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ORIGINAL ARTICLE

Basic Study

Two missense *STK11* gene variations impaired LKB1/adenosine monophosphate-activated protein kinase signaling in Peutz-Jeghers syndrome

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Abstract

BACKGROUND

Peutz-Jeghers syndrome (PJS) is a rare hereditary neoplastic disorder mainly associated with serine/threonine kinase 11 (*STK11/LKB1*) gene mutations. Preimplantation genetic testing can protect a patient's offspring from mutated genes; however, some variations in this gene have been interpreted as variants of uncertain significance (VUS), which complicate reproductive decision-making in genetic counseling.

AIM

To identify the pathogenicity of two missense variants and provide clinical guidance.

METHODS

Whole exome gene sequencing and Sanger sequencing were performed on the peripheral blood of patients with PJS treated at the Reproductive and Genetic Hospital of Citic-Xiangya. Software was employed to predict the protein structure, conservation, and pathogenicity of the two missense variation sites in patients with PJS. Additionally, plasmids were constructed and transfected into HeLa cells to observe cell growth. The differences in signal pathway expression between the variant group and the wild-type group were compared using western blot and immunohistochemistry. Statistical analysis was performed using one-way analysis of variance. $P < 0.05$ was considered statistically significant.

RESULTS

We identified two missense *STK11* gene VUS [c.889A>G (p.Arg297Gly) and

c.733C>T (p.Leu245Phe)] in 9 unrelated PJS families who were seeking reproductive assistance. The two missense VUS were located in the catalytic domain of serine/threonine kinase, which is a key structure of the liver kinase B1 (LKB1) protein. *In vitro* experiments showed that the phosphorylation levels of adenosine monophosphate-activated protein kinase (AMPK) at Thr172 and LKB1 at Ser428 were significantly higher in transfected variation-type cells than in wild-type cells. In addition, the two missense *STK11* variants promoted the proliferation of HeLa cells. Subsequent immunohistochemical analysis showed that phosphorylated-AMPK (Thr172) expression was significantly lower in gastric, colonic, and uterine polyps from PJS patients with missense variations than in non-PJS patients. Our findings indicate that these two missense *STK11* variants are likely pathogenic and inactivate the *STK11* gene, causing it to lose its function of regulating downstream phosphorylated-AMPK (Thr172), which may lead to the development of PJS. The identification of the pathogenic mutations in these two clinically characterized PJS patients has been helpful in guiding them toward the most appropriate mode of pregnancy assistance.

CONCLUSION

These two missense variants can be interpreted as likely pathogenic variants that mediated the onset of PJS in the two patients. These findings not only offer insights for clinical decision-making, but also serve as a foundation for further research and reanalysis of missense VUS in rare diseases.

Key Words: Missense; *STK11*; Peutz-Jeghers syndrome; Rare disease; Genetic counseling; Assisted reproductive technique

Core Tip: These two missense variants, *STK11* c.889A>G (p.Arg297Gly) and *STK11* c.733C>T (p.Leu245Phe), have been found to contribute to the development of Peutz-Jeghers syndrome (PJS) by impairing the *STK11*/adenosine monophosphate-activated protein kinase signaling pathway. It clarifies that these two germline variants, *STK11* c.889A>G (p.Arg297Gly) and *STK11* c.733C>T (p.Leu245Phe), are likely pathogenic mutations, providing valuable information for fertility selection in patients with PJS.

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INTRODUCTION

Peutz-Jeghers syndrome [PJS; Mendelian Inheritance in Man (MIM), #175200, www.ncbi.nlm.gov/OMIM] is a rare hereditary neoplastic disorder characterized by mucocutaneous pigmentation and the development of multiple gastrointestinal (GI) polyps[1]. The annual incidence of PJS ranges from 1: 8300 to 1: 29000 and does not differ by sex, race, or ethnicity[2-4]. PJS has extremely high penetrance and clinical heterogeneity[5] and can lead to the formation of hamartomatous polyps in many organs, such as the jejunum, ileum, duodenum, stomach, colon, and gallbladder[6,7]. Even at a very young age, PJS patients with pathological hamartomatous polyps can experience numerous GI complications, including bleeding, anemia, abdominal pain, intussusception, obstruction, and infarction[8]. Patients with PJS are at high risk for GI and extra-GI cancer[9,10]. The disease is inherited in an autosomal dominant manner and has a 50% chance of being passed on to offspring.

The serine/threonine kinase 11 (*STK11*/*LKB1*; MIM, *602216) gene has been identified as a pathogenic factor of PJS, with germline mutations in this gene listed in the medical records of most PJS patients as a risk factor[3,7]. *STK11* is a tumor suppressor gene located on chromosome 19p13.3 and includes ten exons[11]. *STK11* functions mainly depend on the phosphorylation and activation of adenosine monophosphate-activated protein kinase (AMPK). As an important metabolic regulator, AMPK regulates cell generation by regulating the activity of metabolic enzymes and activating adaptive transcriptional responses[12]. *STK11* is a master stress response regulator[13] and plays an important role in tumorigenesis, implicated in numerous key biological processes, including cell metabolism, cell cycle regulation, cell polarity and motility, and angiogenesis[1].

A search for *STK11* gene variants in the ClinVar database revealed a total of 2481 entries with clinical significance, of which 974 (39.26%) were variants of uncertain significance (VUS), predominantly (79.98%, 779/974) missense variants until December 18, 2023. Many VUS affect diagnosis, clinical treatment, and decision-making processes because their pathogenicity cannot be defined. Some variants have been shown to be frameshift or nonsense mutations that result in abnormal protein truncation and subsequent loss of kinase activity and are therefore classified as pathogenic or potentially pathogenic. However, missense variants usually only lead to the formation of incorrect amino acid residues at the corresponding position in the translated product, leading to an abnormal protein. Whether this protein alteration affects the normal function of the gene needs to be tested experimentally. Understanding how the function of *STK11* is

disrupted may contribute to a better understanding of the molecular mechanisms involved in the pathogenesis and carcinogenesis of PJS. Clinical fertility counseling and reproductive assistance need to target specific variant sites, so determining the pathogenicity of VUS is urgent and important for patients.

MATERIALS AND METHODS

Patient

Peripheral blood samples were collected from 9 PJS probands and 19 family members, all of whom were Chinese Han. These patients with PJS were diagnosed according to the PJS diagnostic criteria published by the European consensus in 2007[14]. When ≥ 2 individuals are affected within the same family, they are considered family cases. Patients without a family history of PJS are defined as sporadic cases. Patient information such as family history and clinical characteristics were collected from the patient's medical records. All patients provided informed consent for this study. Clinical data collection included demographics, medical history, family history, symptoms, and surgical interventions related to PJS. Some patients underwent endoscopic or surgical polypectomy. Tissues were fixed with formalin and embedded in paraffin. The wax blocks were successively sliced and prepared for subsequent hematoxylin-eosin (HE) staining and immunohistochemical (IHC) experiments.

Whole-exome sequencing and Sanger sequencing

The peripheral blood of all probands was sent to Beijing Genomics Research Institute for Whole-exome sequencing (WES), and the tumor-related gene exons and adjacent ± 10 bp intron variations were detected. The results were verified by polymerase chain reaction (PCR)-Sanger sequencing. The identified variations were further tested in all available family members to confirm co-segregation of these variations with the disease. We used the online site primer 3 to design primers. Variation analysis was mainly based on the American Society for Medical Genetics and Genomics (ACMG) guidelines for interpreting sequence variation[15].

