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ORIGINAL ARTICLE ***Reproductive genetics***

Chromosomal instability in women with primary ovarian insufficiency

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STUDY QUESTION: What is the prevalence of somatic chromosomal instability among women with idiopathic primary ovarian insufficiency (POI)?

SUMMARY ANSWER: A subset of women with idiopathic POI may have functional impairment in DNA repair leading to chromosomal instability in their soma.

WHAT IS KNOWN ALREADY: The formation and repair of DNA double-strand breaks during meiotic recombination are fundamental processes of gametogenesis. Oocytes with compromised DNA integrity are susceptible to apoptosis which could trigger premature ovarian aging and accelerated wastage of the human follicle reserve. Genomewide association studies, as well as whole exome sequencing, have implicated multiple genes involved in DNA damage repair. However, the prevalence of defective DNA damage repair in the soma of women with POI is unknown.

STUDY DESIGN, SIZE, DURATION: In total, 46 women with POI and 15 family members were evaluated for excessive mitomycin-C (MMC)-induced chromosome breakage. Healthy fertile females ($n = 20$) and two lymphoblastoid cell lines served as negative and as positive controls, respectively.

PARTICIPANTS/MATERIALS, SETTING, METHODS: We performed a pilot functional study utilizing MMC to assess chromosomal instability in the peripheral blood of participants. A high-resolution array comparative genomic hybridization (aCGH) was performed on 16 POI patients to identify copy number variations (CNVs) for a set of 341 targeted genes implicated in DNA repair.

MAIN RESULTS AND THE ROLE OF CHANCE: Array CGH revealed three POI patients (3/16, 18.8%) with pathogenic CNVs. Excessive chromosomal breakage suggestive of a constitutional deficiency in DNA repair was detected in one POI patient with the 16p12.3 duplication. In two patients with negative chromosome breakage analysis, aCGH detected a Xq28 deletion comprising the Centrin EF-hand Protein 2 (CETN2) and HAUS Augmin Like Complex Subunit 7 (*HAUS7*) genes essential for meiotic DNA repair, and a duplication in the 3p22.2 region comprising a part of the ATPase domain of the MutL Homolog 1 (*MLH1*) gene.

LIMITATIONS REASONS FOR CAUTION: Peripheral lymphocytes, used as a surrogate tissue to quantify induced chromosome damage, may not be representative of all the affected tissues. Another limitation pertains to the MMC assay which detects homologous repair pathway defects and does not test deficiencies in other DNA repair pathways.

WIDER IMPLICATIONS OF THE FINDINGS: Our results provide evidence for functional impairment of DNA repair in idiopathic POI, which may predispose the patients to other DNA repair-related conditions such as accelerated aging and/or cancer susceptibility.

[†]These two authors contributed equally to this article.

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Key words: primary ovarian insufficiency / DNA repair / chromosomal breakage / mitomycin-C / ovarian dysfunction / aging

Introduction

Primary ovarian insufficiency (POI) is a reproductive and endocrine dysfunction characterized in women younger than 40 years by irregular menses, hypoestrogenism and menopausal-level serum gonadotrophins (Cooper *et al.*, 2011). A long-term consequence of POI is a 50% higher risk of overall mortality as compared to women who reach natural menopause, mostly due to ischemic heart disease and premature osteoporosis (Brand *et al.*, 2013). The molecular mechanisms linking POI and adverse health outcomes are currently not clear. Recent application of whole exome sequencing in POI families have identified new POI causing genes including Synaptonemal Complex Central Element Protein 1 (*SYCE1*) (de Vries *et al.*, 2014), Stromal Antigen 3 (STAG3) (Caburet *et al.*, 2014), HFM1 ATP Dependent DNA Helicase Homolog (*HFM1*) (Wang *et al.*, 2014), Minichromosome Maintenance 8 Homologous Recombination Repair Factor (*MCM8*) (AlAsiri *et al.*, 2015; Tenenbaum-Rakover *et al.*, 2015), Minichromosome Maintenance 9 Homologous Recombination Repair Factor (*MCM9*) (Wood-Trageser *et al.*, 2014), RCC1 And BTB Domain Containing Protein 1 (*RCBTB1*) (Coppieters *et al.*, 2016), Shugoshin 2 (*SGO2*) (Faridi *et al.*, 2017), Proteasome 26 S Subunit, ATPase 3 Interacting Protein (*PSMC3IP*) (Zangen *et al.*, 2011) and Nucleoporin 107 (*NUP107*) (Weinberg-Shukron *et al.*, 2015) that are mainly involved in meiosis and DNA repair and damage response (DDR).

