early and carefully designed clinical diagnosis of POF syndrome in women suffering from some interference of their menstrual cycle is therefore of utmost clinical importance. It will not only aid in the early prognosis of the woman's fertility, but also prevent the long term consequences of an hypoestrogenic state, such as the development of genital atrophy, loss of bone mass and the occurrence of cardiovascular diseases.

Usually, premature ovarian failure is diagnosed when patients suffer from secondary amenorrhea, i.e., when the FSH levels are constantly beyond 40 IU/L and when the level of estradiol is low. Nevertheless, spontaneous follicular recovery could be observed in 50% of women with high FSH levels [5] suggesting that also strong non-genetic factors are affecting the maturation of female germ cells. These factors include metabolic diseases, autoimmune processes, infections, and iatrogenic damages caused by radiotherapy or chemotherapy. It is also well known that despite having amenorrhea and markedly elevated serum gonadotropin levels, some women have ovarian follicles that function intermittently [4] although there is a poor correlation between follicle diameter and serum estradiol

levels in these cases. Despite the variability of these phenotypes, genetic abnormalities were estimated to be the likely cause for POF in 31% of all cases [6]. However, in many cases the underlying genetic mutation(s) remains unknown or questionable [7].

Genetic predispositions for POF often follow an X-chromosomal inheritance pattern [8, 9]. On average, these families suffer from an early onset of POF syndrome at the age of 31 years [6, 10]. The description of X-chromosomal rearrangements and X gene defects which cause different folliculogenesis disorders will therefore be one focus of this review. Additionally, we will describe some key autosomal genes which are known to have an essential function in human female germ cell metabolism and of which some, due to distinct molecular mutations, are also known to cause the POF pathology. Based on this knowledge, a detailed diagnostic schedule for the identification of POF patient genetic subgroups is presented including first results from our large outpatient clinic.

2. STRUCTURAL X CHROMOSOME CHANGES IMPAIRING FEMALE FERTILITY

The most prominent example of X-chromosomal changes that impact fertility in women is the complete absence of the second X chromosome, causing a pathology known as Turner's syndrome (TS). Physical findings in patients with Turner's syndrome (TS patients) often include congenital lymphedema, short stature and gonadal dysgenesis [11, 12, 13]. The rate of atresia, which reduces the number of oocytes in the ovary of every female fetus to roughly one million before birth, is strongly increased in TS patients [14]. Consequently, infants with Turner's syndrome with a sufficient number of follicles formed prenatally suffer from a depletion of nearly all oocytes at the time of birth. This

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results in primary amenorrhea and in ovaries that exist only as fibrous streaks. It has thus been hypothesized that female fertility and normal ovarian function relies on the presence of two functional X chromosomes and that X genes, functional for early ovarian maintenance, must be expressed in a double dose [15,16].

It has been stated that deletions of the short arm of the X chromosome usually result in primary amenorrhea, whereas deletions of the long arm of the X chromosome result in either primary or secondary ovarian failure [13]. Secondary ovarian failure is characterized by follicular dysfunction or premature follicular depletion after normal puberty [17]. Consequently, both the short arm and the long arm of the X chromosome seem to contain genes important for ovarian function. By mapping the breakpoints of X-chromosomal autosomal translocations in patients with POF, two major regions in Xq were identified which should contain putative POF candidate genes: *POF1* (Xq26-q28) and *POF2* (Xq13.3-q22) (Fig. (1)).

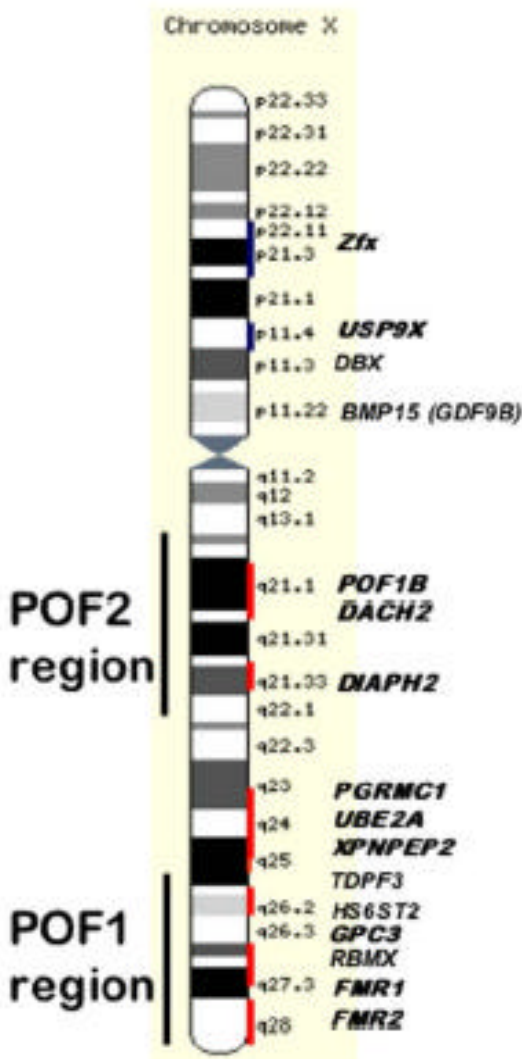


Fig. (1). Schematic view on the G-banding structure of the human X chromosome. On the left the putative extensions of the POF1 and POF2 regions are indicated. On the right a number of putative POF candidate genes have been mapped according to the literature. For further discussion see text.

2.1. *POF1* Region (Xq26.2-q28, OMIM: 311360)

More than 20 years ago, small deletions detected in the long arm of the X chromosome in patients with POF syndrome presented first evidence for the existence of a locus functional in human folliculogenesis in the distal long arm of the X chromosome (Xq26-qter, [18]). This finding was corroborated in subsequent studies which identified more POF patients with deletions in distal Xq [19, 20]. Molecular deletion analysis with probes from the Xq region in six patients with POF narrowed this so called *POF1* region to Xq26.2-q28 [21]. The *POF1* sequence domain covers about 22 Mb, and, according to sequence analysis, contains about 190 known genes (http://www.ensembl.org/Homo_sapiens/mapview?chr=X) which by location are all putative POF candidate genes. The most prominent *POF1* candidate gene among these is the *Fragile site Mental Retardation 1 (FMR1)* gene [22]. A molecular breakpoint analysis conducted in a POF family with the karyotype 46,X,delX(q26) identified three further prominent *POF1* candidate genes, namely *Heparan Sulfate 6-O-Sulfotransferase 2 (HS6ST2)*, *Transcription Factor DP Family, member 3 (TDPF3)* and *Glypican 3 (GPC3)* [23].

2.2. *POF2* Region (Xq13.3-q22, OMIM: 300511)

The second POF locus (*POF2*) in Xq13.3-q22 was first described in a patient with a paternally derived balanced translocation 46,X,t(X;6)(q13.3-21;p12) [24]. Molecular studies in more POF patients with X-autosome translocations revealed the *POF2* candidate genes *Diaphanous Homolog 2 (DIAPH2)* [25], *Dachshund Homolog 2 (DACH2)* and *Premature Ovarian Failure, 1B (POF1B)* [26] by breakpoint mapping (Fig. (1)). The first of these genes, *DIAPH2*, spans about 1 MB and is located in Xq22. Alternative splicing results in two protein isoforms. Furthermore, each splice variant undergoes additional splicing in the 3' UTR (HUGO-Gene-ID: 1730; Locus: HGNC:2877; MIM: 300108). A deletion of the homologous *Drosophila Diaphanous* gene affects spermatogenesis and oogenesis and leads to infertility in the fruit fly. Therefore, it is most likely that chromosomal disruption of the human *DIAPH2* gene causes the associated POF syndrome [25].

The *POF1B* and *DACH2* genes are located 700 kb apart, proximal to the centromere in Xq21 (Fig. (1)). *POF1B* comprises 17 exons and spans about 100 kb of genomic DNA; *DACH2* contains 11 exons and spans about 700 kb of genomic DNA (*POF1B*: Gene-ID: 79983 Locus: HGNC:13711; *DACH2*: Gene-ID: 117154; Locus: HGNC:16814). Both genes were found to be interrupted by X-autosomal translocations in patients with POF [27, 28]. Putative functions of these genes are expressed during early ovarian development (*POF1B*) and during the follicular differentiation process (*DACH2*) [26]. Molecular genetic mutation analyses confirmed the involvement of *DACH2*, but not yet of *POF1B* in the POF phenotype [26].

