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ORIGINAL RESEARCH

Hemizygous *IL2RG* Variants Impair IL-2-Induced STAT5 Phosphorylation and Transcriptional Activity Causing X-Linked Severe Combined Immunodeficiency

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Purpose: X-linked severe combined immunodeficiency (X-SCID) is an inherited immune disorder caused by pathogenic variants in the *IL2RG* gene, leading to recurrent infections. Identifying these variants and elucidating their pathogenic mechanisms are crucial for precise diagnosis and treatment, prenatal diagnosis, and preimplantation genetic testing (PGT). This study aimed to identify candidate variants in four families with suspected immunodeficiency, assess their pathogenicity, elucidate their pathogenic mechanisms, and provide a basis for precise treatment, prenatal diagnosis, and PGT.

Patients and Methods: Four families with suspected immunodeficiency were recruited from the Reproductive and Genetic Hospital of CITIC-Xiangya. Whole exome sequencing (WES) was used to identify the genetic etiology. Functional experiments were performed to assess the pathogenicity of the identified *IL2RG* variants, and to elucidate their pathogenic mechanisms.

Results: WES identified four *IL2RG* variants: three hemizygous (c.569G>C:p.R190P, c.515T>C:p.L172P, c.217A>C:p.T73P) and one heterozygous (c.1091C>T:p.T364I) variants. Three of these variants were novel. Initially three variants (p.R190P, p.T73P, and p.T364I) were classified as variants of uncertain significance (VUS) and one (p.L172P) was likely pathogenic (LP) according to ACMG/AMP guidelines. Functional analyses revealed reduced STAT5 phosphorylation and transcriptional activity across all variants, supporting the reclassification of three variants (p.R190P, p.L172P, and p.T73P) as likely pathogenic (LP), and one variant (p.T364I) as VUS with a Bayesian score of 5. Furthermore, IP-MS analysis revealed that the mutant IL2RG resulted in reduced cell-surface expression and abnormal nuclear localization. Therefore, the identified *IL2RG* variants impair IL-2-induced STAT5 phosphorylation and transcriptional activity to cause X-linked severe combined immunodeficiency in these families.

Conclusion: This study highlights the critical role of functional analysis in clarifying variant pathogenicity and provides a clear example of pathogenicity assessment for *IL2RG* variants. Integrating genomic and functional data enhance diagnostic precision and informs precise treatment strategies, genetic counseling, prenatal diagnosis, and PGT for X-SCID.

Keywords: IL2RG, X-linked severe combined immunodeficiency, IL2RG localization, STAT5 phosphorylation, transcriptional activity

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Introduction

Severe combined immunodeficiency (SCID) is an inherited immune disorder characterized by profound defects in both cellular and humoral immunity. X-SCID (OMIM300400) is the most common form of SCID.[1,2] Patients with X-SCID are highly susceptible to recurrent, severe infections in early infancy. Immunologically, X-SCID patients lack or have significantly reduced T and natural killer (NK) cells, as well as non-functional B cells (T^- B^+ NK^-).[3–5] This profound immunodeficiency impairs effective immune responses to infections, leading to severe morbidity or death if untreated.

X-SCID results from pathogenic *IL2RG* variants that impair γc chain-mediated signaling.[6] The γc chain is a critical component of multiple cytokine receptors, essential for the downstream signaling pathways that regulate immune cell functions. The *IL2RG* gene, located on human chromosome Xq13.1, encodes the γc chain, a transmembrane protein. When it binds to a γc family cytokine, the intracellular region initiates a signaling cascade. This cascade activates Janus kinases (Jak1 and Jak3), leading to the tyrosine phosphorylation of STAT (signal transducer and activator of transcription) proteins, particularly STAT5. This signaling pathway is crucial for the development, proliferation, activation, and differentiation of immune cells, including T cells, B cells, and NK cells.[7,8] Pathogenic variants in *IL2RG* impair γc chain-mediated cytokine signaling, resulting in X-SCID. Although the mechanism by which *IL2RG* gene defects cause X-SCID is well established, clinical heterogeneity remains due to different variants giving rise to diverse phenotypes.[5,9] Therefore, for a *IL2RG* variant of uncertain significance (VUS), further functional analyses are necessary to determine whether it affects IL2RG function and to elucidate the underlying mechanism of functional impairment.[10]

In this study, we identified three VUSs and one likely pathogenic (LP) in the *IL2RG* gene in four families with suspected X-SCID. Comprehensive in vitro functional analyses demonstrated that all four *IL2RG* variants impaired IL2RG-mediated STAT5 phosphorylation and transcriptional activity. Consequently, three variants (p.R190P, p.L172P, and p.T73P) were reclassified as LP, and one variant (p.T364I) was VUS with a Bayesian score of 5. Our study provides a theoretical basis for diagnosis and treatment, offering new insights for prenatal diagnosis and preimplantation genetic testing in affected families.

Materials and Methods

Patients

Four families were recruited from the Reproductive and Genetic Hospital of CITIC-Xiangya, a hospital jointly established in 2002 by China International Trust and Investment Corporation and Central South University. Patients from the first three families have received a preliminary diagnosis of X-SCID based on the following criteria: 1) absent or significantly reduced T cell numbers ($CD3^+$ T cells < 300/μL) and no or very low T cell function (< 10% of the lower limit of normal), as measured by their response to phytohemagglutinin (PHA); 2) reduced $CD3^+$ T cells for age (<1000/μL for <2 years, <800/μL for 2–4 years, <600/μL for >4years) and< 30% of the lower limit of normal T cell function (measured by PHA response); or 3) males with *IL2RG* hemizygous variants identified by molecular genetic testing.[11] In Family IV, the woman's younger female cousin had a male child with suspected immunodeficiency. The families of these patients hope to clarify the etiology of their children's conditions, and to have a healthy child in the future. This study was approved by the institutional ethics committees of the Reproductive and Genetic Hospital of CITIC-Xiangya and Central South University (LL-SC-2019-033). Written informed consent was obtained from all participants or their guardians.

