

Research Article

Cytogenetics of Premature Ovarian Failure: An Investigation on 269 Affected Women

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Received 14 September 2010; Revised 11 November 2010; Accepted 14 December 2010

Academic Editor: Ricardo Benavente

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The importance of X chromosome in the aetiology of premature ovarian failure (POF) is well-known but in many cases POF still remains idiopathic. Chromosome aneuploidy increase is a physiological phenomenon related to aging, but the role of low-level sex chromosome mosaicism in ovarian function is still undiscovered. Standard cytogenetic analysis was carried out in a total of 269 patients affected by POF: 27 chromosomal abnormalities were identified, including X chromosome and autosomal structural and numerical abnormalities. In 47 patients with 46,XX karyotype we performed interphase FISH using X alpha-satellite probe in order to identify X chromosome mosaicism rate. Aneuploidy rate in the patient group was significantly higher than the general population group. These findings underline the importance of X chromosome in the aetiology of POF and highlight the potential role of low-level sex chromosome mosaicism in ovarian aging that may lead to a premature onset of menopause.

1. Introduction

The increase of chromosome aneuploidy in human lymphocytes with aging has been described since early 1960s [1, 2]. Numerous reports confirmed the observations of Jacobs and colleagues and showed that hypodiploidy increases with age and is more common than hyperdiploidy [3–6]. Subsequent studies on metaphase spreads from peripheral blood lymphocytes demonstrated that there was a preferential loss of X and Y chromosomes in female and male, respectively [7–10], suggesting that loss of sex chromosomes follows an upward trend according to aging. An increase in micronucleus formation with age has been highlighted in many studies and in particular a high overrepresentation of the X chromosome in lymphocyte micronuclei of women [6, 11–14].

Premature ovarian failure (POF, OMIM 311360) is defined as the cessation of ovarian function before the age

of 40, associated with elevated gonadotropins serum levels (FSH \geq 40 UI/l) and affects at least 1%–3% of women of reproductive age [15]. The aetiology of POF is highly heterogeneous including genetic, autoimmune, metabolic and infectious causes, but in most cases the aetiology is still unknown [16, 17]. The most common genetic causes of POF are X chromosome abnormalities [18, 19], ranging from numerical defects, deletions, X-autosome translocations, and isochromosomes [17]. Turner syndrome, associated to X monosomy, leads to ovarian dysgenesis and accelerated follicular atresia, showing that two intact X chromosomes are essential for the maintenance of ovarian function as many genes, probably involved in ovarian function, escape X inactivation and are required for a normal ovarian development and maintenance [16, 20–22]. On the other hand, X trisomy seems to be related to ovarian dysfunction as 47,XXX women might experience oligomenorrhea, secondary amenorrhea,

and early menopause [23]. Moreover the role of low-level sex chromosome mosaicism in ovarian function is still unknown, in most cases they escape diagnosis because they do not seem to cause any phenotypic effect [24, 25].

The aim of this work is to carry out a study on a group of 269 patients affected by POF, through a cytogenetic investigation, in order to identify chromosomal abnormalities. Moreover, we assessed an analysis on X chromosome aneuploidy, by means of FISH on interphase nuclei, to evaluate low-level sex chromosome mosaicism rate, trying to understand if there is a correlation between advanced and increased loss of X chromosome and POF.

2. Materials and Methods

2.1. Clinical Population. A total of 269 patients affected by POF referred to genetic laboratory for conventional cytogenetic analysis. A subpart of this case group has been already published in previous works by Vegetti et al. (1998); Tibiletti et al. (1999) and Marozzi et al. (2000) [26–28]. In this study all of the patients had the cessation of menses for a duration of 6 months or longer, with FSH levels \geq of 40 IU/l, before or at the age of 40 (POF1), or between the age of 41 and 45 years (POF2). Also patients affected by primary amenorrhea were included in the study group, as this clinical condition can be considered the most severe cause of ovarian insufficiency [29, 30]. These patients did not show any typical features of Turner syndrome. All of the patients underwent a complete clinical assessment, including complete medical and gynaecological history, in order to exclude any other related pathology. Informed consent was obtained from all participants.