Alterations in DNA repair genes such as Ataxia Telangiectasia Mutated (*ATM*), Nibrin (*NBN*), Bloom Syndrome RecQ Like Helicase (*BLM*), Werner Syndrome RecQ Like Helicase (*WRN*) and RecQ Like Helicase 4 (*RECQL4*) are known to result in chromosome breakage disorders such as ataxia telengiecstasia, Nijmegen breakage, Bloom, Werner and Rothmund-Thomson syndromes, respectively, syndromic conditions associated with increased risk for cancer, premature aging and POI. Furthermore, patients with genetic defects in members of the Fanconi anemia complementation group (FANC), which function in a post-replication DNA repair processes, may present with reduced fertility and POI (Giri *et al.*, 2007).

POI may also be observed as a non-syndromic condition in individuals with defects in DNA repair genes. We and others recently described a subset of POI patients with pathogenic variants, inherited in autosomal recessive fashion, in *MCM8* and *MCM9* genes who present with POI (Wood-Trageser *et al.*, 2014; AlAsiri *et al.*, 2015; Goldberg *et al.*, 2015; Tenenbaum-Rakover *et al.*, 2015; Fauchereau *et al.*, 2016; Desai *et al.*, 2017). The *MCM8* locus and probably its associated interacting protein *MCM9*, are important determinants of ovarian aging from gonadal development to menopause. Defective DDR can associate with endocrine dysfunction and cancer, however, the prevalence of these disorders and penetrance of non-reproductive phenotypes is unknown. Multiple genome-wide association studies conducted on the age of menopause have also shown association with loci that harbor DDR genes. The strongest genetic association for the age of menopause among Caucasian women involves a one non-synonymous single nucleotide polymorphism (SNP), rs16991615, in the *MCM8* gene (He *et al.*, 2009; Murray *et al.*, 2011; Chen *et al.*, 2012; Stolk *et al.*, 2009, 2012). Individuals with *MCM8* and *MCM9* pathogenic variants, despite showing seemingly normal health in their reproductive years, have defective DDR (Wood-Trageser *et al.*, 2014; AlAsiri *et al.*, 2015; Tenenbaum-Rakover *et al.*, 2015; Bouali *et al.*, 2017) when chromosomal breaks were quantitated in the presence of the DNA damaging agent mitomycin C (MMC) (Nishimura *et al.*, 2012). We hypothesized that a subset of women with POI have defective DDR in their somatic tissues, and we screened a cohort of women with idiopathic POI for excessive chromosomal breakage in the peripheral lymphocytes exposed to MMC. We also performed high-resolution array comparative genomic hybridization (aCGH) analysis to determine copy number variations (CNV) for a panel of 341 known and putative DNA repair genes in POI and control females. Our study identified 1 in 46 individuals with an overt deficiency in DDR to MMC, as well as CNV revealed by microarray analysis in three individuals.

Materials and Methods

Patients and samples

This pilot study was approved by the Institutional Review Board of The University of Pittsburgh (PRO09080427). The study cohort included 46 women diagnosed with non-syndromic POI. In total, 30 POI subjects had a concurrent karyotype analysis which revealed a normal female 46,XX karyotype in 29 females and a 47,XXX chromosome complement in one patient. Of all participants, 78% (36/46) were Caucasian and 9% were African American ($n = 4$), 4% were Asian ($n = 2$), 4% were Asian Indians ($n = 2$) and 4% were of mixed ethnicity ($n = 2$) (see Supplementary Table S1). Nine patients (9/46, 20%) presented with primary amenorrhea while the remaining women (37/46, 80%) had secondary amenorrhea. POI subjects completed a detailed questionnaire about their medical, surgical, social, medication and family history.

