Array Comparative Genomic Hybridization Profiling Analysis Reveals Deoxyribonucleic Acid Copy Number Variations Associated with Premature Ovarian Failure

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Introduction: Premature ovarian failure (POF) is defined by amenorrhea of at least 4- to 6-month duration, occurring before 40 yr of age, with two FSH levels in the postmenopausal range. Its etiology remains unknown in more than 80% of cases. Standard karyotypes, having a resolution of 5–10 Mb, have identified critical chromosomal regions, mainly located on the long arm of the X chromosome. Array comparative genomic hybridization (a-CGH) analysis is able to detect submicroscopic chromosomal rearrangements with a higher genomic resolution. We searched for copy number variations (CNVs), using a-CGH analysis with a resolution of approximately 0.7 Mb, in a cohort of patients with POF.

Patients and Methods: We prospectively included 99 women. Our study included a conventional karyotype and DNA microarrays comprising 4500 bacterial artificial chromosome clones spread on the entire genome.

Results: Thirty-one CNVs have been observed, three on the X chromosome and 28 on autosomal chromosomes. Data have been compared to control populations obtained from the Database of Genomic Variants (http://projects.tcag.ca/variation). Eight statistically significantly different CNVs have been identified in chromosomal regions 1p21.1, 5p14.3, 5q13.2, 6p25.3, 14q32.33, 16p11.2, 17q12, and Xq28.

Conclusion: We report the first study of CNV analysis in a large cohort of Caucasian POF patients. In the eight statistically significant CNVs we report, we found five genes involved in reproduction, thus representing potential candidate genes in POF. The current study along with emerging information regarding CNVs, as well as data on their potential association with human diseases, emphasizes the importance of assessing CNVs in cohorts of POF women. **(*J Clin Endocrinol Metab* 94: 4540–4546, 2009)**

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Abbreviations: a-CGH, Array comparative genomic hybridization; BAC, bacterial artificial chromosome; BPES, blepharophimosis ptosis epicanthus syndrome; CNV, copy number variation; POF, premature ovarian failure.

The average age for menopause in developed countries is approximately 51 yr. Premature ovarian failure (POF) is classically defined as secondary amenorrhea for at least 4- to 6-month duration in women less than 40 yr of age, associated with two FSH levels in the postmenopausal range (1). Different mechanisms have been identified to explain POF such as environmental, genetic, autoimmune, infectious, and iatrogenic causes (1). Among genetic causes, the most common one involves the X chromosome, as in Turner syndrome, partial X deletions, as well as X-autosome translocations. Two chromosomal regions involved in POF have been located on the long arm of the X chromosome at Xq13-21 and at Xq26-28. Several candidate genes have been identified on the X chromosome, such as *DIAPH2*, *XPNEP2*, *DACH2*, *POF1B*, or *ZFX*. However, only two genes located in the X chromosome have a proven role in POF: *FMR1* premutations (2), and *BMP15* mutations (3, 4). Autosomal genes can also be involved in POF. Indeed, mutations of the *FSHR* (5, 6), *ATM*, *AIRE*, *GALT1*, *GDF9* (4, 7), *FOXO3a* (8), *NOBOX* (9), *INHA*, *NOG*, *FOXL2* (10), and *NR5A1* (11) have been described. Two studies have suggested that PTH-responsive B1 gene and progesterone receptor membrane component-1 could be associated with POF (12, 13). However, the cause of POF remains unknown in more than 80% of cases.

Our working hypothesis was that in patients with POF, a routine conventional karyotype might miss subtle chromosomal abnormalities. Indeed, in recent years, microarray comparative genomic hybridization (a-CGH) technology has been applied to constitutional chromosomal abnormalities to detect submicroscopic chromosomal aberrations with a higher resolution (14, 15). Recent studies have suggested associations between copy number variations (CNVs) and different common disorders and rare diseases (16–20). Thus, exploring the role of CNVs in many traits and diseases becomes essential (21). Therefore, in the current study, we assessed the presence and the prevalence of CNVs, using a-CGH analysis comprising 4500 clones spread on the entire genome, in a cohort of 99 patients with POF.

