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RESEARCH ARTICLE

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The variations in human orphan G protein-coupled receptor QRFPR affect PI3K-AKT-mTOR signaling

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Abstract

Background: *QRFPR* is a recently identified member of the G protein-coupled receptor and is an orphan receptor for 26Rfa, which plays important role in the regulation of many physiological functions.

Methods: Here, we employed whole exome sequencing (WES) to examine the patients with intellectual disability (ID) and difficulty in feeding. We performed SIFT and PolyPhen2 predictions for the variants. The structure model was built from scratch by I-TASSER. Here, results derived from a number of cell-based functional assays, including shRNA experiment, intracellular Ca^{2+} measurement, the expression of PI3 K-AKT-mTOR, and phosphorylation. The functional effect of *QRFPR* variants on PI3K-AKT-mTOR signaling was evaluated in vitro transfection experiments.

Result: Here, we identified two *QRFPR* variants at c.202 T>C (p.Y68H) and c.1111C>T (p.R371W) in 2 unrelated individuals. Structural analysis revealed that p.Y68H and p.R371W variants may affect the side chain structure of adjacent amino acids causing reduced binding of QRFPR to 26Rfa. The results show that QRFPR stimulated by 26Rfa leading to the transient rise of intracellular Ca^{2+}. The *QRFPR* variations p.Y68H and p.R371 W can reduce the mobilization of intracellular Ca^{2+}. The phosphorylation levels of the PI3K, Akt, and mTOR were significantly up- or downregulated by QRFPR overexpression or silencing, respectively. The *QRFPR* variations inhibited PI3K-AKT-mTOR signaling, resulting in downregulation of p-mTOR.

Conclusions: Our findings suggest that QRFPR acts as important role in neurodevelopment, and the effects of *QRFPR* are likely to be mediated by the Ca^{2+}-dependent PI3K-AKT-mTOR pathways. Importantly, these findings provide a foundation for future elucidation of GPCR-mediated signaling and the physiological implications.

KEYWORDS
Ca^{2+}, G protein-coupled receptor (GPCR), neurodevelopment, PI3K-AKT-mTOR signaling, QRFPR

Huanzheng Li and Ran Lou contributed equally to this paper.

1 | INTRODUCTION

QRPFR is a member of the G protein-coupled receptor (GPCR) family and is a specific receptor for the neuropeptide 26Rfa. So far, QRFPR is the largest cell membrane receptor family, with seven transmembrane helices and rings linking transmembrane regions, including three intracellular rings and three extracellular rings. QRFPR can activate intracellular signal transduction and cell response through extracellular hormones, neurotransmitters, and various sensory stimuli, which can lead to a series of changes in the cell, including regulating human chemotaxis, nerve transmission, cell communication, vision, taste, smell, and other important physiological processes.[1]

In this study, we have identified two naturally occurred variants of QRFPR, a heterozygous T→C transversion at c.202 resulting in a Y68H missense substitution and a homozygous C→T substitution at c.1111 leading to a R371W variant, in 2 unrelated individuals with intellectual disability (ID) and difficulty in feeding. QRPFR, which is highly expressed in the brain, particularly in the hypothalamus and extra-hypothalamic regions, has been deorphanized as the cognate receptor for the neuropeptide 26RFa.[2] 26Rfa is predominantly present in the nervous system and is distributed throughout the body, thus playing various roles as neurohormones and neurotransmitters. 26Rfa also contributes to the regulation of various physiological functions at various stages of development.[3] Moreover, 26RFA-deficient mice showed reduced in food intake, body weight as well as wakefulness time during the dark period, and increase in anxiety-like behavior.[4] Moreover, recent studies provide evidence that 26RFa and its receptor QRFPR play important roles in promoting sleep, regulating bone formation and modulating nociceptive transmission.[5–7] However, characterization of QRFPR-mediated signaling and its associated physiological functions are still to be assessed.

