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ARTICLE

A susceptibility gene for premature ovarian failure (POF) maps to proximal Xq28

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Terminal deletions of the long arm of the human X chromosome have been described in women with premature ovarian failure (POF). We report here the molecular characterization of an inherited deletion in two affected women and in their mother. The two daughters presented secondary amenorrhea at 17 or 22 years respectively, while the mother was fertile. She had four children, but she eventually had premature menopause at 43 years of age. The fine molecular analysis of the deletion showed that the three women carried an identical deletion. We conclude that the phenotypic difference within the family must be attributed to genetic or environmental factors and not to the presence of different extent deletions. By comparison with other deletions in the region, we map a susceptibility gene for POF to 4.5 Mb, in the distal part of Xq.

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Introduction

Genes for ovulation have been hypothesized on the X chromosome. X chromosome monosomy, or Turner syndrome (TS), is the only chromosome monosomy compatible with life and is characterized by short stature, ovarian dysgenesis and a number of anatomical and physiological manifestations.[1] It was suggested that TS is due to monosomy for genes required in double dose in female subjects.[2,3] Accordingly, it was shown that short stature is due to monosomy for a gene, SHOX, in the short arm pseudoautosomal region.[4] The causes of ovarian dysgenesis are not clarified. In TS, the gonads differentiate normally until about the third month of gestation when the oocytes start to accelerate their apoptosis resulting in a decrease in the final number of oocytes, an increase in ovarian fibrosis and in ovarian failure. As a consequence, in the majority of adult TS female subjects ovaries appear as streaks. Partial monosomies of the long or of the short arm are also associated with ovarian failure: the phenotype is less severe and they are commonly associated with secondary amenorrhea, presence of reduced size ovaries and of a reduced number of follicles.[5,6] As different regions of the chromosome may be deleted, alterations in gene dosage of several genes along the whole chromosome appear responsible for ovarian failure. Many genes escaping X chromosome inactivation are in Xp, and they may be responsible for ovarian failure due to Xp monosomy.[7] In Xq, fewer genes escape X inactivation, but ovarian function may still be impaired by monosomy for genes required in double dose after X chromosome reactivation for germ-cell development.

Some of the genes for POF may be localized in the 'critical region for premature ovarian failure', spanning from Xq13 to Xq26/27 and defined from partial deletions,

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X-autosome balanced translocations and other chromosome rearrangements, occurring on the long arm of the chromosome and frequently associated with the disorder.[5,6] However, whether the same genes are involved in X-autosome translocations and in monosomies is an open question, mainly as several independent studies have shown that most balanced translocations do not interrupt genes.[8–10]

The partial X chromosome monosomies are always associated with preferential inactivation of the deleted chromosome and frequently involve most of the q- or p-arm and some TS stigmata. Interstitial deletions and terminal deletions only associated with POF have also been described.[11–13] The smallest deletions were described on the distal long arm of the X chromosome, with breakpoints in Xq26/27.[14–16] None of the carriers of this group of deletions displayed TS or any other TS stigmata, but the phenotypes were rather variable. Some of the carriers had numerous children. Some had irregular menstruation and secondary amenorrhea. Few had premature menopause after 40 years of age. One case had primary amenorrhea. In few instances they were found in families, segregating with different temporal onset of ovarian failure. Since they were described many years ago, no detailed molecular characterization is available and we cannot exclude that the phenotypic differences are due to differences in the extent of the deletions and of the number of genes involved.

One family from Italy, family M, was previously reported as 46,X, del(X)(q27).[16] The three women in the family did not have signs of TS. Two affected women carried a terminal deletion of Xq and presented secondary amenorrhea at 17 or 22 years of age, after a period of oligomenorrhea. The deletion was inherited from the mother who had four children but eventually had premature menopause at 43 years of age. The deleted X was inactivated. We have analyzed in detail the deletion in this family and we now report the molecular characterization leading to suggestion of a susceptibility gene for POF in Xq28.