Whole-Exome Sequencing (WES)

Genomic DNA was extracted from the peripheral blood of patients using the QIAamp DNA Blood Mini Kit (Qiagen, 51185, Hilden, Germany). WES was performed by BGI in Shenzhen, following the protocol described in our previous study.[12] Candidate variants were identified using the variant filtering strategy outlined in our previous study.[13] The pathogenicity of the variants was classified according to the American College of Medical Genetics and Genomics and the Association for Molecular Pathology (ACMG/AMP) guidelines, based on various sources of evidence, including population data, computational data, functional data, and segregation data.[14]

Plasmid Constructs

A polynucleotide fragment containing the entire coding region of human *IL2RG* (NM_000206.3) was amplified by PCR and ligated into the mammalian expression vector pCMV with a 3×Flag tag at the 5'end, forming the pCMV-3×Flag-WT-IL2RG construct. The identified variants were introduced into the pCMV-3×Flag-WT-IL2RG plasmid vector using the Mut Express II Fast Mutagenesis Kit V2 (Vazyme Biotech, C214, Nanjing, Jiangsu, China). The sequences of all wild-type and mutant constructs (c.569G>C, c.515T>C, c.217A>C and c.1091C>T) were confirmed by Sanger sequencing. Additionally, the pCMV-Myc-JAK3 plasmid was constructed using the same procedure.

Cell Culture, Transfection, and Surface Staining

HEK293 cells (ATCC, Manassas, VA, USA) were cultured at 37 °C with 5% CO_2 in cell culture flasks supplemented with SMM 293-TII (Sino Biological Inc, M293TII, Beijing, China). Cells were transiently transfected with either the pCMV-3×Flag-WT-IL2RG or the mutant plasmids using the Neofect™ DNA transfection reagent (Neofect, TF20121201, Beijing, China) when they reached 70–80% confluence. After 48 hours post-transfection, the surface expression of IL2RG (CD132) was assessed using a Flag tag antibody (Abways, AB0008, Shanghai, China) and Goat anti-Mouse IgG (H+L) Highly Cross-Adsorbed Secondary Antibody, Alexa Fluor™ 488 (Invitrogen, A-11029, Carlsbad, CA, USA). Briefly, antibodies were directly added to 100μL of HEK293 cells transfected with plasmids, resuspended in PBS, and pre-cooled to 4 °C. After a 30-minute incubation, the cells were analyzed by flow cytometry (Cytek Biosciences), with data processed using FlowJo software (version 10.8.1).

Western Blotting and Co-Immunoprecipitation Assay

To confirm the expression of the pCMV-3×Flag-WT-IL2RG and mutant plasmids, Western blotting was performed. Briefly, HEK293 cells were transiently transfected with either pCMV-3×Flag-WT-IL2RG or the mutant plasmids, and harvested 48 hours after transfection. Proteins extracted from the transfected cells were blotted onto a polyvinylidene difluoride (PVDF, Sigma-Aldrich, IPVH00010, St. Louis, MO, USA) membrane and incubated overnight at 4 °C with anti-DYKDDDDK Tag antibody (1:1000, Cell Signaling Technology, 8146, Danvers, MA, USA). The following day, the membranes were incubated with secondary antibodies (1:10000, Affinity Biosciences, S0001/S0002, Liyang, Jiangsu, China). Finally, the blots were developed using an Omni-ECL™ Femto Light Chemiluminescence Kit (Epizyme, SQ201, Shanghai, China).

To investigate the interaction between IL2RG and JAK3, we first predicted potential protein interactions using AlphaFold (https://alphafoldserver.com/). Subsequently, HEK293 cells were co-transfected with either pCMV-3×Flag-WT-IL2RG or mutant plasmids, along with the pCMV-Myc-JAK3 plasmid. At 48 hours after transfection, the cells were lysed with NP40 buffer (Beyotime Biotechnology, P0013F, Shanghai, China) containing phenylmethylsulfonyl fluoride (PMSF, Solarbio, P0100, Beijing, China) and a protein phosphatase inhibitor (Solarbio, P1260, Beijing, China). The cell lysates were incubated with Anti-DYKDDDDK beads (Biolinkedin, L-1011, Shanghai, China) or Anti-Myc beads (Biolinkedin, L-1010, Shanghai, China) for 4 hours at 4 °C. The beads were washed three times and subjected to Western blot analysis.

STAT5 Phosphorylation and Luciferase Assays

STAT5 phosphorylation was assessed in HEK293 cells co-transfected with Flag-tagged IL2RG (WT or mutant) plasmids and Myc-tagged JAK3 vectors, followed by 30 min of stimulation with Recombinant Human IL-2 (1000 U/mL, PeproTech, 200–02, Rock Hill, NJ, USA). IL-2-stimulated cells were harvested and lysed in RIPA buffer (Solarbio, R0020, Beijing, China) containing PMSF and a protein phosphatase inhibitor (Servicebio, G2007-1ML, Wuhan, Hubei, China). The lysates were centrifuged at 12,000 × g for 10 minutes at 4 °C, and the supernatants were used for immunoblot analysis, following a previously described protocol. Membranes were incubated overnight at 4 °C with primary antibodies against Flag-tag, Myc-Tag (1:1000, Abways Technology, AB0001, Shanghai, China), Stat5 (D2O6Y) Rabbit mAb (1:1000, Cell Signaling Technology, 94205, Danvers, MA, USA), Phospho-Stat5 (Tyr694) (D47E7) XP

Rabbit mAb (1:1000, Cell Signaling Technology, 4322, Danvers, MA, USA), and GAPDH (1:1000, Affinity Biosciences, T0004, Liyang, Jiangsu, China).