The general population group was composed by 357 uncultured female amniocytes derived from routinely diagnostic procedures, in order to establish the best unbiased reference group. All foetuses showed normal female karyotype after culture using standard cytogenetic techniques.

2.2. Conventional Cytogenetics. Metaphase-chromosome spreads were obtained from phytohaemagglutinin-stimulated peripheral blood lymphocytes using standard methods. The chromosomes were QFQ-banded using quinacrine mustard, and slides were mounted in McIlvaine buffer. A range of 30–50 cells were analyzed for karyotype following the guidelines of the International System for Chromosome Nomenclature 2009 (ISCN 2009) with the exclusion of mosaicism at 10%–6% grade, with 95% confidential level [31].

2.3. FISH Analysis on Interphase Nuclei. FISH analysis on interphase nuclei from lymphocytes was assessed on patients ($n = 47$) with normal constitutional karyotype and on uncultured amniocytes. The mean age of the patients at the time of the study was 34 years (range 12–45). FISH study was performed using alpha satellite probes of chromosomes X, Y, and 18 (AneuVysion Multicolor DNA Probe Kit, Vysis, Abbott Molecular) and performed according to the manufacturer's instructions. Chromosome 18 centromeric

probe was used as reference signal in order to evaluate hybridization efficiency. A range of 40–210 nuclei was analyzed for each case. Only monosomic and trisomic cells for X chromosome were taken into account to determine the aneuploidy rate, since other anomalous categories are small and so irrelevant [32].

2.4. Statistical Analysis. χ^2 test was carried out on raw data using a Microsoft Excel spreadsheet (Microsoft Corporation, Redmond, WA). Data analysis between the two groups analyzed was considered significant, setting a threshold equal to $P < .05$.

3. Results

High-resolution cytogenetic analysis (QFQ banding) of peripheral blood lymphocytes from 269 patients revealed 27 chromosomal abnormalities (Table 1 and Figure 1). There were 13 nonmosaic X chromosome structural abnormalities (48.2% of detected abnormalities), including X-autosome translocations, X;Y translocations, Xq deletions, and pseudodicentric chromosomes. The 46,X,der(X)t(X;Y)(q26.2;q11.223) was a maternal inherited translocation and the mother ceased her menses at 40 years of age. The 46,X,der(X)t(X;19)(p21.1;13.42) case is a maternal inherited translocation, but the sister of this patient, who has inherited the same aberration is not affected by POF [33]. X chromosome was also involved in 3 (11.1%) cases of 45,X mosaic with the other cell line characterized by a pseudodicentric X chromosome. 6 (22.2%) patients showed X chromosome aneuploidy, in particular one patient was nonmosaic 47,XXX, one patient nonmosaic 45,X, 3 patients were 45,X/46,XX mosaic and one patient was 46,XX/47,XXX mosaic. In addition, 3 (11.1%) autosomal structural abnormalities were identified, one involving chromosomes 4 and 5 and a patient was mosaic for an isochromosome 9p. The patient with the 46,XX,t(3;7)(q23;p12) karyotype was characterized by primary amenorrhea and bilateral euryblepharon. The chromosome 3 breakpoint fell under the FOXL2 locus and she was not affected by the blepharophimosis-ptosis-epicanthus inversus syndrome. Autosomal numerical abnormalities were found in 2 patients (7.4%) including a 47,XX,+21 case and a 46,XX/47,XX+18 mosaic case successively described by Bettio et al. (2003) [34].