The primary expression of both 26RFa and its receptor QRFPR in hypothalamic nuclei suggests that 26RFa and QRFPR play important role in the control of feeding behavior.[2] 26RFa induces adrenal steroidogenesis by regulating key steroidogenic enzymes involving Ca^{2+} signaling pathways, and QRFPR has also been found to activate CREB in ERK1/2 signaling pathways. It suggested that QRFPR is a pivotal factor in the regulation of a wide range of biological activities.[8] Previous studies have shown that GPCRs can uncouple from G proteins and form complexes with downstream signaling molecules to regulate phosphatidylinositol-4, 5-bisphosphate 3-kinase (PI3K), mitogen-activated protein (MAP) kinase, and mTOR signaling pathways.[9–11] mTOR can be activated by the upstream PI3K-AKT pathway to regulate neuroprotein synthesis, and the PI3K-AKT-mTOR signaling pathway has been revealed to regulate normal cell growth, metabolism, and survival. mTOR plays a vital role in neurodevelopment and regulation of energy balance.[12,13] As the last specific receptor for the neuropeptide 26Rfa to be identified, little is known about the structure, function, and signal transduction mechanism of QRFPR. Therefore, given the important role of QRFPR and mTOR in regulation of neurodevelopment, we simulated the QRFPR structure, analyzed the effect of *QRFPR* variations on protein structure, and explored the role of *QRFPR* in the PI3K-AKT-mTOR signaling pathway. This is the first time that we report the role of *QRFPR* in PI3K-AKT-mTOR signaling pathway, which reveals underlying mechanism of QRFPR in the neural activity.

2 | MATERIALS AND METHODS

2.1 | Case presentation

We investigated a patient diagnosed with ID and difficulty in feeding in a Chinese family from Zhejiang Province. The patient was an 11-year-old boy with mentally retarded and difficulty in feeding, who was diagnosed at age 7. Other clinical features include cerebral palsy, delayed motor, and poor weight gaining. He has problem with speaking, swallowing and eating, and daily onset of nodding sign (Table 1, Figure 1A). Clinical information was collected through medical records or completed by a genetic counseling physician, and written consent was obtained from the subject' parents in accordance with a protocol approved by the ethics committee of Wenzhou Central Hospital (L2020-03-004 by the ethics committee of Wenzhou Central Hospital).

2.2 | Whole exome sequencing (WES) Analysis

WES was performed in this family. Following SNP detection by samtools, GATK, and other software, reliable SNP loci were filtered and selected. SNP variant loci were evaluated with Annovar software, and variant frequency analysis was performed with reference to dbSNP, Thousand Genomes, ExAC, and gnomAD databases to identify potential disease-associated causative variants. The genes known to be implicated in ID were intensively examined. A heterozygous variant c.202T>C (p.Y68H) and homozygous variant c.1111C>T (p.R371W) were identified in the *QRFPR* gene in the patient 1 (Figure 1B). In addition, we screened the *QRFPR* variations in other 127 ID child, whose WES tests were negative.

2.3 | Structural calculations for QRFPR variants

We performed SIFT and PolyPhen2 predictions for the variants. The structure model was built from scratch by I-TASSER. Both 2 ks9.pdb and 5zbq.pdb template were integrated into the model. The structure of the QRFPR was schematically plotted by PyMol, and the variant structure was optimized in Gromacs to obtain RMSD values. Rosetta's Relax module was used to optimize the ligand 26Rfa and QRPFR structures, and RosettaDock was used to dock 26Rfa to QRPFR 10,000 times, using the model with the lowest score among them as the docking result. The docking results were subjected to structural observation by PyMol, and the ligand and receptor-bound hydrogen bonds were calculated.

TABLE 1 Clinical and genetic findings in two patients

Subject	Patient no.1	Patient no.2
Genetic variation	*QRFPR*, heterozygous variant c.202T>C (p.Y68H) and homozygous variant c.1111C>T (p.R371W)	*QRFPR*, compound heterozygous variants c.202T>C (p.Y68H) and homozygous variant c.1111C>T (p.R371W)
Inheritance	Heterozygous variant c.202 T>C (p.Y68H) inherited from mother, homozygous variant c.1111C>T (p.R371W) inherited from parents	Heterozygous variant c.202T>C (p.Y68H) inherited from father, heterozygous variant c.1111C>T (p.R371W) inherited from mother
Age at diagnosis	Male, 7 Years	Male,4 Years
DD/ID	Severe ID	Severe ID
Language	Delay	Extremely severe
Social communication	Poor	Poor
Walking ability	Uncoordinated	Delay
Motor development	Delay	Delay
Autistic behaviors	+	+
Motor skills	Delay	Delay
Ophthalmic Diseases	Normal	Hypertelorism
Hypomyotonia	-	+
Seizures	+	-
Dysmorphic facial	-	+
Macrocephaly	-	-
Microcephalus	+	+
Feeding	Difficulty	Difficulty

2.4 | Plasmids and antibodies

pLVX-Puro-Myc plasmid was purchased from PPL (Public Protein/Plasmid Librar). pLVX-Puro-Myc-QRFPR expression vector, QRFPR variations (p.Y68H and p.R371W) vectors, and shRNA plasmids (pLVX-shRNA-Neo) were all synthesized and bought from PPL. The sequences 21 bp long for shRNAs targeting QRFPR mRNA were designed based on the sequence of GenBank™ entry NM_198179.3, as follows: GCTTCGAACTATTCATGGAAA.