Female folliculogenesis genes, which if mutated can cause the POF syndrome, seem to be concentrated on the X chromosome (Fig. (1)). This emphasizes a major role of the female sex chromosomes in maintaining both folliculogenesis -the physiological processes promoting ovarian

function- and oogenesis -the germ cell maturation process- leading to a mature oocyte which is fully competent to undergo fertilization and embryo development. A number of female fertility genes are also spread on autosomes. However, although these X and autosome genes are without any doubt functional in human folliculogenesis, their role in the pathogenesis of POF is mostly unknown because of the lack of gene-specific “de novo” mutations that means being found only in the gene of the patient and associated with a specific ovarian pathology. Some prominent human folliculogenesis genes with and without any mutation defects including the POF syndrome are presented below.

3. TRANSFORMING GROWTH FACTOR-β (TGF-β) GENE SUPERFAMILY

In addition to the coordination of the ovarian function by the endocrine hypothalamic-pituitary-ovarian system, autocrine and paracrine regulation of the follicular microenvironment in maintaining folliculogenesis and oogenesis is important as well. Several of the key regulator factors of these processes expressed in oocyte and granulosa cells are members of the *Transforming Growth Factor-β* (*TGF-β*) superfamily [29, 30]. It includes 35 members of the *activin/inhibin*, of the *TGF-β*, of the *Bone Morphogenetic Protein (BMP)* and of the *Growth Differentiation Factor*

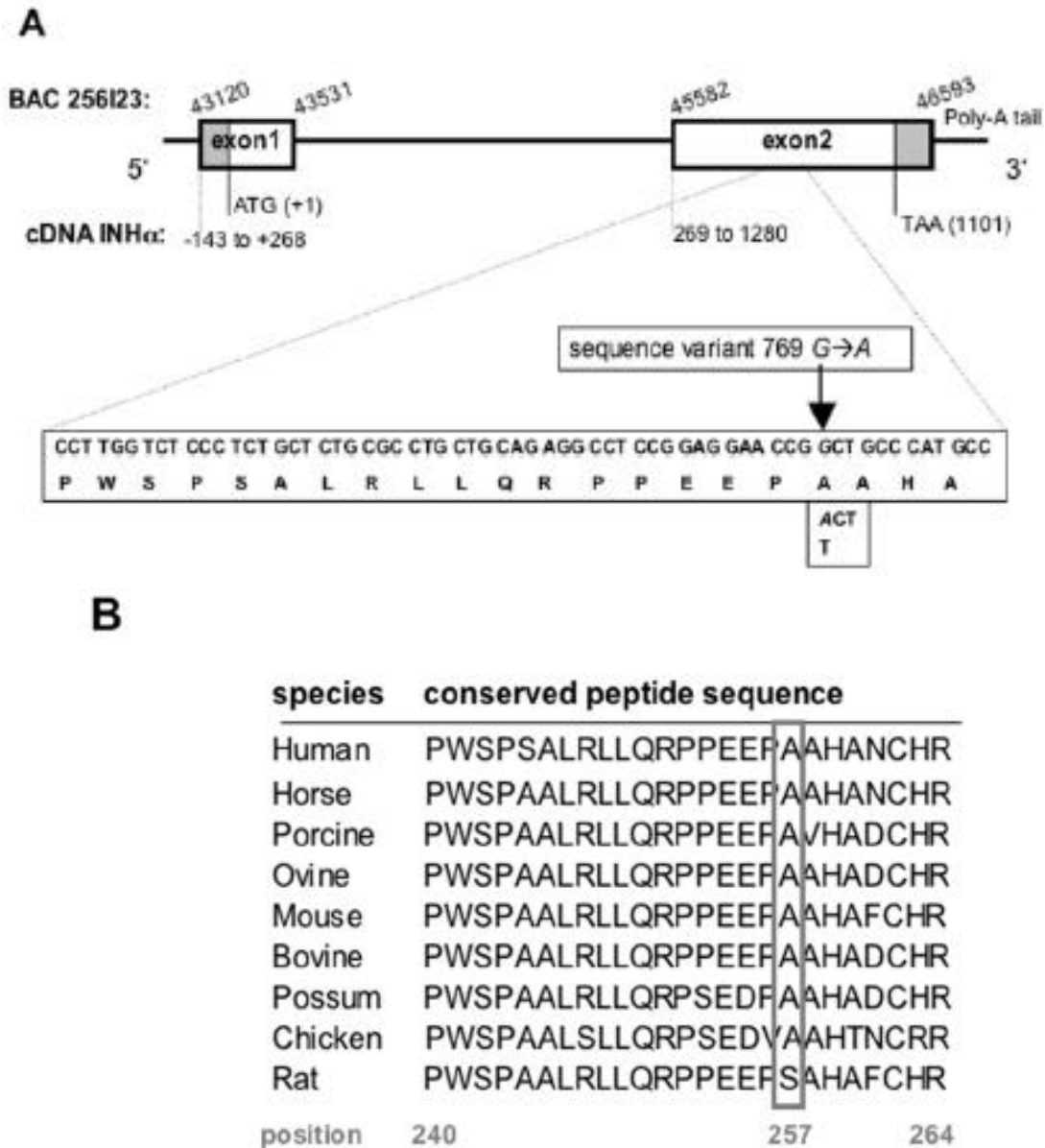


Fig. (2). Structure and sequence variant of the *INHα* gene. In (A) the exon-intron structure of the *INHα* cDNA is schematically shown in relation to its location on the genomic sequence in RP-11-BAC 256I23 (GenBank accession number: AC009955.4). Numbering the cDNA sequence set the begin of the translated sequence part (ATG) to +1. The flanking 5'UTR and 3'UTR sequence regions are shadowed. In exon 2 the sequence is expanded between 718-780 and displayed with the corresponding amino acid sequence in order to focus on the sequence variant in position 769. The G to A transition at that site replaces alanine (A) with threonine (T) in the corresponding *INH* protein sequence as indicated. (B) Comparison of the same *INH* peptide sequence in different species indicates its high conservation during vertebrate evolution [44]. Alanine at position 257 is replaced by serine only in rat.

(*GDF*) subfamilies [31]. Many of these members play an important biological role in human folliculogenesis. A description of the most prominent POF candidate genes in this superfamily, namely *BMP15*, *GDF9* and *INH α* is given separately.

3.1. Bone Morphogenetic Protein 15 Gene (*BMP15*, OMIM: 300247)

The *Bone Morphogenetic Protein 15 (BMP15)* gene, also named *GDF9B*, is encoded by two exons and maps to the X chromosome in Xp11.2 (Fig. (1)) [32]. *BMP15* expression is restricted to male and female gonads. The *BMP15* protein is localized in oocytes of large primary follicles, but not yet in those of small primary follicles [33]. *BMP15* protein expression in oocytes then increases steadily throughout further follicular development [34].

The impact of the *BMP15* gene on oogenesis in humans was recently recognised in the context of a case study describing two sisters with a normal female karyotype (46,XX) but with hypoplastic gonads and primary amenorrhea. A heterozygous variant with an amino acid exchange Tyr235-Cys in the *BMP15* gene was found as the putative causative agent. The non-affected mother had the genomic *BMP15* sequence of the general population, whereas the healthy father was hemizygous for the mutated variant [35]. Function of the *BMP15* gene is, therefore, most likely not restricted to the female germ cell but also expressed in the associated gonad tissue.