Predicting the impact of identified VUS

Amino acid conservation analysis was performed using UGENE software. Five online software programs, AlphaMissense (https://alphamissense.hegelab.org/search), PROVEAN (http://provean.jcvi.org/seq_submit.php), mutation Taster (https://www.mutationtaster.org/), PolyPhen-2 (http://genetics.bwh.harvard.edu/pph2/), and Swiss-model (http://swissmodel.expasy.org/), were used to predict the functional and structural significance of these variations.

Tissue staining

Tissue sections were incubated with liver kinase B1 (LKB1), phosphorylated (p)-LKB1 (Ser428), AMPK, p-AMPK (Thr172) antibodies. The IHC kit used was from Leica Biosystems (catalog No.RE7280-k, Germany) and was operated according to the reagent instructions. Polyp tissue sections were stained by HE staining. An experienced pathologist reviewed these specimens and determined the histological types of these polyps. The staining of the sliced tissue with a brownish yellow color indicates positivity. Statistical analysis of IHC results was based on a direct count of the number of positive cells in five random visual fields at 70 × magnification or a visual assessment of the proportion of positive cells. Each group was repeated three times.

Cell experiments

Five plasmids including enhanced green fluorescent protein (EGFP)-N1-vector (EGFP-Empty), PEGFP-N1-STK11 (EGFP-STK11), PEGFP-N1-*STK11* c.889A>G (p.Arg297Gly), PEGFP-N1-*STK11* c.733C>T (p.Leu245Phe) and PEGFP-N1-*STK11* c.910C>T(p.Arg304Trp) were transfected into HeLa cells with lipofectamine 3000. Cells were collected 48 h after transfection, subsequent PCR product gel electrophoresis, Western blotting (WB), Reverse transcription quantitative PCR (RT-qPCR), and Cell Counting Kit 8 (CCK 8) experiments were performed. Transfected cells were counted and seeded in 6-well plates (1000 cells/well). Cell proliferation was detected at 12 h, 24 h, 36 h, 48 h, 60 h, and 72 h after transfection. Each group was repeated three times.

Total RNA was extracted using Trizol, and cDNA was synthesized by reverse transcription from 1 μg of total RNA using oligo (dT) primers. RT-qPCR was performed using the All-in-One™ qPCR mix on a LightCycler 480 system (Roche, Switzerland). Primers were performed according to the primer website (https://www.ncbi.nlm.nih.gov/tools/primer). Glyceraldehyde 3-phosphate dehydrogenase (GAPDH) was used as an internal control for normalization. The level of expression endogenous, exogenous, and total mRNA of *STK11* was calculated using the $2^{-\Delta\Delta Ct}$ method.

Total protein was extracted using radioimmunoprecipitation assay lysis buffer and detected using bicinchoninic acid kits. Total protein was separated on 10% SDS-PAGE. After separation, the protein was elactrotransferred to a polyvinylidene difluoride membrane, and then blocked in 5% skimmed milk for 1 h at room temperature. The membranes were incubated with primary antibodies against *STK11*, p-*STK11*, AMPK, p-AMPK and GAPDH overnight at 4˚C. GAPDH served as the loading control. Membranes were subsequently incubated with secondary antibodies for 2 h at room temperature. Protein expression was detected using an enhanced chemiluminescence kit (Thermo Fisher Scientific, United States).

Statistical analysis

All statistical analyzes were performed with SPSS (version 26.0, United States) and GraphPad Prism (verison 9.0, United

States). The data are expressed as mean ± SD unless otherwise noted. Comparisons among multiple groups were determined using one-way analysis of variance, followed by least significant difference or Dunnett's T3 test. $P < 0.05$ was considered statistically significant.

RESULTS

Clinical features of patients

Some patients with PJS and a clinical phenotype seek genetic and reproductive assistance when giving birth. The demographics and clinical characteristics of 9 patients with PJS are shown in Table 1. At the time of the last follow-up, 8 patients were married, and 1 was unmarried. All patients with PJS had skin and mucosa pigmentation. The median age of the 9 PJS patients when they visited our hospital for the first time was 30 years (ranging from 23 to 45 years), of whom 5 were male (55.6%). The age at diagnosis varied widely, with some patients being diagnosed in the first few years of life and others demonstrating a later onset and milder symptoms. Eight of the 9 patients (89.0%) with PJS developed colon polyps and underwent polypectomy, and the mean age at the onset of the first GI polyp was 19.38 years (19.38 ± 4.39). The burden of polyps (cumulative number of polyps) varied from approximately 10 to several hundred. PJS polyps were distributed in the stomach in 5 patients (55.6%), in the small intestine in 7 (77.8%), and in the colorectum in 4 (44.4%). Two patients (22.2%) had uterine polyps, 1 (11.1%) had gallbladder polyps, and 1 (11.1%) had nasal polyps. Six (66.67%) were initially treated before the age of 20 years, and all patients received initial treatment before the age of 30 years. Patients with a family history of PJS were treated at a later age than those without; however, the difference was not significant ($P = 0.4059$). The age at first treatment differed by sex, with the first treatment occurring later in men than in women; however, the difference was not significant ($P = 0.4521$).

WES identified heterozygous STK11 variations

WES or *STK11* variant detection was performed for the 9 unrelated PJS patients. Ten (111%) different germline variations in the *STK11* gene were identified. Clinical and sociodemographic data were collected from 26 family members, 19 of whom underwent pedigree analysis for the *STK11* gene (Supplementary Table 1). A total of 4 *STK11* variations were identified in familial cases and 6 in sporadic cases. Of the 10 variants identified, three were nonsense variants, three were splicing variants, three were missense variants, and one was a frameshift variant. According the ACMG guidelines, the variant pathogenicity assessment indicated that 5 were pathogenic, 2 were likely pathogenic, 1 was likely benign, and 2 were VUS. Two patients with missense VUS underwent GI endoscopy at our hospital, and one had polyps removed from the stomach, colon, and uterus (Figure 1A-F). Two of the variations [c.889A>G (p.Arg297Gly) and c.733C>T (p.Leu245Phe)] were identified by Sanger sequencing (Figure 1G-J). A schematic representation of the localization of 10 *STK11* variations is shown in Figure 1K; most of the variations were concentrated in the exon 1 region.