Chromosome breakage analysis

Chromosome breakage studies were performed on fresh peripheral blood samples collected in a tube containing sodium heparin using previously described protocols (Oostra *et al.*, 2012; Auerbach, 2015). Briefly, whole blood samples were cultured in complete RPMI medium with phytohemagglutinin to induce T-cell division in the presence of 0, 50 and 150 nM of the cross-linking agent MMC (Sigma, St. Louis, MO, USA). Analysis was conducted on 46 women with POI and 15 family members through 20 runs. Lymphoblastoid cell lines positive for Fanconi anemia (GM16749) and Nijmegen breakage syndrome (GM15812) were obtained from the Coriell Repository (Camden, NJ, USA) and used as a positive control in each experiment. Blood from an unrelated healthy fertile individual was used as a negative control. Cultures were incubated at 37°C for 72 h and harvested after 30 min treatment with colcemid. A minimum of two slides were prepared for every culture and subsequently stained with Giemsa. Metaphase images were then captured utilizing a Metafer scanning and capturing platform (MetaSystems, Germany). A total of 50 consecutive

metaphase cells from a minimum of two slides from cultures treated with 150 nM MMC were evaluated for aberrations (Supplementary Table SII). Overall, 25 metaphase spreads were screened in healthy fertile controls to establish a baseline for random chromosome breakage. Scoring was performed by a trained cytotechnologist followed by review of the results by two cytogeneticists. Each metaphase was assessed for chromosomal aberrations including chromatid gap, chromatid break, triradial/quadriradial chromosomes and other chromatid interchange figures (Oostra *et al.*, 2012). For each participant, details on type and number of aberrations per cell, total number of aberrations and percentage of affected cells were recorded and compared to the findings for negative and positive controls.

Microarray analysis

We designed a custom microarray to examine submicroscopic deletions and duplications with a high-resolution of 1–2 kb involving a targeted set of known and candidate genes implicated in DNA repair. Genomic DNA was extracted from peripheral blood samples (collected in EDTA tubes) using Qiagen kits (Qiagen, Valencia, CA, USA). DNA from 16 women with POI was tested for CNV using oligonucleotide-based 4×60 K aCGH (Agilent Inc., Santa Clara, CA, USA). Microarrays were scanned into image files using an Agilent Microarray Scanner (PN G2565BA). Aberrant copy number segments were detected and displayed by Cytogenomics 4.0 software (Agilent). Microarray analysis was performed as previously described (Yatsenko *et al.*, 2016). For any given region, duplications and deletions were reported in a minimum of five consecutive probes, with an absolute average $\log_2$ ratio of >0.3 and <−0.5, respectively. CNV intervals reported in the Database of Genomic Variants (DGV) (http://projects.tcag.ca/variation/) as constitutional polymorphic regions were classified as 'likely benign CNVs' and excluded from further analysis.

Statistical analysis

We used the Statistical Package for Social Sciences version 24 (IBM, NY, USA) and Microsoft Excel 365 Pro Plus spreadsheet (Microsoft, WA, USA) for data entry and analysis. The mean number of chromosomal defects was compared between the groups. A Kruskal–Wallis test was performed to determine statistical significance. A *P*-value of <0.05 is considered significant.

Results

Chromosome breakage analysis

We evaluated DNA-repair capabilities of the cultured lymphocytes to MMC exposure from 46 women diagnosed with POI. The total numbers of chromosome/chromatid break and complex rearrangements were analyzed in patients and positive and negative controls (Fig. 1). Positive controls included lymphoblastoid cell lines from patients with Fanconi anemia and Nijmegen breakage syndrome. One of 46 (2.2%) POI patients (CBP34) demonstrated a significantly increased number of chromosomal breaks (Fig. 1A). CBP34 also had higher number of complex chromosomal defects such as tri/tetra-radial structures. Cluster analysis showed a co-segregation of CBP34 results with the positive control group, suggesting susceptibility to DNA damage and genomic instability in CBP34 (Fig. 1B). In the 45 remaining POI individuals, the frequency of chromosomal breaks and complex defects per hundred cells were not significantly different from negative controls (Fig. 1C and D).

CBP34 is a 40 years old, nulliparous Caucasian woman who attained menarche at age 13 years. Her height was 162 cm with a BMI of 33 kg/m^2. She developed symptoms of vaginal dryness, hot flashes, night sweats, sleep disturbances and menstrual irregularity at age 28 years and eventually had cessation of menses at age 31 years. CBP34 was diagnosed with hypergonadotrophic hypogonadism, with elevated gonadotrophins (FSH 128 mIU/ml, LH 75 mIU/ml) and low estradiol (7 pg/ml). She had a normal 46,XX karyotype and was negative for Fragile X premutation, and heterozygous for Factor V Leiden G1691A variant. Her medical history was otherwise only significant for a lower extremity deep vein thrombosis complicated by pulmonary embolism after an ankle surgery for which she was therapeutically anticoagulated for 6 months after the event. Interestingly, her family history (Fig. 2A) was significant for ectodermal dysplasia in her twin brother and myelodysplastic syndrome in her younger sister who died from acute myeloid leukemia. Analysis of her lymphocyte cultures treated with 150 nM MMC showed an excessive chromosomal breakage (334 ± 43 breaks/100 cells), which was reproduced by repeat testing on an independent blood draw. Chromosome breakage analysis revealed numerous tri- and tetra-radial figures (Fig. 2B), extensive chromatid breaks, gaps and aberrant chromatid interchanges. CBP34 siblings and parents were tested for chromosomal breakage analysis and their results were normal.

