MOLECULAR PATHOGENESIS OF GENETIC AND INHERITED DISEASES

CAPS Mutations Are Potentially Associated with Unexplained Recurrent Pregnancy Loss

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Accepted for publication
September 10, 2018.

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Recurrent pregnancy loss (RPL) is a major concern for women's reproductive health. Several studies have proved that genetics is a major factor leading to unexplained RPL, but the maternal pathogenic genes involved in RPL remain largely unknown. A consanguineous family, including the parents who were cousins and their three daughters who had been diagnosed as having nonsyndromic unexplained RPL, was recruited in this study. A rare homozygous variant in calcyphosine (*CAPS*; ENST00000588776: c.377delC, p.Leu127Trpfs) might be the potential candidate variant for this RPL family through whole-exome sequencing. Sanger sequencing confirmed that the three affected sisters carried the homozygous p.Leu127Trpfs, whereas their parents carried the heterozygous p.Leu127Trpfs. *CAPS* encodes a Ca^{2+} -binding protein and may play a role in the regulation of Ca^{2+} transport. Although the precise underlying mechanisms remain unclear, the previous study suggested that they may be involved in cross talk between Ca^{2+} signaling and cAMP–protein kinase A pathways, which are crucial to embryo implantation and pregnancy maintenance. Knockdown of *CAPS* expression might promote the expression of secreted phosphoprotein 1 and matrix metalloproteinase 9, and the release of prostaglandin E_2 , which all played important roles in embryo implantation and early pregnancy maintenance. These results indicated that the autosomal recessive homozygous mutation, p.Leu127Trpfs, in *CAPS* might be a maternal effect causative mutation of RPL pathogenesis. (*Am J Pathol* 2019, 189: 124–131; <https://doi.org/10.1016/j.ajpath.2018.09.010>)

Recurrent pregnancy loss (RPL) is a serious reproductive health problem; according to the American Society for Reproductive Medicine, RPL is defined as two or more clinical pregnancy losses that affect 2% to 5% of all couples.^{1,2} The causes of RPL are various, including uterine factors, antiphospholipid syndrome, inherited thrombophilias, and endocrine, cytogenetic, environmental, and psychological factors.² However, after testing for all these causes, some women have unidentified causes and are considered as having unexplained RPL (URPL).³

Many studies have proved that genetics is a major factor leading to URPL, and many candidate genes have been identified. A recent meta-analysis, including 428 case-control studies, identified 21 variants of 13 genes that might contribute to URPL.⁴ However, owing to the large

differences in sample collection between the studies, the associations were all moderate with RPL. Recently, whole-exome sequencing (WES) was used to identify novel causative genes in RPL patients. A study using WES identified compound heterozygous mutations in dynein cytoplasmic 2 heavy chain 1 (*DYNC2H1*; NM_001080463)

Supported by the National Key Research and Development Program of China grants 2018YFC1002801 (B.W.) and 2016YFC1000307 (X.M.), the National Natural Science Foundation of China grant 81501232 (H.X.), the Central Public Interest Scientific Institution Basal Research Fund grant 2018GJM03 (H.P.), and the National Infrastructure of Chinese Genetic Resources grant YCZYPT (2017)01-6 (B.W.).

H.P. and H.X. contributed equally to this work.

X.M., Y.C., and B.W. contributed equally to this work as senior authors.

Disclosures: None declared.

and arachidonate 15-lipoxygenase (*ALOX15*; NM_001140) in miscarriages from two RPL families.⁵ Another recent study using WES in 49 independent URPL patients identified 27 coding heterozygous variants in 22 genes that belonged to distinct pathways related to implantation and pregnancy outcomes.⁶ However, owing to miscarriages or the lack of segregation analysis, limited progress has been made in identifying the maternal pathogenic genes for RPL.

Calcyphosine (*CAPS*; NM_004058) encodes a Ca^{2+} -binding protein, belonging to the EF hand motif family, that may play a role in the regulation of Ca^{2+} transport.⁷ Although the precise mechanism of *CAPS* remains unclear, the previous study suggested that it may be involved in the cross talk between Ca^{2+} signaling and cAMP–protein kinase A (PKA) pathways.⁸ Therefore, *CAPS* might be related to RPL because of the regulation of Ca^{2+} signaling and cAMP-PKA pathways, which are crucial to embryo implantation and pregnancy maintenance.^{9,10}

Herein, a consanguineous family, including the parents who were cousins and all three daughters who had been diagnosed as having nonsyndromic URPL, was recruited. The phenotype of this family suggested a maternal homozygous mutation shared by the three sisters. To identify the likely causal mutation, WES was performed in the three affected sisters for shared rare homozygous variants, and an autosomal recessive frameshift mutation in *CAPS* was identified. The aim of this study is to identify a potential maternal effect causative mutation of RPL pathogenesis using WES technology in an RPL family.