For STAT5 reporter assays, HEK293 cells were plated in 24-well plates and transiently co-transfected with IL2RG (WT or mutant) plasmids, Myc-tagged JAK3 vectors, and the pGL4.52 (*Luc2P*/STAT5RE/Hygro) reporter plasmid (Promega, E4651, Madison, WI, USA). After 36 hours, IL-2 (1000 U/mL) was added to the cultures and incubated for 40 min. The cells were then lysed, and luciferase activity was measured using the ONE-Glo™ Luciferase Assay System (Promega, E6110, Madison, WI, USA), according to the manufacturer's recommendations.

Immunoprecipitation-Mass Spectrometry

HEK293 cells were transfected with either pCMV-3×Flag-WT-IL2RG or mutant plasmids, along with the pCMV-Myc-JAK3 plasmid, and cultured for 48 hours. The cells were then lysed with NP40 buffer containing PMSF and protein phosphatase inhibitor. Co-immunoprecipitation (Co-IP) was performed as described in the "Western Blotting and Co-Immunoprecipitation Assay" section, with the protein samples resuspended in PBS without denaturation in the final step. For MS analysis of the Co-IP eluents, the beads binding the target proteins were washed three times with 50 mM Triethylammonium bicarbonate buffer (Sigma-Aldrich, T7408, St. Louis, MO, USA) and then digested with 0.05 μg/μL trypsin (Promega, V5280, Madison, WI, USA) at 37 °C for 4 hours with shaking at 400 rpm. The supernatant was transferred to a fresh tube for overnight trypsin digestion at 37 °C. After adding 1 μL of formic acid, the digested peptides were analyzed using an Orbitrap Eclipse Tribrid Mass Spectrometer (Thermo Fisher), as described in our recent study.[15] Proteome Discoverer Software (version 2.5, San Jose, CA, USA) was used to process raw files for feature detection, database searching, and protein/peptide quantification. The MS/MS spectra were searched against the UniProt human database (June 27, 2022; 79,435 entries). Missing values were imputed using DEP (version 1.28.0, R package).[16] Differential protein expression between groups was analyzed by limma package (version 3.62.2, R),[17] and significantly differential proteins were identified based on criteria of $p \leq 0.05$ and log2FoldChange > 1.3.

Statistical Analysis

Statistical analyses were performed using Student's *t*-test and one-way analysis of variance (ANOVA) with SPSS software (version 20.0, Chicago, IL, USA).

Results

Case Presentation

The patient from Family I (II-1), a four-month-old boy (Figure 1A), presented with unexplained high fever and swelling in both lower limbs, which progressed to severe generalized edema, skin redness, chills, and bloody stools. Pathogenetic tests revealed positive nucleic acid results for adenoviruses and *Streptococcus pneumoniae*. Despite anti-infective and symptomatic treatment, recurrent high fever persisted. Blood tests revealed significantly elevated levels of inflammatory markers (C-reactive protein [CRP], serum amyloid A [SAA], and IL-6) and persistently low platelet and lymphocyte counts (Figure S1A–E). Lymphocyte subset analysis revealed decreased levels of $CD3^+$ T cells (3.82%), $CD3^+$ $CD4^+$ T cells (2.32%), $CD3^-$ $CD16^+$ $CD56^+$ NK cells (6.14%), and $CD3^+$ $CD8^+$ T cells (0.36%), while $CD4^+/CD8^+$ T cell ratios (6.38) and $CD3^-$ $CD19^+$ B cells (86.86%) were increased (Table 1). The patient was suspected of having primary immunodeficiency and is currently undergoing hematopoietic stem cell transplantation.

The patient from Family II (II-1) received pentavalent and rotavirus vaccines for 2.5 months of age (Figure 1A). At 3 months of age, the child developed a runny nose and fever with chills, and was hospitalized with elevated infection markers, diarrhea, red rash, cicatricial macules on the back, and yellow crusted exudate. Despite anti-infective treatment, recurrent fever persisted. Blood tests showed a normal white blood cell count (13,440 cells/μL) but a decreased lymphocyte count (1310 cells/μL), elevated neutrophils (10,040 cells/μL), and monocytes (2050 cells/μL), along with increased levels of inflammatory markers (CRP, SAA, and IL-6) (Figure S1F–H). Lymphocyte subset analysis revealed reduced $CD3^+$ T cells (46.38%), $CD3^+$ $CD4^+$ T cells (8.33%), $CD3^-$ $CD16^+$ $CD56^+$ NK cells (2.33%), and a low $CD4^+/CD8^+$ ratio (0.24), while $CD3^+$ $CD8^+$ T cells (34.42%) and $CD3^-$ $CD19^+$ B cells (51.16%) were elevated (Table 1). By 5