Targeted microarray analysis of DNA repair genes

We performed custom microarray analysis on 16 of the 46 POI samples from patients with a diagnosis of primary amenorrhea or early onset secondary amenorrhea suggestive of underlying genetic etiology. A total of 23 CNV (5 losses and 18 gains) were identified in 12 of the 16 POI samples after exclusion of known benign CNVs observed with high frequency in a normal population. Overall, 20 CNVs comprising intergenic and intronic DNA sequences, and those reported in the DGV database were interpreted as likely benign. In three patients (3/16, 18.8%), CBP34, CBP16 and CBP43, we detected pathogenic submicroscopic chromosomal imbalances (Table I).

Patient CBP34, who had the abnormal chromosomal breakage assay, had a 3.4 Mb duplication in the short arm of chromosome 16 (Fig. 2C) involving at least 18 known and Refseq genes as well as a cluster of microRNAs. To date, none of the genes in the region have been associated with DNA repair or DNA damage control. This microduplication is likely mediated by a non-allelic homologous recombination between NODAL modulator 1 (*NOMO1*) and NODAL modulator 2 (*NOMO2*) highly similar genes flanking the duplication breakpoints. Further analysis showed that the observed microduplication was inherited from the patient's father and was also detected in the patient's twin brother with ectodermal dysplasia.

Patient CBP16 showed a loss of a 2.6 Mb segment in the X chromosome involving the Xq28 region (Table I, Fig. 2D), suggestive of a mosaicism. Fluorescence *in situ* hybridization (FISH) analysis on the 72 h lymphocyte culture showed 89% of cells with Xq28 deletion, ~5% of cells with two normal X chromosomes, and ~5% of cells with monosomy X. CBP16 was a 30-year-old Caucasian of Eastern European descent. She attained menarche at age 12 years and presented with secondary amenorrhea at age 28 years, but otherwise was healthy with an unremarkable medical and family history. Her height was 160 cm. Clinical evaluation for secondary amenorrhea revealed hypergonadotrophic hypogonadism with 46,XX karyotype and normal fragile X mental