Materials and Methods

Study Subjects

All subjects were recruited from the Center for Reproductive Medicine at The First Affiliated Hospital of Anhui Medical University between 2015 and 2018. According to the American Society for Reproductive Medicine's criteria, the peripheral blood of the three sisters diagnosed as having nonsyndromic URPL and their parents, who were cousins, was collected. In addition, 264 independent RPL patients were included in the study. All clinical information available is shown in Table 1. The decidual and placental villi tissues from five independent URPL patients (cases) and five deliberate pregnancy termination cases with no history of spontaneous abortions (controls) were also collected. The study was approved by the Institute Ethics Committee, National Research Institute for Family Planning, and written informed consent was obtained from all subjects.

Whole-Exome Sequencing

Genomic DNA samples from the three sisters were used to perform WES. Exomes were captured with SureSelect Human All Exon V5 Kit (Agilent Technologies, Santa Clara, CA), and DNA sequencing was performed on the

HiSeq 2500 platform (Illumina, San Diego, CA). BWA version 0.7.12 (<http://bio-bwa.sourceforge.net>; OMICS_00654)¹¹ was used for mapping. The variants were referenced to hg19 genome (<http://genome.ucsc.edu>, last accessed May 2, 2018) and annotated using SAMtools version 0.1.19 (<http://samtools.sourceforge.net>).¹² Rare homozygous mutations [missense, nonsense, frameshift, or splice site variants; frequency, <1% in 1000 Genomes Project database, Single Nucleotide Polymorphism Database, National Heart, Lung, and Blood Institute Exome Sequencing Project database, Exome Variant Server (ExAC) database, and Genome Aggregation Database r2.0.2 (<http://gnomad.broadinstitute.org>)] were retained. Then, the genotypes of the candidate variants were confirmed in the family members by Sanger sequencing. All the coding regions of candidate *CAPS* gene were sequenced in another 264 independent RPL patients. The primer sequences are shown in Table 2. Mutationtaster (<http://www.mutationtaster.org>, last accessed April 14, 2018),¹³ PolyPhen-2 version 2.2.2 (<http://genetics.bwh.harvard.edu/pph2>, last accessed April 14, 2018),¹⁴ and PROVEAN version 1.1.3 (<http://provean.jcvi.org/index.php>, last accessed August 17, 2018)¹⁵ were used to predict the effect of the mutations on protein structure and function. Conservation analysis was performed using Clustal Omega (<https://www.ebi.ac.uk/Tools/msa/clustalo>, last accessed August 14, 2018).¹⁶

Detection of *CAPS* Expression in Decidual and Placental Villi Tissues

The mRNA levels of *CAPS* in decidual and placental villi tissues from the five URPL patients and the five controls were analyzed by real-time RT-PCR (RT-qPCR). Each sample was analyzed in triplicate, and data were analyzed using the $2^{-\Delta\Delta\text{Ct}}$ method. Melting curve analysis and agarose gel electrophoresis confirmed the amplification specificity. Primers were picked from PrimerBank (<https://pga.mgh.harvard.edu/primerbank>, last accessed April 5, 2018), and the sequences are shown in Table 2.

Cell Culture and Transient Transfection

The human endometrial epithelial RL95-2 (CRL 1671; ATCC, Manassas, VA) and human placenta choriocarcinoma JAR (HTB 144; ATCC) cell lines were maintained in medium supplemented with 10% fetal bovine serum, 100 mg/mL penicillin, and 100 mg/mL streptomycin in 5% CO_2 at 37°C. For transient transfection, the cells were seeded in 6-well culture dishes and transfected with siRNA-*CAPS* using Lipofectamine RNAiMAX (Invitrogen, Carlsbad, CA), according to the manufacturer's protocol.

Table 1 Clinical Features of Familial and Independent RPL Patients with Mutations in *CAPS*

	<i>CAPS</i> mutation (ENST00000588776)		Genotype	Spontaneous abortion, <i>n</i>	Age at diagnosis, years	Menstrual history	Uterus
Patient number	Nucleotide	Amino acid					
Family members							
II-1	c.377delC	p.Leu127Trpfs	HOM	5	30	Normal	Normal
II-2	c.377delC	p.Leu127Trpfs	HOM	2	26	Normal	Normal
II-3	c.377delC	p.Leu127Trpfs	HOM	2	23	Normal	Normal
Independent RPL cases							
1	c.274G>A	p.A92T	HET	2	26	Normal	Normal
2	c.685C>T	p.R229C	HET	2	29	Normal	Normal
3	c.764_765insT	p.S256Efs*8	HET	2	31	Normal	Normal
<i>(table continues)</i>							

(table continues)

—, negative; ACA, anticardiolipin antibody; ANA, antinuclear antibody; dsDNA, double-stranded DNA; β2GP1, β2 glycoprotein 1; HET, heterozygous; HOM, homozygous; RPL, recurrent pregnancy loss; TORCH, *Toxoplasma gondii* others rubella virus cytomegalovirus herpes simplex viruses 1 and 2.