The distribution of aneuploidy rate for the patients and reference group is given in Tables 2 and 3. FISH analysis on interphase nuclei from amniocytes on the general population group revealed X chromosome monosomy in 2.7% (range 0%–11.4%) of cases and triple X signal in 0.6% (range 0%–6.1%) of cells. POF patient group showed higher aneuploidy values than reference group, in particular the percentage of cells with only one detectable signal was 7.5% (range 2.0%–19.2%), while a triple X chromosome signals were found in 3.3% (0%–12%) of cells. All these data were referred to cells with 2 visible 18 chromosome signals in order to evaluate only the cells with right hybridization efficiency. We established three 18:X categories and in detail: (i) 2:1 (two 18

TABLE 1: Summary of the chromosomal abnormalities found in the POF patient group.

X chromosome structural abnormalities	nonmosaic	46,X,t(X;2)(q21.33;q14.3)
	nonmosaic	46,X,der(X)t(X;9)(q21.33;p22.3)*
	nonmosaic	46,X,der(X)t(X;19)(p21.1;13.42)#
	nonmosaic	46,X,der(X)t(X,X)(q21.3;p21)*
	nonmosaic	46,X,del(X)(q21.2 → qter)*
	nonmosaic	46,X,del(X)(q22.3 → q27)*
	nonmosaic	46,X,del(X)(q26.2 → qter)*
	nonmosaic	46,X,del(X)(p21 → pter?)
	nonmosaic	46,X,del(X)(p21.2)
	nonmosaic	46,X,psudic(X)(q10;q10)
	nonmosaic	46,X,del(X)(q21.1;q21.3)
	nonmosaic	46,X,der(Y)t(X;Y)(q13.1;q11.223)†
	nonmosaic	46,X,der(X)t(X;Y)(q26.2;q11.223)
	mosaic	46,X,psudic(X)(q23;q23)[75]/45,X[25]
	mosaic	45,X[97]/46,X,psudic(X)(q22;q22)[3]*
	mosaic	45,X[29]/46,X,der(X)t(X;X)(q21.2;p22.33)[47]
X chromosome numerical abnormalities	nonmosaic	47,XXX
	nonmosaic	45,X
	mosaic	46,XX[99]/45(X)[24]
	mosaic	46,XX[96]/47,XXX[4]
	mosaic	46,X[96]/45,X[4]
Autosomal structural abnormalities	nonmosaic	46,XX,t(4;5)
	nonmosaic	46,XX,t(3;7)(q23;p12)
	mosaic	47,XX,+i(9)(p10)[72]/46,XX[28]
Autosomal numerical abnormalities	nonmosaic	47,XX,+21
	mosaic	46,XX[54]/47,XX+18[36]§

* Marozzi et al. (2000) [28]; # Maraschio and Fraccaro (1983) [33]; § Bettio et al. (2003) [34]; † Lissoni et al. (2009) [35]. Square brackets, placed after the karyotype description, are used to designate the absolute number of cells in each clone, as stated by the International System for Human Cytogenetic Nomenclature 2009.

signals and one X signal), (ii) 2:2, and (iii) 2:3 (Figure 2). All the cells that did not fall within these three categories were classified as “other” and their percentage was 5.1% and 4.8% for the reference and patient groups, respectively.

X chromosome aneuploidy comparison of raw data between the two groups by the means of statistical analysis using the χ^2 test for variance showed a significant difference between the data distribution of the two groups ($P < .001$). Interestingly, there was no difference in the distribution of so-called “other” signals, so the difference of X chromosome aneuploidy between the two groups can not be attributed to different signal dispersion (Table 3).

4. Discussion

Cytogenetic analysis on blood lymphocytes derived from POF patients is an important tool in the detection of cytogenetic abnormalities that lead to premature ovarian

insufficiency. In this study we identified 27 chromosomal abnormalities out of 269 cases of POF occurred to our attention. Cytogenetic findings include X chromosome and autosomal structural and numerical abnormalities. Our data show a prevalence of X structural abnormalities (16 chromosomal abnormalities out of 27 found, equal to 59.3%), highlighting the importance of X chromosome in ovarian function and POF aetiology as described in the literature [18, 19]. Conventional cytogenetic analysis through QFQ banding is a powerful tool for a first round of screening, but a wide range of POF cases remains defined as idiopathic. Some of these cases may be explained by FRAXA premutation or mutations in BMP15 or inhibin alpha gene [36–38] but a large group of cases still remains with unknown cause.