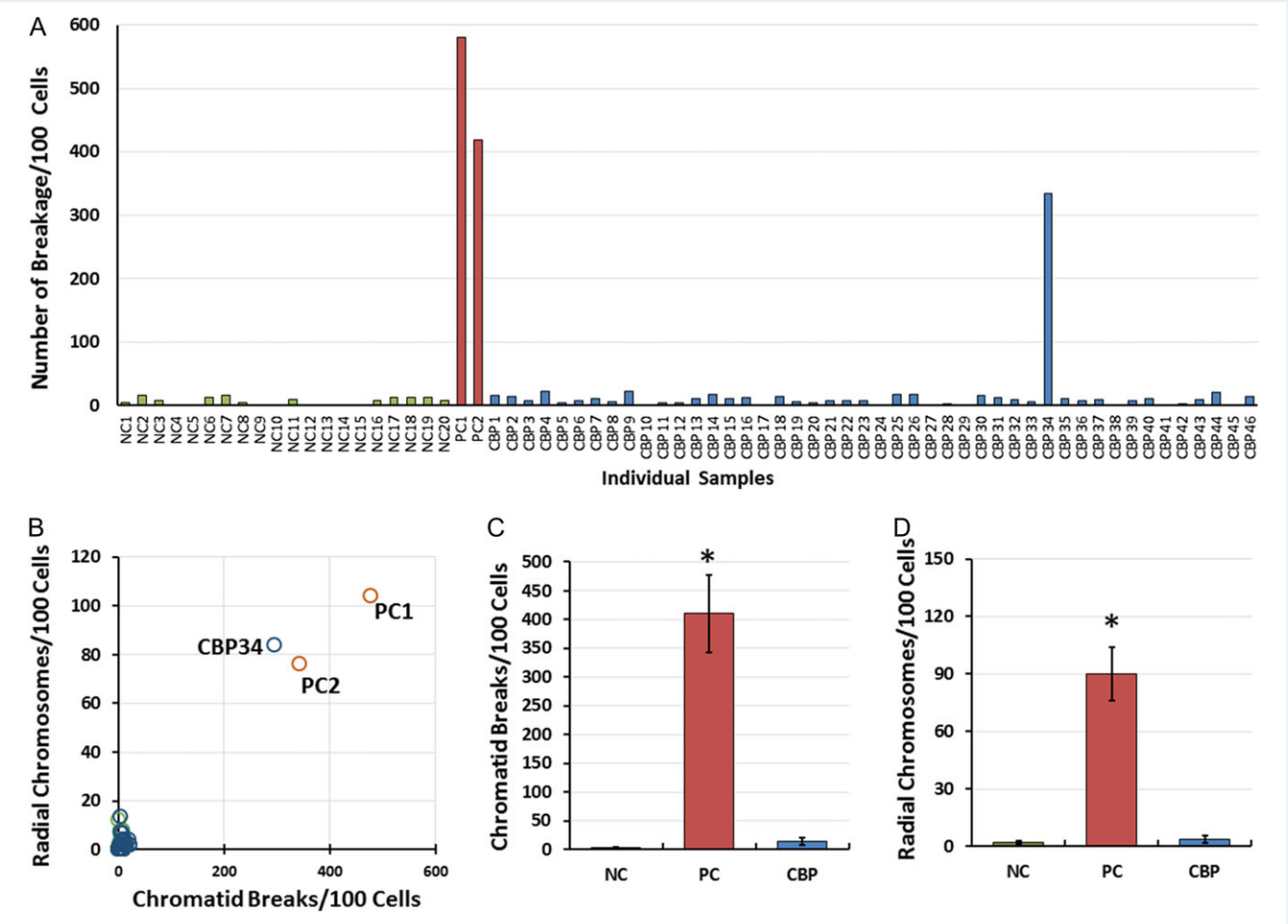

Figure 1 DNA damage response to mitomycin C in peripheral lymphocytes among women diagnosed with primary ovarian insufficiency. (**A**) Total number of breaks per 100 cells observed for individual negative controls (NC1-20, green), positive controls (PC1 and PC2, red) and patients with primary ovarian insufficiency (POI) (CBP1-46, blue). (**B**) Scatter plot shows the number of tri-/tetra-radial chromosomes and chromatid breaks per 100 cells in individual participants from the NC, PC and POI groups. Note the three outliers, positive control samples (PC1 and PC2) and POI patient (CBP34) demonstrate extensive chromosomal breakage. (**C**, **D**) The mean (±SEM) of chromatid break (C) and radial chromosome (D) numbers in 100 cells among positive controls was higher than patients and negative controls. Asterisk in each graph shows significant difference of PC as compared to the other two groups (Kruskal–Wallis test, $P < 0.05$).

retardation 1 (*FMR1*) CGG repeat number. Two highly conserved genes, *CETN2* (OMIM*300006) and *HAUS7* (OMIM*300540) involved in mitotic spindle formation, are localized to the deleted region in this patient. Her MMC assay did not demonstrate excess chromosomal breakage with a total of 12 ± 4 (mean ± SD) chromosomal aberrations in 50 metaphases.

In patient CBP43, the microarray detected a 14 kb gain in the 3p22.2 region encompassing the promoter and exons 1–5 of mismatch repair gene *MLH1* (Table I, Fig. 2E). A gain of this region has not been observed among healthy individuals and is not listed in the database (DGV) of benign CNV. Partial duplication of the *MLH1* is predicted to result in a truncated protein, containing a part of the ATPase domain, and therefore, we consider this gain as a deleterious CNV. This patient was diagnosed with POI at age 31 years, but had menstrual abnormalities 3 years preceding her diagnosis. Her family history was significant for metastatic colon cancer in the maternal grandfather who died at age 55 years.

Discussion

In this pilot study, we detected an overt deficiency in DNA damage response to MMC as well as abnormal microarray with likely pathogenic CNVs in POI patients. The utilization of new genomic technologies has uncovered novel genetic contributors, such as *MCM8*, *MCM9*, X-ray repair complementing defective repair in Chinese hamster cells 4 (*XRCC4*) and other genes, linking POI to genomic instability and dysfunctional DNA repair. The function of these genes involves DNA replication, DNA damage repair and cell-cycle checkpoint control, extending POI etiologies beyond the defects in gonadal development, X chromosome aberrations and hormonal dysregulation. POI is an inevitable reproductive phenotype in many chromosomal instability syndromes, demonstrating the sensitivity of gonads to DNA injuries and oocyte depletion as a secondary cellular defense against damaged cells.

