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Genomic analysis using high-resolution single nucleotide polymorphism arrays reveals novel microdeletions associated with premature ovarian failure

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Structured Abstract

Objective—To analyze DNA from women with premature ovarian failure (POF) for genome-wide copy number variations (CNVs), focusing on novel autosomal microdeletions.

Design—Case-control genetic association study.

Setting—Department of Obstetrics and Gynecology, Baylor College of Medicine, Houston, TX, USA.

Patients—Of 89 POF patients, eight experienced primary amenorrhea while 81 exhibited secondary amenorrhea prior to age 40.

Interventions—Genomic DNA from peripheral blood samples was analyzed for CNVs using high-resolution single nucleotide polymorphism (SNP) arrays.

Main outcome measures—Identification of novel CNVs in 89 POF cases, using the Database of Genomic Variants as a control population.

Results—A total of 198 autosomal CNVs were detected by SNP arrays, ranging in size from 0.1 Mb to 3.4 Mb. These CNVs included seventeen novel microduplications and seven novel microdeletions larger than 0.1 Mb, six of which contained coding regions: 8q24.13, 10p15-p14, 10q23.31, 10q26.3, 15q25.2, and 18q21.32. Most of the novel CNVs were derived from autosomes rather than the X chromosome.

Conclusions—The present pilot study revealed novel microdeletions/microduplications in POF. Two novel microdeletions caused haploinsufficiency for *SYCE1* and *CPEB1*, genes known to cause ovarian failure in knockout mouse models. Chromosomal microarrays may be a useful adjunct to conventional karyotyping when evaluating genomic imbalances in women with POF.

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Keywords

premature ovarian failure; POF; copy number; CNV; microdeletion and microduplication; SNP microarray; POI; array CGH; chromosomal microarray

INTRODUCTION

Menopause, or the permanent cessation of menses, naturally occurs in most American women between 46 and 54 years of age (1). Approximately 1–5% of women in the world experience cessation of their menstrual cycle prior to age 40 (2), a condition known as premature ovarian failure (POF) or premature ovarian insufficiency (POI). POF encompasses a broad spectrum of individuals, ranging from women with primary amenorrhea to women who have reproduced but experience loss of menstrual cycles prior to age 40, coupled with menopausal serum FSH levels (3–4). Unlike menopause, however, this condition does not always result in permanent loss of ovarian function, as nearly 5–10% of women are able to conceive after the onset of POF (5).

Most POF cases are classified as idiopathic, and these cases are further divided into sporadic and familial forms. Genetics is known to contribute significantly to idiopathic POF cases (6), and approximately 10–15% of diagnosed women have an affected first degree relative (5). Initial genetic interest has focused on the X chromosome due to Turner syndrome, as well as published associations of POF cases with X chromosome deletions and translocations (7–10). Genes specifically expressed from the X chromosome that have been implicated in POF include *FMR1* (premutation; Fragile X Syndrome), *BMP15*, and *POF1B* (11–14).

In addition to X chromosome abnormalities, POF has been associated with autosomal gene aberrations. *FSHR* mutations are associated with POF in Finnish women, although such mutations are rare outside of Finland (15). Other autosomal genes that have been more widely associated with POF include *GDF9*, *FIGLA*, *FOXO1a*, *FOXO3a*, and *NOBOX* (13,16–18).

Current genetic guidelines in the evaluation of POF cases recommend karyotyping and *FMR1* premutation carrier screening. Conventional karyotyping has been a mainstay to determine genomic imbalances in POF individuals; however, it cannot detect genomic imbalances smaller than 3–5 Mb in size (microdeletions and microduplications, also known as copy number variations or CNVs) due to banding resolution. Recent studies utilizing oligonucleotide and single nucleotide polymorphism (SNP) microarray technologies have shown that genomic imbalances involving as few as 1,000 base pairs can be detected (19). It is possible that some of the idiopathic POF cases are due to CNVs. Therefore, we utilized a SNP based array to determine if women with POF exhibit novel CNVs.

MATERIALS AND METHODS

Participant population, blood collection and genomic DNA isolation

The study was composed of 89 women with POF: eight women who exhibited primary amenorrhea and 81 who exhibited secondary amenorrhea for more than six months prior to the age of 40, with FSH serum levels greater than 40 IU/L. Women with a history of pelvic surgery, cancer, radiation exposure, smoking, and genetic syndromes were excluded from the study. Peripheral blood samples were collected from all women, and genomic DNA (gDNA) was extracted from 5 mL of whole blood (Qiagen Puregene protocol, Valencia, CA, USA), as previously described (20). One of the eight women with primary amenorrhea

failed genotyping quality control and was excluded from analysis. Therefore, a total of 88 women were available for analysis. This study was approved by the Institutional Review Board at Baylor College of Medicine.