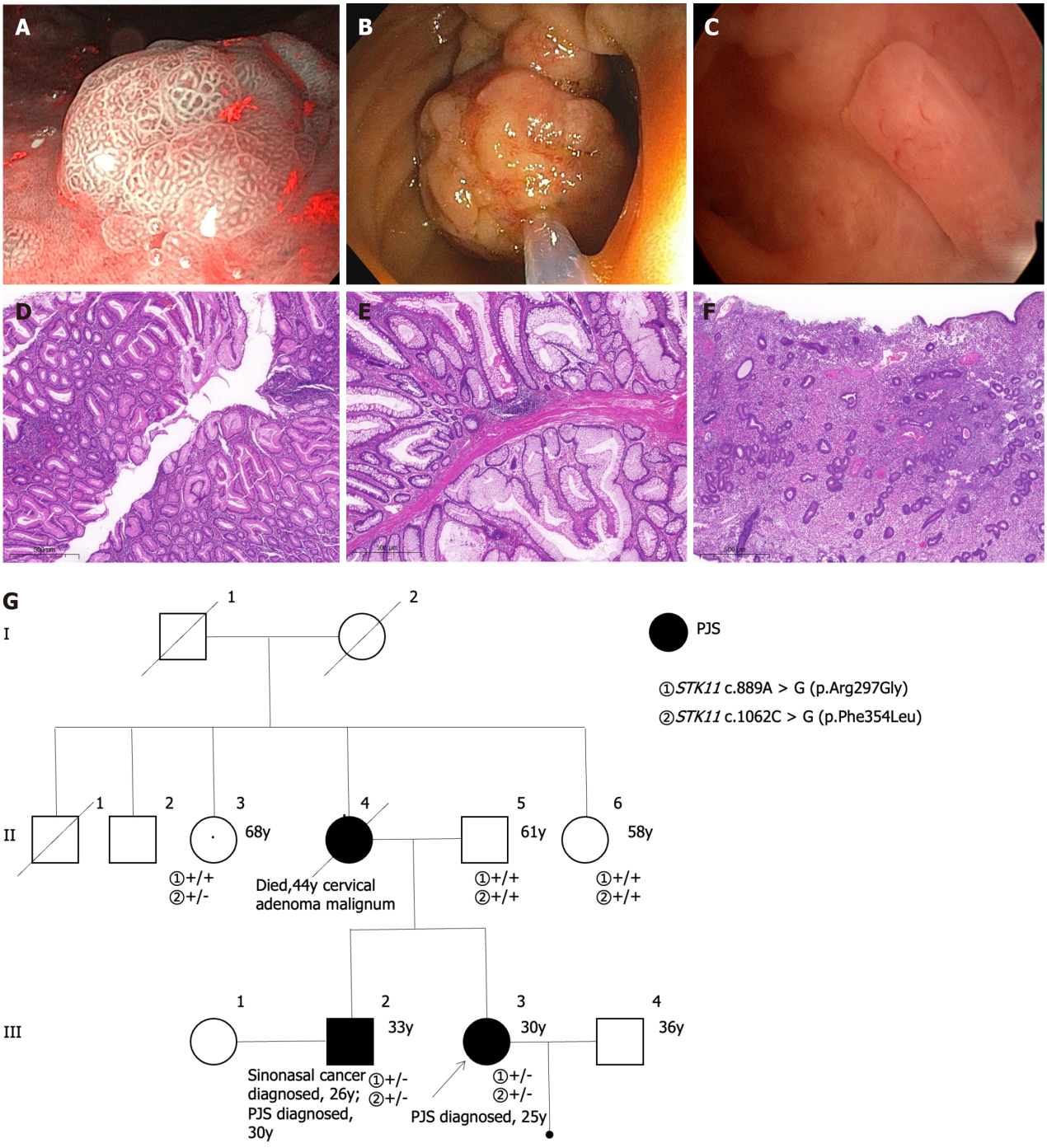
Three-dimensional structure and function prediction of proteins resulting from missense STK11 VUS

Swiss-model software was used to construct a three-dimensional (3D) model of the protein structure of *STK11* (Figure 2A-D). The *STK11* c.889A>G (p.Arg297Gly) variant resulted in the substitution of arginine for glycine. Arginine has a large molecular weight, electrically charged side chain, hydrophilic properties, and an alkaline R group. It plays an important role in the structure and function of proteins. Glycine has a small molecular weight, nonpolar side chain, hydrophobic properties, and a neutral R group containing only one hydrogen atom, as well as a large degree of freedom. The *STK11* c.733C>T (p.Leu245Phe) variant substituted leucine with phenylalanine. Both amino acids have nonpolar side chains and are hydrophobic, but leucine is a side branched-chain amino acid, while phenylalanine has an aromatic ring in the side chain. Therefore, the variation between these two amino acids may lead to large structural changes. The two amino acids were evolutionarily conserved by UGENE (Figure 2E), while Mutation Taster, AlphaMissese, PolyPhen-2, and SIFT predicted the two missense variations (p.Arg297Gly and p.Leu245Phe) to be deleterious (Figure 2F).

Functional analysis of the two missense STK11 variations in HeLa cells

To verify whether these two missense variants affect *STK11* gene transcription and translation, *STK11* plasmids were established [an empty vector, wild-type vector, *STK11* c.889A>G (p.Arg297Gly) variant vector, and c.733C>T (p.Leu245Phe) variant vector]. One missense variant c.910C>T (p.Arg304Trp) was interpreted as pathogenic in ClinVar; thus, a plasmid with this mutation was used as the positive control. PCR and WB were used to detect endogenous *STK11* expression levels in HeLa and HEK-293T cell lines. *STK11* expression levels were nearly undetectable in HeLa cells *vs* HEK-293T cell lines. Thus, HeLa cells were used for further studies. All plasmids were verified by gel electrophoresis and PCR product sequencing; since all carried GFP constructs, successful transfection was detected by fluorescence microscopy (Figure 3A-E). *STK11* expression was examined by WB and RT-qPCR analysis 48 h after transfection. The protein and mRNA expression levels of *STK11* in HeLa cells transfected with the *STK11* variation and wild-type vectors were significantly increased in comparison with those in cells transfected with the empty vector. However, there was no significant difference in *STK11* expression in cells transfected with the wildtype vector and the *STK11* variant vectors (Figure 3F and G). These results suggest that the two missense *STK11* variants have negligible effects on *STK11* transcription and translation.

We next tested LKB1, p-LKB1, AMPK, and p-AMPK protein levels to detect the effects of these two missense VUS on LKB1 protein function and whether they play a role through the downstream AMPK signaling pathway. p-LKB1 and p-AMPK levels were significantly lower in all cells transfected with variant plasmids than in wild-type cells (Figure 3H).


A
B
C
D
E
F
G
I
1
2
PJS
①STK11 c.889A > G (p.Arg297Gly)
②STK11 c.1062C > G (p.Phe354Leu)
II
1
2
3
68y
①+/+
②+/-
4
Died,44y cervical adenoma malignum
5
61y
①+/+
②+/+
6
58y
①+/+
②+/+
III
1
2
33y
Sinonasal cancer diagnosed, 26y; PJS diagnosed, 30y
①+/-
②+/-
3
30y
①+/-
②+/-
PJS diagnosed, 25y
4
36y
H
c.889A > G
C C A A G G G G T T C T
III-3
c.889A > G
C C A A G G G G T T C T
III-2