The following antibodies were used in biochemical experiments: anti-QRFPR (Proteintech, 25247-1-AP, 1:1000), anti-Phospho-mTOR (Cell Signaling Technology, 2971S, 1:1000), anti-mTOR (Cell Signaling Technology, 2983S, 1:1000), anti-Phospho-PI3K p85 (Cell Signaling Technology, #17366, 1:1000), anti-PI3 Kinase p110α (Cell Signaling Technology, #4255, 1:1000), anti-Phospho-AKT (Cell Signaling Technology, #4060, 1:1000), anti-AKT (Cell Signaling Technology, #4691, 1:1000), and anti-β-Actin (Cell Signaling Technology, 5174S, 1:1000). All antibodies were diluted in PBS containing 5% semi-skimmed milk and 0.1% Tween-20.

2.5 | Cell Culture and transfection

Human embryonic kidney cell line (HEK293) was cultured in DMEM containing 10% fetal bovine serum, 100 IU/ml penicillin, and 100 μg/ml streptomycin. HEK293 cells were grown in 6-well plates and were incubated in a saturated humidity incubator at 37°C with 5% CO_2. The QRFPR expression plasmid, mutant plasmid, and shRNA plasmid were transfected into HEK293 cells using Lipofectamine 3,000 according to the instructions.

2.6 | Measurement of intracellular calcium ion concentration

Fluo-3-Am, a fluorescent indicator of calcium ion (Invitrogen, F1242), was used to determine the intracellular calcium concentration. Transiently transfected HEK293 cells were collected, washed once by PBS, and then resuspended by PBS; Fluo-3-Am (10 μmol/L) was added to the cells, and incubation was continued for 1 h in a CO_2 incubator with slight shaking 3 times during the incubation; cells were collected by centrifugation, washed 3 times by calcium-free PBS, and then resuspended by calcium-free PBS; finally, the calcium ion concentration was calculated from the fluorescence intensity of the cells, which was excited at an excitation wavelength of 488 nm for 60 s in a flow cytometer.

2.7 | Immunoassay

Immunoblot analysis: cells with shRNA, wild-type or mutant expression plasmids were transfected in 6-well plates, and after 24 hours, cells were lysed. Lysates were analyzed by immunoblotting according to a standard protocol.