Single nucleotide polymorphism arrays and data analysis

Illumina's (San Diego, CA, USA) HumanCNV370-Duo DNA Analysis BeadChip with more than 318,000 tag SNP markers and 52,000 markers targeting additional CNV regions (5.5 kb median marker spacing) was used to examine CNVs among gDNA for 38 individuals on the BeadArray 500GX. Illumina's Human660W-Quad v1 DNA Analysis BeadChip with 550,000 tag SNPs and an additional 100,000 markers targeting common CNV regions (2.5 kb median marker spacing) was used for an additional 51 individuals on the iScan System.

The array data was analyzed using Illumina's GenomeStudio Genotyping Module software. A call rate of >99% was accepted as the validity cutoff for each sample on both chips, which indicates >99% of the probes successfully identified a genotype. Using the GenomeStudio software, the log of the signal intensity (log R ratio) highlighted CNVs as an increase or decrease from the baseline intensity which was standardized to zero. The B-allele frequency, standardized at 0.5, was coupled with the log R ratio values to examine areas of DNA loss/gain. The concurrent use of these two tools enabled CNVs to be differentiated from regions of copy-neutral loss of heterozygosity.

All CNVs were screened against polymorphisms noted in the Database of Genomic Variants (DGV; <http://projects.tcag.ca/variation>); those already listed with a high population frequency in the DGV were regarded as benign polymorphisms and subsequently excluded from further analyses.

Quantitative real-time PCR

Quantitative real-time PCR (qPCR) was utilized to corroborate SNP array findings (20). Emphasis was placed on unique microdeletions that were not present in the DGV database. Where possible, a relevant protein-coding gene within each deleted region was chosen as a target, and oligonucleotides were designed for qPCR analysis (Table 1). Primers were custom-designed through Primer3Plus (<http://frodo.wi.mit.edu/primer3>).

Quantitative real-time PCR was conducted using 100 ng of gDNA, 0.4 μ M forward and reverse primers, 1X iQTM SYBR Green Supermix (Bio-Rad, Hercules, CA, USA) and nuclease free water, with a total volume of 20 μ L per well. Five replicates were run for each POF individual for the targeted gene within each respective deleted region. A pooled human gDNA sample from five women was used as a control (Promega, Cat # G1521). Threshold cycle (Ct) values were calculated and averaged for each set of five replicates; these values were then used to calculate the standard error of the mean. The $2^{-\Delta \Delta C_t}$ method of quantification was used to analyze fold changes in copy number between control (2n) and deleted regions (1n or absent).

RESULTS

We analyzed the genomic DNA of 89 women with POF utilizing high-resolution SNP arrays to determine the prevalence of novel CNVs (microdeletions/microduplications) which would not be detected by conventional karyotype methods. Eighty-eight samples were successfully arrayed. Microarray data revealed a total of 50 microduplications and 148 microdeletions on autosomal chromosomes, ranging in size from 0.1 Mb to 3.4 Mb. These CNVs occurred in 72 out of 88 genotyped POF individuals, and they spanned every autosome. In order to distinguish novel CNVs from seemingly benign, non-pathogenic imbalances, we compared

our data to the Database of Genomic Variants. The DGV is a compilation of common CNVs detected in more than 10,000 healthy individuals, and it has been used as a control population in several studies (21–23). We found 33/50 microduplications and 141/148 microdeletions in the control population of the DGV. Therefore, 24 autosomal CNVs in a total of nineteen women were considered novel. There were seven novel autosomal microdeletions (Table 2), one in each of seven different women, and seventeen novel autosomal microduplications (Table 3), one in each of eleven women and two in each of three women.

We specifically focused on microdeletions, as genomic losses are more likely to result in a clinical phenotype than genomic gains (24). Seven women with POF exhibited novel hemizygous microdeletions greater than 0.1 Mb in size, across the autosomes. Six of the novel autosomal microdeletions contained coding regions, and five of the deletions were subsequently verified by qPCR analysis: 8q24.13, 10p15-p14, 10q26.3, 15q25.2, and 18q21.32 (Table 2). Control amplifications for regions of no change in copy number did not show detectable Ct shifts. A $2^{-\Delta\Delta C_t}$ (fold change) quantification analysis was also performed (Figure 1), with the standard error of the mean Ct difference for each group of replicates given as the error bars. The fold change was plotted against the mean intensity shift (log R ratio), as measured on the array. All plotted microdeletions showed considerable fold reductions, as indicated by the top bars that lie below the shaded region ($2n = \text{diploid}$). *SYCE1* and *CPEB1* were genes included in the verified microdeletions. These genes are known to cause ovarian failure in knockout mouse models (25–26).