CAPS-Specific siRNA

The siRNA set for *CAPS*, including three pairs of siRNA-*CAPS*, positive control, negative control (NC), and FAM-labeled NC, was purchased from Genepharma (Shanghai, China). Real-time RT-PCR and Western blot analysis were used to detect the expression of *CAPS*. Each assay was run in triplicate with each set of primers, and data were analyzed using the $2^{-\Delta\Delta C_t}$ method. Melting curve analysis and Sanger sequencing confirmed the amplification specificity. The mRNA level of each sample was normalized to β-actin mRNA. Densitometry of bands was analyzed using the ImageJ software version k 1.45 (NIH, Bethesda, MD; <https://imagej.nih.gov/ij>). The *CAPS*—homo-919 duplexes were as follows: 5'-GGAUCUGGAGGAGUUCCUUTT-3' (forward) and 5'-AAGGAACUCCUCCAGAUCCTT-3' (reverse). Cells were seeded in 6-well plates and transfected with *CAPS*—homo-919 and NC in the following assays.

Detection of Secreted Phosphoprotein 1 and Matrix Metalloproteinase 9 Expression

The human endometrial epithelial RL95-2 cell line was used to detect the biomarkers related to endometrial receptivity and implantation using real-time RT-PCR. Each assay was run in duplicate with each set of primers. The mRNA level of each sample was normalized to β-actin mRNA. The sequences of the primers for secreted phosphoprotein 1 (*SPPI*; NM_001251830) and matrix metalloproteinase-9 (*MMP9*; NM_004994) are listed in Table 2.

Prostaglandin E₂ Measurement

The human endometrial epithelial RL95-2 cell line was used to detect the content of prostaglandin E₂ (PGE₂) release. After treatment, cell-free supernatant was collected and PGE₂ content was measured using a PGE₂ enzyme-linked

Table 2 Primers for Sanger Sequencing and Real-Time RT-PCR

Variable	Identification	Sequences	Length, bp
Sanger sequencing	CAPS-1F	5'-GAGCCAGCCCACTAAGTCACGT-3'	903
	CAPS-1R	5'-CTGTGGCACTGCATCCCTTCCT-3'	
	CAPS-2F	5'-CTGTCCAGGGTCACACAGGAATCAT-3'	
	CAPS-2R	5'-CTCCGCATCTCAGCATCCTCTGT-3'	858
	CAPS-3F	5'-GGACTCCTCCCTAAGAGCTGCTGT-3'	
	CAPS-3R	5'-CTGGTCAGCATGGCGTGGGT-3'	
	CAPS-4F	5'-GCAGGACTGGCTAGACCAGGT-3'	790
	CAPS-4R	5'-CCAGCTAGTGACGAGTACAACGAGAT-3'	
	β-Actin-F	5'-CTCCATCCTGGCCTCGCTGT-3'	
Real-time RT-PCR		5'-ACTAAGTCATAGTCCGCCTAGA-3'	117
	CAPS-qPCR-F	5'-GTCCAGGGTCACACAGGAATC-3'	184
		5'-GACTGCTTACTCAACTGCCAG-3'	
	SPP1-qPCR-F	5'-CTCCATTGACTCGAACGACTC-3'	230
		5'-CAGGTCTGCGAAACTTCTTAGAT-3'	
	MMP9-qPCR-F	5'-TGTACCCTATGGTTACACTCG-3'	97
		5'-GGCAGGACAGTTGCTTCT-3'	

Table 1 (continued)

Hormonal	Karyotype	TORCH	Anti-β2GP1	ACA, ANA, and anti-dsDNA	Hepatitis	Infections	Childbearing	Male factor
Normal	46, XX	—	—	—	—	No	1	None
Normal	46, XX	—	—	—	—	No	1	None
Normal	46, XX	—	—	—	—	No	0	None
Normal	46, XX	—	—	—	—	No	1	None
Normal	46, XX	—	—	—	—	No	0	None
Normal	46, XX	—	—	—	—	No	0	None

immunosorbent assay kit, monoclonal (Cayman Chemical, Ann Arbor, MI).

Transwell Migration and Invasion Assays

Fetal bovine serum-free JAR cells were added to the upper chamber, and 500 μ L medium with 25% fetal bovine serum was placed in the lower compartment. After incubation, the nonmigrated cells in the upper chamber were removed with a cotton swab. The migrated cells were fixed with 100% methanol, stained with 0.5% crystal violet, and washed with phosphate-buffered saline. The membranes were observed under a light microscope, and three fields were randomly selected to represent each membrane. As for the invasion assay, 70 μ L Matrigel diluted with Dulbecco's modified Eagle's medium was used to plate each transwell chamber and was incubated for 4 hours at 37°C, and the same procedure for migration assay was conducted.

Statistical Analysis

Statistical analysis was performed using GraphPad Prism 5 (GraphPad Inc., San Diego, CA). The independent-sample *t*-test was applied to compare the data, and *P* < 0.05 was considered statistically significant.