Turner syndrome is the chromosomal disorder most commonly associated with POF, but the correlation between ovarian function and karyotype is less clear [39, 40]. Nevertheless, the highest number of follicles were found in subjects

TABLE 2: Detailed list of 18:X chromosome signals found in the patient group.

Case no.	Diagnosis	Age at menopause	Number of signals (18:X)			Other	Total
			2:1	2:2	2:3		
1	POF2-FAM2	45	5	51	5	0	61
2	PA	/	7	54	3	0	64
3	POF1-FAM2	40	11	69	10	0	90
4	POF1	27	6	86	8	0	100
5	POF1	33	13	99	7	0	119
6	POF2	42	16	82	2	0	100
7	POF1-FAM2	36	3	138	9	0	150
8	PA-FAM1	/	12	56	11	20	99
9	POF2-FAM1	41	7	81	3	9	100
10	POF1-FAM2	35	16	72	3	8	99
11	POF1-FAM1	34	12	76	3	8	99
12	POF1	12	10	74	9	8	101
13	POF2	42	17	74	2	11	104
14	POF1-FAM1	33	8	72	3	13	96
15	POF1-FAM1	38	19	62	3	15	99
16	PA	/	15	74	2	9	100
17	POF1	32	5	83	2	11	101
18	POF1	35	11	75	4	10	100
19	POF1	37	10	74	6	9	99
20	POF1-FAM1	25	4	88	6	2	100
21	POF1	40	6	89	1	4	100
22	POF1-FAM1	37	10	76	6	8	100
23	POF1	34	6	32	8	54	100
24	POF1-FAM2	34	4	35	6	5	50
25	POF1	37	6	80	10	4	100
26	POF1-FAM1	18	9	87	4	5	105
27	POF1-FAM1	34	12	176	6	14	208
28	POF1	30	11	167	0	6	184
29	POF1-FAM2	35	16	167	5	12	200
30	POF1	34	13	168	6	7	194
31	POF1	27	9	180	7	3	199
32	POF1	40	14	183	8	2	207
33	POF1	40	6	190	4	3	203
34	POF1-FAM1	40	13	182	4	5	204
35	POF2	41	15	177	5	7	204
36	POF1	26	12	182	4	5	203
37	POF2	44	12	188	2	2	204
38	POF1-FAM2	30	10	187	4	4	205
39	POF1-FAM2	33	4	176	8	14	202
40	PA	/	23	177	2	3	205
41	PA	/	12	192	2	2	208
42	POF1-FAM1	38	12	192	1	4	209
43	POF1	38	13	191	5	5	214
44	POF1-FAM1	18	10	190	2	3	205
45	POF1	38	15	181	6	7	209
46	POF1-FAM1	17	15	185	3	3	206
47	POF1-FAM1/2	31	17	184	4	5	210
%			7.4	84.4	3.3	4.8	

POF1: menopause before or at the age of 40; POF2: menopause between 41 and 45 years of age; PA: primary amenorrhea; FAM1: at least one relative with POF1; FAM2: at least one relative with POF2.