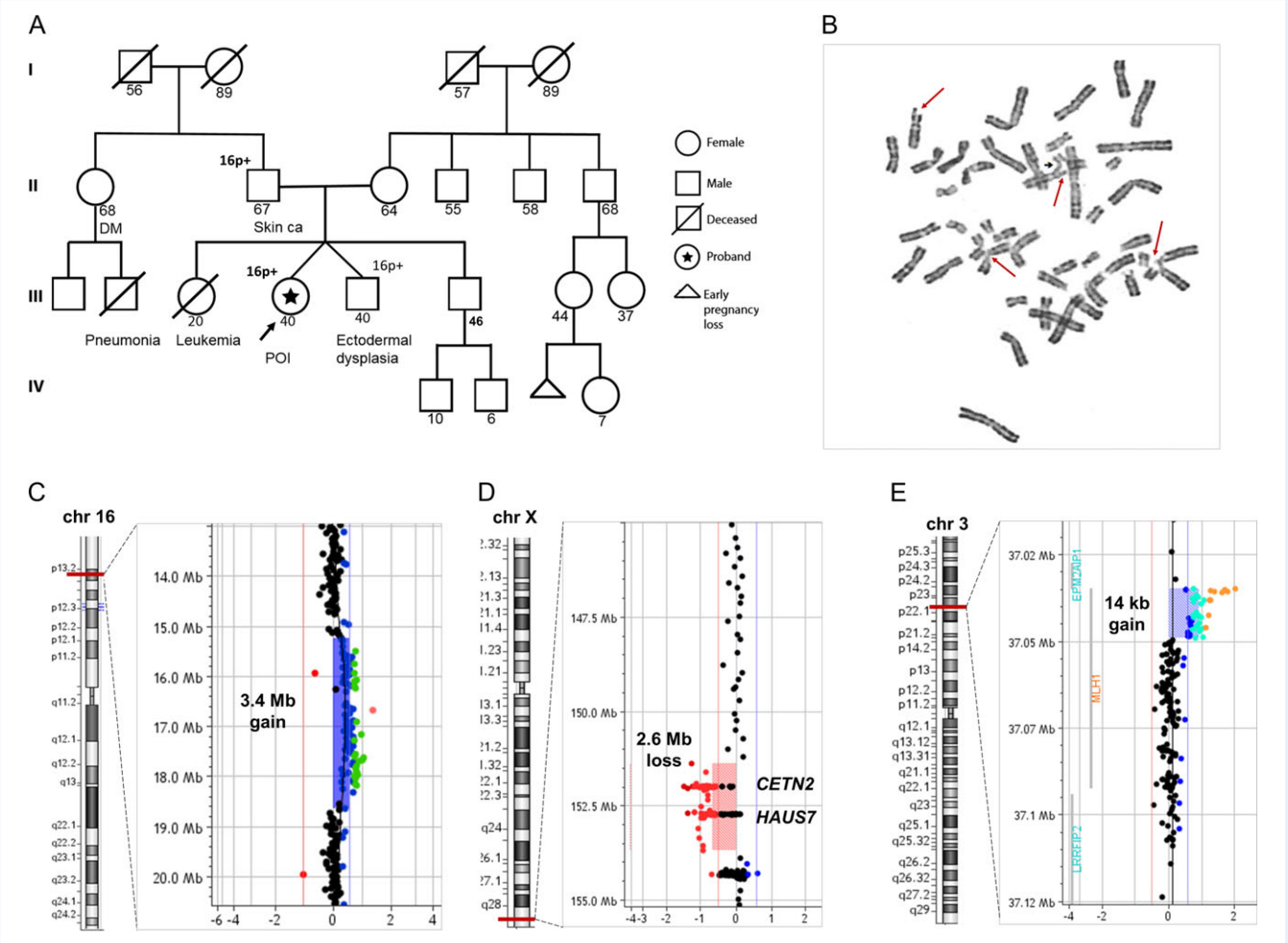

Figure 2 POI patients with abnormal DNA damage response and copy number aberrations detected by array comparative genomic hybridization. (**A**) Pedigree in patient CBP34. Horizontal lines signify marriage, and vertical lines lineage. Age and relevant medical history is written beneath each individual. DM- diabetes mellitus. (**B**) A representative metaphase cell with excessive chromosomal defects observed in a lymphocyte culture of patient CBP34 treated with 150 nM mitomycin-C. Chromatid breaks, gaps, triradial figures and chromatid fusions are indicated by arrows. (**C–E**) Array comparative genomic hybridization revealed a 3.4 Mb duplication involving the 16p13.11-p12.3 region in patient CBP34 (C), a loss of 2.6 Mb in the Xq28 region including the Centrin EF-hand Protein 2 (*CETN2*) and HAUS Augmin Like Complex Subunit 7 (*HAUS7*) genes in patient CBP16 (D), and a 14 kb gain comprising the promoter and exons 1–5 of the MutL Homolog 1 (*MLH1*) gene in patient CBP43 (E).

Table 1 **Copy number variants detected in patients with primary ovarian insufficiency.**

Patient	CNV co-ordinates (hg19)	Loss/ gain	Size (Mb)	DNA repair genes	Age (years)	Presentation	MMC assay	Clinical history
CBP16	chrX:151365798_153675233	Mosaic loss	2.6	Centrin EF-hand Protein 2 (*CETN2*), HAUS Augmin Like Complex Subunit 7 *(HAUS7)*	32	Secondary amenorrhea at 28 years	Normal	Not significant
CBP34	chr16:15234433_18631981	Gain	3.4	None known	40	Secondary amenorrhea at 30 years	Abnormal	Younger sibling with MDS/AML, twin brother with ectodermal dysplasia
CBP43	chr3:37034779_37049084	Gain	0.014	MutL Homolog 1 (*MLH1*) Exons 1-5	37	Secondary amenorrhea at 31 years	Normal	Maternal grandfather died with metastatic colon cancer at age 55 years

CNV, copy number variants; AML, acute myelogenous leukemia; MDS, myelodysplastic syndrome; MMC, mitomycin C.

Previously, our group identified pathogenic variants in *MCM8* and *MCM9* in association with chromosomal instability in non-syndromic, familial POI (Desai *et al.*, 2017). Other investigators have also shown a pathogenic variant in *XRCC4* in two siblings with syndromic POI who also demonstrated impaired efficiency of the non-homologous end joining DNA repair pathway (de Bruin *et al.