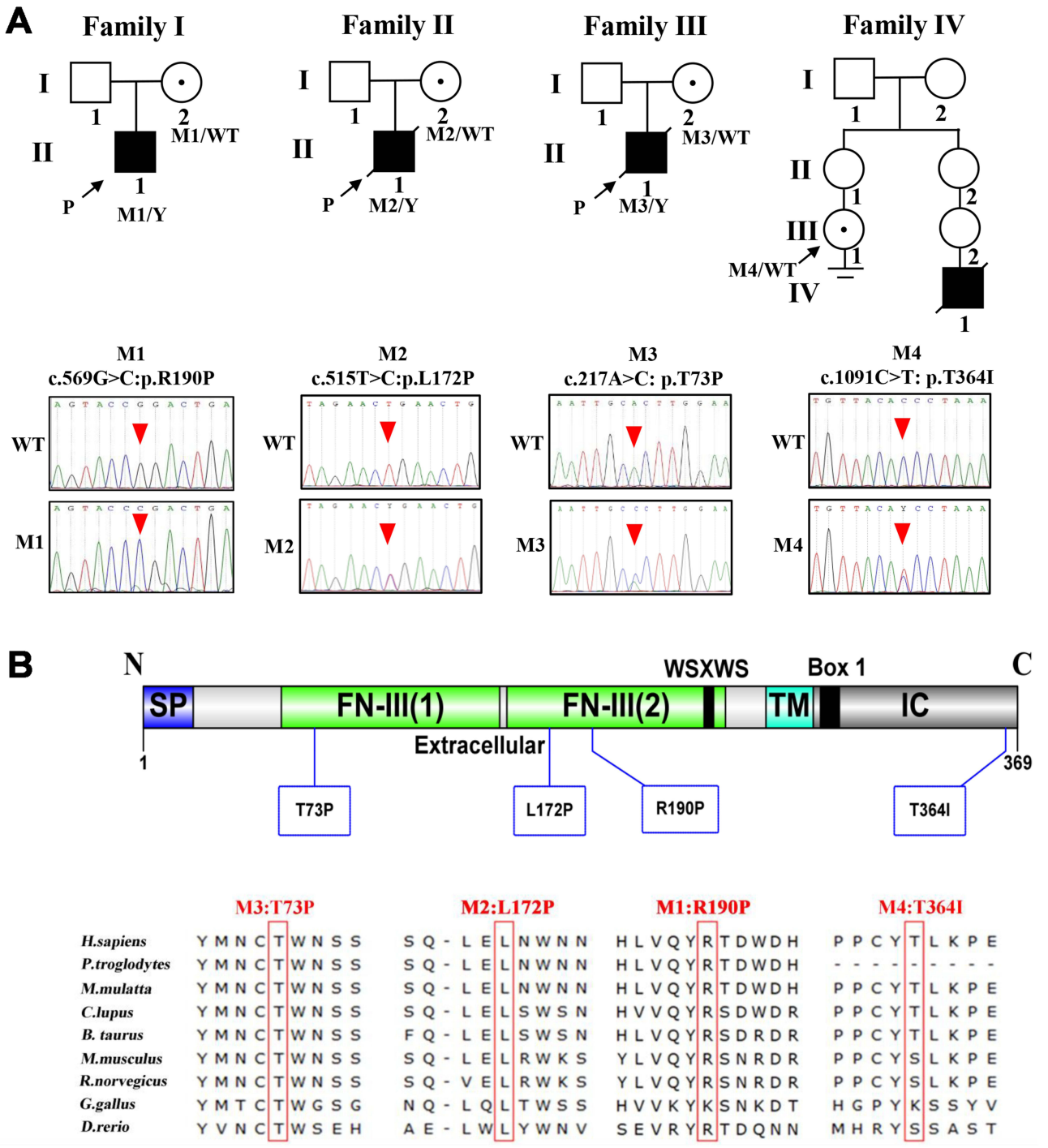

Figure 1 Family pedigree and conservation analysis of *IL2RG* variant sites. (**A**) Pedigrees of the four X-SCID families. The Sanger sequencing chromatogram of Family I from the proband (II-1), Families II and III from the proband's mother (I-2), and Family IV from the proband's aunt (III-1). Arrows indicate probands, black symbols indicate patients, white symbols represent unaffected individuals, and black spots indicate carriers. (**B**) Conservation analysis of *IL2RG* variants. Human IL2RG encodes a 369-amino acid protein. The protein contains two extracellular FN-III structural domains, FN-III (1) and FN-III (2), that facilitate its binding to cytokines. Cross-species alignment revealed that the aspartic acid residues at positions 73 and 172 in IL2RG are highly conserved across species, and residues at positions 190 and 364 are relatively conserved.

months of age, the child exhibited hepatomegaly, hyperlipidemia, hypofibrinogenemia, and a progressive decline in hemoglobin and platelet levels. Despite symptomatic treatment, the patient's condition worsened, and he tragically died at 6 months and 17 days.

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Table 1 Immunologic Characteristics of the Patients

Parameters	II-1 from Family I	II-1 from Family II	Normal Range
White blood cells (cells/μL)	3150 ↓	13440	4800–14600
Lymphocytes (cells/μL)	198 ↓	1310 ↓	2400–9500
Neutrophils (cells/μL)	2860	10040↑	600–7500
Monocytes (cells/μL)	90 ↓	2050↑	150–1560
Red blood cells ($\times 10^6$ cells/μL)	3.24 ↓	3.7	3.3–5.2
Hemoglobin (g/dL)	9.2 ↓	9.7	9.7–18.3
Platelet ($\times 10^3$ cells/μL)	54 ↓	702↑	100–300
IgG (mg/dL)	161 ↓	2426↑	200–550
IgA (mg/dL)	26	8	5–34
IgM (mg/dL)	17	45	17–66
IgE (IU/mL)	5	–	<15
$CD3^+$ T cells %	3.82 ↓	46.38 ↓	60.8–75.4
$CD3^+$ $CD4^+$ T cells %	2.32 ↓	8.33 ↓	29.4–45.8
$CD3^+$ $CD8^+$ T cells %	0.36 ↓	34.42↑	18.2–32.8
$CD4^+/CD8^+$ Ratio	6.38↑	0.24 ↓	0.9–3.6
$CD3^-$ $CD19^+$ B cells %	86.86↑	51.16↑	6–25
$CD3^-$ $CD16^+$ $CD56^+$ NK cells %	6.14	2.33 ↓	5–20
$CD3^-$ $CD16^+$ $CD56^+$ NK cells (cells/μL)	28 ↓	–	290–780