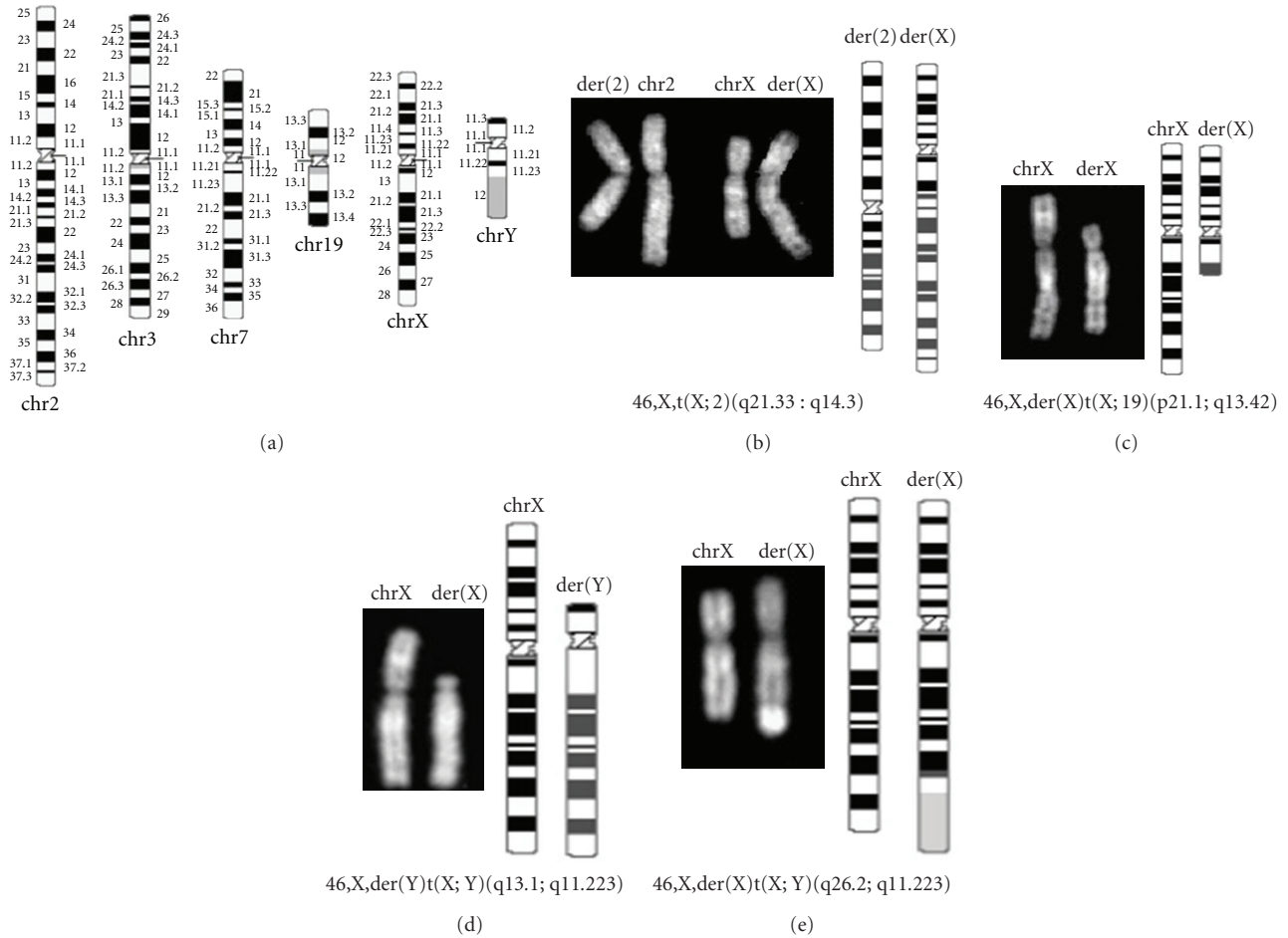


FIGURE 1: Conventional cytogenetic analysis. (a) Ideograms of the normal chromosomes (550-band level). (b)–(f) Chromosomal abnormalities found in 5 patients affected by POF. Partial Q-banding karyotype on the left and ideograms of derivative chromosome on the right.

TABLE 3: FISH analysis on interphase nuclei data and statistical analysis.