3.2. Growth Differentiation Factor 9 Gene (*GDF9*, OMIM: 601918)

The *Growth Differentiation Factor 9 (GDF9)* gene is also encoded by two exons and maps to chromosome 5 (5q23.3). In mice, *Gdf9* is expressed exclusively in mouse oocytes in all stages of folliculogenesis, except in primordial follicles [36]. Female *Gdf9* knockout mice are infertile because they can only develop primordial and primary follicles. The fact that further stages of follicle maturation are missing shows that *Gdf9* is a key gene required for the development of secondary follicles [37]. Human *GDF9* transcripts are expressed in ovarian and nonovarian tissues, including uterus, pituitary and bone marrow [38]. Translation of *GDF9* to the respective protein occurs in oocytes during early folliculogenesis and precedes expression of the closely related *BMP15* protein in primary follicles (see paragraph 3.1 above). Recent studies reveal that *GDF9* and *BMP15* proteins are most likely growth factors co-operating synergistically during folliculogenesis to regulate proliferation and gonadotropin-induced differentiation of granulosa cells in mammals [39].

3.3. Inhibin α Gene (*INH α* , OMIM: 147380)

Inhibin is one of the most important regulators of the female reproductive cycle. It is composed of an α subunit and one of the two β subunits, either A or B. Depending from the β subunit obtained, the protein is named Inhibin A or Inhibin B. The protein is produced in the granulosa cells of the ovary reducing hypophysial FSH secretion [40]. Apart from this hormone function, inhibin may also play a role as

a growth and differentiation factor [29] and in the regulation of follistatin expression [41]. In pre-menopausal women, where clinical symptoms of menopause have not yet become apparent, inhibin levels in the blood serum are decreasing due to a considerable reduction of follicles in the ovary. Inhibin can thus serve as a serum marker for indicating the follicular capacity of the ovary [42].

Betaglycan and *InhBP/p120* have been discussed as two putative inhibin receptors since both proteins are able to promote inhibin-mediated antagonism of activin signalling [43]. However, since both do not generate inhibin-specific intracellular signals, their classification as an inhibin receptor in ovarian cells is still doubtful.

The *Inhibin α (INH α)* gene is a two exon gene located on chromosome 2 (2q33-qter). Screening for mutations of the *INH α* sequence in POF patients revealed a polymorphism in the second exon of the gene sequence (769 G \rightarrow A) (Fig. (2A)), occurring in three of the 43 POF patients examined [44]. The variant G \rightarrow A substitutes alanine for threonine at position 257 in a highly conserved sequence region of the *INH* protein (Fig. (2B)). This suggests a functional role of this mutation site in receptor binding since the putative receptor binding site is close to it [44]. Since the same sequence variant was also found in only one of 150 healthy controls, an association between this sequence mutation and the occurrence of POF in these patients was therefore postulated. These findings were indeed confirmed in a larger Italian patient collective [45] and in Indian patients [46]. However, in Korean patients the polymorphism was found neither in POF patients nor in controls [47].

4. AUTOIMMUNE REGULATOR GENE (*AIRE*, OMIM: 607358)

The *AutoImmune REgulator* gene (*AIRE*) gene consists of 14 exons spanning 11.9 kb of genomic DNA and is located on chromosome 21 (21q22.3) [48]. The *AIRE* protein was found to be located in the nucleus in multiple human tissues, suggesting that it probably functions as a regulator of gene transcription. In mice, wildtype *Aire* protein is a strong activator of transcription [49, 50]. It is also involved in the ubiquitin proteasome pathway, functioning as an E3 ubiquitin ligase with its PHD-type zinc finger motifs [51].

Mutations in the *AIRE* gene cause APECED (autoimmune polyendocrinopathy-candidiasis-ectodermal dystrophy), a clinical pathology characterized by the typical triad: hypoparathyroidism, primary adrenocortical failure and chronic mucocutaneous candidiasis [52]. In addition, the clinical phenotype consists of a combination of other autoimmune reactions towards multiple organs, among which are the gonads and pancreas. The prevalence of ovarian failure in this patient group is estimated to be 60% [52].

5. DELETED IN AZOOSPERMIA LIKE GENE (*DAZL*, OMIM: 601486)

The *Deleted in AZoospermia Like (DAZL)* gene consists of 10 exons and is located on chromosome 3 (3p24). Together with *BOULE* on chromosome 2 (2q33) and *DAZ*

on the long arm of the Y chromosome (Yq11.23), it forms the *DAZ* gene family. These genes encode a conserved family of RNA-binding proteins which are expressed specifically in germ cells and if deleted would impair male and female fertility [53].

In mouse models it has been demonstrated that both male and female homozygous *Dazl* knockout mice are infertile [54]. In embryonic and pre-pubertal ovaries, *Dazl* protein is found in the cytoplasm of the oocyte and in granulosa cells. In adult follicles the location is consistent with that of the zona pellucida produced by the maturing oocyte. In humans, *DAZL* mRNA can be found abundantly in testis and ovarian tissue but not in other tissues such as heart, liver or brain [55]. The protein appears in the cytoplasm of the oocyte in primary, secondary and tertiary follicles (Fig. (3)) [55, 56] and in the corpus luteum [57]. It can therefore be assumed that the *DAZL* gene plays a key role in male and female early germ cell development, and that its absence will express a pathogenic POF phenotype.

6. DEAD-BOX 3, X-LINKED GENE (DBX OR DDX3X, OMIM: 300160)

The *DEAD-Box 3, X-linked (DBX)* gene consists of 17 exons and is located on the short arm of the X chromosome (Fig. (1)) in Xp11.23-p11.3 [58]. It belongs to the large family of DEAD-box proteins. These are RNA helicases containing the conserved sequence motif Asp-Glu-Ala-Asp (DEAD) functional as ATP binding domain [59]. They are involved in all processes of the cellular RNA metabolism, e.g. transcription, splicing, ribosomal biogenesis, RNA export and translation. All human DEAD-box RNA helicases have been now categorised under the *DDX* gene family symbol adding a distinct number for each specific DEAD-box gene [60].

The *DBX* gene is highly conserved and homologues are found from yeast to human. The homologous *Drosophila*

protein *belle* is required for male and female fertility and is essential for larval growth in the fruit fly [61]. In *Xenopus*, mRNA expression of the homologous *DBX* gene (*An3*) is found in oocytes. The subcellular localization of the respective protein is variable during oocyte development. In earlier stages, *An3* protein is found in both the nucleus and the cytoplasm, whereas in the last stage, *An3* protein is only detectable in the cytoplasm. This differential expression throughout oogenesis suggests a role in the production, processing and transport of RNA during oogenesis [62, 63]. In zebra fish and mice, the *DBX* homologous RNA helicase *zPL10* and *Dbx*, are also involved in germ cell metabolism. Expression of the *zPL10* gene is restricted to primordial germ cells [64] whereas expression of the mouse *Dbx* gene was also found in mature oocytes [65].

Expression of the *DBX* homologous gene in the female germ line of *Drosophila*, frog, zebrafish and mouse strongly suggests that this function is conserved and that also the human *DBX* gene most likely plays a functional role in human folliculogenesis. The impairment of female fertility such as POF can therefore be postulated in case of a *DBX* deletion or gene mutation disrupting the *DBX* female germ line function.