Results

Clinical Features of Familial and Independent RPL Patients

Three sisters (II-1, II-2, and II-3), from a consanguineous Chinese family (Figure 1A), experienced unexplained RPL. Their parents, who were cousins, had given birth to three daughters and one son, without a history of spontaneous miscarriages. The peripheral blood and clinical information from all of them were collected in 2015 (Table 1). The proband (II-1) with the age at diagnosis of 30 years, who had been married for 8 years and had a healthy girl during her second pregnancy, miscarried her first and third to sixth pregnancies for unknown reasons. Her sister (II-2) with the age at diagnosis of 26 years, who had been married for 6 years and had a healthy girl during her second pregnancy, miscarried her first and third pregnancies for unknown reasons. Another sister (II-3) with the age at diagnosis of 23 years, who had been married for 2 years, miscarried twice for unknown reasons. They all had normal karyotype, hormone levels, menstrual history, and uterus. They were negative for TORCH (*Toxoplasma gondii* others rubella virus cytomegalovirus herpes simplex viruses 1 and 2), anti-β2 glycoprotein 1, anticardiolipin antibody, antinuclear antibody, anti—

Figure 1 Pedigree of a family with three sisters experiencing unexpected recurrent pregnancy loss (URPL), with homozygous *CAPS* p.Leu127Trpfs variant. **A:** The pedigree of the URPL family; the parents were cousins. Whole-exome sequencing was performed on the three affected sisters (II-1, II-2, and II-3). **Arrow** indicates the proband. **B:** DNA chromatogram of the *CAPS* (ENST00000588776: c.377delC, p.Leu127Trpfs) mutation, compared with the wild type (WT); the three sisters carried a homozygous (HOM) variant, and the parents carried a heterozygous (HET) variant. **C:** This mutation is highly conserved across human, chimpanzee, cattle, rabbit, and dog. **Blue boxes** indicate the location of the mutated nucleotide base or amino acid residue; **asterisks**, amino acid residues are the same between different species.

double-stranded DNA, hepatitis, and infections during screening. Male factor was excluded as well. In addition, the mean age of the 264 independent RPL patients was 29.26 years, and they all experienced at least two spontaneous miscarriages with unknown reasons (Table 1).

Homozygous Frameshift Mutation in *CAPS* Identified in URPL Family

In the consanguineous RPL family, three affected sisters (II-1, II-2, and II-3) were chosen for WES. Pedigree analysis suggested the presence of a recessive inheritance model. Therefore, rare homozygous mutations (missense, nonsense, frameshift, or splice site variants; frequency, <1% in 1000 Genomes Project database, Single Nucleotide Polymorphism Database, Exome Sequencing Project database, ExAC database, and Genome Aggregation Database) that were shared by all three sisters were retained (Supplemental Table S1). Six nonsynonymous homozygous variants in *CAPS* (NM_004058), patatin-like phospholipase domain containing 6 (*PNPLA6*; NM_001166114), semaphorin 6B (*SEMA6B*; NM_032108), TBC1 domain family member 25 (*TBC1D25*; NM_002536), WWC family member 3 (*WWC3*; NM_015691), and zinc finger protein 441 (*ZNF441*, NM_152355) were identified (Supplemental Table S2^{8,17–21}).

Given the frequency and location of the mutations, the effect on protein structure and function predicted by MutationTaster, PolyPhen-2, PROVEAN, and SIFT, the gene expression profile, the conservation among several species, the published phenotype of knockout mice from Mouse Genome Informatics, the variants reported in the ClinVar database (<https://www.ncbi.nlm.nih.gov/clinvar>), the function of the genes, and the three sisters' phenotypes, all six rare nonsynonymous homozygous variants were thoroughly screened. The *SEMA6B* and *WWC3* variants were predicted to not alter the protein structure and function and were not conserved among species. The *ZNF441* variant was not conserved and had not been previously reported. The *TBC1D25* variant was located near the C-terminal end of the TBC1D25 protein, which may not affect the protein features. Furthermore, all these variants have not been reported to relate to human reproductive diseases so far. *PNPLA6* was thought to function in the nervous system, and *PNPLA6* variants were always related to severe syndromes, such as spastic paraplegia 39 [Online Mendelian Inheritance in Man (OMIM) number 612020],²² Boucher-Neuhauser syndrome (OMIM number 215470),¹⁷ Laurence-Moon syndrome (OMIM number 245800),²³ Oliver-McFarlane syndrome (OMIM number 275400),²³ and Gordon Holmes syndrome (OMIM number 212840).²⁴ However, the three sisters were healthy and had no sign of any identified disease, except URPL. Furthermore, hypogonadotropic hypogonadism is one of the classic characteristics of Boucher-Neuhauser syndrome and

Gordon Holmes syndrome, which is associated with underdevelopment of the gonads and infertility.^{17,24}