Interestingly, microdeletions were enriched in women with primary amenorrhea; two of the seven genotyped women with primary amenorrhea (29%) had novel microdeletions as compared to 6/81 women with secondary amenorrhea (7%). Although our study focused on autosomes, we detected one novel interstitial microdeletion on the X chromosome in a woman with secondary amenorrhea, which mapped to Xp11.23-p11.22 where the *BMP15* gene is located (Table 2).

DISCUSSION

Animal models indicate that the majority of candidate genes for ovarian failure are located on autosomes (13,16–18). Although initial genetic screening studies for POF focused on the X chromosome, recent genome-wide studies in humans have found associations with autosomal chromosomes, as well (21,27–29). These findings offer further motivation to continue looking more closely across the entire genome for genomic imbalances that could contribute to the phenotypic spectrum of POF.

Previous studies have highlighted the possible role of CNVs in the pathogenesis of several common and rare diseases (30–32). Haploinsufficiency encountered in microdeletions is a well-known cause of human genetic disorders; these microdeletions can occur sporadically, or they can be inherited. Sporadic cases likely arise from non-allelic homologous recombination or unequal crossing-over between large, highly identical segmental duplications. It has also become clear that the severity of clinical presentations varies widely, with identical microdeletions causing no apparent symptoms in carrier parents, yet severe clinical presentation in the offspring (33). The causes of such variable expression are unclear but likely include predisposing polymorphisms in the affected individuals. The objective of our study was to utilize a genome-wide approach to detect microdeletions and microduplications in a cohort of 89 women diagnosed with POF. We hypothesized that novel CNVs associate with POF.

We identified seven novel autosomal microdeletions among 88 successfully arrayed POF women. Quantitative real-time PCR was used to corroborate five of the six novel autosomal microdeletions that contained known coding regions. In the case of POF-66, the deletion (0.24 Mb) was in a genomic region devoid of known genes, miRNAs or other non-coding RNAs. Although it is easy to dismiss genomic regions that do not contain known transcriptional units, recent data shows that transcriptionally inactive regions can account for an abnormal phenotype when disrupted (34).

Among the genes found within the novel autosomal microdeletions, two have been strongly linked to abnormal reproductive phenotypes in knockout mouse models – *SYCE1* (10q26.3) and *CPEB1* (15q25.2). *SYCE1* has been shown to disrupt the repair of DNA double-strand breaks during meiosis, which leads to apoptosis of germ cells and subsequent infertility of both male and female knockout mice (25). Based on this null phenotype in female mice, *SYCE1* haploinsufficiency in humans may have a similar effect, leading to loss of oocytes and POF (35). The woman who presented with a hemizygous deletion of *SYCE1* had menarche at 14 and experienced cessation of menstrual cycles at age 21. Another gene, *CPEB1*, is preferentially expressed in the oocyte and encodes a protein that regulates the translation of mRNAs for proteins involved in synaptenomal complex formation during oocyte maturation (35). Adult female mice deficient in *CPEB1* lack follicles due to loss of oocytes, while mid-gestation female embryos show oocytes arrested at the pachytene stage (36). *CPEB1* has also been found to regulate mitotic cell progression in the S and M phases, which are especially important during embryonic cell divisions (26). *CPEB1* haploinsufficiency in humans may accelerate germ cell loss during the reproductive lifespan, as is the case in POF. This hypothesis could support the fact that the woman who presented with a hemizygous deletion of *CPEB1* exhibited ovarian failure at an earlier age; she never experienced menarche (primary amenorrhea).

Other microdeletions identified in our screen harbor genes that are not known to play a role in reproductive biology. POF-119 exhibited a deletion that resulted in haploinsufficiency of *CCBE1* and *PMAIP1*. We used microarray data generated from wild-type newborn mouse ovaries (37), as well as NCBI EST databases (Unigene and Gene Expression Omnibus), to determine whether *CCBE1* and *PMAIP1* are expressed in mammalian ovaries. Based on these data sets, both *CCBE1* and *PMAIP1* are expressed in mouse ovaries. Similarly, *CTNNA3* (POF-85), *ANKRD22* and *STAMBPL1* (POF-58) transcripts are present in the murine ovarian transcriptome. It is possible that such genes may play a yet-to-be-defined role in ovarian development.