7. FRAGILE-SITE-MENTAL-RETARDATION 1 GENE (FMR1, OMIM: 309550)

The *Fragile-site-Mental-Retardation 1 (FMR1)* gene is located in the *POF1* region in Xq27.3 (Fig. (1)). It spans 38 kb and encodes a 4.4 kb transcript consisting of 17 exons. Alternative splicing of some terminal exons produces a number of *FMR1* protein (*FMRP*) isoforms (Fig. (4)) [66, 67, 68]. Dysfunction of the *FMR1* gene results in the clinical phenotype of the Fragile-X-syndrome also called Martin-Bell syndrome (OMIM: 309550). It is characterized by mental retardation, macroorchidism and an abnormal facies. Its molecular origin is an expansion of the CGG trinucleotide repeat to more than 200 units in the 5'

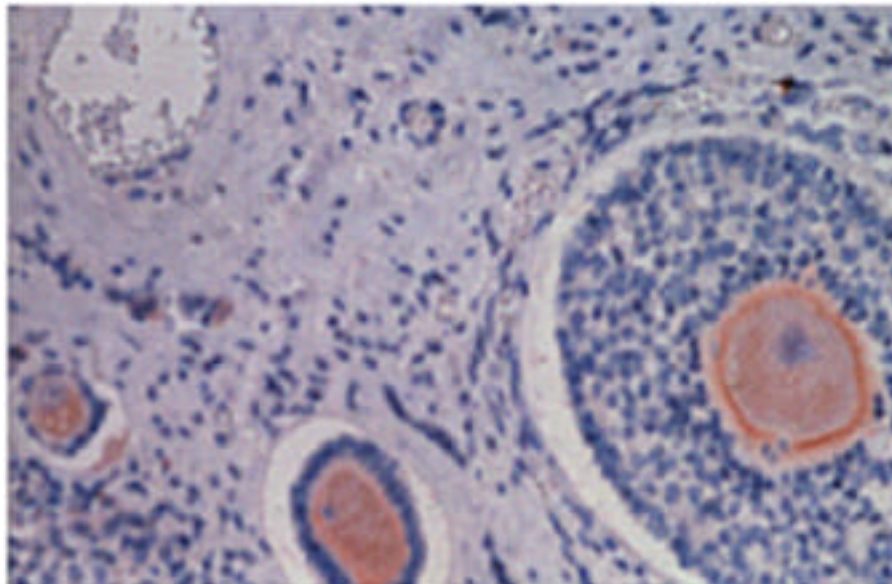


Fig. (3). Immunohistochemical staining pattern of human ovarian tissue section with a polyclonal antiserum specific for human *DAZL* protein (anti-*DAZL1*). The *DAZL* protein is shown to be expressed in the oocyte in primordial, primary and secondary follicles.

untranslated region of exon 1 of the *FMRI* gene causing hypermethylation of the underlying CpG island which starts upstream of exon 1 and extends to intron 1 of the *FMRI* gene (Fig. (4)). Hypermethylation inhibits *FMRI* transcription and consequently stops the synthesis of FMRP [69]. Consistent with the primary features of the clinical phenotype, *FMRI* mRNA is highly expressed in the male gonads and in the fetal and adult brain [70].

Male and female carriers of the so called premutation of the fragile-X-syndrome are characterized by 60-200 repeats of the CCG triplet in the *FMRI* 5'UTR-sequence. Although these individuals do not have phenotypic symptoms of the fragile-X-syndrome, the increased number of CGG repeats is rather unstable extending further in the following generation and therefore leading to the full fragile-X phenotype one to three generations later, a phenomenon known as anticipation.

Interestingly, ~20% of premutation carriers suffer from the POF syndrome [71]. A screening of 147 patients with POF found six women with premutations, but no woman with the full mutation [71]. In 395 women from fragile X families who were carriers of the premutation, 16% experienced menopause prior to the age of 40, compared with 0% among carriers of the full mutation and 0.4% in controls [72]. The fragile X premutation can therefore be considered as a significant risk factor for POF. An analysis of the effect of the *FMRI* CGG repeat size on ovarian function revealed a significant positive association between repeat size and ovarian pathology. It has been postulated that an 5'UTR *FMRI* CCG repeat size exceeding the threshold of 80 repeats contributes to a significant risk for ovarian dysfunction by an increased transcription level of the *FMRI* gene leading to reduction of FMRP protein in the female gonad [22, 69, 73].

8. FORKHEAD-TRANSCRIPTION-FACTOR-LIKE-2 GENE (FOXL2, OMIM-NR.: 605597)

The *Forkhead-Transcription-Factor-Like 2* (*FOXL2*) gene is located on chromosome 3 (3q23) and codes for a

transcription factor belonging to the winged helix/forkhead family. Forkhead proteins can be found in all eukaryotes playing important roles in the development of the body axis and in the development of embryonic tissue. Over 20 forkhead proteins have been discovered in humans so far, the dysfunction of which is also involved in the aetiology of various tumours [74]. *FOXL2* is expressed exclusively in the ovary and in the developing eyelid, the two organ systems which consequently are affected in case of mutation [75]. A great number of mutations in the *FOXL2* gene cause a complex phenotype commonly described as BPES (blepharophimosis-ptosis-epicanthus inversus syndrome) [76, 77, 78]. BPES occurs in two variants: BPES type I affected patients show characteristic facial dysmorphia: a reduction in horizontal fissure length, a drooping upper eyelid, epicanthus inversus and the POF syndrome. Affected patients with BPES type II only show the facial dysmorphia. In the first case (BPES I) nonsense-mutations in *FOXL2* and deletions lead to synthesis of a truncated protein, in the latter case (BPES II) missense and frame shift mutations or duplications within the *FOXL2* gene produce a larger protein [75]. However, the genotype-phenotype correlation is variable, meaning that the kind of mutation does not always permit a definite conclusion about the corresponding clinical pathology [78]. Despite this variability, an isolated POF syndrome without facial dysmorphia caused by *FOXL2* mutations was not yet found. In three studies, which altogether screened 220 patients with isolated POF syndrome for *FOXL2* mutations, no gene mutation could be identified [76, 79, 80].

It might therefore be concluded that patients with dysfunction of the *FOXL2* gene only suffer from POF when they also suffer from BPES. POF patients in the clinic should therefore always be carefully diagnosed for the typical BPES facial dysmorphic phenotype because this phenotype would predict that a *FOXL2* gene mutation is most likely the causative agent of the patient's POF syndrome. This can then be confirmed by analysis of the patient's *FOXL2* exon sequence.

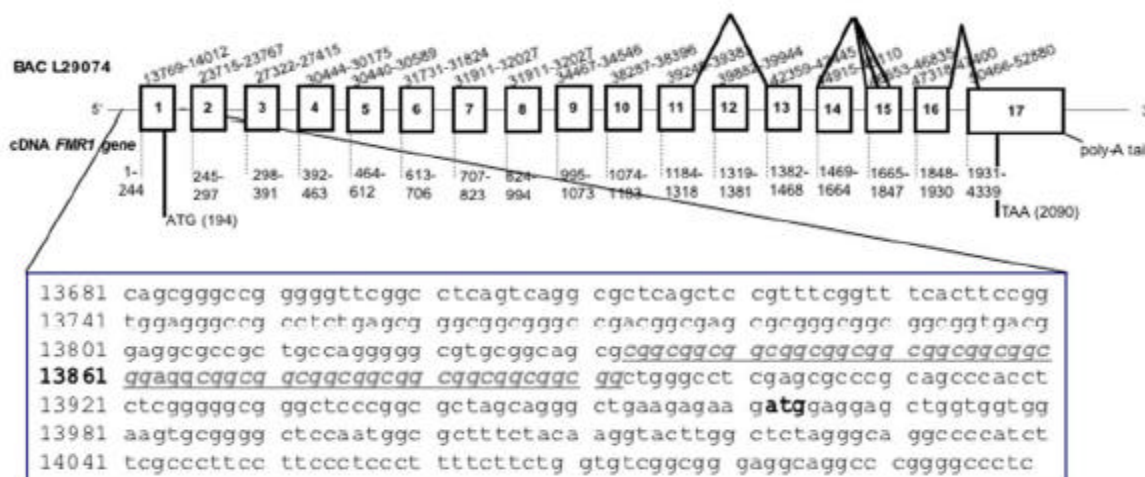


Fig. (4). Alternative splicing events of *FMRI* primary transcripts. Schematic view on the exon-intron structure of the *FMRI* gene located on the genomic sequence of BAC L29074 (GenBank accession number: L29074). Numbering of the cDNA sequence (Ensembl accession number: ENST 342305) starts with 1 at exon 1, the translation start codon ATG is located at nucleotide position 194. Below the *FMRI* exon structure the genomic BAC sequence around exon1 is expanded to the nucleotide format to display the repetitive CCG repeat (marked in *italic*) which can be variable in its extension. Above the number of 200 repeats it causes repression of *FMRI* gene transcription resulting in the pathology of the Fragile X syndrome. For further description see text.

9. FOLLICLE STIMULATING HORMONE RECEPTOR GENE (FSHR, OMIM: 136435)

The *FSHR* gene encoding the FSH (Follicle Stimulating Hormone) receptor protein is located on chromosome 2 (2p21-p16). It contains 10 exons and spans 54 kb of genomic DNA [81]. The FSHR protein contains 7 putative trans-membrane segments and binds FSH, which is one of the most important hormones for maintenance and control of the male and female reproduction cycle.