	Number of signals (18:X)				Total
	2:1	2:2	2:3	Other	
Patients (<i>n</i> = 47)	512 (7.5%)	5754 (84.4%)	224 (3.3%)	329 (4.8%)	6819
Reference group (<i>n</i> = 357)	596 (2.7%)	20020 (91.5%)	139 (0.6%)	1121 (5.1%)	21876
<i>P</i> -value* patients versus reference group	.001	.001	.001	.161	/

* χ^2 test.

with mosaic Turner syndrome and, above all, in subjects with the lowest percentage of cells with the 45,X karyotype [41]. These data strengthen the importance of X chromosome in ovarian function, showing that a double dose of some genes located on the X chromosome is essential in ovary maintenance, preventing follicle apoptosis and atresia [22]. Evaluating the implication of 45,X low level mosaicism in POF patients could give some clues in understanding the role

of low level sex chromosome mosaicism in ovarian function even if the precise role is still unknown [24, 25].

In order to evaluate X chromosome aneuploidy we performed FISH analysis using alpha satellite probes on interphase cells because the exclusive analysis of metaphase chromosome may provide only partial information since the analysis is restricted to a specific type of cycling cells and in this way nondividing cells would remain undetected [42].

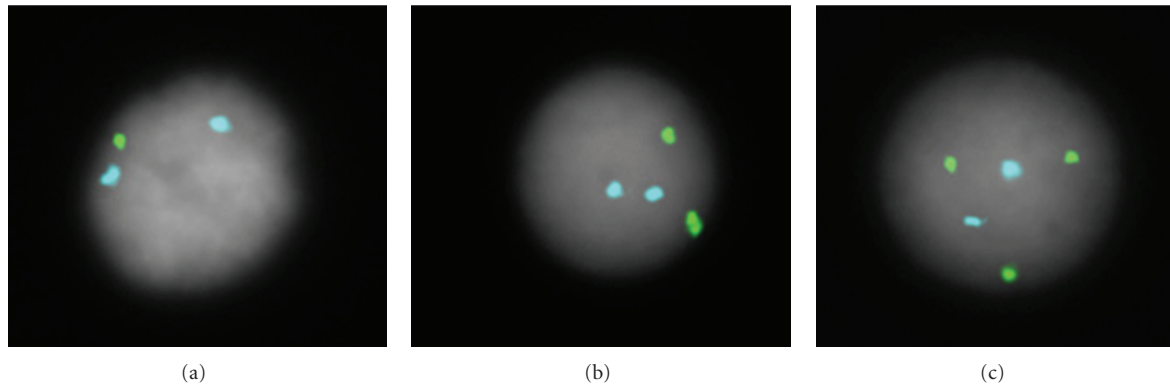


FIGURE 2: FISH analysis on interphase nuclei. FISH was performed using alpha satellite probes of X (green) and 18 (aqua) chromosomes. The images show the three different categories of signals detected: (a) X monosomy (two 18 signals and one X signal); (b) X disomy and (c) X trisomy.

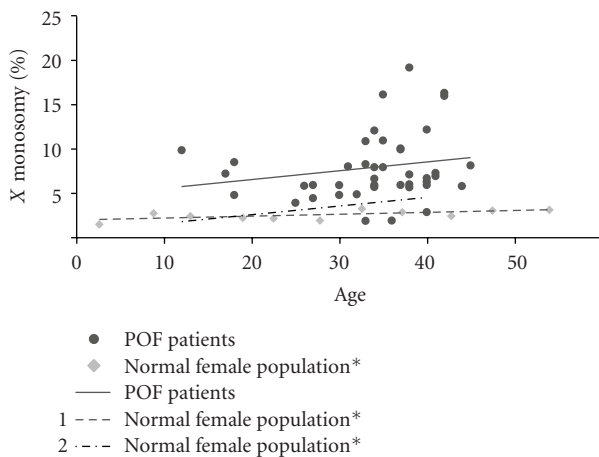


FIGURE 3: Percentage of X monosomy related to aging. The graph shows the linear trend of X monosomy both in POF patient group and in two different reference groups described in two reports from the literature: * (1) Guttenbach et al. (1995) [10] and (2) Lakhal et al. (2010) [25].