We also identified seventeen autosomal microduplications which have not been reported in the DGV. The role of duplications in human disorders is less compelling, since corresponding animal models are lacking or difficult to interpret. Two duplications on chromosome one were nearly identical in two distinct POF individuals (POF-21 and POF-106), and they included genes for hydroxy-delta-5-steroid dehydrogenase, 3 beta- and steroid delta-isomerase 2 (*HSD3B2*) and hydroxyacid oxidase 2 (*HAO2*). The role of *HAO2* in ovarian biology is unknown, despite its expression in the mouse ovary. Conversely, *HSD3B2* is preferentially expressed in the adrenal glands and ovaries of mice, according to the Unigene and BioGPS databases (38–39). *HSD3B2* is essential for steroid hormone production in humans, and deficiencies cause a severe form of congenital adrenal hyperplasia (40). Furthermore, additional studies report increased expression of *HSD3B2* in theca cells of women with polycystic ovary syndrome, a frequent cause of infertility in women (41). It is possible that duplication of *HSD3B2* may lead to androgen and estrogen imbalances within the ovary with subsequent disruption of folliculogenesis, but this hypothesis remains to be tested.

We discovered seven novel autosomal microdeletions and seventeen novel autosomal microduplications; in addition, we identified one novel microdeletion located on the X chromosome. Our results add to the growing body of recent literature supporting our hypothesis that CNVs associate with POF. A recent study on French women used a lower resolution BAC array (0.7 Mb mean spatial resolution) to identify CNVs that statistically associate with POF (21). As with our study, there was an excess of CNVs on the autosomes as compared to the X chromosome. Eight CNVs showed statistical association with POF in their study, (seven autosomal and one X chromosomal), but no novel CNVs were presented. A second study focused only on the X chromosome (22). A BAC tiling array for X/Y chromosomes was utilized, and the authors discovered new, discrete X chromosome intervals associated with POF. It is important to note that there may be additional CNVs that went undetected by our arrays and those from previous studies, as even the highest resolution arrays may only tag approximately 50% of CNVs in the genome (42). In addition to technological limitations, our study was limited by the lack of family members and the small sample size. However, due to the high variability in the clinical phenotype of carriers and affected family members with microdeletions (33), the presence of such microdeletions in a fertile parent would not rule out the pathogenic role of such genomic imbalances in ovarian function in the offspring. Future studies with larger populations will be necessary to determine whether recurring genomic imbalances are present in women with POF and to discern the utility of molecular karyotyping methods, such as array CGH, in replacing conventional karyotyping. Moreover, animal models that mimic novel human deletions and/or duplications may be useful in further delineating the importance of such regions in mammalian reproductive biology.

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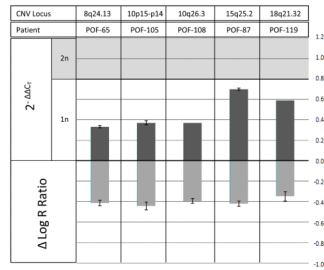


Figure 1. Data from SNP arrays and qPCR plotted as $\Delta \log R$ ratio and $2^{-\Delta \Delta C_t}$, respectively, for five autosomal microdeletions in women with POF

Regions of interest were initially determined based on log R ratio shifts, where reported copy number reductions in gDNA from peripheral blood samples are indicated above by a negative $\Delta \log R$ ratio. $2^{-\Delta \Delta C_t}$ values (fold changes) were then calculated from qPCR output for the target regions. Fold change values lying within the shaded region are indicative of a normal diploid copy number of 2, whereas those below the shaded region are indicative of a copy number reduction of 1, a fold change of approximately 0.5 (1/2). Hemizygous deletions were corroborated on 8q24.13 in POF-65, 10p15-p14 in POF-105, 10q26.3 in POF-108, 15q25.2 in POF-87 and 18q21.32 in POF-119.

Table 1

Oligonucleotides designed for quantitative real-time PCR corroboration of novel autosomal microdeletions with coding regions

Microdeletion locus	Patient	Targeted gene	Forward primer (5' -> 3')	Reverse primer (5' -> 3')
8q24.13	POF-65	<i>FER1L6</i>	CGA ACC ACA GTG CAG AAG AA	AGG GCT AGG TCA TTA ATG CT
10p15-p14	POF-105	<i>AKR1C1</i>	TGT GGA TGG TGA CAC AGA GG	TCT TTC CTT TCT GGC CAA TG
10q23.31 ^a	POF-58	<i>STAMBPL1</i>	TAA GCC CAG AAG AGC GAG TC	CGC CAT CCT CTC CAT CTC TA
10q26.3	POF-108	<i>SYCE1</i>	CCA GTG ACA TGG TGG AGT TG	GGT GTC TTC AIT GCC ACT CA
15q25.2	POF-87	<i>CPEB1</i>	CCT GGG TAT TAG CCG ACA GT	AAT CCC GGC ATA CAC CAC T
18q21.32	POF-119	<i>PMAIP1</i>	CAC CGT GTG TAG ITG GCA TC	CCT TCT TCC CAG GCA TCT

