Transcription Factor *FIGLA* is Mutated in Patients with Premature Ovarian Failure

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Premature Ovarian Failure (POF) is a genetically heterogenous disorder that leads to hypergonadotropic ovarian failure and infertility. We screened 100 Chinese women with POF for mutations in the oocyte-specific gene *FIGLA* and identified three variants in four women: missense mutation c.11C → A (p.A4E) was found in two women; deletion c. 15–36 del (p.G6fsX66), resulting in a frameshift that leads to haploinsufficiency, was found in one woman; and deletion c.419–421 delACA (p.140 delN) was found in one. Functional analyses by the yeast two-hybrid assay demonstrated that the p.140 delN mutation disrupted FIGLA binding to the TCF3 helix-loop-helix (HLH) domain. Our findings show that a subset of Chinese women with sporadic, premature ovarian failure harbor mutations in *FIGLA*.

Premature Ovarian Failure (POF [MIM 311360]) is defined as a primary ovarian defect characterized by premature depletion of ovarian follicles before the age of 40 years.[1] POF has an estimated prevalence of one in 10,000 women by age 20, one in 1000 women by age 30, and one in 100 women by age 40.[2] Premature ovarian failure can be heritable, but few mechanisms have been identified.[3–5] Known nongenetic mechanisms include gonadotoxic chemotherapy, radiation treatment, and autoimmune oophoritis, but in most cases POF is idiopathic and probably genetic.[6] Besides chromosome abnormalities involving X or autosomes,[7] single-gene-mutation associations with POF include fragile X mental retardation 1 (*FMR1* [MIM 309550]),[8–12] forkhead box L2 (*FOXL2*[MIM 605597]),[13–15] premature ovarian failure 1B (*POF1B* [MIM 300603]),[16,17] inhibin alpha (*INHA* [MIM 147380]),[18–23] NOBOX oogenesis homeobox (*NOBOX* [MIM 610934],[24] growth differentiation factor 9 (*GDF9* [MIM 601918]), and bone morphogenetic protein 15 (*BMP15* [MIM 300247]).[25–30] *FMR1* premutation carriers have a 15% risk of developing POF, whereas the prevalence of *FMR1* premutation among women with sporadic POF is less than 10%.[31] The prevalence of other gene mutations in women with POF is not well known. Mouse models[32–37] of ovarian failure closely mimic human ovarian failure, as exemplified by mutations in the human and mouse follicle-stimulating hormone (FSH) receptor (*FSHR* [MIM 136435]).[32–35] Targeted deletion of mouse genes indicates that there are more than 200 genes that can cause reproductive dysfunction.[38]

Factor in germline alpha (*FIGLA* [MIM 608697]), located on human chromosome 2p13.3, is a germ cell-specific basic helix-loop-helix (bHLH) transcription factor that regulates expression of zona pellucida genes as well as that of other oocyte-specific genes.[37,39,40] bHLH proteins are a group of highly conserved transcription factors that typically bind to a consensus sequence (CANNTG), called an E-box, to promote selective genes.[41,42] In both mouse and human, *FIGLA* is expressed in the embryonic gonad, heterodimerizes with ubiquitously expressed bHLH protein TCF3 (transcription factor 3 [MIM 147141]), and binds to the zona pellucida gene promoters.[37,40] *Figla* knockout mice cannot form primordial follicles and lose oocytes rapidly after birth, whereas male gonads are unaffected.[36] The ovarian-failure phenotype in mice makes *FIGLA* a candidate gene for premature ovarian failure in humans. We therefore examined whether mutations in *FIGLA* are present in women with premature ovarian failure, specifically a Han Chinese cohort, compared with an ethnically matched control cohort.

In this study, 100 Chinese women with POF were recruited from the Reproductive Medical Center, Shandong Provincial Hospital Affiliated to Shandong University in Jinan, Shandong, China. Controls were healthy women chosen randomly between 30 and 62 years of age, with regular menstrual cycles and no known history of infertility. Institutional review boards at both Shandong Provincial Hospital and Baylor College of Medicine approved the study. Written informed consent was obtained from all participants. Inclusion criteria were defined as cessation of menstrual cycles before 40 years of age,[43] two serum FSH concentrations greater than 40 IU/L, and normal 46, XX karyotype. Women with associated endocrinopathies or autoimmune disorders were also excluded.