Materials and methods

Patients and DNAs

The three women carrying the deletion in family M were described previously (patients 12–14 from Maraschio *et al*).[16]They had normal height (160–168 cm) and none of the TS traits. The mother, I-1, had menarche at 14 years of age. She had periods of amenorrhea alternating with regular menstruation. This allowed her to have four children at 20, 25, 27 and 32 years of age. At 43 years of age she entered menopause. The daughter II-1, had menarche at 15 years of age and she had always irregular cycles until 17 years of age when she entered menopause. The second daughter, II-2, had menarche at 13, irregular cycles until the age of 19 years when she started taking the pill. At 22 years, she stopped the pill and she had amenorrhea thereafter. The three women referred to have had high FSH and LH levels at diagnosis, but the data were not available at the time of the study. The last daughter, II-4, had a normal caryotype. She had menarche at 14 years of age and she always had regular cycles. She has two children.

DNA was prepared from blood or lymphoblastoid cell cultures using standard methods. For pulsed field gel electrophoresis (PFGE) analysis, the DNAs were digested with the enzymes indicated in the conditions suggested by the suppliers (Promega) and fractionated in 1% agarose gels at 180 V/170 mA for 20 h and 5 s pulses.

Heterozygosity mapping

The extent of the deletion was determined by heterozygosity mapping using microsatellites. Some of the microsatellites analyzed (DXS1227, DXS8043, DXS8091, DXS1073) were from the ABIPrism^{TM} linkage mapping set. The PCRs were carried out as suggested by the supplier (ABI) and the analysis was performed by capillary electrophoresis, using the MegaBace and analyzed by the MegaBace Genetic Profiler (Amersham Pharmacia Biotech).

Additional microsatellites were selected using the Ensembl browser (http://www.ensembl.org/). Primer sequence, PCR amplification conditions and heterozygosity of the microsatellites used are described in Table 1. Amplification products were analyzed by radioactive labeling the PCR reaction using ^{32}P dCTP(0.25 μCi/25 μl reaction) and fractionation in 7% denaturing acrylamide gel electrophoresis. Position of the microsatellites and of the genomic clones used in FISH experiments was derived from Ensemble (release of 4-11-2003).

FISH analysis

BAC and PAC clones were selected from Ensembl (http://www.ensembl. org/). Cosmid clones were characterized in our laboratory.[17,18] DNA was extracted from BAC and PAC clones using the PhasePrep BAC DNA kit (SIGMA) and labeled by nick translation (Roche Diagnostic, IN, USA) with biotin-dUTP or dig-dUTP (Roche). FISH experiments were performed by conventional methods. Briefly, the probes for each slide were combined as required (400 ng per slide), dried down and suspended in 50% formamide, 1% Tween-20, 20% dextran sulfate along with salmon sperm DNA and Cot- 1 DNA, then denatured at 70°C for 10 min, preannealed at 37°C for 15 min, and hybridized with denatured slides at 37°C. Post-hybridization washes were 50% formamide at 42°C for 15 min, 2xSSC at 37°C for 8 min, and 1xPBD at room temperature for 2 min. The signals were amplified with FICT-avidin (Sigma) and anti-avidin (Sigma) for the biotin-dUTP, and with anti-digoxigenin monoclonal antibody, anti-mouse IgG-digoxigenin and anti-digoxigenin-rhodamine FAB fragments (all from Sigma) for dig-dUTP. Slides were counterstained with DAPI

Table 1 Primers for heterozygosity mapping

	Primer sequence	Annealing temp.(°C)	Het[a]
DXS6751	F: 5′-CAGAGCTCATCACACATAGG-3′ R: 5′-GTGCACATGGCATAAAATCCG-3′	53	ND
AFMB323XG1	F: 5′-GAGTTCCTACATATGTACACC-3′ R: 5′-CCCAGATGTGAGCTGTTGAC-3′	55	ND
DXS8106	F: 5′-AATTCATCTGCTCCTAATGC-3′ R: 5′-AGCTGTAGAGTTGAGGAATG-3′	57	0.6917
DXS8069	F: 5′-AACAGTCATTGTAGGCATCG-3′ R: 5′-GAATTGCCAGTCATCCC-3′	58	0.62
DXS1684	F: 5′-AGCACCCAGTAAGAGACTGAAC-3′ R: 5′-CCTCAGTGGCAACCACTCAAG-3′	57	0.8176
DXS8061	F: 5′-GCTTGAAGTGTCCATGAGGTATC-3′ R: 5′-AGAAGCTGATGTGCTCCCTG-3′	57	0.6903
DXS15	F: 5′-AGCACATGGTATAATGAACCTCCACG-3′ R: 5′-CAGTGTGAGTAGCATGCTAGCATTT-3′	65	0.84
DXS1108	F: 5′-ATTCATCATATGTGATTTCCACAGCC-3′ R: 5′-ACTAGGCGACTAATACAGTGGTGC-3′	65	0.75