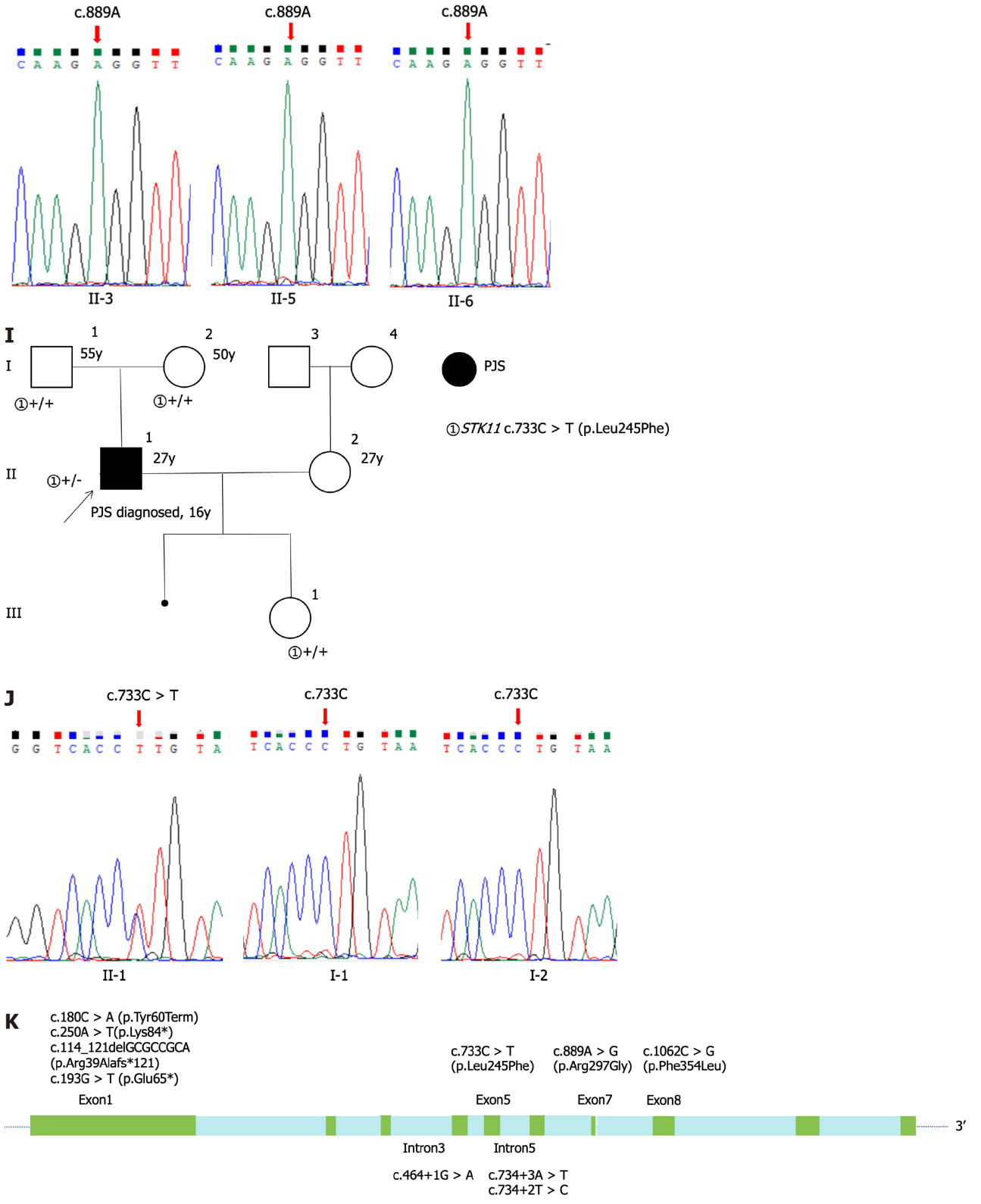


Figure 1 Clinical features and sequencing data of patients with Peutz-Jeghers syndrome. A-F: Polyps and hematoxylin-eosin (HE) staining of a patient with Peutz-Jeghers syndrome (PJS). Gastroenteroscopy and hysteroscopy revealed and resected multiple polyps in the stomach, intestines, and uterus. Gastric polyp and HE (A and D), colon polyp and HE (B and E), endometrial polyp and HE (C and F), magnification, 5 × (D-F); G-J: Family pedigree and sequencing maps of two missense *STK11* variants of uncertain significance (VUS) in two PJS patients; Family pedigree of a female patient with PJS who had *STK11* c.889A>G (VUS) and c.1062C>G (likely benign) variations (G); The patient and her older brother had *STK11* c.889A>G variation, but her father and two aunts were undetected (H); Family pedigree of a male patient with PJS who had *STK11* c.733C>T (VUS) variations (I); The patient had *STK11* c.733C>T variations, but his father and mother were undetected (J); K: Schematic representation of the *STK11* gene structure and variations in 9 PJS families. Arg: Arginine; Gly: Glycine; Phe: Phenylalanine; Leu: Leucine; y: Years; Tyr: Tyrosine; Term: Termination; Lys: Lysine; del: Deletion; Glu: Glutamic acid.