Genomic DNA was extracted from blood samples, and the five exons coding for *FIGLA* (GeneBank accession number NM_001004311) were amplified with polymerase chain reaction (PCR) with four pairs of *FIGLA*-specific primers (Table S1, available online). Sequencing was performed via ABI Prism Sequencer 3130XL (Applied Biosystems) as previously described.[44] The PCR products were sequenced with BigDye Sequencing reagent V3.1 (Applied Biosystems). PCR and sequencing products were purified

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Table 1. *FIGLA* Mutations in 100 Han Chinese Women with POF

dbSNP ID	Exon	Sequence Variation	Amino Acid Variation	Frequency (POF)	Frequency (Controls)
ss99307022	1	c.11C → A	p.Ala4Glu	2/100	1/304
ss99307023	1	c. 15–36 del	p.G6fsX66	1/100	0/304
rs7566476	3	c.422C → G	p.Ser141Thr	12/100	35/304
rs7566541	3	c.552A → G	Silent	12/100	35/304
ss99307024	3	c.419–21 delACA	p.140 delAsn	1/100	0/304

POF = Premature Ovarian Failure; *FIGLA* = Factor in the Germline alpha.

with the use of Performa Ultra 96-well PCR plates (Edge Biosystems). Sequence analyses were performed with Sequencher 4.2 software (Gene Codes).

Among the 100 Chinese POF subjects, we identified in the *FIGLA* gene two single-nucleotide polymorphisms (SNPs), c.422C → G and c.552A → C, in both controls and POF subjects. These polymorphisms have already been reported in the online dbSNP database (dbSNP accession numbers rs7566476 and rs7566541, respectively) and did not show a statistically significant difference between controls and women with POF. We also detected three novel heterozygous variants in four individuals (Table 1): c.11C → A (p.A4E), c.15–36 del (p. G6fsX66), and c.419–421 del ACA (p.140 delN), which have been deposited into the dbSNP database. The c.11C → A changes alanine for glutamic acid and was present in two individuals with POF (S8 and S58; details are provided in Table 2) but was also present in one of the 304 control subjects. The c.11C → A thus probably represents a rare polymorphism. However, c.15–36 del and c.419–421 delACA mutations were detected only in women with POF and in none of the 304 controls.

The 22 base pair (22 bp) deletion, c.15–36 del (p.G6fsX66) in case S13 (Figure 1), is predicted to cause a frame shift at the 6th codon and change glycine to arginine to create a new open-reading frame ending in a stop codon at position 66 (G6fsX66).This effectively creates *FIGLA* haploinsufficiency. wild-type (WT) *FIGLA* encodes 219 amino acids, whereas truncated FIGLA protein shares only a five-amino-acid homology with the wild-type. The c.15–36 delta was not found among the entire 304 control cohort. The deleted nucleic acids are flanked by identical nine-nucleotide-long repeats (CCCCGCGCC). We hypothesize that misalignment between the first and the second repeat during recombination causes the 22bp deletion.

S13 presented with secondary amenorrhea at 36 years of age (Table 2). Menarche occurred when she was 14 years old, and her subsequent menstrual cycles were irregular. At 23 years of age, she gave birth to a girl who is now healthy at the age of 16 years. S13 presented with hot flashes, excessive sweating, lassitude, emotional lability, and vaginal dryness. Her initial FSH concentration was 48.1 IU/L, and a subsequent concentration was 50.6 IU/L. Pelvic ultrasonography showed a small uterus (55×35×30 mm) and bilateral-streak ovaries devoid of follicles. The proband's 15–36 del mutation was inherited from her father, who has no evident somatic anomalies. S13's mother and daughter do not carry the deletion and have no reproductive or menstrual dysfunction.

The c.419–421 delACA variant (p.140 delN) causes a loss in asparagine at position 140 (Figure 2). The proband (S69) was a 29-year-old woman presenting with secondary amenorrhea and infertility. Menarche occurred when she was 14 years old, and menstrual cycles were normal until 27 years of age, at which time secondary amenorrhea ensued. FSH concentrations were highly elevated on two separate interval measurements, at 70.4IU/L and 89.6IU/L, respectively. Transvaginal ultrasonography showed a small uterus (45 × 35 mm), an atrophic endometrium, and a small right ovary (12 × 10 mm) devoid of follicles. The left ovary was not visualized. She had no other medical problems and lacked dysmorphic features. Her parents are healthy; no other family members had POF.