Patients and Methods

Patients

Women were consecutively recruited from three French hospitals: Hôpital Saint-Antoine, Paris; Hôpital Antoine Béclère, Clamart; and Hôpital Jeanne de Flandre, Lille. Inclusion criteria were primary amenorrhea or secondary amenorrhea for more than 6 months, occurring before the age of 35, with FSH serum level higher than 40 mIU/ml. Women with previous ovarian surgery, chemotherapy, or radiotherapy were excluded from the study. We chose to recruit women with an amenorrhea occurring before the age of 35 to increase the probability of a genetic disorder potentially involved in POF. FSH was measured in duplicate using an immunofluorescent assay (Immulite, Siemens, Germany). The study was approved by the institutional review board of Saint-Antoine Hospital, and all participants gave their written informed consent.

Blood samples

A total of 25 ml was collected for each patient to perform karyotype analysis, hormonal level measurements, and DNA extraction. DNA was extracted from peripheral blood, using the QiaAmp DNA blood mini kit (QIAGEN, Courtaboeuf, France). Samples were centralized and numbered from 1 to 99, consecutively according to the date of the patient's inclusion in the study.

Cytogenetic analysis

Peripheral blood samples were collected, and metaphase chromosomes were prepared using standard and molecular cytogenetic methods. Twenty metaphase spreads, GTG or RHG banded, and 200 interphase nuclei were routinely analyzed from each patient. X chromosome mosaicism has been searched by fluorescent *in situ* hybridization using X centromeric probe.

Array CGH analysis

Array CGH analysis was performed according to the manufacturer's protocol on a genomic DNA array containing 4500 bacterial artificial chromosome (BAC) clones and P1-derived artificial chromosome clones spotted in duplicate (Integragen, Evry, France) (22). This microarray provides a mean spatial resolution of approximately 0.7 Mb on chromosomes to detect chromosomal imbalances throughout the whole genome. The genome positions of all clones on the array have been determined by BAC end sequencing.

Genomic DNA from the patients (test DNA) were digested with EcoR1 for 16 h at 37 C and repurified by Zymo Research's Clean and Concentrator (Zymo Research, Orange, CA). The reference and test DNAs were labeled with Cy3 and Cy5 by BioPrime random labeling kit (Invitrogen, San Diego, CA). The labeled reference DNA and labeled test DNA samples were combined with 50 μg of human Cot-1 DNA and 30 μg of sheared salmon sperm DNA. The labeled DNAs were denatured at 72 C for 10 min, followed by incubation at 37 C for 30 min to block repetitive sequences. Hybridization was performed for 48 h at 37 C. Slides were washed at room temperature in 2×SSC for 3–5 min, then, washed at 50 C for 25 min in 50% formamide/2×SSC.

Array CGH were scanned on Agilent DNA microarrays scanner (Agilent Technologies, Massy, France) and analyzed with Genocensus software (Integragen, Evry, France). DNA CNVs were mentioned as gain or loss as a linear ratio of at least 1.2 or no more than 0.8, respectively. In our study, gain or loss of at least two adjacent clones was considered significant. The length of each variation was given in megabases (Mb). All the CNVs identified in this study were assessed by screening them against the Database of Genomic Variants (http://projects.tcag.ca/variation). Statistically significant CNVs were verified by fluorescence *in situ* hybridization technique using corresponding BAC clones from the array.

Statistical analysis

The CNVs identified in our study were compared with CNVs reported in phenotypically normal individuals from control populations from the web site Database of Genomic Variants (23–32). Control populations published in this database are usually used as references to determine CNV frequencies. They include healthy women and men from different ethnic origins (Caucasian, Asian, and African). We first estimated the prevalence of CNVs in our sample of each woman with POF. For each chromosomal region potentially involved in POF, we compared the proportion of CNVs (*i.e.* percentage of patients with loss and percentage of patients with gain, respectively) in our sample to the proportion of CNVs (*i.e.* percentage of women with loss and percentage of women gain, respectively) in control populations using a χ^2 or Fisher's exact test when appropriate.

Results

Patients

Among the 99 patients, 33 and 66 presented with primary or secondary amenorrhea, respectively. In the group with secondary amenorrhea, the age of the last spontaneous period varied from 13 to 34 yr. Six patients stopped cycling before the age of 15, 10 between the ages of 16 and 20 yr, 14 between 21 and 25 yr, 24 between 26 and 30 yr, and 12 between the ages of 31 and 35. Among women with an amenorrhea occurring after the age of 30, 10 of 12 presented a "post pill amenorrhea." In those cases, the date of ovarian failure might have been overestimated because the pill might have hidden the clinical phenotype of POF for several months or even several years. All women were of Caucasian origin. Familial cases, defined by at least two cases of POF in the family, including the index patient, were present in 20% of cases. In our study, we only included the index case of each family. In the cohort, upon examination, six patients presented with clinical abnormalities in addition to amenorrhea. Two of them suffered from hearing loss although they did not present any neurological disorders referring to Perrault's syndrome. Another patient had an ogival palate, one presented with upslanting palpebral fissures without any other symptoms of BPES (blepharophimosis ptosis epicanthus syndrome). One patient had a cubitus valgus, bradymetacarpia of the fourth fingers, with a height of 164 cm. The last one had a shield chest and inverted mammelons, with a height of 155 cm.