Mutations in the *FSHR* gene can cause various alterations of its physiological function, including premature ovarian failure. Dependent on the grade of *FSHR* malfunction, its mutations can result in primary or secondary amenorrhea and in a variable development of the secondary sex characteristics [82]. The first variant identified, ALA189VAL, was found to be associated with the occurrence of hypergonadotrophic ovarian dysgenesis in some Finnish patients with normal karyotype [83, 84]. The compound heterozygous ILE160THR and ARG573CYS mutations in an Armenian woman caused premature ovarian failure at the age of sixteen years although the development of secondary sex characteristics was normal [85]. Other allelic variants of the *FSHR* gene associated with the occurrence of ovarian dysgenesis are ALA419THR [86] and PRO519THR [87]. In these cases, functional *FSHR* testing revealed an altered signal transduction after binding of FSH with a very low cAMP second messenger response.

Some allelic *FSHR* sequence variants, THR449ILE [88], ASP567ASN [89], and THR449ALA [90], cause ovarian hyperstimulation. Association of the *FSHR* variant, PHE591SER, with an increased rate for sex cord tumours [91] and observation of a high prevalence of dizygotic twinning associated with the THR307ALA variant [92] indicate that function of the *FSHR* protein is not restricted to the female germ line but may also be involved in some other important signal pathways functional during early human female embryogenesis.

10. GALACTOSE-1-PHOSPHATE URIDYLYLTRANSFERASE GENE (GALT, OMIM: 606999)

The *Galactose-1-Phosphate Uridyltransferase (GALT)* gene is located on chromosome 9 (9p13) [93]. It consists of

11 exons and is about 4 kb long [94]. The enzyme, galactose-1-phosphate uridylyltransferase catalyzes the inter-conversion of galactose-1-phosphate and glucose-1-phosphate via transfer of uridine monophosphate. Mutations in the *GALT* gene cause galactosemia, a condition characterized by growth and mental retardation, unless galactose is omitted from the nutrition. More than 150 *GALT* gene mutations causing galactosemia are reported. Of those, mainly the homozygous mutant genotype Q188R/Q188R is associated with the occurrence of POF syndrome [95]. Several reports state that women with galactosemia have generally a higher risk of developing POF [95, 96, 97]. The underlying pathogenetic mechanism most likely is expressed already during fetal folliculogenesis when an impaired migration of germ cells is caused by a high level of galactose or metabolites of galactose that are toxic at this phase of development [98].

11. UBIQUITIN-SPECIFIC PROTEASE 9, X-LINKED GENE (USP9X, OMIM: 300072)

The *Ubiquitin-Specific Protease 9, X-linked (USP9X)* gene is located on the X chromosome near the *DBX* gene in Xp11.4 (Fig. (1)) and encodes an ubiquitin-specific protease. Deubiquitinating enzymes such as *USP9X* are substrate specific cysteine-proteases involved in regulation of the cellular ubiquitin metabolism which controls many intracellular processes e.g. cell cycle, apoptosis and signal transduction pathways [99]. In contrast to the large class of non-specific ubiquitin proteases, *USP9X* catalyze the deubiquitination of specific substrates. The *USP9X* gene is the human homolog of the *Drosophila* gene *faf* (*faf* facets) [100] and of the mouse gene *Fam* [101, 102]. In mice, the *Fam* protein interacts specifically with the substrates -catenin and AF-6 [103]. Without expression of *Fam* during the preimplantation stage, development of the mouse embryo into a blastocyst is not possible [104]. In *Drosophila*, *faf* is necessary to regulate the development of eyes and oocytes [105, 106]. In *Drosophila* pole cells the germ line specific DEAD-box RNA helicase Vasa is specifically stabilized by *Faf* protein [107]. Animal models therefore suggest an important conserved function of *USP9X* also expressed in human folliculogenesis. Like *DBX*, the *USP9X* gene is

Table 1. POF Candidate Genes Expressed In Folliculogenesis

gene	OMIM-nr.	prognosis of clinical pathology in case of dysfunction or deletion
AIRE	607358	autoimmune-polyendocrinopathia-candidiasis-ectodermal-dystrophia (APECED; OMIM: 240300)
BMP15	300247	gonadal dysgenesis
DAZL	601486	primary amenorrhea
DBX	300160	primary amenorrhea
FMR1	309550	Fragile X syndrome and POF (premutation)
FOXL2	605597	Blepharophimosis-ptosis-epicanthus-inversus syndrome and POF
FSHR	136435	ovarian dysgenesis
GALT	606999	galactosaemia (OMIM-Nr.: 230400) and POF
GDF9	601918	primary or secondary amenorrhea
INH	147380	secondary amenorrhea
USP9X	300072	primary amenorrhea

located in a region of the X chromosome (Xp11.4) which escapes X inactivation. It is therefore believed that both these genes also play a critical role in the pathogenesis of the Turner-syndrome.

An alphabetical list of the POF candidate genes discussed above with description of their follicular pathology to be expected in the case of dysfunction is given in Table 1. For a better understanding of their functional contribution to the genetic aetiology of POF, an interdisciplinary scheme for their diagnostic analysis will now be presented in the form of a pilot study focussed on chromosome analyses and the expression analysis of some major POF candidate genes (*DAZL*, *DBX*, *FOXL2*; *INH α* , *GDF9*, *USP9X*) in the leukocytes of 101 POF patients. It starts with a comprehensive and significantly improved clinical diagnostic program to subdivide this large and heterogenous patient group.

12. MOLECULAR CLINICAL DIAGNOSTIC SCHEME FOR REVEALING THE GENETIC COMPLEXITY OF POF

In order to study a putative contribution of one of the genetic factors listed above to the pathology of POF, first a sophisticated clinical questionnaire is required (*step 1*) to distinguish clinical subgroups [108]. This is followed by chromosome analysis (karyotyping) to select all cytogenetically visible genetic abnormalities (*step 2*). The subsequent molecular diagnostic analysis is then focussed on the expression analysis of the key folliculogenesis genes expressed in leukocytes and ovarian tissue samples (*step 3*).

12.1. Clinical Evaluation Questionnaire (*Step 1*)

An extensive clinical history protocol including pedigree analysis and guidelines for a comprehensive clinical examination of the patient has been developed to distinguish genetic from non-genetic factors causing the patient's POF syndrome. It starts with evaluation of the general and of the gynecological clinical history of each POF candidate patient including a thorough inspection of the patient's amenorrhea. Patients were also asked if they themselves or a member of their family were affected by some autoimmune diseases (e.g. Addison's disease) or metabolic diseases (e.g. galactosemia) known to be associated with POF. A careful inspection for any facial dysmorphic phenotypes reminding the BPES syndrome (see paragraph 8 above) would define the *FOXL2* POF patient subgroup and focus the subsequent

genetic analysis on a mutation screen in the patient's *FOXL2* exon sequence.

Iatrogenic causes of POF were excluded by asking the patients about past and present gynecological and oncological diseases and their treatment. If the patient has been treated by chemotherapy or radiotherapy, this can be a cause of POF, since such a therapy usually affects also the gonads [17]. For the same reason, a possible exposure to infectious and toxic agents has to be clarified as well. POF patients with a positive record after these questions represent the major "non-genetic" group of POF patients.

In all cases the gynecological examination of each POF patient is complemented by sonography to carefully evaluate the morphology of the uterus and ovaries and to distinguish between atfollicular and follicular forms of POF [109]. Biochemical analyses of the patient's blood samples include estimations of the level of FSH, LH, inhibin and estradiol hormones. POF is positively diagnosed after finding a repeated hypergonadotrophic hormone status with FSH values of >40 IU/L [17] and significantly decreased inhibin levels in the blood serum [42].

In the first molecular clinical screening program of 101 patients with a putative POF pathology enrolled from 2002 till 2005 in our outpatient clinic, 22 were identified as "non-genetic" based on this clinical evaluation record. This suggests a major contribution of genetic factors to the clinical occurrence of the POF syndrome.