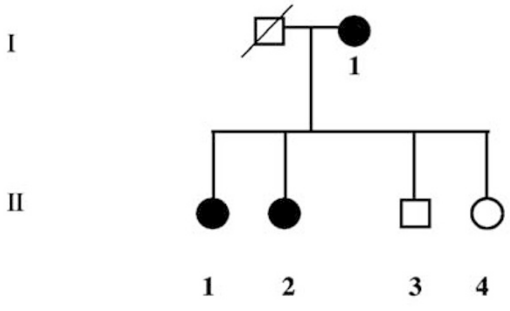
[a]Het indicates Heterozygote frequency among Caucasians.

(Sigma) and mounted in Vectashield antifading medium (Vector Laboratories, USA). Signals were visualized under a Nikon E1000 microscope equipped with a cooled charge-coupled device (CCD) camera and the Genikon image analysis software.

Results

Heterozygosity mapping

The extent of the deletions in the three affected women of family M was defined by heterozygosity mapping using microsatellite markers spanning Xq26-Xq28 (Figure 1). The DNA of the three affected women, of a normal daughter and of a normal son, were analyzed. The three women carrying the deletion were heterozygotes for marker AFMB323Xg1, but they had only one of the alleles for marker DXS8106 in Xq27.3, at 140.9 Mb and of all the other tested until DXS15 in Xq28, at 151.0 Mb. The last two markers tested, DXS1073 and DXS1108 were both present in heterozygote state in the three women. The deletion is therefore interstitial and the last 3 Mb of Xq28 are present in the three patients.



DXS1227	1/1	1/1	1/2	2	1/2	139.5 Mb
DXS6751	2/1	2/1	1/2	2	2/2	139.7 Mb
AFMB323XG1	2/3	2/3	3/1	1	2/1	139.9 Mb
DXS8106	2	2	1	1	2/1	140.9 Mb
DXS8043	1	1	2	//	1/2	142.7 Mb
DXS8091	1	1	2	2	1/2	146.3 Mb
DXS8069	2	2	1	1	//	148.3 Mb
DSX1684	2	2	1	1	2/1	148.8 Mb
DXS8061	1	1	2	2	1/2	150.6 Mb
DXS15	1	1	2	2	1/2	151.0 Mb
DXS1073	1/2	1/2	1/2	1	1/1	152.3 Mb
DXS1108	2/3	2/3	3/1	1	2/1	153.3 Mb

Figure 1 Heterozygosity mapping with microsatellites. Under the family tree are the genotypes of each member of family M. Different alleles have different numbers and the position in Mb of each microsatellite along the X chromosome sequence is indicated.

FISH mapping

To further define the deletion BAC, PAC and cosmid clones spanning the breakpoint regions were used in FISH analysis (Table 2 and Figure 2). The proximal breakpoint in all the three women was mapped in Xq27.3 between the BACs RP11-485I9, which is present on both chromosomes, and RP11-156M16, which is absent from the deleted chromosome. The two BACs define a breakpoint interval of 200 kb, identical in the three women, that was not defined further as analysis of the region showed that it is gene free. The breakpoint is 900 kb distal from the *MAGEE1* gene and 500 kb from the gene *NM-173078* of unknown function, which is deleted.

In Xq28, the breakpoint was mapped between cosmid Qc-12B2, that is absent and the overlapping cosmids Qc-N2215 and Qc-P0113, both present. Accordingly, two-color FISH on the deleted chromosome (Figure 2) shows merging signals with clones RP11-485I9 and Qc-P0113 flanking the deletion (Table 2) and no signals with clones RP5-1185M13 and Qc-L074, both in the deletion (Table 2). The sequence of the deleted cosmid, Qc-12B2, is included in the contig U52112, between 151.58 and 151.76 Mb of the X chromo-

Table 2 FISH analysis

Clone name	Position (Mb)	RT	MM	MS1
RP11-73D6	140.3	+/+	+/+	+/+
RP11-260C6	140.4	+/+	+/+	+/+
RP11-485I9	140.5	+/+	+/+	+/+
RP11-156M16	140.8	+/−	+/−	+/−
RP5-1185M13	140.9	+/−	+/−	+/−
Qc-12B2	151.7	+/−	+/−	+/−
Qc-L074	151.8	+/−	+/−	+/−
Qc-N2215	151.98	+/+	+/+	+/+
Qc-P0113	152.02	+/+	+/+	+/+

+/+ = signal on both chromosomes; +/− = signal on one chromosome.