Therefore, the homozygous variant in *CAPS* (ENST00000588776: c.377delC, p.Leu127Trpfs), a frameshift mutation caused by a single-base deletion, was the potential candidate for this RPL family (Figure 1B). The *CAPS* variant was not previously reported in 1000 Genomes Project, Exome Sequencing Project, and ExAC databases, and was likely to cause a loss-of-function mutation. This amino acid residue is highly conserved across human, chimpanzee, cattle, rabbit, and dog (Figure 1C). Furthermore, no homozygous loss-of-function mutation has been registered in the ExAC database, and the minor allele frequency of any loss-of-function mutation in *CAPS* is <0.0001894, which is low enough to explain the autosomal recessive model of a rare condition. Moreover, *CAPS* might be involved in both Ca²⁺ signals and the cAMP-PKA pathway, which are essential for implantation and placental development.^{9,10}

Furthermore, Sanger sequencing for all coding exons for *CAPS* in independent cases with RPL identified three additional heterozygous mutations (ENST00000588776: c.274G>A, p.A92T; c.685C>T, p.R229C; c.764_765insT, p.S256Efs*8) in three patients; however, no homozygous or compound heterozygous variants were found (Table 1). The p.A92T and p.S256Efs*8 variants were not previously reported. The p.R229C variant was reported once in the ExAC database. Among them, a truncated protein would result from the p.S256Efs*8 variant.

Low Expression Levels of *CAPS* in Decidua and Placental Villi from Independent RPL Patients

The decidual and placental villi tissues were collected from five independent URPL patients who had experienced two consecutive spontaneous abortions because of unknown reasons. The couples' karyotypes were all normal. Tissues were also collected from five deliberate pregnancy termination cases that had no history of spontaneous abortions as controls. Compared with controls, the expression of *CAPS* was down-regulated in decidual and placental villous tissues of RPL patients ($P < 0.05$) (Figure 2).

The Most Effective *CAPS*-Specific siRNA, *CAPS*—Homo-919

Real-time RT-PCR and Western blot analysis were used to detect *CAPS* expression and select the most effective *CAPS* siRNA. According to the data, *CAPS* expression in CAPS—homo-919—transfected cells showed a significant decrease compared with the other groups, and the effectiveness of CAPS—homo-919 was best among the three *CAPS* siRNAs (data not shown). Therefore, CAPS—homo-919 was used for *CAPS* knockdown in the following experiments.

Figure 2 The mRNA levels of *CAPS* in decidual and placental villi tissues from independent unexpected recurrent pregnancy loss patients. **A:** Compared with the control, *CAPS* mRNA expression in decida is significantly down-regulated in the cases. **B:** Compared with the control, *CAPS* mRNA expression in placental villi is significantly down-regulated in the cases. * $P < 0.05$ (t -test).

Effects of Knockdown *CAPS* on *SPP1* and *MMP9* Expression

To explore the possible role of *CAPS* in embryo implantation, the expression of several endometrial receptivity markers was detected after *CAPS*—homo-919 transfection. Compared with controls, Real-time RT-PCR showed that the expression of *SPP1* and *MMP9* was up-regulated in RL95-2 cells after down-regulation of *CAPS* expression (Figure 3A). *SPP1* and *MMP9* play important roles in implantation and early pregnancy establishment.^{25,26} The expression levels of *SPP1* and *MMP9* were up-regulated by 2.04- and 2.82-fold, respectively.

Knockdown *CAPS* Increases PGE₂ Release from RL95-2 Cells

At the epithelial-stromal interface, PGE₂, released from endometrial epithelial cells driven by Ca²⁺ influx, was shown to be essential for stromal cell decidualization and embryo implantation.²⁷ The human endometrial epithelial RL95-2

cells are generally used as a model for providing secretory products and receptive epithelial cells.²⁸ An enzyme-linked immunosorbent assay showed that *CAPS*—homo-919 could induce a dramatic increase in PGE₂ release from the RL95-2 cells ($P < 0.05$), compared with NC (Figure 3B).

Knockdown *CAPS* Inhibits JAR Cell Migration and Invasion

Trophoblast migration and invasion are critical features of implantation and placental development. To examine the possible effect of *CAPS* knockdown on cell migration and invasion, the transwell assays were performed using JAR cells transfected with *CAPS*—homo-919 or NC. The transwell assay showed that *CAPS*—homo-919 could inhibit JAR cell migration compared with NC ($P < 0.01$) (Supplemental Figure S1A). In addition, the transwell assay with Matrigel revealed that *CAPS*—homo-919 could affect JAR cell invasion compared with NC ($P < 0.01$) (Supplemental Figure S1B). These results indicated that knockdown of *CAPS* might lead to a slight decrease in JAR cell migration and invasion.

Figure 3 Effects of *CAPS* knockdown on human endometrial epithelial RL95-2 cell line. **A:** Knockdown of *CAPS* expression significantly increases the expression of *SPP1* and *MMP9*, which are factors related to endometrial receptivity. **B:** Knockdown of *CAPS* expression significantly promotes prostaglandin E₂ (PGE₂) release from the epithelial culture. Data are expressed as means $\pm$ SEM (A and B). * $P < 0.05$, *** $P < 0.001$ versus NC (t -test). NC, negative control.