Moreover, FISH analysis on interphase cells is a sensitive method for detecting low-level sex chromosome mosaicism and also avoids artifacts and problems that may be associated to metaphase chromosome preparations [24, 43]. Indeed, metaphase chromosome spreads only allow the exclusion of 10 to 6% of mosaicism at confidence level of 95% [31] while the detection of low-level mosaicism increases from 30%–40% of cases to 74% using standard karyotyping and molecular techniques (FISHs), respectively [39].

Many reports describe the increase of X chromosome aneuploidy with aging and that the increasing age-related loss of sex chromosome loss associates with a higher level of micronuclei formation [6, 10, 13, 14, 33]. Instead, few works try to correlate X chromosome loss and POF [24, 25, 44] or at most, it is possible to find some studies that are more general and amenorrhea or aneuploidy only represent a secondary aspects [45, 46].

We studied a group of 47 POF patients with normal karyotype among our case group, using interphase FISH analysis,

in order to detect low-level X chromosome mosaicism and to understand if there is a correlation between increased rate of sex chromosome aneuploidy and POF condition.

The identification of the suitable reference group is tricky both for the type of disorder itself (POF) and the kind of analysis (X chromosome aneuploidy). The study on the impact of X chromosome mosaicism on fertility is very difficult to assess due to the lack of a normal fertile reference group [47]. In the literature, the rate of X monosomy and aging has already been determined by several works [10, 25, 48] and so we decided to use a new reference group based on the most possible random and unbiased group. Thus we established a general population group composed by uncultured amniocytes derived from routinely diagnostic procedures and analyzed during the same period of POF patients. This group can be considered a sort of rational reference group (a general population) as, in this specific case, a real reference group can not be formed, due to the characteristics of the disease itself: POF can occur till the age of 40(POF1)–45(POF2) and does not show any early biochemical or phenotypic signal, furthermore using a general female population, other time-related variables should be considered, such as personal habits or occupational exposure. Thus, an age-matched female group could determine a pre-established bias [49]. Moreover, women with a regular ovarian function normally cease their menses between 45 and 55 years of age and so their lymphocytes are already predisposed to X chromosome aneuploidy as it is a physiological phenomenon related to aging [50].

In this study we report a higher percentage of X aneuploidy rate in the POF patient group than in the general population group and we found also that the percentage of monosomic nuclei is much higher than trisomic nuclei. In particular, in the general population group, the percentage of 45,X cells was 2.7% and 47,XXX was 0.6%. POF group showed a statistical significant higher percentage rate equal to 7.5% and 3.3%, respectively ($P < .001$, χ^2 test). We compared also the percentage of one X chromosome signal in interphase cells between our patient group and two normal female population described in the literature in two reports

by Guttenbach and colleagues in 1995 and Lakhal et al. (2010) (Figure 3). Guttenbach et al. analyzed X chromosome mosaicism in a group of female aged 1 week to 91 years and in particular they found that the percentage of X monosomy in female aged 16–50 years was 3,13% [10, 25, 48]. The comparison between this literature age-matched control group and our patient group showed a significant increase of X monosomy in POF patient group (7.5% of X monosomy, $P < .001$). Similar results were obtained comparing our patient control group and the control group described by Lakhal and colleagues (2.33% of X monosomy) [25]. These data suggest that low-level X chromosome mosaicism may contribute to POF pathology and these findings are very interesting because POF is a premature cessation of ovarian function and probably the lack of adequate number of follicles may be due to an earlier oocyte aging in these patients that could lead to premature follicular atresia [24, 25]. In particular, Lakhal and colleagues found a correlation between X chromosome monosomy and the age of POF installation in a subgroup (11.5%) of patients, suggesting that mosaicism in these patients was pathological and linked to POF [25].