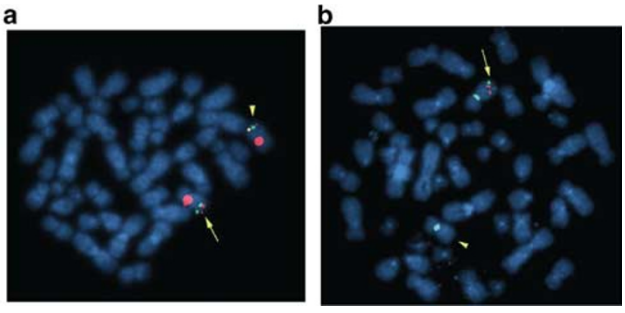


Figure 2 Two-color FISH analysis of the deletion. (**a**) *In situ* hybridization with clones RP11-485I9 (green) and Qc-PO113 (red) delimiting the deletion. The yellow arrow indicates the normal X chromosome with two distinct signals. The yellow arrowhead shows the deleted chromosome X with merging signals. An alpha-X specific clone was hybridized as a control probe (red). (**b**) *In situ* hybridization with clones RP5-1185M13 (red) and Qc-LO74 (green) located within the deleted region. The yellow arrow indicates the normal X chromosome with two distinct signals. The yellow arrowhead shows the deleted chromosome X with no signals. An alpha-X specific clone was hybridized as a control probe (green).

some. The ends of cosmids Qc-N2215 and Qc-P0113 were sequenced. Comparison with the human genomic sequence positioned the cosmids 4 kb distal to the 3′ end of the color vision gene cluster. In Xq28 the distal breakpoints was therefore mapped to a 200 kb region containing the *MECP2* gene and the *OPN*, and the *CXorf2* genes cluster (Figure 3a).

PFGE analysis of the Xq28 breakpoint

The breakpoint in Xq28 was further refined by PFGE and Southern blot analysis. From the restriction map of the region, the DNAs of the three patients and of male and female controls were digested with *Eco*RV and *Kpn*I and hybridized with the probes indicated in Figure 3a. All the probes tested (MIR2–IR6) were deleted in the patients as

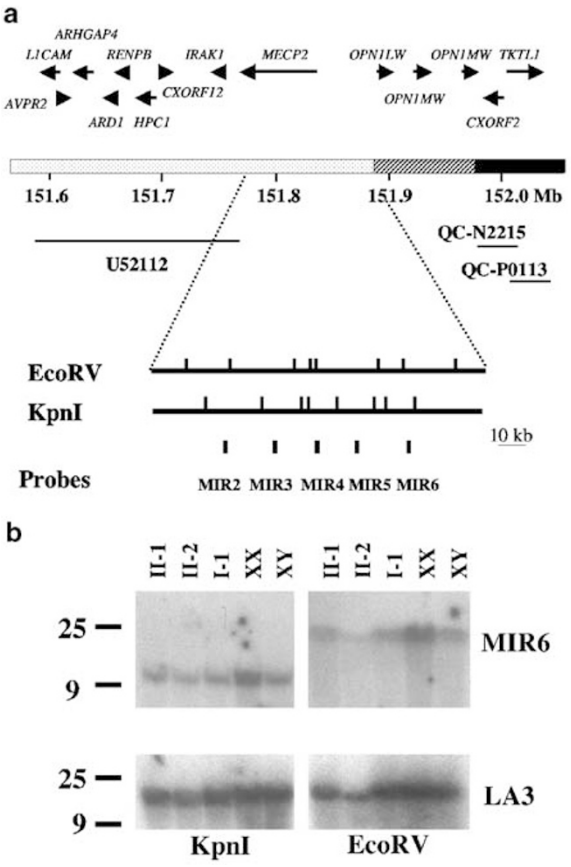


Figure 3 Fine analysis of the Xq28 deletion breakpoint. (**a**) map of the region: the dotted bar indicates the deleted region, the striped bar is the region of the breakpoint, in black is the nondeleted region. Above are the genes of the region. Below is the position of the restriction enzyme site used in PFGE, and the probes used. (**b**) Southern blot analysis of the Xq28 breakpoint. Above are the DNA used in the analysis and below are the enzymes used for DNA digestion. MIR6 is the most distal probe used that identified the region flanking the OPN1LW gene. LA3 is a probe from chromosome 18 hybridized to the same filter to control for the amount of DNA in each lane.

