Molecular cytogenetic studies of Xq critical regions in premature ovarian failure patients

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BACKGROUND: Premature ovarian failure (POF) is defined as amenorrhoea for more than 6 months occurring before the age of 40, with an FSH serum level higher than 40 mIU/ml. Cytogenetically visible rearrangements of the X chromosome are associated with POF. Our hypothesis was that cryptic Xq chromosomal rearrangements could be an important etiological contributor of POF. METHODS: Ninety POF women were recruited and compared to 20 control women. Peripheral blood samples were collected and metaphase chromosomes were prepared using standard cytogenetic methods. To detect Xq chromosomal micro-rearrangements, fluorescence in situ hybridization (FISH) analysis was performed using a selection of 30 bacterial artificial chromosome (BAC) and P1 artificial chromosome clones, spanning Xq13–q27. We further localized the translocation breakpoints by FISH with additional BAC clones. RESULTS: Chromosomal abnormalities were identified in 8.8% of our 90 patients (one triple X, three large Xq deletions 46, X, del(X)(q22.3), 46, X, del(X)(q21.2) and 46, X, del(X)(q21.32), two balanced X;autosome translocations 46, X, t(X;1)(q21.1;q32) and 46, X, t(X;9)(q21.31;q21.2) and two Robertsonian translocations 45, XX, der(15;22)(q10;q10) and 45, XX, der(14;21)(q10;q10)). The two Xq translocation breakpoints were among a cluster of repetitive elements without any known genes. FISH analysis did not reveal any Xq chromosomal micro-rearrangement. CONCLUSIONS: Karyotyping is definitely helpful in the evaluation of POF patients. No submicroscopic chromosomal rearrangements affecting Xq region were identified. Further analysis using DNA microarrays should help delineate Xq regions involved in POF.

Key words: deletion/molecular cytogenetic/premature ovarian failure/translocation/X chromosome

Introduction

Premature ovarian failure (POF; OMIM 311360) is defined as amenorrhea for more than 6 months in the presence of raised gonadotrophins, FSH serum level higher than 40 mIU/ml, occurring before the age of 40 (Coulam et al., 1986; Vegetti et al., 2000). Ovarian failure can be induced by viral or autoimmune oophoritis, environmental toxins, pelvic surgery, radiations or chemotherapy. Mutations in several genes such as ATM, AIRE, FSH receptor, GALTL1, BMP15, FOXL2 and inhibin α genes and FMR1 premutation have been related to POF (see review in Goswami and Conway, 2005). Furthermore, abnormalities of the X chromosome have been reported in POF patients. Cytogenetically visible rearrangements of the X chromosome are associated with POF. Many of those rearrangements occur in specific Xq regions. Two main critical regions have been located on the long arm of the X chromosome, at Xq13–q21 (Powell et al., 1994) and at Xq26–q27 (Krauss et al., 1987). However, conventional karyotyping has a resolution of 5–10 Mb and does not detect all chromosomal alterations. In clinical cytogenetics, several new methods such as fluorescence in situ hybridization (FISH) and other molecular techniques have been developed, to detect submicroscopic rearrangements, not visible by routine chromosome analysis. This strategy identified previously unrecognized chromosomal causes of mental retardation (Knight et al., 1999). Therefore, our hypothesis was that cryptic chromosomal rearrangements affecting Xq region could be an important etiological contributor of POF.

The purpose of this study was to uncover submicroscopic chromosomal anomalies by testing loci potentially involved in the pathogenesis of POF. In this study, 90 patients with POF and 20 control women were analysed, using FISH with bacterial artificial chromosome (BAC) and P1 artificial chromosome (PAC) clones, spanning areas of Xq chromosome known to be rearranged in POF patients.
Materials and methods

Patients

Women were recruited from three clinical centres: Hôpital Saint-Antoine, Paris, Hôpital Jeanne de Flandres, Lille, and Hôpital Antoine Béclère, Clamart. Inclusion criteria were primary amenorrhea or secondary amenorrhea for more than 6 months, occurring before the age of 40, with an FSH serum level higher than 40 mIU/ml. The study was approved by the institutional review board of Saint-Antoine hospital, and all participants gave their written informed consent.