Many studies suggest that the incidence of cells lacking sex chromosome, however, might be caused by a preferential cell survival rate than preferential X chromosome loss. In fact, the loss of sex chromosomes (late replicating X chromosome for female and Y chromosome for male) should not affect cell survival as these chromosomes should not play a critical role in lymphocyte survival [6, 49, 50]. X chromosome loss could be accounted by aneuploidy of the late replicating X [51, 52]. Anyway, even if X chromosome is not fundamental for lymphocyte or other type of cell survival, it might have an essential role in the ovary, since various X chromosome abnormalities are associated with POF and many studies suggest that two intact X chromosome are essential for proper ovarian function [16, 20–22]. X chromosome has a pivotal role in ovarian development and maintenance, so the 45,X mosaicism may influence survival rate and be related to accelerated aging of ovarian cells. Follicular deficit of POF patients may be explained by decreased germ cell number, accelerated oocyte atresia, and postnatal destruction of germ cells [53]. The X chromosome appears to play an essential role in the mechanism that leads to POF phenotype, as females lacking an X chromosome, or showing extra copies of the X chromosome, are predisposed to developing POF [54]. In this regard, even trisomy seems to be related to ovarian dysfunction: although normal ovarian function and fertility are reported in most 47,XXX females, some of these patients experience delayed menarche or premature ovarian failure, late onset menarche, and oligomenorrhea [23, 55].

The prevalence of X monosomy can not be attributed to culture artifacts since this phenomenon has been described both in vitro and in vivo [11–13, 56] and it is unlikely that the observed hypodiploidy can be due primarily to technical factors, considering X loss rate in the younger and older age groups [57]. Peripheral blood lymphocytes still remain the standard method in cytogenetic diagnosis procedures because of their accessibility, but it is important

to highlight that the karyotypic pattern of the gonad may be different [46]. Anyway, blood lymphocytes provide the best indicator in the detection of senescence, and the increasing of chromosome aneuploidy with aging was assessed also in skin fibroblasts (reviewed in [48]). So it is reasonable to use blood lymphocytes as a model to study chromosome aneuploidy as the specific tissue of interest is often not available.

Fitzgerald in 1975 suggested a mechanism for X chromosome loss based on premature centromeric division (PCD), linked to a subsequent chromosome nondisjunction that may result in cells with an extra X chromosome or with only one sex chromosome, with a great majority of 45,X cells [58]. Burgoyne and Baker in 1984 suggested that 45,X/46,XX/47,XXX mosaicism may accelerate follicular atresia by different mechanisms: (i) aberrant chromosome pairing during meiosis; (ii) deficiency or overexpression of specific gene products on the X chromosome may influence oocyte quality; (iii) an overall impaired genetic control could be related to X chromosome mosaicism leading to defects in meiosis-mitosis process, resulting in gonadal damage, aberrant meiosis, and oocyte atresia [59].

Considering the high rate of X chromosome loss in POF patients, it is reasonable to hypothesize that POF represents a disease spectrum with various degrees, maybe related to X chromosome mosaicism. In fact, considering our data it is possible to suggest that women with X chromosome mosaicism can experience premature menopause [24, 25, 44]. The underlying pathological mechanism may be explained by accelerated oocyte aging due to increased mosaicism rate that leads to premature follicular atresia.

5. Conclusion

We performed a study on a large group of POF patients and we identified 27 chromosomal abnormalities associated with POF. Moreover, we assessed a higher frequency of X chromosome aneuploidy rate in POF patients than in the general population group, in particular an increased rate of X chromosome loss, observed by FISH on interphase nuclei. These findings confirm the importance of X chromosome in POF aetiology and the incidence of low-level X chromosome mosaicism in POF patients, as sex chromosome mosaicism may account for some “idiopathic” POF cases.

Acknowledgment

The authors gratefully acknowledge Telethon Foundation, Italy (Grant no. GGP09126 to Professor A. Marozzi).

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