shown by the decreased intensity of he band compared to the control DNAs (Figure 3b). The results show that the distal breakpoint in all three patients is identical and is localized within the *OPN/Cxorf2* gene cluster.

Discussion

We report the fine mapping of an interstitial deletion of the X chromosome in family M.[16] The deletion was associated with early onset ovarian failure and sterility in two sisters. A third sister had a normal karyotype. She had regular cycles and two children. The deletion was inherited

from the mother who had a much milder phenotype as she had four children. The mother had, however, irregular menses and early menopause at the age of 43 years. We show here that the three women carry an identical deletion. Despite their very different phenotype, the results exclude the possibility that recombination occurring in the mother has produced a larger deletion responsible for the earlier onset of POF in the two daughters.

Few other deletions in distal Xq have been reported that were associated with POF. Two sisters[14] presented a deletion similar to that in family M, from Xq26/27 to qter: one sister, KS, entered menopause at 21 years of age, the other (MK) had very irregular menstrual periods for many years and was still irregularly menstruating at 40 years of age, the time of the report. She had a normal son. The mother was dead when the family was studied, but she was reported as normal except for early onset of menopause at 25 years of age. A third family was described by Trunca *et al*:[15] the patient had irregular menses for few years and amenorrhea at 18 years of age. One sister and the mother had the same deletion. The sister was still menstruating regularly at 29 years of age and the mother had menopause at 35 years of age. Mosaics were tentatively excluded in all cases.

The characteristics of the three families and the similar extent of the three deletions led us to conclude that POF may be due to the deletion of the distal portion of Xq, and we suggests that a susceptibility gene for POF maps to distal Xq. The deletion in family M is rather large (>11 Mb), but it could be compared to that in a published family identified from a male proband presenting FRAXA mental retardation.[19] The deletion was present in the mother who had skewed inactivation and was healthy and intellectually normal. It was reported that she had undergone menopause at the normal age of 48 years. The deletion contained the *FMR1* gene and should not contain any gene for POF. Its distal breakpoint was mapped between DXS1193 at 147.08 Mb and the gene *IDS* at 147.27 Mb, <300 kb apart. The two deletions, in family M and in Wolff *et al*[19], partly overlap in their proximal portion. By comparison, we can therefore reduce the region of susceptibility to POF to 4.5 MB between the IDS gene and the CV genes. The region is very gene rich as more than 60 genes are mapped there. Studies of additional terminal deletions may reduce the number of genes and analysis of candidate genes in the region may allow identification of a novel POF gene. This may determine whether a POF gene is indeed present or POF is due to other causes such as X inactivation or alterations in chromosome pairing, as suggested.[20]

The results show that the POF phenotype depends form the deletion and from additional factors. One could be X chromosome inactivation. It is not known at which time of embryo development inactivation nor skewing of inactivation exactly occur. The distal breakpoint of the deletion in family M was mapped within the *OPN* gene cluster, encoding the genes for Color Vision. The *MECP2* gene, responsible for RETT syndrome,[21] is the first of many genes deleted in the patients. Some (*LICAM*, *AVPR2*, *ABCD1*, *DKC1*, *IDS* and others) are known to cause severe disorders (http://www.ncbi.nlm.nih.gov/entrez). Thus, the complete skewing for X inactivation that is characteristics of this type of deletions and was previously reported also for family M must occur early enough in order to produce a normal phenotype, devoid of any other symptom, as found in family M.[16] However, small differences in the time of skewing of X inactivation in germ cells or in granulosa cells may alter the number of ovarian follicles and determine the age of onset of POF.

Other possibilities may explain the different phenotypes as other environmental factors, such as those known to affect ovulation[20,22] and the inheritance of a modifying genetic factor. No evidence for the latter hypothesis could be obtained from the family history, and further experiments are needed to clarify this point.

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