Discussion

In a consanguineous Chinese family of three sisters with nonsyndromic URPL, an autosomal recessive frameshift mutation was identified in *CAPS* (ENST00000588776: c.377delC, p.Leu127Trpfs) by WES, which is a possible link to URPL. The results indicated that the homozygous mutation, p.Leu127Trpfs, in *CAPS* might be the maternal effect causative mutation of RPL pathogenesis.

These data demonstrated that *CAPS* might be the maternal recessive causative mutation in this nonsyndromic URPL family. Three sisters from a consanguineous Chinese family experienced at least two spontaneous miscarriages for unknown reasons. Pedigree analysis suggested there might be a maternal recessive homozygous mutation shared by the three sisters. Through WES and subsequent bioinformatic analysis, it was found that the homozygous variant in *CAPS*, a frameshift mutation caused by a single-base deletion, was the candidate causative mutation in this RPL family. The *CAPS* variant (ENST00000588776: c.377delC, p.Leu127Trpfs) had never been reported in 1000 Genomes Project, Exome Sequencing Project, and ExAC databases, and was likely to cause a loss-of-function mutation. Besides, no homozygous loss-of-function mutation has been registered in the ExAC database, and the minor allele frequency of any loss-of-function mutation in *CAPS* is low enough to explain the autosomal recessive model of this rare condition. To determine the contribution of the *CAPS* gene to RPL, all the exons of *CAPS* were screened by Sanger sequencing in 264 independent cases with RPL, and no homozygous or compound heterozygous variants were identified. Three heterozygous mutations in three independent cases with RPL were identified, which might not be responsible for RPL pathogenesis because of heterozygosity because the mother of the three sisters also carried the heterozygous p.Leu127Trpfs and gave birth to four children without a history of RPL. Thus, these data suggested that a biallelic *CAPS* mutation is a possible link to URPL and that further screening the biallelic *CAPS* mutation from unrelated families would strengthen the genetic evidence.

The Ca^{2+} signaling pathway is known to participate in the regulation of several implantation-related events, such as adhesion of the blastocyst to the endometrium,²⁹ prostaglandin release from endometrial epithelial cells and the process of stromal cell decidualization,²⁷ and the expression of implantation factors.³⁰ Similar to the Ca^{2+} signaling pathway, the cAMP-PKA pathway is also essential for embryo implantation progression.³¹ *CAPS* encodes a Ca^{2+} -binding protein, which might be involved in cross-signaling between Ca^{2+} signaling and the cAMP-PKA pathway.⁸ Moreover, real-time RT-PCR results revealed that the expression of *CAPS* might be down-regulated in decidual and placental villous tissues of RPL patients. These data suggested that *CAPS* might be involved in embryo implantation and early pregnancy maintenance.

The prolonged receptivity state will cause poor embryo implantation and miscarriage. Thus, a proper transient window of endometrial receptivity is essential for embryo

implantation.^{32,33} *SPP1*, expressed by endometrial epithelial cells, was reported to act as an important component of the uteroplacental microenvironment and play an essential role in implantation.²⁵ Highly expressed *MMP9* can promote the degradation of endometrial extracellular matrix during embryo implantation and may play an important role in the occurrence of RPL.^{34,35} In addition, increased expression of *SPP1* may increase the expression of *MMP9*.³⁶ Using the human endometrial epithelial RL95-2 cell line, which is generally used as a model for providing secretory products and receptive epithelial cells, knockdown of *CAPS* expression was found to increase the expression of *SPP1* and *MMP9*. Besides, the enzyme-linked immunosorbent assay also showed that knockdown of *CAPS* expression could induce a significant increase in PGE_2 , an important factor for embryo implantation²⁷ and endometrial decidualization, release from the epithelial RL95-2 cells. Therefore, these results indicated that *CAPS* might be a link to URPL, by regulating the expression of *SPP1* and *MMP9* and the release of PGE_2 . However, the pathways and mechanisms linking *CAPS* and RPL require further elucidation.

Besides, to determine the effect of *CAPS* knockdown on trophoblast cells, a first-trimester trophoblast cell model of JAR choriocarcinoma cells was used. The results confirmed that knockdown of *CAPS* expression in JAR cells could lead to a slight decrease in cell migration and invasion, which might impair trophoblast infiltration ability. Because the mother of the three sisters gave birth to three daughters carrying homozygous p.L127Wfs*73, the effect of this mutation from the placental side might be limited.

In sum, the results indicated that the autosomal recessive homozygous mutation, p.Leu127Trpfs, in *CAPS* might be a maternal effect causative mutation of RPL pathogenesis.

Acknowledgment

We thank the patients and their family members for participating in this study.

Supplemental Data

Supplemental material for this article can be found at <https://doi.org/10.1016/j.ajpath.2018.09.010>.