FIGLA is known to heterodimerize with TCF3.[37, 40] We tested whether the missense or deleted (c.11C → A or c.419–421 delACA) alleles affect the ability of FIGLA to dimerize with itself or TCF3 by utilizing a yeast two-hybrid strategy to study protein-protein interactions. Proteins under study were either fused to *GAL4* DNA-binding domain as a bait or fused to *GAL4*-activation domain as a prey. If

Table 2. Phenotype of Women with POF Carrying *FIGLA* Mutations

Patient	Variant	Age* (yrs)	Karyotype	FSH (IU/L)	LH (IU/L)	E_2 (pg/ml)	Additional Information
S8	p.Ala4Glu	22	46,XX	70.0[a]; 78.5[b]	19.5[a]; 24.7[b]	44.2[a]; 31.8[b]	Perimenopausal syndrome; TV-B: Streak left ovary; right ovary not visualized
S13	p.G6fsX66	36	46,XX	48.1[a]; 50.6[b]	32.1[a]; 11.5[b]	28.0[a]; 22.0[b]	Perimenopausal syndrome; TV-B: Ovaries not visualized
S58	p.Ala4Glu	32	46,XX	95.7[a]; 135.7[b]	42.9[a]; 47.1[b]	42.0[a]; 27.3[b]	TV-B: Small uterus and bilateral streak ovaries
S69	p.140 delAsn	27	46,XX	70.4[a]; 89.6[b]	28.8[a]; 45.2[b]	17.0[a]; 11.2[b]	TV-B: Small uterus and small right ovary devoid of follicles; left ovary not visualized

FIGLA denotes factor in the germline alpha; FSH denotes follicle-stimulating hormone; LH denotes luteinizing hormone; E_2 denotes estradiol; TV-B denotes Transvaginal ultrasonography.

* Age at exposure.

[a] Serum levels; initial value.

[b] Serum levels; repeat value.

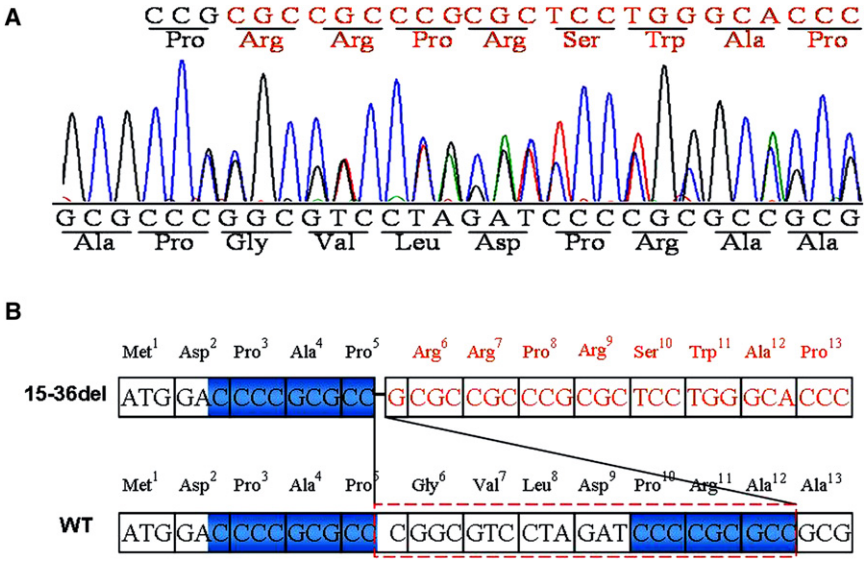

Figure 1. *FIGLA* Mutation c.15–36 del (G6fsX66)
(A) Partial nucleotide sequences of the mutant and WT alleles are shown on top and bottom of the sequence, respectively. (B) The 22 bp deletion (dashed box) causes a frame shift beginning at the 6th codon and creates a new open reading frame (red boxes). The deletion is flanked by two identical nine-nucleotide sequences (CCCCGCGCC) (blue shade).

bait and prey interact physically when brought together in a yeast reporter strain, *GAL4* promoter-driven reporter genes (*LacZ*, *ADE2*, *HIS3*, and *MEL1*) will be transcribed, thus resulting in blue colonies on synthetic droupout (SD) plates selective for −Ade, −His, −Leu, −Trp, and X-a-gal (Figure 3A). In this study, full-length wild-type human *FIGLA* cDNA was purchased from ATCC (Manassas, VA, USA), and *TCF3* cDNA was a gift from Rosemary A.L. Bayne (Centre for Reproductive Biology, University of Edinburgh, Edinburgh, UK[40]). *FIGLA* and *TCF3* (Ensembl accession number ENSG00000071564) constructs were subcloned into the MatchMaker *GAL4* two-hybrid pGBKT7 and pGADT7 vectors (Clontech), and primer sequences are shown in Table S1.