Ninety patients participated in the study. Women with clinical stigmata of Turner’s syndrome were excluded from the study, as well as two patients with blepharophimosis syndrome. Fourteen patients presented with primary amenorrhea (15.6%). Among the remaining patients, the mean age at first menses was 13.02 years, ranging from 10 to 18 years. The mean age at menopause was 29.4 ± 6.5 years (mean ± SD). A family history of POF was found in 18 patients (20%). A personal history of autoimmunity was identified in seven patients (7.78%).

Controls

Women with regular menses (28–32 days) having at least one child and no personal history of infertility or autoimmune disease were selected as controls. Twenty control women were recruited and gave their informed consent.

Conventional cytogenetic analysis

Peripheral blood samples were collected and metaphase chromosomes were prepared using standard cytogenetic methods. Twenty metaphase spreads, GTG or RHG banded, were routinely analysed from each patient. Karyotypes of peripheral lymphocytes in patients and controls were analysed. When possible, samples from the parents of the patients carrying anomalies were also cytogenetically analysed.

FISH

FISH analysis was performed and processed, by standard procedures, using DNA fluorescent probes for the DXZ1-alpha satellite probe (Vysis, Inc., Downers Grove, IL, USA) for detecting X chromosome mosaicism. Fifty metaphases and 200 interphase cells were analysed from each patient.

To exclude a potential complex rearrangement in Xq deletions and X;autosome translocations, chromosome painting using whole X, 1 and 9 chromosome probes (Vysis) was performed. Subtelomeric Xq chromosome FISH analysis was performed to characterize Xq deletions.

To detect Xq chromosomal micro-rearrangements, FISH analysis was performed using a selection of 30 BAC and PAC clones (from the Wellcome Trust Sanger Institute, UK, and from M. Rocchi, University of Bari, Italy), spanning Xq13–q27. Clones across Xq13–q21 and Xq26–q27 were spaced at ∼1 Mb interval (Figure 1). To define our translocation breakpoints, 33 additional BAC and PAC clones were used in our FISH analysis.

Clones were plated and propagated, and glycerol stocks were prepared. Isolation and purification of DNA were performed using the Qiagen plasmid Maxi Kit (Qiagen SA; Courtaboeuf, France), according to the manufacturer’s instructions. FISH probes were created using purified BAC and PAC DNA by using a nick translation kit to incorporate Spectrum Green or Spectrum Red dUTP, as per manufacturer’s instruction (Vysis). The labelled DNA probes were applied to interphase and metaphase cells obtained from the patients and controls. For each probe, 10 metaphases were analysed.

Results

Cytogenetic investigations

A total of 90 cases of POF-affected women and 20 female controls were analysed.

Eight chromosome rearrangements were identified in POF patients (8.8%) by conventional cytogenetic analysis of GTG- or RHG-banded chromosomes from peripheral lymphocytes. One patient had triple X, five patients had structural anomalies involving the long arm of X chromosome and two patients were carrying a Robertsonian translocation, with 45,XX,der(15;22)(q10;q10) and 45,XX,der(14;21)(q10;q10) karyotypes. The structural abnormalities of the Xq chromosome showed three large Xq deletions 46,X,del(X)(q22.3), 46,X,del(X)(q21.2) and 46,X,del(X)(q21.3) and two (X;autosome) balanced translocations 46,X,t(X;1)(q21.1;q32) and 46,X,t(X;9)(q21.3;q21.2) (Figure 2). 45,X/46,XX and 46,XX/47,XXX mosaicisms or other complex mosaicsisms involving X chromosome were not observed in any patients or controls. The Xq21.2 and Xq22.3 deletions occurred de novo. The mode of inheritance was not identified for the other abnormalities, because of non-availability of parental karyotypes.