In Family III, a male child with a suspected immunodeficiency died at approximately 10 months of age (Figure 1A). This prompted his parents to undergo genetic testing to identify potential genetic variants associated with immunodeficiency.

In Family IV, a woman's younger female cousin had a male child with suspected immunodeficiency who died at the age of 8 months (Figure 1A). The woman requested genetic testing to identify potential genetic variants associated with immunodeficiency.

Genetic Analysis

WES and segregation analyses identified three hemizygous variants in the *IL2RG* gene in the patients from Families I, II, and III, respectively: c.569G>C:p.R190P, c.515T>C:p.L172P, c.217A>C:p.T73P. The mothers of these patients were confirmed to be carriers of the respective variants. In Family IV, a heterozygous *IL2RG* variant (c.1091C>T:p.T364I) was identified in the patient's aunt (III-1) (Figure 1A). Three of these variants (p.R190P, p.T73P, and p.T364I) are novel. All four missense variants occurred at evolutionarily conserved sites across the species. The p.L172P and p.R190P variants are located in the fibronectin type III (2) domain of IL2RG. The other two variants (p.T73P and p.T364I) are located in the fibronectin type III (1) domain and cytoplasmic tail region of IL2RG, respectively (Figure 1B). Bioinformatics tools, including PolyPhen-2, CADD, and Mutation Taster, predicted three of these variants to be pathogenic and one (p.T364I) to be benign (Table S1). However, according to the ACMG/AMP standards and guidelines for interpreting sequence variants, three variants (p.R190P, p.T73P, and p.T364I) were classified as variants of uncertain significance (VUS) and one (p.L172P) was likely pathogenic (LP).

IL2RG Variants Reduce IL-2-Induced Phosphorylation and Transcriptional Activity of STAT5

IL2RG receptor-mediated signaling depends on JAK3 binding to the intracellular structural domain of IL2RG. To assess the impact of *IL2RG* variants on this interaction, a series of in vitro functional assays was performed using five plasmid constructs (WT, p.R190P, p.L172P, p.T73P, and p.T364I) (Figure 2A). AlphaFold predicted that the four mutant IL2RG proteins interact with JAK3 (Figure S2). Co-IP analysis showed significantly reduced binding between JAK3 and the mutant IL2RG proteins (p.R190P, p.L172P, and p.T364I) while the T73P mutant protein exhibited only a modest reduction in binding to JAK3 compared to wild-type IL2RG (Figure 2B). Next, the DynaMut2 tool was used to predict