References

1. Practice Committee of American Society for Reproductive Medicine: Evaluation and treatment of recurrent pregnancy loss: a committee opinion. *Fertil Steril* 2012, 98:1103–1111
2. El Hachem H, Crepau V, May-Panloup P, Descamps P, Legendre G, Bouet PE: Recurrent pregnancy loss: current perspectives. *Int J Womens Health* 2017, 9:331–345
3. Kutteh WH: Novel strategies for the management of recurrent pregnancy loss. *Semin Reprod Med* 2015, 33:161–168
4. Perez N, Ostojic S, Kapovic M, Peterlin B: Systematic review and meta-analysis of genetic association studies in idiopathic recurrent spontaneous abortion. *Fertil Steril* 2017, 107:150–159.e2

5. Qiao Y, Wen J, Tang F, Martell S, Shomer N, Leung PC, Stephenson MD, Rajcan-Separovic E: Whole exome sequencing in recurrent early pregnancy loss. *Mol Hum Reprod* 2016, 22:364–372
6. Quintero-Ronderos P, Mercier E, Fukuda M, Gonzalez R, Suarez CF, Patarroyo MA, Vaiman D, Gris JC, Laissue P: Novel genes and mutations in patients affected by recurrent pregnancy loss. *PLoS One* 2017, 12:e0186149
7. Dong H, Li X, Lou Z, Xu X, Su D, Zhou X, Zhou W, Bartlam M, Rao Z: Crystal-structure and biochemical characterization of recombinant human calyphosine delineates a novel EF-hand-containing protein family. *J Mol Biol* 2008, 383:455–464
8. Lefort A, Lecocq R, Libert F, Lamy F, Swillens S, Vassart G, Dumont JE: Cloning and sequencing of a calcium-binding protein regulated by cyclic AMP in the thyroid. *EMBO J* 1989, 8:111–116
9. Kusama K, Yoshie M, Tamura K, Imakawa K, Isaka K, Tachikawa E: Regulatory action of calcium ion on cyclic AMP-enhanced expression of implantation-related factors in human endometrial cells. *PLoS One* 2015, 10:e0132017
10. Salker MS, Singh Y, Durairaj R, Yan J, Alauddin M, Zeng N, Steel JH, Zhang S, Nautiyal J, Webster Z, Brucker SY, Wallwiener D, Anne CB, Brosens JJ, Lang F: LEFTY2 inhibits endometrial receptivity by downregulating Orail expression and store-operated Ca(2+) entry. *J Mol Med* 2018, 96:173–182
11. Li H, Durbin R: Fast and accurate short read alignment with Burrows-Wheeler transform. *Bioinformatics* 2009, 25:1754–1760
12. Li H, Handsaker B, Wysoker A, Fennell T, Ruan J, Homer N, Marth G, Abecasis G, Durbin R: 1000 Genome project data processing subroup. The sequence alignment/map (SAM) format and SAMtools. *Bioinformatics* 2009, 25:2078–2079
13. Schwarz JM, Cooper DN, Schuelke M, Seelow D: MutationTaster2: mutation prediction for the deep-sequencing age. *Nat Methods* 2014, 11:361–362
14. Adzhubei IA, Schmidt S, Peshkin L, Ramensky VE, Gerasimova A, Bork P, Kondrashov AS, Sunyaev SR: A method and server for predicting damaging missense mutations. *Nat Methods* 2010, 7:248–249
15. Choi Y, Sims GE, Murphy S, Miller JR, Chan AP: Predicting the functional effect of amino acid substitutions and indels. *PLoS ONE* 2012, 7:e46688
16. Sievers F, Wilm A, Dineen DG, Gibson TJ, Karplus K, Li W, Lopez R, McWilliam H, Remmert M, Söding J, Thompson JD, Higgins DG: Fast, scalable generation of high-quality protein multiple sequence alignments using Clustal Omega. *Mol Syst Biol* 2011, 7:539
17. Synofzik M, Gonzalez MA, Lourenco CM, Coutelier M, Haack TB, Rebelo A, Hannequin D, Strom TM, Prokisch H, Kernstock C, Durr A, Schols L, Lima-Martinez MM, Farooq A, Schule R, Stevanin G, Marques WJ, Züchner S: PNPLA6 mutations cause Boucher-Neuhauser and Gordon Holmes syndromes as part of a broad neurodegenerative spectrum. *Brain* 2014, 137:69–77
18. Collet P, Domenjoud L, Devignes MD, Murad H, Schohn H, Dauca M: The human semaphorin 6B gene is down regulated by PPARs. *Genomics* 2004, 83:1141–1150
19. Itoh T, Kanno E, Uemura T, Waguri S, Fukuda M: OATL1, a novel autophagosome-resident Rab33B-GAP, regulates autophagosomal maturation. *J Cell Biol* 2011, 192:839–853