Mutant constructs carrying c.11C → A and c.419–421 delACA were created with the Quik-Change multi-site-directed mutagenesis kit (Stratagene) according to the manufacturer's instructions.

We studied protein-protein interactions by transforming the Y187 yeast strain with mutant (11C → A and 419–421 del) and wild-type *FIGLA* constructs subcloned into the bait vector (pGBKT7) as previously described.[44] The corresponding prey (pGADT7) vectors containing wild-type *FIGLA*, *FIGLA* HLH domain, or *TCF3* HLH domain were transformed into the AH109 yeast strain. Y187 and AH109 cells transformed with the bait and prey constructs were selected on SD plates lacking either tryptophan (SD/−Trp) or leucine (SD/−Leu), respectively, and the two strains were mated according to the manufacturer's instructions. Mating mixtures were spread onto double-dropout plates (SD[−Leu/−Trp]) for the growing of diploids. Diploid strains were streaked onto quadruple-dropout plates (SD[−Ade/−His/−Leu/−Trp/X-a-gal]), and positive interactions scored as blue colonies. Each experiment was performed in three replicates. In this study, SV40 T antigen with p53 and SV40 T antigen with Lamin C were used as positive and negative controls, respectively.

We mated human wild-type *FIGLA* with itself or with *TCF3* HLH domain containing prey constructs. Blue colonies grew on selective plates only by the mating of yeast bait wild-type (WT) *FIGLA* (full-length or HLH domain) with prey *TCF3* HLH domain, indicating that human FIGLA can heterodimerize with TCF3 HLH domain but does not homodimerize with itself. We also studied whether TCF3 HLH domain can interact with FIGLA mutant proteins A4E and 140 delN. As shown in Figure 3B, blue colonies were detected within three days when *TCF3* HLH domain was mated with WT *FIGLA* or *FIGLA* mutant

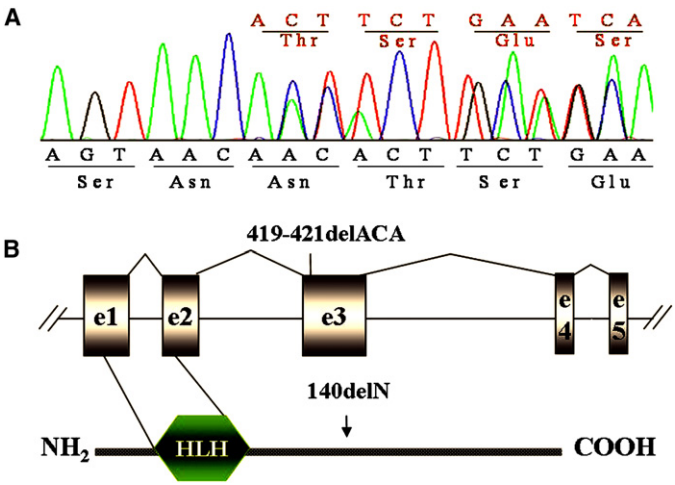

Figure 2. *FIGLA* Mutation c.419–421 del ACA (p.140 delN)
(A) The mutant-allele partial sequence is shown in red, and the WT-allele partial sequence is shown in black on the top and the bottom of the sequence chromatogram, respectively.
(B) Human *FIGLA* gene is schematically drawn with five known exons (gray boxes). The three deleted nucleotides ACA are located in the *FIGLA* exon3 and cause a loss in asparagine at position 140, which is downstream of the *FLGLA* HLH domain (green rhombus).