12.2. Karyotype Analysis (*Step 2*)

The first step for the identification of POF patients with a putative genetic origin is the determination of the patients' chromosome constitution (karyotype). It selects the first "major" genetic subgroup of POF patients and distinguishes patients with the 45,X0 karyotype associated with Turner's syndrome. Major chromosome aberrations associated with POF are especially X-autosomal translocations mapped with their breakpoint in one of the POF regions (POF1 and POF2) of the X chromosome as described above (Fig. (I)).

We selected 79 patients with a putative genetic cause of the POF syndrome from the clinical evaluation of the 101 POF patients for the subsequent karyotype analysis. A normal karyotype (46,XX) was found in 71 patients (90%) after evaluation of 40-50 metaphases in the patients' leukocyte nuclei. The abnormal karyotypes in the remaining eight patients (10%) were mainly X chromosomal mosaics beside some autosomal structural variants. Most interesting

Table 2. Molecular Clinical Diagnostic Schedule For POF Patients

step	diagnostic procedure	aim	consequence
1	clinical evaluation according to detailed and specific questionnaire	selection of idiopathic, genetic and non-genetic POF patients	all patients with non-genetic POF syndrome can be excluded
2	karyotype analysis	detection of possible chromosomal aberrations	patients with any chromosomal abnormalities are excluded (e.g. Turner's syndrome)
3	RNA expression analysis in leukocytes and ovarian tissue samples	identification of quantitative variability or failure in expression of POF candidate genes	sequence analyses of genes with abnormal expression profiles

was identification of POF75 with a breakpoint in the *POF2* region of the X chromosome (Table 3). A similar frequency of chromosomal abnormalities especially of the sex chromosome was also found in other studies ranging from 13.3% in a screen of 30 patients [110] to 21.3% in a screen of 47 patients [111] (for further references see paragraph 2 above).

Whether the low X-chromosomal aneuploidies found in POF10, POF19, POF85 and POF102 are causing the patients' POF syndrome is difficult to judge because the number of X aneuploid cells in the patients' leukocytes was generally low (2-6%). However, since quantitative, tissue-specific differences in the number of these aneuploid cells are well known in the literature the number of X aneuploid cells present in the patients ovary may be different from that in leukocytes. This patient subgroup will therefore now be asked for an ovarian tissue sample in order to estimate the number of X chromosomes also in the cells of the target organ.

The structural variants of chromosome 14 observed in POF4 and of chromosome 9 in POF25 and POF67 are especially interesting because they are not yet described as being associated with the POF syndrome. Unfortunately, we were not yet able to study the karyotype of some family members of these patients' pedigrees in order to exclude a possible polymorphic inheritance pattern of these chromosome variants. Only if presence of these variants would be restricted to the patients' karyotype ("*de novo*" mutation) a possible association with occurrence of the patients' POF syndrome might be considered.

12.3. Molecular Expression Analysis of POF Candidate Genes in Leukocytes (Step 3)

RNA expression analysis of POF candidate genes in POF patient's leukocytes is an attractive diagnostic tool for recognising folliculogenesis gene defects already in blood cells. It also shifts the possible need for a tissue sample of the patient's ovary to a later time-point. After leukocyte analysis has strongly indicated that the expression of a specific folliculogenesis gene is modified or disrupted, studies on the functional consequences of this genetic dysfunction in the patient's folliculogenesis can be focussed on this particular POF candidate gene. This clinical

diagnostic schedule also promises to be more practical because the patient would be more willing to agree to an ovarian biopsy *after* the detection of a putative functional POF gene mutation in her leukocytes. Expression analysis of the dysfunctional POF gene in the patient's ovary tissue sample will then be able to confirm the patient's leukocyte result and eventually be extended by an immunohistochemical analysis of the encoded protein using some follicular tissue sections for incubation with specific antisera.

However, not all genes functioning in the ovary are also transcriptionally expressed in leukocytes at a measurable level. From the POF candidate genes included in our leukocyte expression assays, *DAZL* and *FOXL2* have a restricted germ line transcription profile, i.e., being functionally expressed only in the ovary and the testis [55] and the developing eyelid (only *FOXL2* gene [75]). Although *INH* protein can be easily analysed in blood cells, transcription of the *INH α* gene seems to be also restricted to the ovarian tissue cells.

Despite of this, it is well known that each gene has a so called "basal expression level" in each cell type, including peripheral blood cells, which is independent of its functional status in this cell. This phenomenon also known as "illegitimate" or "ectopic" transcriptional gene activity [112] allows the transcriptional analysis of each gene also in leukocytes and also when this gene has no function in the blood cell. The method of ectopic gene transcription analysis in leukocytes has been used successfully in a study for the identification of genetic abnormalities in patients with haemorrhagic diseases such as Glanzmann thrombasthenia [113], in a study of patients with a metabolic disease such as phenylketonuria [114] and in a study of patients with Duchenne muscular dystrophy [115]. The transcriptional analysis of some candidate genes probably responsible for a distinct, genetically caused disease is therefore not necessarily dependent on the preparative isolation of an RNA sample from the often inaccessible target tissue [116].

Consequently, as long as there is no knowledge of a gene-specific mutation site, the transcriptional analysis of a disease candidate gene from the RNA population of the patient's leukocytes is quicker and more informative than its sequence analysis. RNA transcript analyses not only focus on the detection of functional gene mutants but also is able

Table 3. Abnormal Karyotypes In POF Patients

patient's lab code	karyotype	interpretation
POF 4	46, XX, 14p+	structural variant
POF 10	mos 45, X[2]/46 XX[47]/47,XXX[1]	low mosaic
POF 14	mos 45, X[2]/46 XX[47]	low mosaic
POF 25	46, XX, inv(9)(p11,q13)	structural variant
POF 67	46, XX, inv(9)(p11,q13),v3q11.2+	structural variant
POF 75	46, X,t(X,3)(q22,p26)	<i>POF2</i> locus breakpoint
POF 85	mos 46 XX[48]/47,XXX[1]/49,XXXX[1]	low mosaic
POF 102	mos 45, X0[3]/46 XX[47]	low mosaic

to detect promoter defects and possible splicing errors, becoming visible by modified gene expression profiles. Whether promoter defects are due to changes of chromatin condensation (“histon code”) or due to DNA-methylation of some target sites functional in binding specific transcription factors or transcriptional co-activators, or are direct mutations of the promoter sequence can then be analysed by appropriate experiments focussed on this gene region. Splicing errors usually result in the synthesis of a shorter or longer mRNA which can be simply identified by appropriate sequence analysis of the RT-PCR products.

We first examined 6 POF candidate genes listed in Table 1 for their expression profiles in the leukocytes of women without POF syndrome (positive control population). Three of them, *DAZL*, *INH α* and *FOXL2*, were known with a restricted transcription profile mainly observed only in the germ line. Three of them, *DBX*, *GDF9* and *USP9X*, were known to be expressed in multiple tissues including leukocytes [38, 100, 116]. All six POF candidate genes are readily expressed in leukocytes (Fig. (5)). However, we failed to establish a reproducible, semi-quantitative RT-PCR assay of the ectopic expression levels of the *INH α* and *FOXL2* gene in our female control samples. Expression was visible only by increasing the number of PCR cycles to 40 which is a borderline number and not advised for the semi-quantitative analysis of any specific gene transcription profile. Experiments with three cDNA aliquots prepared from the same RNA pool of a given positive control person always resulted in a variable and, therefore, non-reproducible amplification of the *INH α* and *FOXL2* leukocyte cDNAs. We therefore decided to exclude these POF candidate genes from our further leukocyte expression assays.