20. Han Q, Lin X, Zhang X, Jiang G, Zhang Y, Miao Y, Rong X, Zheng X, Han Y, Han X, Wu J, Kremerskothen J, Wang E: WWC3 regulates the Wnt and Hippo pathways via Dishevelled proteins and large tumour suppressor 1, to suppress lung cancer invasion and metastasis. *J Pathol* 2017, 242:435–447
21. Wang Y, Jiang M, Yao Y, Cai Z: WWC3 inhibits glioma cell proliferation through suppressing the Wnt/beta-catenin signaling pathway. *DNA Cell Biol* 2018, 37:31–37
22. Rainier S, Bui M, Mark E, Thomas D, Tokarz D, Ming L, Delaney C, Richardson RJ, Albers JW, Matsunami N, Stevens J, Coon H, Leppert M, Fink JK: Neuropathy target esterase gene mutations cause motor neuron disease. *Am J Hum Genet* 2008, 82:780–785
23. Hufnagel RB, Arno G, Hein ND, Hersheson J, Prasad M, Anderson Y, Krueger LA, Gregory LC, Stoetzel C, Jaworek TJ, Hull S, Li A, Plagnol V, Willen CM, Morgan TM, Prows CA, Hegde RS, Riazuddin S, Grabowski GA, Richardson RJ, Dieterich K, Huang T, Revesz T, Martinez-Barbera JP, Sisk RA, Jefferies C, Houlden H, Dattani MT, Fink JK, Dollfus H, Moore AT, Ahmed ZM: Neuropathy target esterase impairments cause Oliver-McFarlane and Laurence-Moon syndromes. *J Med Genet* 2015, 52:85–94
24. Topaloglu AK, Lomniczi A, Kretzschmar D, Disen GA, Kotan LD, McArdle CA, Koc AF, Hamel BC, Guclu M, Papatya ED, Eren E, Mengen E, Gurbuz F, Cook M, Castellano JM, Kekil MB, Mungan NO, Yuksel B, Ojeda SR: Loss-of-function mutations in PNPLA6 encoding neuropathy target esterase underlie pubertal failure and neurological deficits in Gordon Holmes syndrome. *J Clin Endocrinol Metab* 2014, 99:E2067–E2075
25. Johnson GA, Burghardt RC, Bazer FW, Spencer TE: Osteopontin: roles in implantation and placentation. *Biol Reprod* 2003, 69: 1458–1471
26. Vu TH, Werb Z: Matrix metalloproteinases: effectors of development and normal physiology. *Genes Dev* 2000, 14:2123–2133
27. Ruan YC, Guo JH, Liu X, Zhang R, Tsang LL, Dong JD, Chen H, Yu MK, Jiang X, Zhang XH, Fok KL, Chung YW, Huang H, Zhou WL, Chan HC: Activation of the epithelial Na⁺ channel triggers prostaglandin E(2) release and production required for embryo implantation. *Nat Med* 2012, 18:1112–1117
28. Hannan NJ, Paiva P, Dimitriadis E, Salamonsen LA: Models for study of human embryo implantation: choice of cell lines? *Biol Reprod* 2010, 82:235–245
29. Thie M, Denker HW: In vitro studies on endometrial adhesiveness for trophoblast: cellular dynamics in uterine epithelial cells. *Cells Tissues Organs* 2002, 172:237–252
30. Banerjee P, Ghosh S, Dutta M, Subramani E, Khulpada J, Roychoudhury S, Chakravarty B, Chaudhury K: Identification of key contributory factors responsible for vascular dysfunction in idiopathic recurrent spontaneous miscarriage. *PLoS One* 2013, 8: e80940
31. Kusama K, Yoshie M, Tamura K, Daikoku T, Takarada T, Tachikawa E: Possible roles of the cAMP-mediators EPAC and RAP1 in decidualization of rat uterus. *Reproduction* 2014, 147: 897–906
32. Salker MS, Nautiyal J, Steel JH, Webster Z, Sucurovic S, Nicou M, Singh Y, Lucas ES, Murakami K, Chan YW, James S, Abdallah Y, Christian M, Croy BA, Mulac-Jericevic B, Quenby S, Brosens JJ: Disordered IL-33/ST2 activation in decidualizing stromal cells prolongs uterine receptivity in women with recurrent pregnancy loss. *PLoS One* 2012, 7:e52252
33. Achache H, Revel A: Endometrial receptivity markers, the journey to successful embryo implantation. *Hum Reprod Update* 2006, 12: 731–746
34. Bischof P: Endocrine, paracrine and autocrine regulation of trophoblastic metalloproteinases. *Early Pregnancy* 2001, 5:30–31
35. Perez N, Ostojic S, Volk M, Kapovic M, Peterlin B: Matrix metalloproteinases 1, 2, 3 and 9 functional single-nucleotide polymorphisms in idiopathic recurrent spontaneous abortion. *Reprod Biomed Online* 2012, 24:567–575
36. Desai B, Rogers MJ, Chellaiah MA: Mechanisms of osteopontin and CD44 as metastatic principles in prostate cancer cells. *Mol Cancer* 2007, 6:18