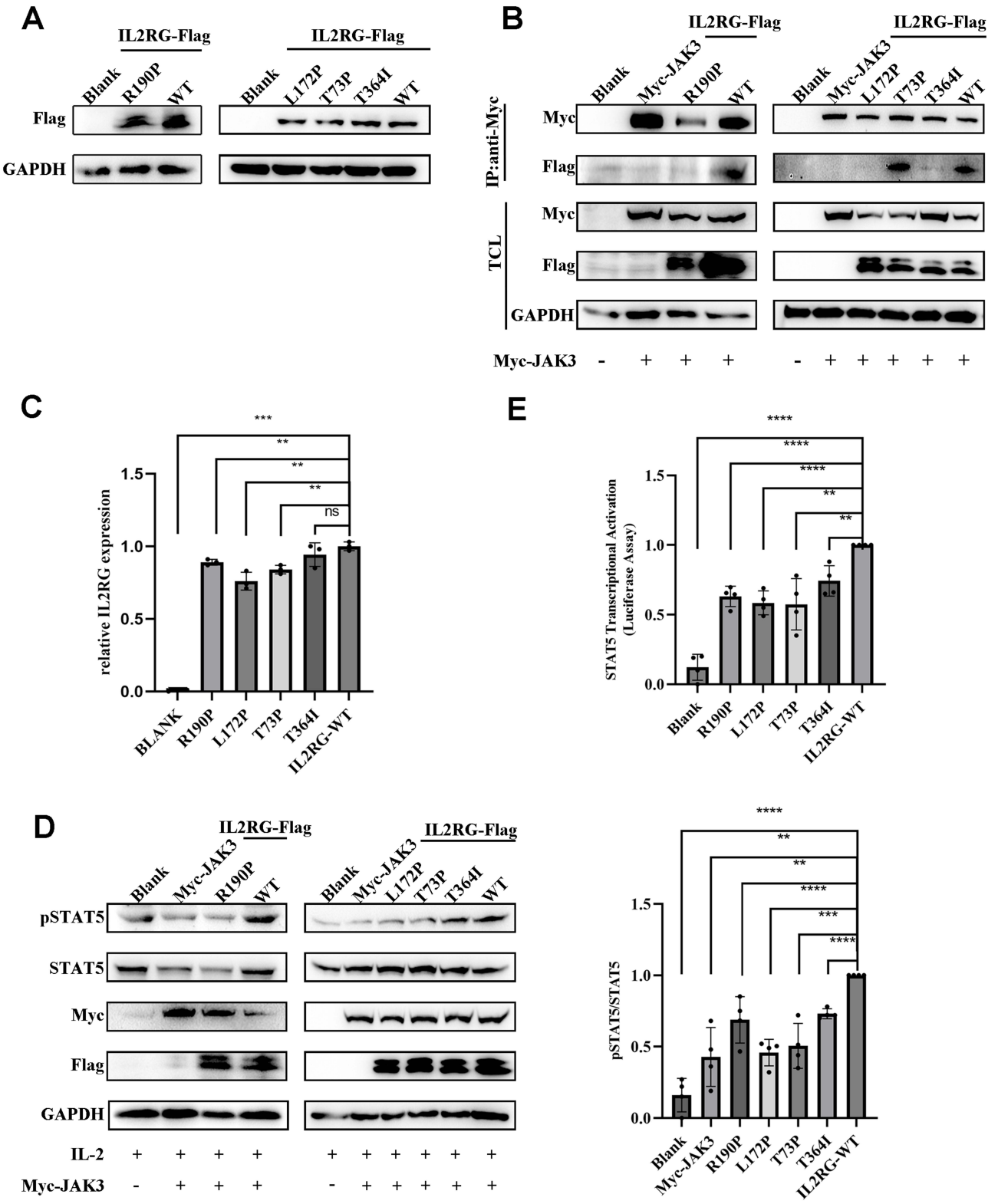

Figure 2 Reduced IL2RG expression on the cell surface affects IL-2-mediated signal transduction. (**A**) IL2RG mutant protein expression in HEK293 cells, as determined by Western blotting. (**B**) Co-IP analysis showed significantly reduced binding between JAK3 and the three IL2RG mutant proteins (p.R190P, p.L172P, and p.T364I), while the p.T73P mutant protein exhibited partial interaction with JAK3. (**C**) Flow cytometry analysis demonstrated that the surface expression of mutant IL2RG proteins was significantly reduced in HEK293 cells compared to that of wild-type IL2RG. (**D**) After stimulation with 1000 U/mL IL-2, STAT5 phosphorylation was substantially decreased in cells expressing mutant IL2RG proteins compared to wild-type IL2RG. (**E**) Luciferase reporter assays showed a significant reduction in STAT5 transcriptional activity in cells expressing the mutant IL2RG proteins compared to those expressing wild-type IL2RG. Cells in the blank control group were untransfected with any plasmid. Data are representative of three or four independent experiments, results are represented as mean ± SD, and statistical analyses were carried out via *t* test. (**p<0.01; ***p<0.001; ****p<0.0001).

Abbreviation: ns, non-significant.

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the stability of mutant IL2RG,[18] indicating that variants p.L172P, p.T73P, and p.T364I lead to protein instability, with p.L172P being the most unstable (Figure S3). Flow cytometry analysis of HEK293 cells expressing IL2RG showed that three mutant IL2RG proteins (p.R190P, p.L172P, and p.T73P) had significantly reduced surface expression compared to wild-type IL2RG (Figure 2C). To investigate the effect of these variants on IL-2R signaling, STAT5 phosphorylation was analyzed after IL-2 stimulation (1000 U/mL). All four IL2RG mutants exhibited a substantial reduction in STAT5 phosphorylation compared to wild-type IL2RG (Figure 2D). A luciferase reporter assay further confirmed the reduced STAT5 transcriptional activity in cells expressing mutant IL2RG (Figure 2E).

Together, these findings demonstrate that all four *IL2RG* variants significantly impair IL2RG signaling by reducing JAK3 interactions, protein stability, surface expression, and downstream STAT5 phosphorylation and transcriptional activity. According to the ACMG/AMP standards and guidelines for interpreting sequence variants, three variants (p.R190P, p.L172P, and p.T73P) were reclassified as LP, and one variant (p.T364I) as VUS with a Bayesian score of 5 (Table 2).

IL2RG Variant Disturbs Its Plasma Membrane Targeting

To investigate how *IL2RG* variants affect protein function, we analyzed the localization of mutant IL2RG proteins using IP-MS. Differentially expressed proteins, defined as those with an absolute log2FoldChange > 1.3, were classified based on their functional localization. The analysis revealed that the four mutant IL2RG proteins showed increased interactions with nuclear proteins and decreased interactions with plasma membrane proteins (Figure 3). Gene ontology (GO) analysis revealed that these differentially expressed proteins were primarily involved in biological processes such as chromatin reorganization, ribosome processing, histone methylation, and rRNA metabolism (Figure 3). These findings suggest that the four mutant IL2RG proteins exhibited enhanced interactions with nuclear proteins and reduced interactions with plasma membrane proteins. This disruption could impact the function of IL-2 receptor common chain by disrupting plasma membrane targeting and potentially resulting in aberrant nuclear localization.

Discussion

In this study, we identified four *IL2RG* variants in four families suspected of having X-SCID. Through a series of in vitro functional assays, including Western blotting, immunoprecipitation (Co-IP), luciferase reporter assays, flow cytometry, and IP-MS, we demonstrated that all four *IL2RG* variants significantly impair IL2RG-mediated signaling, leading to reduced JAK3 interactions, surface expression and downstream STAT5 phosphorylation and transcriptional activity, due to disrupting plasma membrane targeting and aberrant nuclear localization. These findings provide strong evidence that the identified *IL2RG* variants are contribute to the development of X-SCID.