Interestingly, the specifically germ-line expressed *DAZL* gene was always found to be expressed in leukocytes. We

therefore developed a diagnostic duplex RT-PCR protocol for analysis of the leukocyte *DAZL* expression profile, including the ubiquitous expressed *DBX* gene in the same experiment. The density of the amplified PCR product of *DBX* thereby serves as an internal control of the *DAZL* expression level when compared in different RT-PCR assays. Technical problems e.g. due to a bad reverse transcription of the leukocyte RNA pool are easily revealed and eliminated in this way. A schematic view on the schedule of the applied duplex-PCR protocol is displayed in (Fig. (6)). In all cases, we noticed that negative results of the *DAZL* RT-PCR were becoming positive when repeated with a higher amount of the cDNA template or with a novel cDNA preparation reverse-transcribed from another RNA aliquot of the patient’s leukocytes. Expression analysis of POF candidate genes with only an ectopic, although reproducible, expression level in leukocytes such as *DAZL* might therefore entail a greater diagnostic effort by including internal controls.

A quick routine analysis of the expression profile of POF candidate genes in leukocytes can be performed if the gene in question has a ubiquitous expression profile like the *DBX*, *GDF9* and *USP9X* genes analysed in this pilot study. For an economic use of the costly consumables essential for reproducible RNA extraction and cDNA reverse transcription we developed a triplex RT-PCR protocol. This strategy also saves some time, if a large number of POF patients needs to be analysed. In addition, the PCR multiplex approach inherently contains a number of internal controls that quickly disclose technical difficulties which may be associated with some leukocyte samples.

In order to identify not only the absence, but also, the putative variability of the leukocyte expression level of the POF candidate genes, we established a semi-quantitative

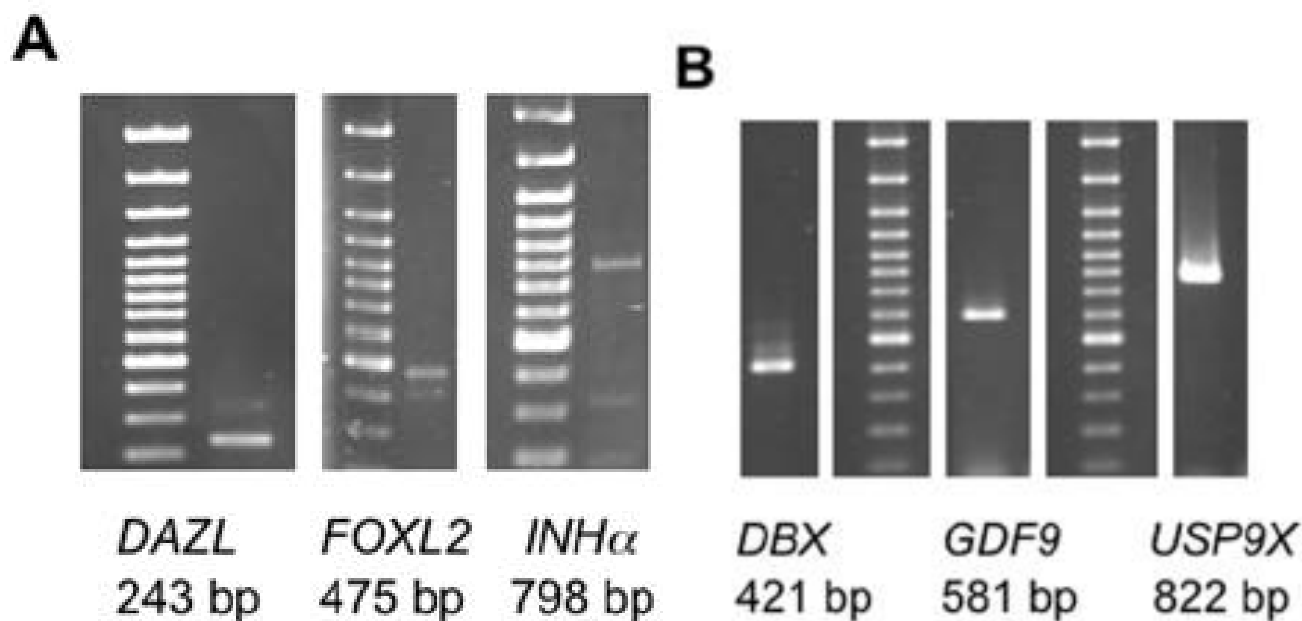


Fig. (5). Expression analysis of six POF candidate genes in leukocytes. The germ line expressed genes *DAZL*, *FOXL2* and *INH α* (A) and the ubiquitous expressed genes *DBX*, *GDF9* and *USP9X* (B) are displayed with the expected RT-PCR fragment lengths below the corresponding gel lanes. To exclude genomic DNA contaminations of the used cDNA samples reverse and forward primers overlapping two neighboured exons were designed for the RT-PCR experiments.

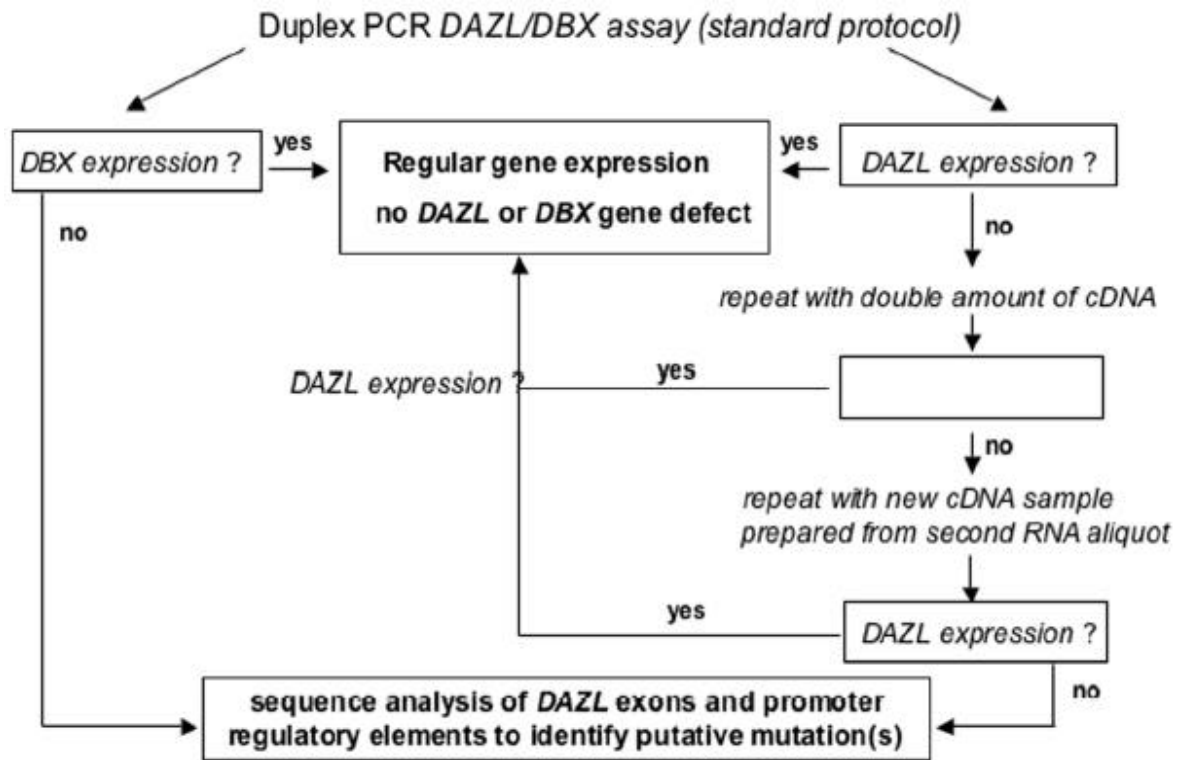


Fig. (6). Experimental schedule for RT-PCR duplex *DAZL-DBX* expression assay in leukocytes. The quantitative transcript level of the ubiquitous expressed *DBX* gene in leukocytes serves as an internal control of the transcript level of the ectopically expressed *DAZL* gene and its reproducibility under the different experimental conditions. Repeated experiments as indicated are necessary when the ectopic transcript level of *DAZL* in leukocytes is variable. After the different control steps to verify absence or variance of *DAZL* transcripts in leukocytes it can be concluded that this POF candidate gene indeed displays an aberrant expression probably because of the patient's POF syndrome. In this case sequence analysis of the *DAZL* exons is recommended including its promoter regulatory domains. For further discussion see text.