^aUnable to corroborate via quantitative real-time PCR

Table 2

Novel hemizygous microdeletions in cohort of women with POF

Microdeletion locus (hg18)	Patient	Length (Mb)	Age at POF onset (years)	Genes within deleted region
chr 8: 124,964,990-125,101,913	POF-65	0.14	36	<i>FER1L6</i>
chr 10: 4,867,522-5,196,096	POF-105	0.33	35	<i>AKRIE2, AKRIC1, AKRIC2, AKRIC3, AKRICLI</i>
chr 10: 90,517,549-90,648,588	POF-58	0.13	36 ^a	<i>LIPN, LIPM, ANKRD22, STAMBPL1</i>
chr 10: 135,092,227-135,256,027 ^b	POF-108	0.16	21	<i>CYP2E1, SYCE1</i>
chr 14: 40,060,168-40,305,161	POF-66	0.24	31	None
chr 15: 80,972,574-82,640,853	POF-87	1.67	PA	<i>CPEB1, AP3B2, FSD2, HOMER2, WHAMM, FAM103A1, C15orf40, BTBD1, TM6SF1, HDGFRP3, BNCL, SH3GL3, ADAMTSL3</i>
chr 18: 55,460,039-55,783,281	POF-119	0.32	PA	<i>CCBE1, PMAIP1</i>
chr X: 48,104,013-52,083,957	POF-37	3.98	34	<i>SSX4.....BMP15..... AK09637^c</i>

^a Age at onset of POF unknown; age at time of POF diagnosis indicated^b Pearson's chi square test was used to show statistical significance:

3/2,802 control individuals showed a variation of this deletion in Database of Genomic Variants at the indicated locus (43-44); P < 0.0001

^c The deletion encompasses a total of 65 genes; the first and last genes in the deleted interval are listed, as well as the *BMP15* gene, known to play critical roles in folliculogenesis

PA = primary amenorrhea (absence of menarche)

Table 3

Novel microduplications in cohort of women with POF

Microduplication locus (hg18)	Patient	Length (Mb)	Genes within duplicated region
chr 1: 119,652,659-119,966,935	POF-106	0.31	<i>HAO2, HSD3B2, HSD3B1</i>
chr 1: 119,719,096-119,953,605	POF-21	0.23	<i>HAO2, HSD3B2, HSD3B1</i>
chr 1: 237,422,778-237,602,136	POF-56	0.18	None
chr 2: 51,536,418-51,865,782	POF-87	0.33	None
chr 3: 7,979,828-8,127,315	POF-87	0.15	None
chr 4: 4,705,258-4,939,891	POF-124	0.23	<i>MSX1</i>
chr 4: 6,003,730-6,247,313	POF-124	0.24	<i>CRMPI, C4orf50, JAKMIP1</i>
chr 6: 57,377,166-57,503,129	POF-34	0.13	<i>PRIM2</i>
chr 7: 70,257,034-70,592,167	POF-37	0.34	<i>WBSCR17, CALN1</i>
chr 7: 75,984,206-79,394,983	POF-83	3.41	<i>PMS2L11, CCDC146, FL2, PION, PTPN12, RSNIL, TMEM60, PHTF2, MAGI2, RPL13AP17</i>
chr 7: 97,252,046-97,393,215	POF-10	0.14	<i>ASNS</i>
chr 8: 509,919-835,807	POF-16	0.33	<i>ERCHI</i>
chr 8: 2,442,385-3,126,569	POF-45	0.68	<i>CSMDI</i>
chr 8: 47,626,500-48,043,110	POF-13	0.42	<i>BEYLA</i>
chr 9: 71,619,570-72,483,319	POF-119	0.86	<i>C9orf135, MAMDC2, SMC5, KLF9, TRPM3</i>
chr 11: 48,556,067-51,447,829	POF-10	2.89	<i>FOLH1, OR4C13, OR4C12, OR4A5, OR4C46</i>
chr 16: 14,983,979-16,367,263	POF-11	1.38	<i>PCXDCl, NTAN1, RRN3, MPV17L, C16orf45, NDE1, MIR484, MYH11, C16orf63, ABCC1, ABCC6, NOMO3</i>