FISH analysis

FISH analysis with the X-specific centromeric probe (DXZ1) showed two positive signals in the total counted 50 metaphases and in at least 97% of the 200 interphase nuclei analysed (Figure 3A). A single X-chromosome signal was found in <3% of the total counted cells, which was not significantly greater than that in the controls. This indicates the absence of mosaic...
constitution in all patients and controls. All cases with chromosome rearrangements were non-mosaic.

The triple X was present in all 50 metaphases and 200 interphase nuclei analysed. Additional FISH study with DXZ1 probe performed on buccal mucosal cells of this patient confirmed the absence of mosaic.

In the three cases of Xq deletions, FISH with BAC clones spanning the breakpoint regions was used (Figure 3B). FISH using the whole X chromosome painting probe revealed complete hybridization on normal and deleted X chromosomes, thus excluding any translocation (Figure 3C). After FISH with BAC clones spanning the breakpoint regions, the deletion breakpoints were located respectively at q21.2, q21.31 and q22.33. FISH analysis using Xqter probe confirmed terminal Xq deletions in all cases.

FISH with the whole chromosome-specific paints of X, 1 and 9 confirmed the balanced (X;autosome) translocations in t(X;1) and t(X;9) patients (Figure 3D). The Xq breakpoint of the (X;1) translocation was mapped in Xq21.1, between RP11-346E8 and RP1-63M23 BACs and between 80.40 and 80.95 Mb of the X chromosome. The two clones defined an interval of 550 kb. This interval was not further defined, as analysis of this region revealed that it does not contain any known gene (http://www.ensembl.org/index.html). The Xq breakpoint of the (X;9) translocation was more distal, as it mapped in Xq21.31. The breakpoint was in the overlapping BAC RP13-428K23, between 89.93 and 90.02 Mb of the X chromosome, in a breakpoint interval of 190 kb. Each Xq breakpoint falls among a cluster of repetitive elements (http://www.ensembl.org/index.html). Thus, the breakpoints fall in DNA segments that are essentially untranscribed, ∼10 Mb apart in Xq21.

FISH analysis was performed in each patient using Xq chromosome locus-specific probes, 30 large insert clones (BAC) spanning Xq13–q27, spaced at ∼1 Mb intervals across Xq13–q21 and Xq26–q27. No Xq chromosomal micro-rearrangement was detected as normal signal patterns on both X chromosomes were present on Xq with the BAC clones used. Therefore, no microdeletion and no microduplication of Xq chromosome were identified within this cohort of 90 POF patients.

Discussion

An association between POF and abnormalities of the X chromosome has been extensively reported in the literature (Goswami and Conway, 2005). A Dutch study has recently suggested that the involvement of the X chromosome may not be limited to POF but may influence the broader spectrum of menopausal age (van Asselt et al., 2004). In POF patients,
mutations have been identified in a cohort of POF women (Bione et al., 2004). Breakpoints associated with POF fall within genes having no obvious relationship with ovarian function (Prueitt et al., 2000, 2002; Bione et al., 2004). Other translocations previously described fall in poorly transcribed regions (Mumm et al., 2001; Schlessinger et al., 2002; Rizzolio et al., in press). Fine mapping of our two Xq breakpoints identified that they fall in regions of repetitive elements in Xq21, ~10 Mb apart in regions without known genes. In theory, gonadal dysfunction related to chromosomal abnormalities can occur for several reasons. The first one is linked to critical gene expression being disrupted by the rearrangement (Sala et al., 1997). The second one is related to temporally inappropriate gene expression following incomplete pairing of X chromosomes at pachytene, leading to meiotic arrest (Sala et al., 1997; Schlessinger et al., 2002). A third potential explanation is a position effect. Rizzolio et al. (in press) suggested that POF should be ascribed to a position effect of the breakpoints on flanking X-linked genes or on genes flanking the autosomal breakpoints. Our findings related to breakpoints falling within a region of repetitive elements in Xq are in favour of alterations in chromosome meiotic pairing or position effect.