Cytogenetic analysis

Conventional karyotypes were abnormal in five of 99 cases. Cytogenetic analysis showed two Xq deletions, 46,X,del(X)(q21.31) and 46,X,del(X)(q21.2); two X-autosome translocations, 46,X,t(X;2)(q22.3;q33) and 46,X,t(X;1)(q21.1;q32); and one 45,X/46,XX mosaicism (8% of monosomic cells). One of the five patients with abnormal karyotype had a cubitus valgus, bradymetacarpia of the fourth fingers, with a height of 164 cm (45,X/46,XX mosaicism).

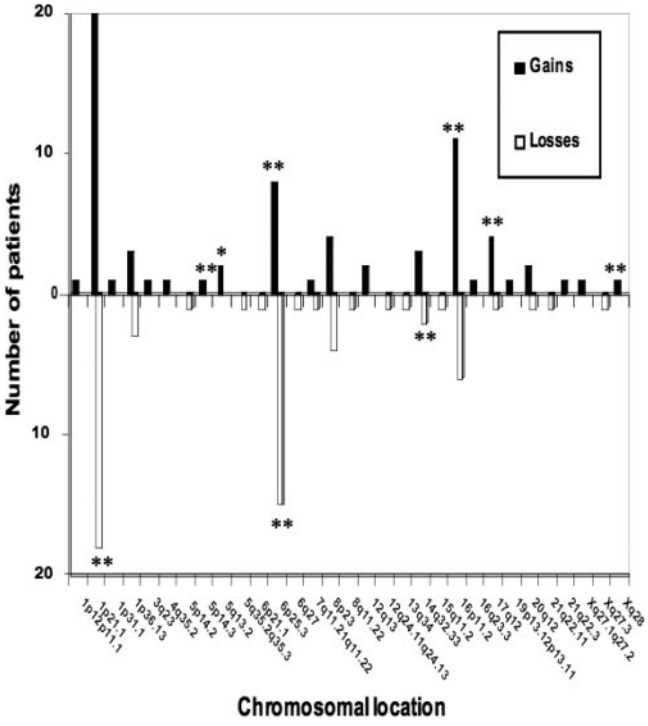

FIG. 1. Number of patients presenting CNVs, according to their different chromosome location. Gains are in *black*, losses in *white* histograms. *Asterisks* indicate CNVs statistically different from controls: *, $P < 0.05$; **, $P < 0.001$.

CGH array

Among the 99 samples, 90 were suitable for analysis. Eighteen of them showed no DNA CNV. In the remaining 72 samples, a total number of 131 gains or losses was identified. As reported in Fig. 1, those variations corresponded to 31 different CNVs. They were spread over the entire genome because 28 were located on autosomes, three on the X chromosome, as shown in Table 1. The CNV sizes varied from 0.151 to 7.134 Mb. The 31 CNVs identified have been previously described in phenotypically normal individuals according to the database of genomic variants. As shown in Tables 1 and 2, among the 31 CNVs, eight were statistically different from control populations. Among them, four were observed as gain (5p14.3, 5q13.2, 17q12, and 16p11.2), three as loss (1p21.1, 14q32.33, and Xq28), and one was reported both as a gain and a loss (6p25.3). Gains or losses identified by our CGH array have been confirmed by fluorescence *in situ* hybridization analysis. No correlation could be made between the CNVs and the phenotype of POF: primary *vs.* secondary amenorrhea, familial *vs.* sporadic cases, due to the low number of patients presenting statistically significant CNVs. Table 3 reports the different genes located in the eight statistically significant regions.