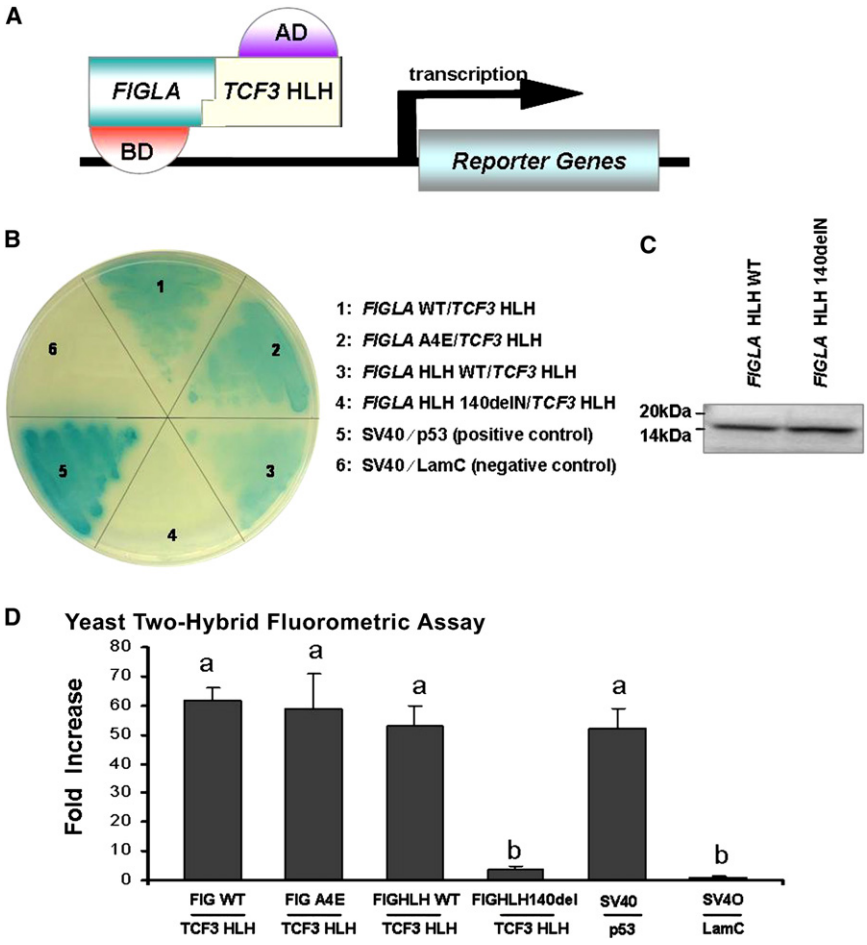

Figure 3. Dimerization of WT and Mutant *FIGLA* with the *TCF3* HLH Domain in Yeast Two-Hybrid Assay

(A) Diagram of the system used in the yeast two-hybrid assay. *FIGLA* fusion to the *GAL4* DNA-binding domain (FIGLA-BD) will drive transcription of reporter genes only if it interacts with *TCF3* HLH fused to the *GAL4*-activation domain (TCF3 HLH-AD). HLH denotes helix-loop-helix domain.

(B and C) Mating strains were streaked onto SD plates selective for −Ade, −His, −Leu, −Trp, and X-a-gal and incubated for three days. Positive protein-protein interactions allow the transcription of reporter genes that permit mating strains to grow on selective media and appear as blue colonies (B). The mating of *FIGLA* HLH mutant 140N to the *TCF3* HLH domain carrying yeast did not yield blue colonies ([B], sector 4), despite the presence of an equivalent amount of mutant HLH 140 delN protein in the yeast (C).

(D) A BioSensor fluorometric assay was performed to confirm interactions observed in (B). Bait-and-prey combination is shown below the corresponding bar. After 32 hr of culturing, fluorescence intensity increased more than 50-fold with colonies containing WT *FIGLA* and mutant A4E. However, yeast colonies containing the mutant 140 delN protein (lane 4) failed to show increase in fluorescence, suggesting little interaction with *TCF3* HLH. Fluorescence was normalized to the baseline at time zero (0 hr). Values are expressed as mean + SEM ($N = 3$). Bars with different superscript letters (^{a} and ^{b}) are significantly different ($p < 0.001$; one-way ANOVA).

A4E, but only a few white colonies appeared when it was mated with *FIGLA* HLH mutant 140 delN despite equivalent amounts of mutant and WT *FIGLA* protein (Figure 3C).