Table 2 Classification of IL2RG Variants According to ACMG Guidelines

Family	Individual	Gene (Mode of Inheritance)	Variants (Reference Transcript Number)	Exon	Protein Changes	Original Classification	Reclassification
I	II-1	*IL2RG* (XLR)	c.569G>C (NM_000206.3)	4	p.R190P	VUS (PM2+PP3+PP4)	LP (PM2+PP3+PP4+PS3)
II	II-1	*IL2RG* (XLR)	c.515T>C (NM_000206.3)	4	p.L172P	LP (PM5+PM2+PP3+PP4)	LP (PM5+PM2+PP3+PP4+PS3)
III	II-1	*IL2RG* (XLR)	c.217A>C (NM_000206.3)	2	p.T73P	VUS (PM2+PP3+PP4)	LP (PM2+PP3+PP4+PS3)
IV	III-1	*IL2RG* (XLR)	c.1091C>T (NM_000206.3)	8	p.T364I	VUS (PM2+BP4)	VUS* (PM2+BP4+PS3)

Notes: PM, PP, PS, the type and strength of the of evidence in the American College of Medical Genetics and Genomics (ACMG) standards and guidelines for the interpretation of variations. *VUS with a Bayesian score of 5.

Abbreviations: XLR, X-linked recessive inheritance; VUS, variant of uncertain significance; LP, Likely pathogenic.

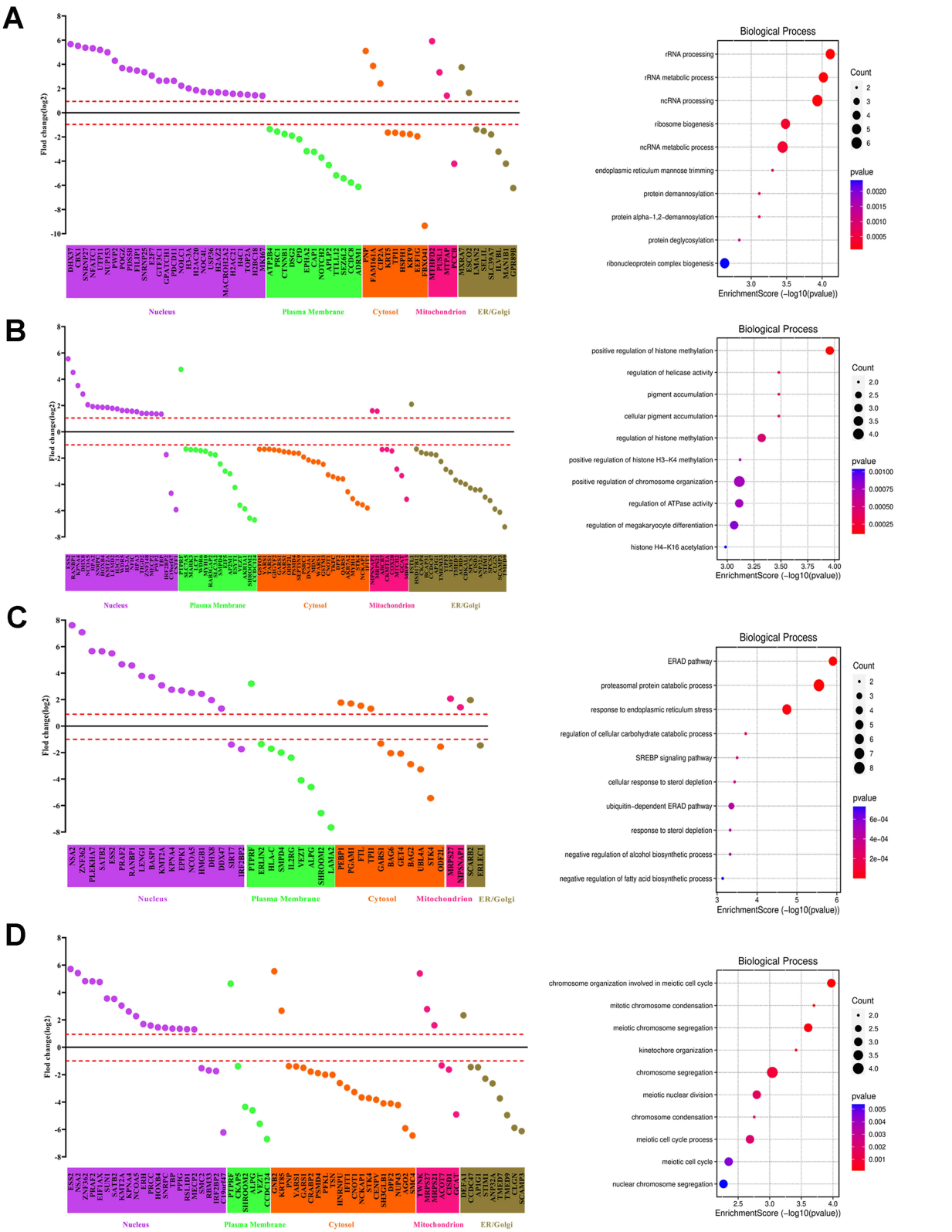

Figure 3 IL2RG mutants display increased interactions with nuclear proteins. (**A**–**D**) GO analysis of WT and four mutant IL2RG proteins. The left panels show that all four IL2RG mutant proteins exhibited enhanced interactions with nuclear proteins and reduced interactions with plasma membrane proteins compared to wild-type IL2RG. The red dashed line indicates a more than 1.3-fold change in the abundance of interacting proteins in the mutant proteins compared to wild-type IL2RG. The right panels show the GO analysis of these differentially expressed proteins related to biological processes. The size of the dot represents the protein count, and the color of each dot corresponds to the *p*-value.