TABLE 1. Nonstatistically significant CNVs identified in our POF population (n = 90) compared to CNVs in control populations

Chromosomal location	Gain		Loss		
	Patients[a]	Controls[b]	Patients[a]	Controls[b]	Refs.[c]
1p12p11.1	1	3/95	0	1/95	24
1p31.1	1	3/30	0	0/30	25
1p36.13	3	22/270	3	17/270	26
3q23	1	1/270	0	1/270	26
4q35.2	0	0/30	1	2/30	25
5p14.2	0	0/36	1	1/36	27
5q35.2q35.3	0	0/30	1	2/30	25
6p21.1	0	0/270	1	4/270	26
6q27	0	1/30	1	0/30	25
7q11.21q11.22	1	1/272	1	1/272	29
8p23.1	4	30/270	4	31/270	26
8q11.22	0	0/270	1	1/270	26
12q13	2	1/270	0	1/270	26
12q24.11q24.13	0	1/272	1	1/272	29
13q34	0	0/30	1	1/30	25
15q11.2	0	3/112	1	3/112	24
16q23.3	1	0/50	0	1/50	28
19p13.12p13.11	1	14/270	0	1/270	26
20q12	2	0/270	1	2/270	26
21q22.11	0	1/248	1	0/248	30
21q22.3	1	4/50	0	0/50	28
Xq27.1q27.2	1	2/270	0	0/270	26
Xq27.3	0	1/270	1	0/270	26

[a] Number of POF patients identified with CNVs.

[b] Number of patients with CNVs in control populations.

[c] References for control populations.

Discussion

Many studies looking for genes in POF patients have focused on the X chromosome. Only two studies so far, have studied all the genome to find candidate genes. Those genome-wide association studies in POF have been reported. The first one in Korean women suggested that PTH-responsive B1 gene could be associated with POF (12). The second study in a Dutch family identified a POF susceptibility locus in 5q14.1-q15 (33).

The aim of our study was to search for chromosomal variations in the whole genome to identify chromosomal regions that could be associated to POF. Recent developments and applications of genome-wide structural variation technologies have led to the identification of thousands of CNVs. CNVs, defined as stretches of DNA larger than 1 kb that display copy number differences in the normal population, have recently gained considerable interest as a source of genetic diversity likely to play a role in functional variation (34). Indeed, these CNVs can influence transcriptional and translational levels of overlapping or nearby genes (25). Particular CNVs have been recently reported to be associated with susceptibility to glomerulonephritis, autism, HIV, BPES-like phenotypes, and medullary thyroid carcinomas (16–20).

Therefore, we performed a genome-wide analysis of 99 patients using microarray CGH technology. Eighteen patients did not present any CNVs. As it has been reported that CNVs make up about 15% of the human genome, often with tens to hundreds of CNVs per individual, our results are in accordance with the fact that our array CGH resolution is 0.7 Mb. Thus some CNVs may have been missed in the present study. However, a total of 31 CNVs spread over the genome have been identified. Among them, three were located on the X chromosome, and the remaining 28 on autosomal chromosomes. Those results emphasize previous data suggesting that X chromosome microdeletions are rare in POF (35) and that autosomal chromosomes can also be involved in POF (36).

Two of the 31 CNVs we identified were localized in chromosomal regions 3q23 and 21q22.3 containing genes known to be associated with POF, *FOXL2* and *AIRE* genes, respectively. The first patient presented with secondary amenorrhea at the age of 16 yr and did not present with BPES. The second patient had secondary amenorrhea at the age of 30 and did not have any feature of autoimmune polyendocrinopathy candidiasis ectodermal dystrophy syndrome. So far, the phenotypic effects of most CNVs remain unknown. Therefore, the report and accu-

TABLE 2. Statistically significant CNVs (c or d) in our POF population compared to CNVs in control populations

Chromosomal location	CNV size (Mb)	Gain		Loss		Refs.
		Patients[a]	Controls[b]	Patients[a]	Controls[b]	
1p21.1	0.176	20	181/269	18[d]	0/269	32
5p14.3	0.968	1[d]	7/30	0	0/30	25
5q13.2	2.076	2[c]	6/47	0	1/47	31
6p25.3	0.266	8[d]	133/270	15[d]	10/270	26
14q32.33	0.417–1.064	3	1/269	2[d]	41/269	32
16p11.2	1.241	11[d]	101/270	6	17/270	26
17q12	0.166–0.565	4[d]	112/270	1	1/270	26
Xq28	0.217	1	5/67	0[d]	62/67	28

[a] Number of POF patients identified with CNVs.

[b] Number of patients with CNVs in control populations.

[c] $P < 0.05$.

[d] $P < 5 \times 10^{-3}$.