We verified the two-hybrid interactions with an oxygen-biosensor assay. In three independent experiments, we measured the fluorescence emitted by the O_2-sensing platform that detects *GAL4*. The amount of fluorescence intensity increases according to the strength of the two-hybrid interactions.[45] With this method used, TCF3 HLH domain showed strong interaction with the WT FIGLA, as well as with mutant FIGLA A4E protein. Mutant FIGLA 140 delN protein showed markedly decreased interaction with the TCF3 HLH domain ($p < 0.001$; ANOVA) (Figure 3D). The data imply that the mutant protein FIGLA140 delN disrupts heterodimer formation between FIGLA and TCF3 whereas A4E missense mutation does not have appreciable effect on the FIGLA and TCF3 interaction in vitro.

In this study, we identified two plausible mutations in the *FIGLA* gene among 100 POF cases (2%), whereas none were present among 304 ethnically matched controls. *FIGLA* is a germ cell-specific transcription factor preferentially expressed in oocytes and critical in oocyte differentiation. Transgenic mouse models replicate known human reproductive phenotypes well, as exemplified by FSH-receptor mutations.[32–35] Mice completely lacking *Figla* expression lose oocytes rapidly after birth, are infertile, and do not display any other anatomic abnormality or dysmorphology.[36] These mice thus represent a good model for the study of nonsyndromic ovarian failure. Our results, as well as supporting data from studies performed with mice, indicate that *FIGLA* mutations contribute to human POF. Given the heterozygous nature of mutations we report here, we cannot rule out the possibility that other genes and/or environmental factors modulate the effects of these mutations on ovarian function.

One of the two heterozygous mutations we detected, G6fsX66, is predicted to prematurely terminate translation of the *FIGLA* transcript after five amino acids. Transcription-factor haploinsufficiency is a well-recognized cause

of many human genetic syndromes.[46] Most mice heterozygous for transcription-factor mutations have no discernable phenotype, and only a homozygous transcription-factor mutation leads to pathology in mice.[46] Detailed characterization of oocyte numbers in adult *Figla* mice is lacking, but given what is known about other mouse models of transcription-factor insufficiency, we postulate that *FIGLA* haploinsufficiency in humans causes premature ovarian failure by disrupting formation of primordial follicles, as observed in *Figla* mouse knockouts, resulting in diminished follicular endowment and accelerated oocyte loss throughout the reproductive lifespan. We cannot exclude the possibility that the new protein created by the G6fsX66 mutation adversely affects WT FIGLA function via dominant negative interaction.

The 140 delN mutation was also not present in the control cohort of women, and yeast two-hybrid analysis shows adverse effects of this mutation on FIGLA interaction with *TCF3*. Abnormal FIGLA function could be due to dominant negative interference of the 140 delN protein on the binding of WT FIGLA to TCF3, or it could be due to decreased levels of functional heterodimers.[44] The interaction between TCF3 and other bHLH transcription regulators occurs mainly via the bHLH domain; however, our study shows that residues outside of the bHLH domain are also important for the interaction of FIGLA with TCF3. The deleted asparagine is carboxy terminal to the bHLH domain. Residues carboxy terminal to the bHLH domain have been shown to affect heterodimerization.[47]

To our knowledge, this is the first study to evaluate mutations in the *FIGLA* gene in women with premature ovarian failure. We identified two plausible mutations in *FIGLA* among 100 Chinese POF subjects. Our study shows that *FIGLA* mutations contribute to the pathophysiology of ovarian failure and adds to the growing evidence that disruption of germ cell-specific pathways adversely affects oocyte survival in humans. Future studies should focus comprehensively on germ cell-specific pathways as contributors to the etiology of POF.

Supplemental Data

One supplemental table is available with this manuscript online at http://www.ajhg.org/.

Acknowledgments

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Web Resources

The URLs for data presented herein are as follows:

GenBank, http://www.ncbi.nlm.nih.gov/Genbank/
Ensembl Genome Browser, http://www.ensembl.org/Homo_Sapiens/
ExPASy Proteomics Server, http://us.expasy.org/
Single nucleotide polymorphism database, http://www.ncbi.nlm.nih.gov/SNP/
Online Mendelian Inheritance in Man (OMIM), http://www.ncbi.nim.nih.gov/Omim/

Accession Numbers

The dbSNP database accession numbers for the c.11C → A (p.A4E), c.15–36 del (p. G6fsX66), and c.419–421 del ACA (p.140 delN) variant sequences reported in this paper are ss99307022, ss99307023, and ss99307024, respectively.

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