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The *IL2RG* gene encodes the γc chain, a critical subunit for multiple cytokine receptors. Deficiency in *IL2RG* disrupts the downstream JAK-STAT signaling pathway, compromising both humoral and cellular immunity and ultimately resulting in X-SCID.[19,20] Although the Clinvar database and HGMD have documented over 400 *IL2RG* variants, including 64 patients from China,[2,5,21–33] (Table S2), not all have been validated as pathogenic through in vitro functional studies and the causal relationship between the variants and the patient's condition has yet to be clarified. Our study identified four *IL2RG* variants, initially classified as VUS or LP, three of which were novel. Further functional analysis confirmed that these variants were LP or VUS with a Bayesian score of 5, demonstrating they impaired γc chain function and serve as the etiological cause of X-SCID in the studied families.

AlphaFold and Co-IP are crucial tools for investigating protein interactions, and are widely used to elucidate the pathogenicity of variants by investigating the interactions between mutant proteins and their interacting partners, such as variants in *IL2RG*[34] In this study, AlphaFold and Co-IP results revealed that the T73P mutant IL2RG proteins exhibited a strong interaction with JAK3, initially suggesting that this variant might be benign. However, further investigation using luciferase reporter assays and phosphorylation analyses demonstrated that the mutant protein (T73P) significantly decreased downstream STAT5 phosphorylation and transcriptional activity. Additionally, it also disrupted plasma membrane targeting. Finally, the T73P variant was reclassified as LP. These findings highlight the importance of using multiple methods to assess variant pathogenicity, rather than relying solely on Co-IP.

Signal transducer and activator of transcription (STAT)-5 proteins play a crucial role in immune regulation, with two homologs in mammals: *STAT5A* and *STAT5B*. STAT5B is predominantly expressed in hematopoietic cells,[35–37] where it mediates three sequential tyrosine phosphorylations triggered by cytokine stimulation via the JAK-STAT pathway. This process is critical for lymphocyte development and differentiation, and its deficiency is associated with typical X-SCID.[36,38] In patients with atypical X-SCID, low concentrations of IL-2 significantly reduced STAT5 phosphorylation in T lymphocytes compared to healthy controls; however, phosphorylation levels were similar in both groups under high IL-2 stimulation.[39] Our study demonstrated that STAT5 phosphorylation in cells transfected with mutant IL2RG plasmids was significantly decreased, even under high IL-2 stimulation, indicating severe impairment in IL-2 signaling. Additionally, luciferase reporter assay assessing STAT5 transcriptional activity showed a significant reduction in cells transfected with mutant IL2RG plasmids. IP-MS revealed that these mutant IL2RG proteins disrupted plasma membrane targeting. Based on these findings, we concluded that the identified variants resulted in decreased STAT5 phosphorylation and transcriptional activity, due to disrupting plasma membrane targeting, aberrant nuclear localization and severely disrupting cytokine signaling.

Prenatal diagnosis and preimplantation genetic testing (PGT) play a crucial role in preventing the birth of children affected by X-SCID. X-SCID is the most common form of SCID, accounting for approximately 50% of all SCID cases.[2] Without HSCT or gene therapy, severe infections often lead to death within the first year.[40] X-SCID is inherited in an X-linked pattern, meaning that women who are heterozygous carriers of the causative *IL2RG* variant have a 25% risk of having an affected son during each pregnancy.[41] There have been reports of the successful birth of more than four healthy children following prenatal diagnosis and PGT.[40,42,43] Our study offers a valuable theoretical basis for conducting prenatal diagnosis and PGT in the four X-SCID families, while also serving as a reference for other families carrying *IL2RG* variants.

Conclusion

In conclusion, this study successfully identified four *IL2RG* variants in four families with X-SCID using WES, three of which were novel. Functional analyses revealed that these variants caused reduced STAT5 phosphorylation and transcriptional activity-key processes for maintaining immune function, due to decreased cell-surface expression and nuclear mislocalization of the mutant IL2RG protein. These findings not only highlight the critical role of comprehensive functional studies in reclassifying variants of uncertain significance (VUS) as likely pathogenic (LP) but also deepen our understanding of the pathogenic mechanisms underlying X-SCID. This work underscores the importance of integrating genetic and functional analyses to enhance precision in diagnosis and treatment, improve genetic counseling, and guide prenatal diagnosis and PGT, thereby potentially improving clinical outcomes for patients with X-SCID and their families.

Abbreviations

X-SCID, X-linked severe combined immunodeficiency; WES, whole exome sequencing; ACMG, American College of Medical Genetics and Genomics; AMP, Association for Molecular Pathology; LP, likely pathogenic; VUS, variant of uncertain significance; NK, natural killer; γc/CD132, common cytokine receptor γ chain; STAT, signal transducer and activator of transcription; Co-IP, co-immunoprecipitation; IP-MS, immunoprecipitation-mass spectrometry; CRP, C-reactive protein; SAA, serum amyloid A; HGMD, Human Gene Mutation Database; PGT, preimplantation genetic testing.

Data Sharing Statement

The sequencing data are available from the corresponding authors upon reasonable request, and the other data is provided within the manuscript or supplementary information files.

Editorial Policy and Ethical Considerations

This study was approved by the institutional ethics committee of Reproductive and Genetic Hospital of CITIC-Xiangya (LL-SC-2019-033) and adhered to the Declaration of Helsinki.

Consent for Publication

The authors obtained written informed consent for genetic testing and anonymous publication of results from the patients or affected patients' legal guardians following applicable local laws.

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Disclosure

The authors report no conflicts of interest in this work.

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