Clinically, the 46,X,t(X;1)(q21.1;q32) patient presented with primary amenorrhea. The 46,X,t(X;9)(q21.31;q21.2) patient had secondary amenorrhea at the age of 25, occurring later than previously described. Indeed, translocations involving this Xq chromosomal region are known to cause amenorrhea between the age of 16 and 21 years (Powell et al., 1994). Therefore, our data emphasize the importance of the Xq proximal region in POF.

Concerning the Robertsonian translocations, very few cases associated with POF have been reported in the literature (Orczyk et al., 1989; Kawano et al., 1998). Our patients presented a translocation between chromosome 15 and chromosome 22 [45,XX,der(15;22)(q10;q10)] and between chromosome 14 and 21 [5,XX,der(14;21)(q10;q10)]. Robertsonian translocations are the most frequent translocations reported in humans (Scriven et al., 2001). Thus, the presence of those translocations could be coincidental. Nevertheless, Robertsonian translocations are associated with spermatogenesis failure in men (Van Assche et al., 1996). The correlation between those Robertsonian translocations and the ovarian phenotype in our patients can only be suggested.

One of our patients presented with triple X. The triple X incidence is estimated to be 1/1000 live born females. Most of them have normal physical appearance and puberty. Several sporadic cases of triple X women associated with POF have been reported (Villanueva and Rebar, 1983; Itu et al., 1990; Holland, 2001). In the study by Goswami et al. (2003), the prevalence of triple X among 52 POF women reached 3.8%. In our population, the prevalence is lower, reaching 1.1%. Our patient presented her first menses at the age of 13 and secondary amenorrhea occurred at the age of 21. Her height was 165 cm with a weight of 80 kg. She had no stigmata of autoimmunity, although hypothyroidism has been previously reported in POF triple X women (Goswami et al., 2003).

In our cohort, we did not identify any X/XXX, XX/XXX mosaicism or any mosaicism involving X chromosome. Previous studies including unexplained secondary hypergonadotrophic
hypogonadism have reported low levels of X/XX mosaicism (Wu et al., 1993; Devi et al., 1998). In 15 patients with a normal 46,XX karyotype, a FISH study revealed a statistically significant increase in the frequency of cells showing a single X-chromosome signal compared to normal women (mean 5.5, \( P < 0.001 \)) (Devi et al., 1998). However, we studied a large amount of cells, 70 metaphases and 200 interphase nuclei excluding low-grade X/XX mosaicism.

In our selected POF population, our extensive molecular cytogenetic study did not reveal any cryptic chromosomal rearrangements affecting critical Xq regions. Although those data may be surprising, as microdeletions are generally rather common, our results suggest that cryptic rearrangements in the Xq critical region are unlikely to be a common cause of POF. We used BACs spanning Xq13–27, spaced at ~1 Mb intervals across Xq13–q21 and Xq26–q27. This resolution is similar to most of the reliable CGH array assays currently available. Furthermore, as we focussed on critical Xq regions involved in POF patients, the number of 30 clones could be considered as sufficiently high to detect most micro-rearrangements in those two regions. Nevertheless, we cannot exclude the possibility that resolution of our study was not high enough to detect potential Xq interstitial chromosomal abnormalities. Moreover, lack of small deletions could also be explained by others factors such as chromatin effects or monosomy for ovary-specific autosomal genes for the POF phenotype (Rizzolio et al., in press).

In conclusion, we identified 8.8% of X chromosome abnormalities in a large population of POF patients, excluding patients with clinical stigmata of Turner’s syndrome, implying that karyotyping is helpful in the evaluation of POF patients. The Xq breakpoints of two balanced X:autosome translocations have no putative or detected gene content. Moreover, in our cohort, no cryptic chromosomal rearrangements affecting Xq region were identified using FISH studies with a 1 Mb resolution. Further studies using DNA microarrays allowing a high chromosomal resolution analysis will help delineate the Xq critical region in POF patients.

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References


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