*, 2015). Accelerated loss of oocytes leading to sterility has been observed in knockout rodent models involving genes [DNA meiotic recombinase 1 (*Dmc1*), mutS homolog 5 (*Msh5*), stromal antigen 1 (*Stag1*), synaptonemal complex protein 1 (*Sycp1*)] that are related to meiosis, homologous recombination, double-strand DNA break (DSB) and DNA repair (Yoshida *et al.*, 1998; Di Giacomo *et al.*, 2005; Wang and Hoog, 2006; Remeseiro *et al.*, 2012). The breast cancer 1, early onset (*BRCA1*) and breast cancer 2, early onset (*BRCA2*) genes are involved in homologous recombination based DSB repair and women who carry mutations in these genes are at risk for POI and cancer susceptibility (Oktay *et al.*, 2015). Based on this evidence from both human data and animal models on inherited defects in DNA repair and syndromic and non-syndromic POI, we hypothesized that women with idiopathic non-syndromic POI are at risk for chromosomal instability, and potentially at risk for adverse health outcomes. In this pilot study, we chose a functional approach to investigate susceptibility to chromosomal breakage in a cohort of women with non-syndromic POI.

There are several methods to measure DNA damage, each with their own limitations. Our assay utilized MMC, a DNA cross-linking agent that induces DSB. During the 72 h of culture, lymphocytes may undergo 1–2 cycles of DNA replication and cell division. Induced breaks in a healthy cell with normal repair mechanisms will have the ability to self-repair and continue with mitosis (Oostra *et al.*, 2012). Chromosomal aberrations due to unrepaired lesions can be observed after treatment with MMC. Of the 46 POI women screened, one patient (CBP34) had an excessive number of chromosomal breaks. Her family history was significant for a sibling with hematologic malignancy and father with skin cancer. Of note, the patient and her twin brother had a 3.4 Mb duplication in the short arm of chromosome 16, which they inherited from their father (Fig. 2A). The significant phenotypic variability observed among the family members could be likely related to gender dimorphism and additional risk factors contributing to an induced DNA damage in individuals with deficient DNA repair abilities.