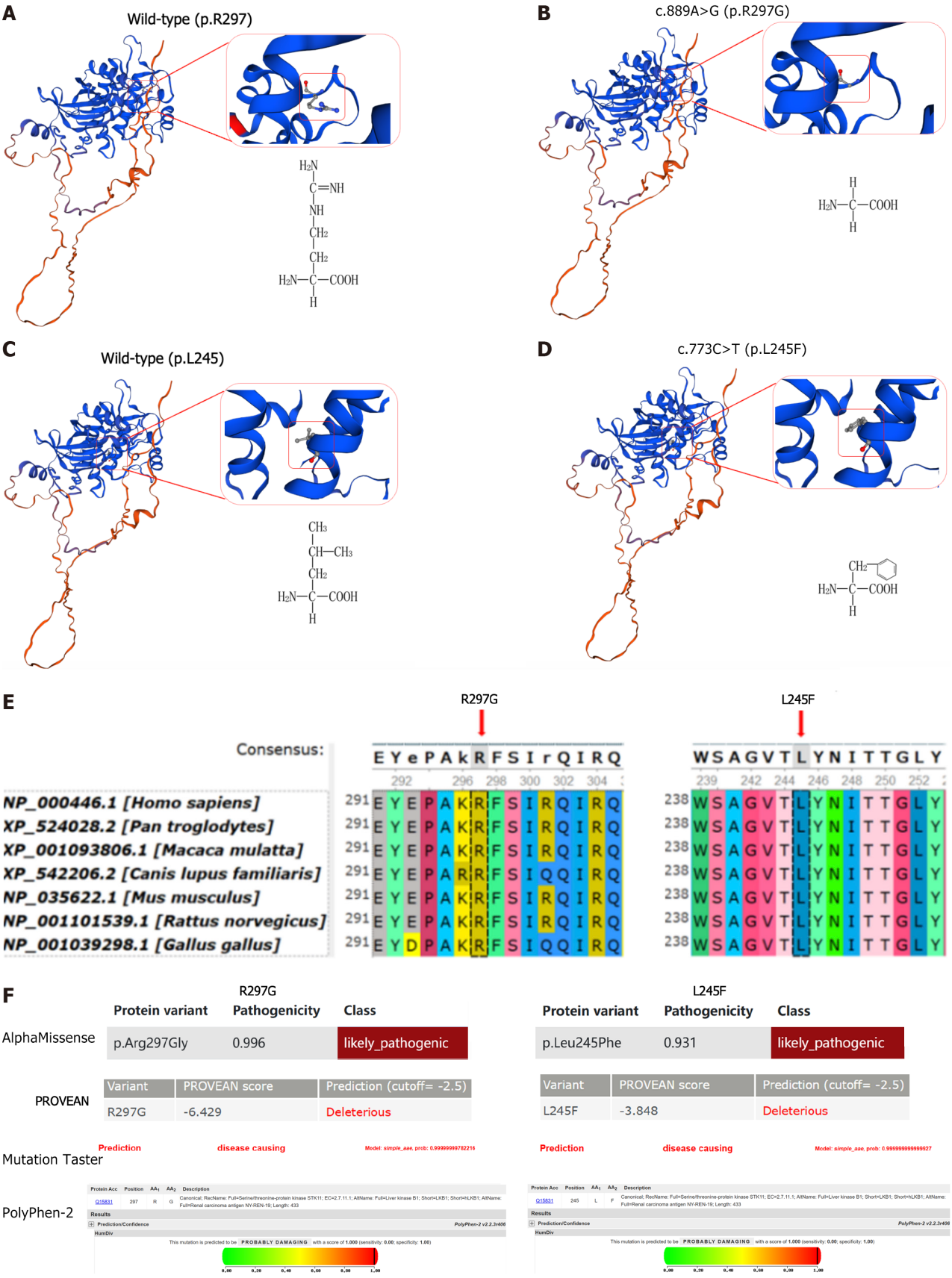
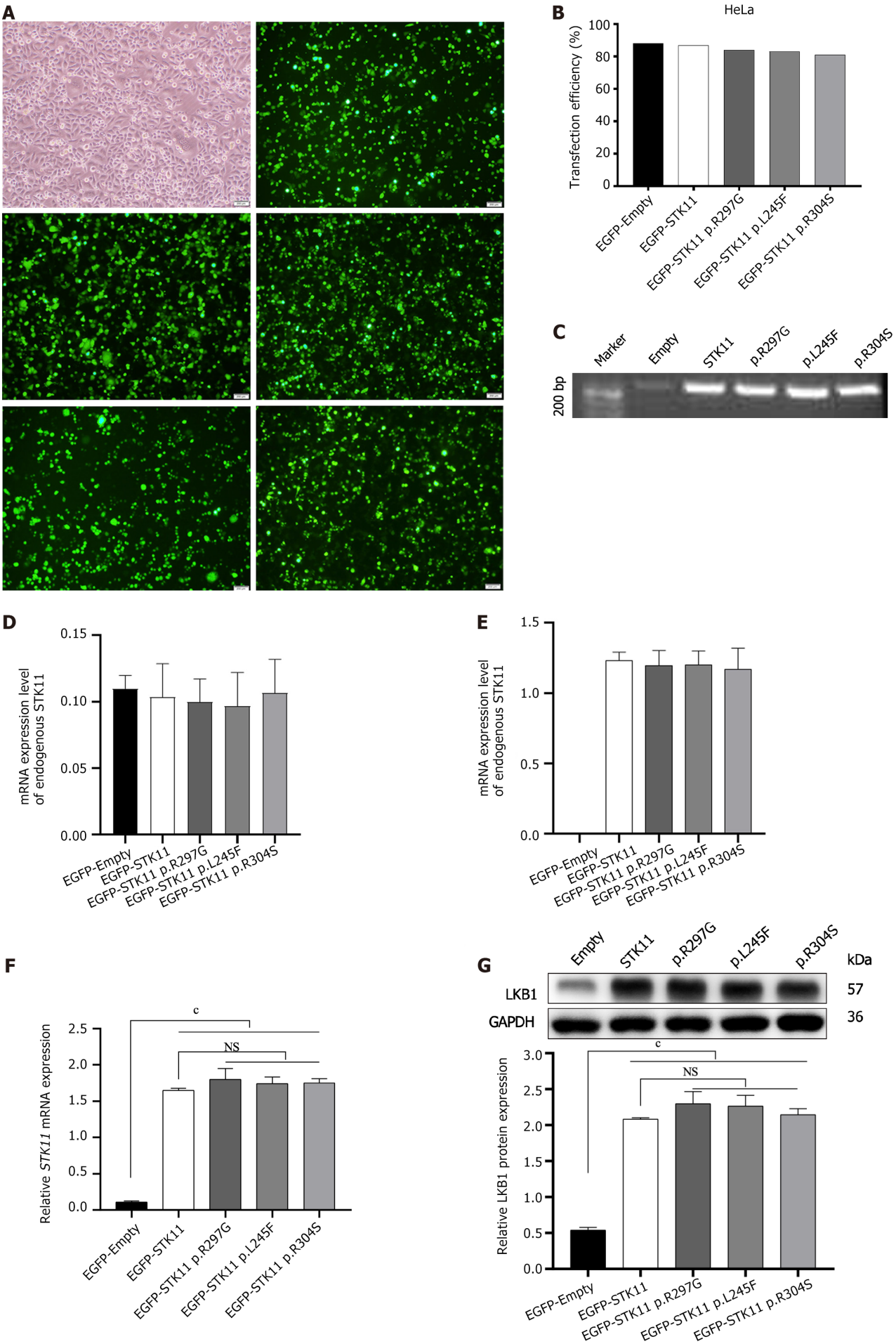


Figure 2 Multiple software predict the pathogenicity of these variations. A-D: Three-dimensional model of the protein structure of liver kinase B1 and amino acid molecular structure formula. These variations result in significant changes in the properties of amino acids, which may be harmful; E: Evolutionary conservation shows that the variation sites (p.R297G and p.L245F) are highly conserved across different species; F: Four software assessed the pathogenicity of these variations, all of which were likely pathogenic or pathogenic. "R" represents arginine; "G" represents glycine; "L" represents leucine; and "F" represents phenylalanine.


A
B
HeLa
Transfection efficiency (%)
100
80
60
40
20
0
EGFP-Empty
EGFP-STK11
EGFP-STK11 p.R297G
EGFP-STK11 p.L245F
EGFP-STK11 p.R304S
C
Marker
Empty
STK11
p.R297G
p.L245F
p.R304S
200 bp
D
mRNA expression level of endogenous STK11
0.15
0.10
0.05
0.00
E
mRNA expression level of endogenous STK11
1.5
1.0
0.5
0.0
F
Relative STK11 mRNA expression
2.5
2.0
1.5
1.0
0.5
0.0
c
NS
G
Empty
STK11
p.R297G
p.L245F
p.R304S
kDa
LKB1
57
GAPDH
36
Relative LKB1 protein expression
3.0
2.5
2.0
1.5
1.0
0.5
0.0
c
NS