TABLE 3. List of genes located in the chromosomal regions containing the eight statistically significant CNVs from control populations

BAC clones and localization	Genes
1p21.2	*AMY2A*, *AMY1C*, *AMY1B*, *LOC6648740 RNPC3*
5p14.3	***DNAH5***
5q13.2	*GTF2H2D*, *GTF2H2C*, *GTF2H2B*, *SMN1*, *SMN2*, *SERF1B*, *SERF1A*, *CCDC125*, *RAD17*, ***NAIP***, *CDK7*, *TAF9*, *MARVELD2*, *OCLN*, *GTF2H2*, *PMCHL2*, *BDP1*
6p25.3	*IRF4*, ***DUSP22***, *HUS1B*, *EXOC2*
14q32.33	*SIVA1*, ***AKT1***, *ZBTB42*, *INF2*, *ADSSL1*, *MGC23270*
16p11.2	*XPO6*, *SULT1A1*, *EIF3CL*, *EIF3C*, *CDC37P1*, *CLN3*, *NPIPL*, *CCDC101*, *SULT1A2*, *ATXN2L*, *ATP2A1*, *SH2B1*, *LAT*, *NFATC2IP*, *RABEP2*, *TUFM*, *SBK1*, ***NUPR1***, *CD19*, *IL27*, *SPNS1*
17q12	*TBC1D3C*, *TBC1D3G*, *GGNPB2*, *ZNHIT3 PIGW*, *MYO19*, *CCL4L1*, *CCL4L2*, *CCL3L1*, *CCL3L3*, *MRM1*, *DHRS11*, *CCL3*, *TBC1D3B*, *CCL4*, *CCL18*, *CCL15*, *CCL14*, *CCL23*
Xq28	*SPRY3*, *TMLHE*, *H2AFB1*, *F8*, *MPP*, *H2AFB2*, *F8A3*, *CLIC2*, *F8A2*, *VBP1*, *FUNDC2*, *H2AFB3*, *MTCP1*, *BRCC3*, *RAB39B*, *F8A1*

Genes involved in reproduction are shown in *bold*.

mulation of such a-CGH data may lead to the identification of pathogenic CNVs. To investigate the potential biological consequences of the observed CNVs, we compared them to the CNVs previously described in phenotypically normal individuals (23). Eight CNVs were statistically different from control populations. Copy number gains and losses were observed in our study. The functional impact of CNV loss or gain associated with diseases actually remains poorly studied. Recent studies in both human and model organisms have revealed that genes in CNV regions are expressed at lower and more variable levels than genes mapping elsewhere, and also that CNVs not only affect the expression of gene varying in copy number, but also have a global influence on the transcriptome (34). Both deletion and duplication can be associated with the same phenotype, suggesting that the simple presence of a structural change at a given position of the human genome may cause perturbation in particular pathways regardless of gene dosage (34). Among those chromosomal regions, we looked for genes known to be involved in reproduction. Although this strategy might misidentify the responsible gene, we chose it as a first approach. Until now, no gene involved in reproduction has been previously reported in 1p21.1, 17q12, and Xq28 regions. However, we found five genes potentially involved in reproduction in the five remaining CNVs. Among these five genes, two are involved in reproductive diseases (*DNAH5* and *NAIP*), two in reproductive endocrinology (*DUSP22* and *NUPR1*), and one gene in folliculogenesis (*AKT1*). *DNAH5* (dynein axonemal heavy chain) gene located in 5p15-p14 has been involved in asthenospermia with primary ciliary dyskinesia (37). In 5q13.2 is located the gene coding for neuronal apoptosis inhibitory protein (*NAIP*). This protein is a recently identified inhibitor of apoptosis, overexpressed in breast cancer patients with unfavorable clinical features (38). Dual specificity phosphatases gene (DUSP22/LMW-DSP2) regulating estrogen receptor α-mediated signaling (39) is located in 6p25.3. *NUPR1* gene coding for nuclear protein 1, also named COM1/P8, is located in 16p11.2. COM-1 plays a tumor suppressor role in breast cancer cells and is involved in estrogen-regulated cell growth (40). Finally, *AKT1* (akt murine thymoma viral oncogene homolog 1) gene located in 14q32 is known to be involved in folliculogenesis (41). Therefore, this first study of CNVs in POF patients has enabled us to point out five genes that could represent new putative candidate genes associated with POF.

Conclusion

Our study and emerging information about CNVs, as well as data on their potential association with human diseases, emphasizes the importance of assessing CNVs in cohorts of POF women. The accumulation and annotation of such a-CGH data can lead to the identification of pathogenic CNVs and genes involved in POF.

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