In patient CBP16, microarray analysis revealed a 2.6 Mb mosaic Xq28 microdeletion comprising two genes implicated in DNA repair, *CETN2* and *HAUS7*. Both genes are highly expressed in ovaries. *CETN2* plays a role in organizing microtubules, regulating cytokinesis and genome nucleotide excision repair and *HAUS7* is required for centriole duplication and correct spindle formation. Terminal deletions involving Xq28 have previously been reported in several women with POI and attributed to haploinsufficiency of important genes involved in ovarian development that normally would escape X-inactivation (Mercer *et al.*, 2013). The negative breakage analysis of CBP16 can be explained by a different X inactivation pattern, dosage compensation, the level of mosaicism or the existence of alternative DNA repair mechanisms in the peripheral blood cells versus the ovarian tissue. On the other hand, the MMC assay may not be ideal to screen for abnormalities in the nucleotide excision repair pathway and may demonstrate chromosome instability only for those POI patients who have alterations in genes implicated in both mitotic and meiotic DNA repair pathways.

Targeted microarray also identified and a 14 kb gain in the 3p22.2 region encompassing exons 1–5 of mismatch repair gene *MLH1* in patient CBP43. *Mlh1*-deficient mice are predisposed to cancers and are sterile due to impaired DNA mismatch repair and interference with chromosomal synapses (Baker *et al.*, 1996; Avdievich *et al.*, 2008). In humans, germline mutations of *MLH1* can result in Lynch syndrome. *MLH1* alterations are associated with male infertility (Ferguson *et al.*, 2009), but there are no data on female infertility or POI. DNA mismatch repair proteins are essential for correction of base mispairs due to replication errors. Insufficient DNA repair increases rates of spontaneous mutation leading to cancer susceptibility and accelerated ageing. Remarkably, most patients with compromised DNA repair also have gonadal dysfunction and shorter reproductive lifespan, presenting a novel paradigm for ovarian ageing as a marker for general health and longevity (Yatsenko and Rajkovic, 2015; Levine *et al.*, 2016; Desai and Rajkovic, 2017). Deficiency in DNA damage response may result in chromosomal instability during meiotic and mitotic divisions of the early stages of embryonic development. Therefore, women who carry pathogenic variants in genes implicated in DNA repair may have a higher incidence of chromosomal aneuploidy and structural chromosome rearrangements leading to infertility, recurrent pregnancy losses or aneuploid live births (Bolor *et al.*, 2009; Wood-Trageser *et al.*, 2014).

To our knowledge, this is the first study that focused on DNA damage susceptibility in a sporadic, idiopathic POI cohort by assessing chromosomal instability using a functional assay and by screening for pathogenic CNVs in DNA repair genes. Some intragenic copy number variants, such as the 14 kb gain involving a part of the *MLH1* gene identified by our custom targeted microarray, would have been missed by conventional array CGH platforms. We utilized peripheral lymphocytes as a surrogate tissue to quantify induced chromosome breaks, as it is not feasible to perform the experiment on oocytes or ovarian somatic cells. It is known that tissue specific repair mechanisms exist (Garinis *et al.*, 2008), and the findings in peripheral lymphocytes may not completely represent gametes and their supporting cells. Another limitation is the ability of the MMC assay to specifically detect defects in the homologous repair pathway. This may lead to a negative MMC studies in patients who indeed harbor deficiencies in other DNA repair pathways. Our assay will therefore only capture a subset of women with deficient DDR. Nevertheless, functional DNA breakage assays may be useful for screening of patients with reproductive problems for the identification of those who have susceptibility to genomic instability, and therefore, the potential for adverse health outcomes.

Our pilot study provides evidence that a subset of women with idiopathic POI may present with genome instability due to defects in DNA repair. It is likely that these women are prone to morbidities that are not alleviated with hormone replacement alone. Identifying these women is essential to provide better care to reduce long-term risks and to screen family members for a predisposition to DNA repair-related disorders.

Supplementary data

Supplementary data are available at *Human Reproduction* online.

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Authors' roles

The aims and study design were formulated by S.K., S.Y. and A.R. in consultation with J.S. and T.Z. M.A, A.K., S.M. and S.Y., performed the chromosomal breakage and microarray analysis. S.K. and M.A. completed the analysis and wrote the article in consultation with S.Y. and A.R. All authors contributed and critiqued all versions of the analyses and articles. All authors have read and approved the final article.

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Conflict of interest

The authors have declared that no conflict of interest exists.

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