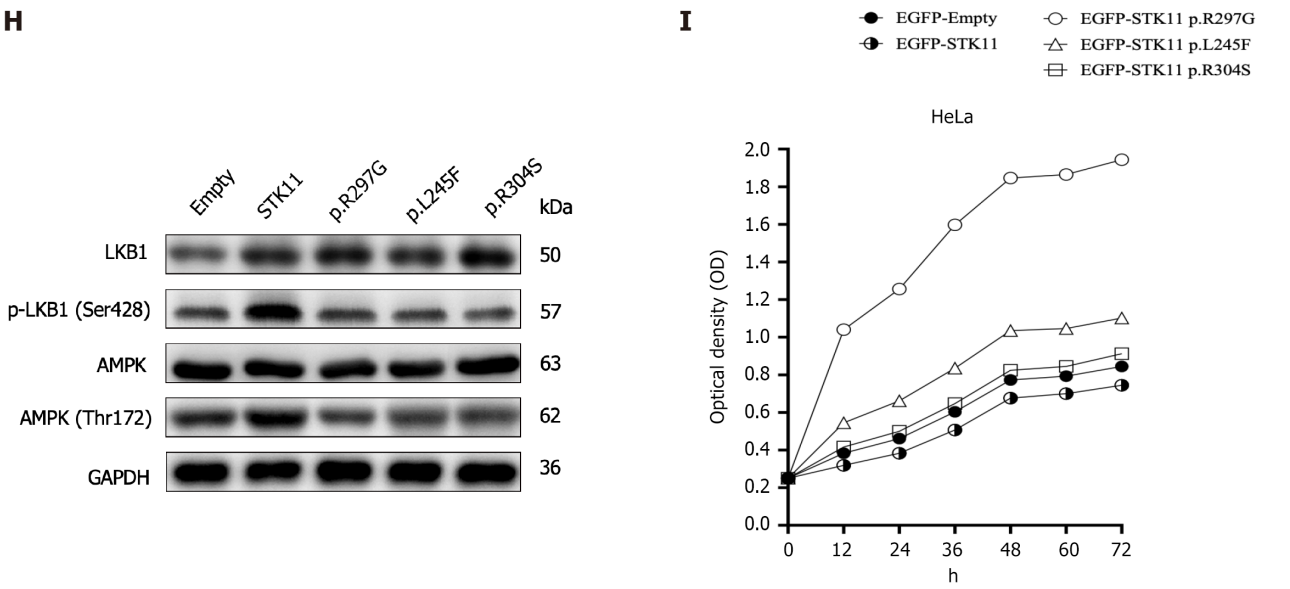


Figure 3 Expression and functional analysis of *STK11*. A: Plasmids were successfully transfected into HeLa cells. HeLa cell-Lipofectamine 3000; and HeLa cell transfected with enhanced green fluorescent protein (EGFP)-Empty, EGFP-N1-*STK11*, EGFP-N1-*STK11* c.889A>G (p.Arg297Gly), EGFP-N1-*STK11* c.733C>T (p.Leu245Phe) and EGFP-N1-*STK11* c.910C>T (p.Arg304Trp) plasmid vectors, Magnification: 10 ×; B: Plasmid transfection efficiency was high; C: PCR gel electrophoresis confirmed that the transfected plasmid was correct; D: The endogenous expression of *STK11* gene was low in HeLa cells; E: The exogenous expression of *STK11* gene in HeLa cells after transfection; F: These missense variants do not affect gene transcription; G: These variations do not affect the function of gene translation into proteins; H: These missense variants disrupted the phosphorylation of liver kinase B1 and adenosine monophosphate-activated protein kinase; I: These missense variants promote cell proliferation, $^{\circ}P < 0.0001$. "R" represents arginine; "G" represents glycine; "L" represents leucine; "F" represents phenylalanine; and "S" represents serine. Independent sample results were obtained after at least three experiments and then obtained statistically. NS: Not significant; GAPDH: Glyceraldehyde 3-phosphate dehydrogenase; LKB1: Serine/threonine kinase 11.

We performed CCK-8 assays to further determine whether these two *STK11* VUS could effectively inhibit cell growth and proliferation. Compared with the wild-type group, the growth of cells transfected with the *STK11* variant vectors was significantly accelerated (Figure 3I). These results indicate that both missense VUS disrupt the endogenous protein kinase activity of *STK11* and inhibit the activation of the AMPK pathway, disrupting the inhibitory function of the *STK11* gene on cell growth, leading to cell proliferation that may contribute to the development of polyps and tumors.

In summary, based on the above results, functional evidence is provided that these two missense *STK11* variants are likely pathogenic according to the ACMG's guidelines.

STK11 gene variation may influence the occurrence of polyps in PJS patients via the LKB1/AMPK pathway

Polyp tissue from non-PJS patients with the same social factors as the PJS patients was used as the control, and the LKB1, p-LKB1, AMPK, and p-AMPK protein expression levels were compared between the two groups. The expression levels of p-*STK11* and p-AMPK were decreased in the stomach, colon, and uterus in patients with PJS, and the differences were statistically significant ($P < 0.05$). Therefore, it is inferred that the *STK11* gene may cause polyps or PJS through the LKB1/AMPK pathway (Figures 4 and 5).

DISCUSSION

STK11 plays a crucial role in regulating cell damage repair, energy metabolism, and tumor immune responses[16]. The LKB1 protein comprises three major domains: The N-terminal non-catalytic domain (encoded by amino acids 1-49), the catalytic kinase domain (encoded by amino acids 49-309), and the C-terminal non-catalytic regulatory domain (encoded by amino acids 309-433)[1,17]. Variations in patients with PJS are predominantly located in the catalytic domain region, leading to kinase activity dysfunction and the disruption of *STK11* function[18]. Germline *STK11* mutation has been confirmed to be a significant cause of PJS[19].

We conducted a review of PJS patients with *STK11* gene variations at our hospital. A total of 10 variations were found, primarily in exon 1, consistent with Li *et al*[20]. While all PJS patients exhibited *STK11* gene variations, some were VUS [21,22], and numerous *STK11* gene VUS exist in the ClinVar database, with the majority being missense variations[23]. Understanding the relationship between these variations and PJS is challenging, complicating clinical decision-making, reproduction decisions, and genetic counseling for the patient[24,25], which may lead to increased psychological stress [26]. Therefore, it is crucial to determine the pathogenicity of these VUS.

Most patients with PJS have *STK11* germline mutations, and their offspring face an elevated risk of inheriting the same mutation and experiencing PJS due to the autosomal dominant inheritance pattern[27]. PJS patients affected by the disease naturally do not want their offspring to experience the same effects[28]. Therefore, some patients opt for