RT-PCR assay according to the protocol of Ditton *et al.* [117]. Using the same amount of gene primers for each gene analysed, quantitative measurements of the optical densities of the different PCR amplification products indicated the highest expression for *USP9X* in leukocytes, whereas *DBX* and *GDF9* transcript levels seemed to be comparable and distinctly lower than that of *USP9X* (Fig. (7A)). For diagnostic purposes we consequently defined the expression of *DAZL* as "low", the expression of *DBX* and *GDF9* as "intermediate" and that of *USP9X* as "high". Combining the three gene primer pairs in a triplex-PCR format the same pattern of the three transcript levels are displayed in one experiment when using the same cDNA amount (Fig. (7B)).

We used this triplex RT-PCR assay for analysis of the transcript levels of the *DBX*, *GDF9* and *USP9X* genes in the leukocytes of the 79 POF patients with a putative genetic cause of their ovarian pathology. We found a reproducible pattern in all cDNA samples analysed with similar expression levels as displayed in (Fig. (7)). We conclude that expression of these three POF candidate genes is most likely not involved in the occurrence of this patients' POF syndrome.

SUMMARY

The genetic complexity causing the POF syndrome can only be revealed step by step and after a comprehensive

analysis of the patients' clinical histories being very divergent. Putative genetic causes of POF are often X chromosomal abnormalities easily identified by normal karyotyping of the patient's leukocyte metaphase chromosomes. In our study population of 101 POF patients we were able to reveal 8 POF patients with a distinct chromosome abnormality (Table 3) suggesting karyotype analysis as an essential diagnostic tool for dividing the genetic complexity of POF in microscopically visible and non-visible genetic abnormalities. Based on our current knowledge of a number of folliculogenesis genes presumed to cause the POF syndrome if functionally disrupted, we set up specific RT-PCR assays for some of them analysing their semi-quantitative expression profiles in the POF patients' leukocytes. This seems to not only be possible for all POF candidate genes with an ubiquitous expression and therefore also in leukocytes, but also, for some genes expressed usually only in the germ line (like here *DAZL*) but also displaying an ectopic transcript level in leukocytes sufficient to obtain a reproducible result when analysed in appropriate duplex RT-PCR experiments with an internal control.

Some POF candidate genes like *INHα* and *FOXL2* seem to not be suitable for any RT-PCR based leukocyte expression assay because their ectopic expression level in these cells is too low. Therefore, we can only recommend an analysis of *FOXL2* exon sequence mutations after the patient's clinical history has revealed the typical BPES I

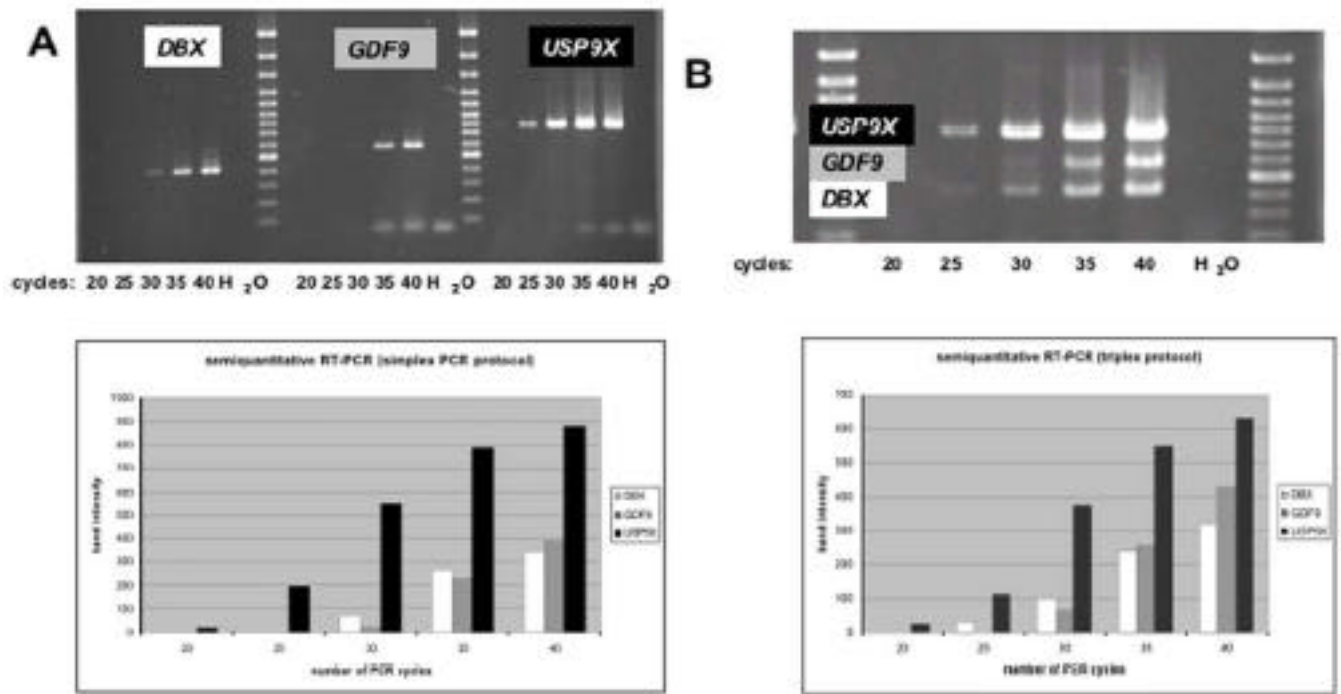


Fig. (7). Semi-quantitative analyses of leukocyte expression of the three POF candidate genes *DBX*, *GDF9* and *USP9X* according to the protocol of Ditton *et al.* [117]. **(A):** Simplex PCR experiment with the same amount of cDNA. An equal amount of primer pairs (12.5 nmol) and template concentration (50 ng) was used in all experiments. A graphical display of the same PCR reactions is displayed below the primary gel picture indicating the highest expression level for the *USP9X* gene. **(B):** Comparison of the semi-quantitative triplex RT-PCR assay for the same genes with the simplex PCR assay in **(A)** shows that both results are matching completely also in their graphical output. For this triplex PCR assay we estimated empirically a primer ratio of: *USP9X*: *GDF9*: *DBX*=1: 4: 2. For further discussion see text.

syndrome associated facial dysmorphism. Analysis of the *INHα* gene might be restricted to an analysis of the *INHα* sequence variant 769 G A described repeatedly to be associated with the POF syndrome although not in all populations.

Our pilot study also points to the fact that it is most important to perform the molecular clinical expression analysis of POF candidate genes only after a detailed clinical questionnaire has excluded the so called “non-genetic” causes of the POF syndrome and after a karyotype analysis has been performed from the patient’s leukocytes. A novel chromosome aberration detected in the POF patient needs to be confirmed by the same karyotype analysis of a member of the patient’s family because only “*de novo*” chromosome aberrations, i.e., restricted to the POF patient, can be considered as a putative agent causing POF syndrome.

Although we have shown that the use of the patient’s leukocytes for RT-PCR expression analysis of POF candidate genes is generally an attractive possibility to diagnose folliculogenesis’ gene defects, we also have shown its limitations. Therefore, in any case where the patient is willing to agree to an ovarian biopsy for analysis of the expression profile of a distinct folliculogenesis gene, the corresponding quantitative real time RT-PCR experiment with a Light Cycler assay is always the method of choice. Moreover, only tissue sections of ovarian biopsies can be used for immunohistochemical analyses of the corresponding protein expression pattern using a specific polyclonal or monoclonal antiserum of which many are commercially

available. Any aberration of the normal ovarian tissue location of a given folliculogenesis protein is a strong indication of the involvement of this protein in the molecular aetiology of the patient’s POF syndrome. Recent utilization of suppressive subtractive hybridization (SSH), PCR amplification, and cDNA microarray analysis techniques alongside established transgenesis mouse models have largely expanded the classification of novel oocyte-specific genes required for reproductive fitness in various species, including human [118]. Obviously, many non-genetic and genetic based aberrations that interfere with the oocyte maturation pathway have yet to be explored before we can reveal more than just the tip of the POF iceberg.

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