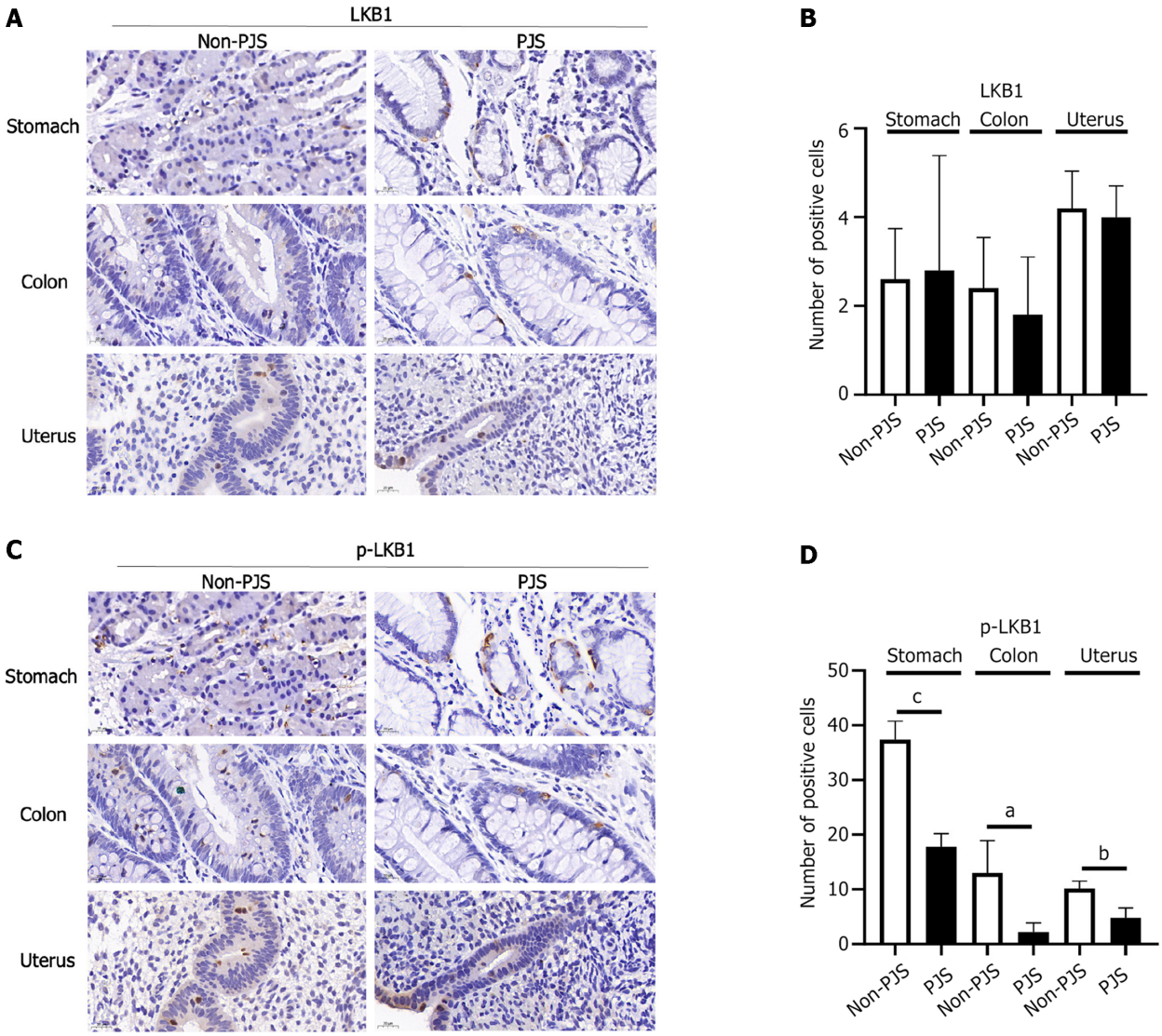


Figure 4 Expression of the serine/threonine kinase 11 in polyps of Peutz-Jeghers syndrome and non-Peutz-Jeghers syndrome patients.
A-D: The phosphorylation levels of liver kinase B1 in Peutz-Jeghers syndrome (PJS) patients polyps were significantly lower than those in non-PJS patients polyps. PJS: Peutz-Jeghers syndrome; LKB1: Liver kinase B1. [a]$P < 0.05$; [b]$P < 0.001$; [c]$P < 0.0001$.

preimplantation genetic testing (PGT)[29] to prevent the transmission of disease-causing mutations to future generations [5,30]. Some PJS patients choose to pursue a natural pregnancy[31]. In cases where the female patient or the male patient's partner becomes pregnant, prenatal diagnosis is performed to predict whether the fetus carries *STK11* or other genetic mutations[32,33]. Parents can terminate the pregnancy if the fetus is a carrier of the *STK11* mutation[34]; therefore, this method also allows healthy children to be born. In our study, one PJS patient chose *in vitro* fertilization-embryo transfer. Unfortunately, prenatal diagnosis at 18 wk of gestation revealed that the fetus carried the *STK11* gene mutation; thus, the pregnancy was terminated. Seven patients (78%) chose PGT to block the transmission of the mutated genes to their offspring. It is worth mentioning that after the two missense VUS were indicated to likely be pathogenic through experiments, both patients chose PGT to assist their pregnancies, and one patient then gave birth to a healthy girl. The other suffered a spontaneous miscarriage in the fifth week of pregnancy. Therefore, identifying the pathogenicity of genetic variations may affect the patient's reproduction decisions.

The two missense variants found in this study are VUS, complicating clinical decision-making. Clinical geneticists and reproductive specialists cannot provide help and guidance to patients with VUS since it is unclear whether the variant is related to the disease. Here, the two missense VUS were predicted by software to be harmful and affect protein structure; however, because the variants were VUS, the patients faced great difficulties in clinical fertility selection. Now that experiments have supported the idea that these variants can be interpreted as being likely pathogenic, reproductive, and genetic physicians will be able to provide better fertility counseling to patients. The findings can also be used for evidence-based genetic and reproductive counseling for PJS families. Finally, we provided valuable insights into the molecular mechanisms of PJS pathogenesis. Two patients with PJS had a child by PGT, and no pathogenic gene mutation was found in the fetus by prenatal diagnosis, suggesting that the combination of molecular and prenatal diagnosis is an effective way to prevent the recurrence or transmission of inherited diseases in these families. However, determining the genetic cause before conception is crucial in this process.

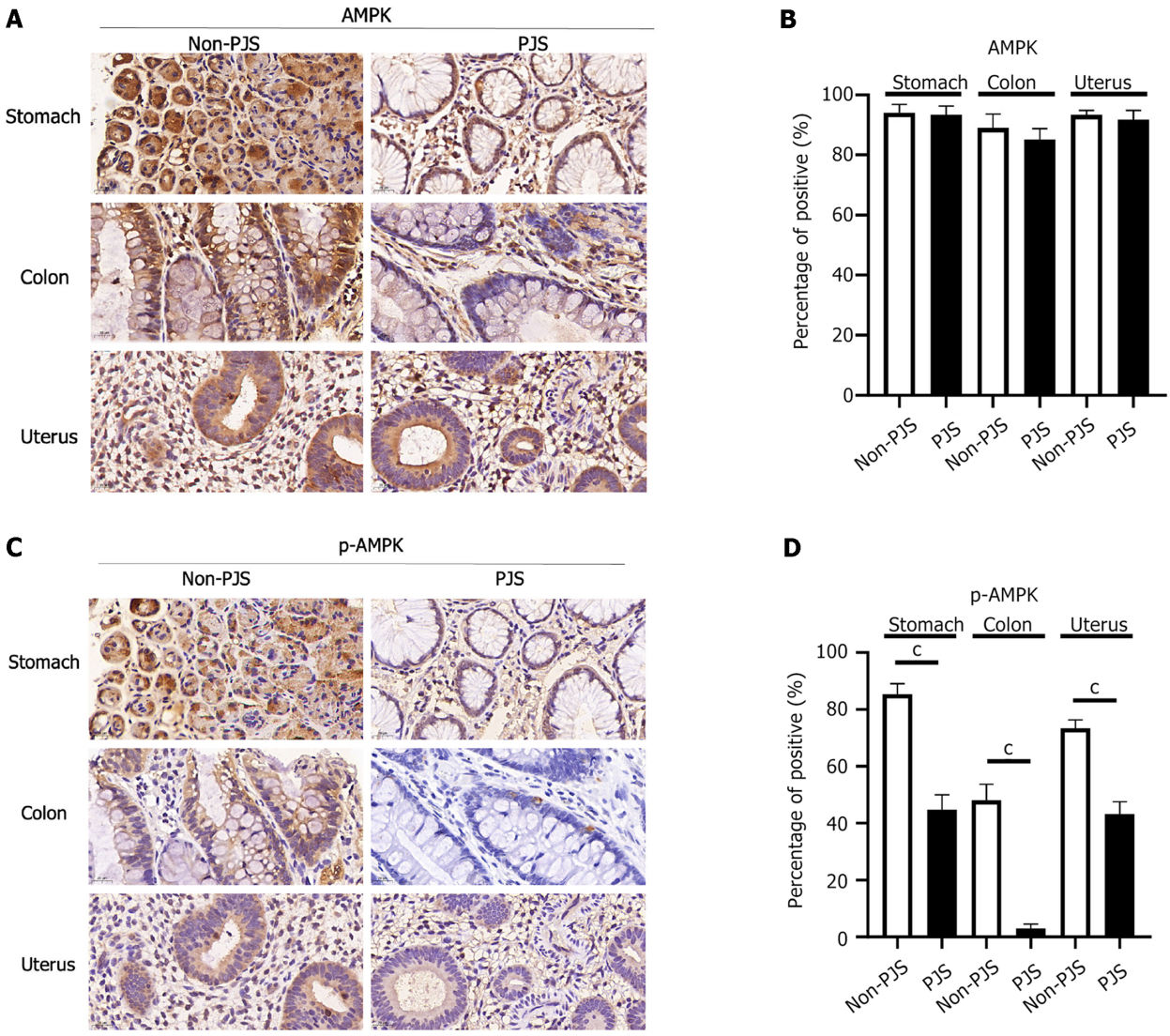


Figure 5 Expression of the adenosine monophosphate-activated protein kinase in polyps of Peutz-Jeghers syndrome and non-Peutz-Jeghers syndrome patients. A-D: The phosphorylation levels of adenosine monophosphate-activated protein kinase in Peutz-Jeghers (PJS) patients polyps were significantly lower than those in non-PJS patients polyps. Magnification: 70 ×. Results of independent samples were calculated by using five randomly selected visual fields. Independent sample results were obtained after at least three experiments and then obtained statistically. PJS: Peutz-Jeghers syndrome; AMPK: Adenosine monophosphate-activated protein kinase. $^{c}P < 0.0001$.

Several limitations should be noted in our study. First, because of the rarity of PJS, the number of patients included is relatively small, which may reduce the statistical analysis power. Second, the follow-up period was short; the longest follow-up was 8 years. Because the patients with PJS were generally young when they visited the doctor, no malignant tumors were found till the date of the final follow-up; thus, the correlation between PJS and cancer development could not be discussed. Third, because our hospital mainly caters to patients who need assisted reproduction and genetic counseling, there may be some bias in the selection of patients included; that is, patients visit the doctor for assisted reproduction, leading to a high proportion of patients choosing PGT.

CONCLUSION

We identified two missense variants in the *STK11* gene that were experimentally evaluated for their functional significance. Our results identify the two missense variations as being likely pathogenic, providing new guidance for genetic counseling, fertility counseling, and prenatal diagnosis in patients with *STK11* gene variants.

Table 1 Spectrum of STK11 mutations and clinical characteristics of patients with Peutz-Jeghers syndrome

Proband family No.	Proband gender, age (yr)	Clinical features	Age at first discovery of polyps	Previous medicine history	Nucleotide alteration, amino acid alteration	Exon/Intron, mutation type	ACMG	PJS family history	Reproductive choice	Current Clinical outcome	Pedigree analysis
1	Man, 34	Pigmentation of skin and mucosa; multiple polyps	19	Intestinal polypectomy	c.180C>A (p.Tyr60Ter)	Exon1, nonsense	P	*De novo*	IVF-ET	The fetus had the same mutation and the pregnancy was terminated	Reject
2	Woman, 30	Pigmentation of skin and mucosa; multiple polyps	25	Polypectomy was performed in the stomach, colon and uterus	c.889A>G (p.Arg297Gly)	Exon7, missense	VUS	Yes, maternal. Mother, brother	PGT	The fetus was 5 wk+ and had a spontaneous abortion	Brother carries *STK11* variation but father doesn't
					c.1062C>G (p.Phe354Leu)	Exon 8, missense	LB				
3	Man, 27	Pigmentation of skin and mucosa; multiple polyps	16	Multiple gastrointestinal polypectomy	c.733C>T (p.Leu245Phe)	Exon5, missense	VUS	*De novo*	PGT	The fetus was prenatally diagnosed as having no mutation and gave birth to a child	Parents had no *STK11* variation
4	Man, 25	Pigmentation of skin and mucosa; multiple polyps	15	Multiple gastrointestinal polypectomy	c.250A>T (p.Lys84*)	Exon1, nonsense	P	*De novo*	PGT	Preoperative preparation for PGT	Parents had no *STK11* variation
5	Woman, 35	Pigmentation on the face and lips; multiple polyps	19	Twice minimally invasive surgery for intestinal polyps and laparotomy operation	c.114_121delGCGCCGCA (p.Arg39Alafs*121)	Exon1, frame shift	P	Yes, paternal. Father, uncle, brother	PGT	The fetus was prenatally diagnosed as having no mutation and gave birth to a child	Mother and son had no *STK11* variation
6	Woman, 33	Pigmentation on the lower lip, fingers, soles of the feet, and oral mucosa; multiple polyps	25	Polypectomy was performed in the small intestine and uterus	c.193G>T(p.Glu65*)	Exon1, nonsense	P	*De novo*	PGT	Preoperative preparation for PGT	Parents had no *STK11* variation
7	Man, 45	Pigmentation on the lower lip, fingers, and oral mucosa; multiple polyps	22	Multiple gastrointestinal polypectomy	c.464+1G>A	Intron 3-4, splicing	LP	*De novo*	PGT	Preoperative preparation for PGT	Parents had no *STK11* variation
8	Man, 23	Pigmentation on the lips	/	Laser treatment for hyperpigmentation at 19 yr, no polyps found on gastrointestinal endoscopy	c.734+3A>T	Intron 5-6, splicing	LP	Yes, paternal. Father, grandfather, sister, and niece	/	Unmarried, under consideration	Father had *STK11* variation
9	Woman, 24	Pigmentation on the limbs and trunk;	13	Multiple gastrointestinal polypectomy; termination	c.734+2T>C	Intron 5-6, splicing	P	*De novo*	PGT	Preoperative preparation for PGT	Parents had no *STK11*

	multiple polyps	because fetal had *STK11* mutation	variation

ACMG: American Society for Medical Genetics and Genomics; P: Pathogenic; IVF-ET: *In vitro* fertilization-embryo transfer; VUS: Variant of uncertain significance; LB: Likely benign; PGT: Preimplantation genetic testing; LP: Likely pathogenic.

ARTICLE HIGHLIGHTS

Research background

Peutz-Jeghers syndrome (PJS) is a rare hereditary tumor disease with autosomal dominant inheritance that primarily results from mutations in the *STK11* gene. While certain missense variations of the *STK11* gene have been identified and classified as pathogenic or likely pathogenic, numerous missense variations still remain as variants of uncertain significance (VUS). This ambiguity makes it challenging to establish the association between these variations and PJS, which poses significant challenges for clinical treatment management and fertility selection.

Research motivation

Among the 9 PJS families evaluated at our hospital, 2 patients with PJS were identified as having missense variations through whole-exome sequencing and were classified as VUS. Understanding how the function of *STK11* is disrupted may contribute to a better understanding of the pathogenesis of PJS and the molecular mechanisms involved in the carcinogenesis process. Clarifying the pathogenicity of variations can offer patients the most appropriate reproductive guidance.

Research objectives

To clarify the pathogenicity of these two missense variations and to better provide patients with health management and fertility guidance.

Research methods

This study used whole-exome gene sequencing and Sanger sequencing methods to identify gene variants, combining bioinformatics analysis, quantitative polymerase chain reaction, immunoblotting, immunohistochemistry, and a variety of *in vitro* and *in vivo* functional assays to investigate pathogenicity.

Research results

Bioinformatics software analysis indicated that these two missense variants are deleterious. The phosphorylation levels of adenosine monophosphate-activated protein kinase (AMPK) and liver kinase B1 in the variant group were significantly lower than those in the wild-type group. Both missense *STK11* variants promoted the proliferation of HeLa cells. The expression of phosphorylated-AMPK was significantly lower in PJS patients with a missense variant in gastric, colon, and uterine polyps compared to non-PJS patients. These missense variants inactivate the *STK11* gene, disrupting the regulation of downstream AMPK proteins, thereby mediating the occurrence of PJS. As a result, they are interpreted as likely pathogenic, providing valuable information for patients in reproductive decision-making.

Research conclusions

These findings provide a basis for further research on and reanalysis of clinical decision making related to rare disease missense VUS.

Research perspectives

Further investigation into the role of *STK11* gene in mediating PJS is required. Identifying the pathogenicity of more missense VUS is crucial to offer more accurate medical services for patients.

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FOOTNOTES

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