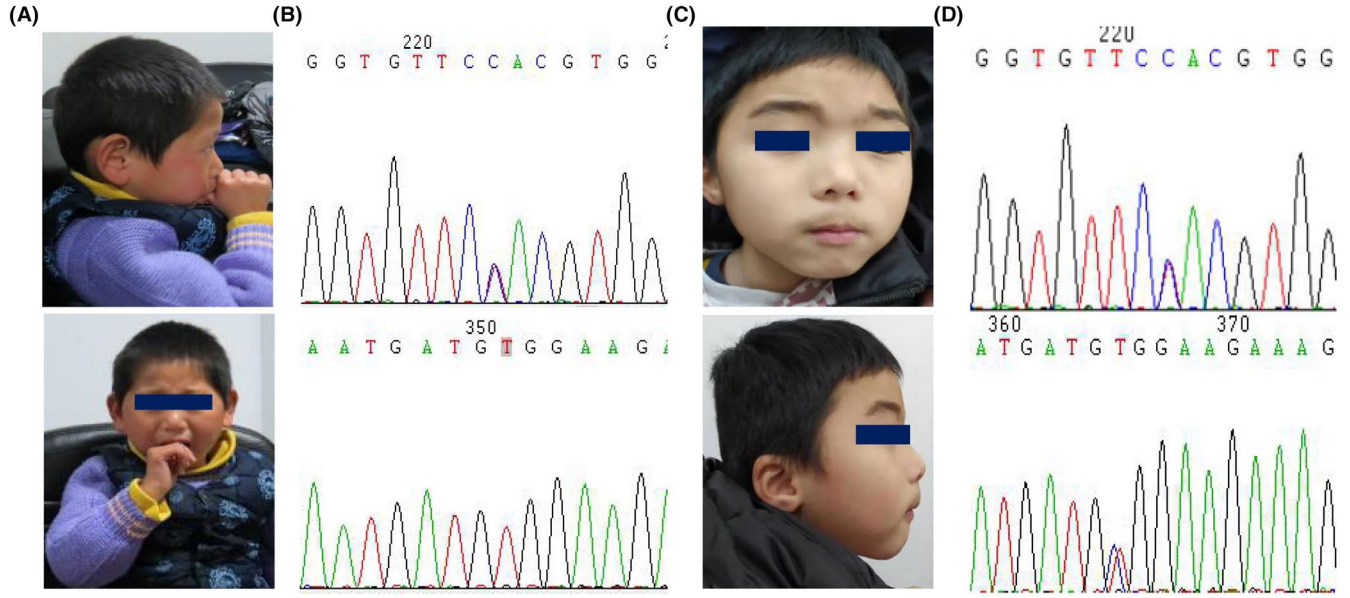

FIGURE 1 Phenotype and variants identified in two subjects (A) Front and side views of subject 1 (at age 7 years), microcephalus and daily nodding episodes; (B) DNA sequencing revealed heterozygous variant c.202T>C (p.Y68H) and homozygous variant c.1111C>T (p.R371W) in the *QRFPR* gene in subject 1, heterozygous variant c.202T>C (p.Y68H) inherited from mother, homozygous variant c.1111C>T (p.R371 W) inherited from parents; (C) Front and side views of subject 2 (at age 4 years), microcephalus, hypertelorism, high-vaulted arch, strabismus and hypertrichosis. (D) DNA sequencing revealed compound heterozygous variants c.202T>C (p.Y68H) and homozygous variant c.1111C>T (p.R371W) in the *QRFPR* gene in subject 2, heterozygous variant c.202T>C (p.Y68H) inherited from father, heterozygous variant c.1111C>T (p.R371W) inherited from mother

2.8 | Statistical analysis

All results are expressed as mean ± SEM. Data were analyzed using GraphPad prism 8 software with two-tailed paired *t* tests (*$p < 0.05$) for statistical significance.

3 | RESULTS

3.1 | The new genetic variants identified by exome sequencing in patient

Whole exome sequencing revealed the presence of two variants in the *QRFPR*, namely the heterozygous variant c.202T>C (p.Y68H) located in the first transmembrane α-helix near the intracellular end and the homozygous variant c.1111C>T (p.R371W) located in the C-terminal end near the cytosol (Figure 1B). No other candidate variants were identified in this patient. For screening the *QRFPR* variation, we found another case who carried the compound heterozygous variants c.202T>C (p.Y68H) and c.1111C>T (p.R371W) in the *QRFPR* (Figure 1D). Patient 2, male, 4 years old, presented clinically with microcephaly, small eyes, wide eye spacing, bilateral intraocular strabismus, hairy thighs and back, mental retardation, and delayed language development. He has also problem with swallowing and eating (Table 1, Figure 1C).

QRFPR has no associated disease documented in either OMIM or the Human Mutation Database (HGMD). Given the important regulatory role of *QRFPR* in physiological processes such as neurotransmission, we subsequently performed structural and functional analyses of the observed non-synonymous variations of the *QRFPR*, which were c.202T>C (p.Y68H) and c.1111C>T (p.R371W).

3.2 | c.202 T>C (p.Y68H) and c.1111C>T (p.R371W) disrupt the structural stability of the QRFPR

We performed SIFT and PolyPhen2 predictions for the c.202T>C (p.Y68H) and c.1111C>T (p.R371W), suggesting that the variations are disruptive to *QRFPR* function. To assess the effect of variations on *QRFPR* function, we established a structural model of QRFPR by integrating both 2 ks9.pdb and 5zbq.pdb. Our model shows that Y68 is located on the β-fold sheet of the QRFPR's tertiary structure and R371 is located on the loop (Figure 2A); the RMSD value of the p.Y68H variation structural model is 0.537, and the model shows that the replacement of tyrosine by histidine changes an uncharged polar amino acid to a positively charged basic amino acid, which is also located on the solvent-exposed surface. This change may affect the spatial site barrier at amino acid sites 9, 38, 39, 40, and 371 of the QRFPR, leading to instability of the complex. Similarly, the structural model of the p.R371 W variations, which has an RMSD value of 0.579, replaces the hydrophobic side chain arginine with a positively charged side chain tryptophan and also alters the spatial sites of the 2, 38, 39, and 40 amino acid sites of the QRFPR (Figure 2A), disrupting the protein structure.

To test the effect of c.202T>C (p.Y68H) and c.1111C>T (p.R371W) variants on ligand and receptor binding, we performed 26Rfa docking with QRPFR using RosettaDock and PyMol to observe the

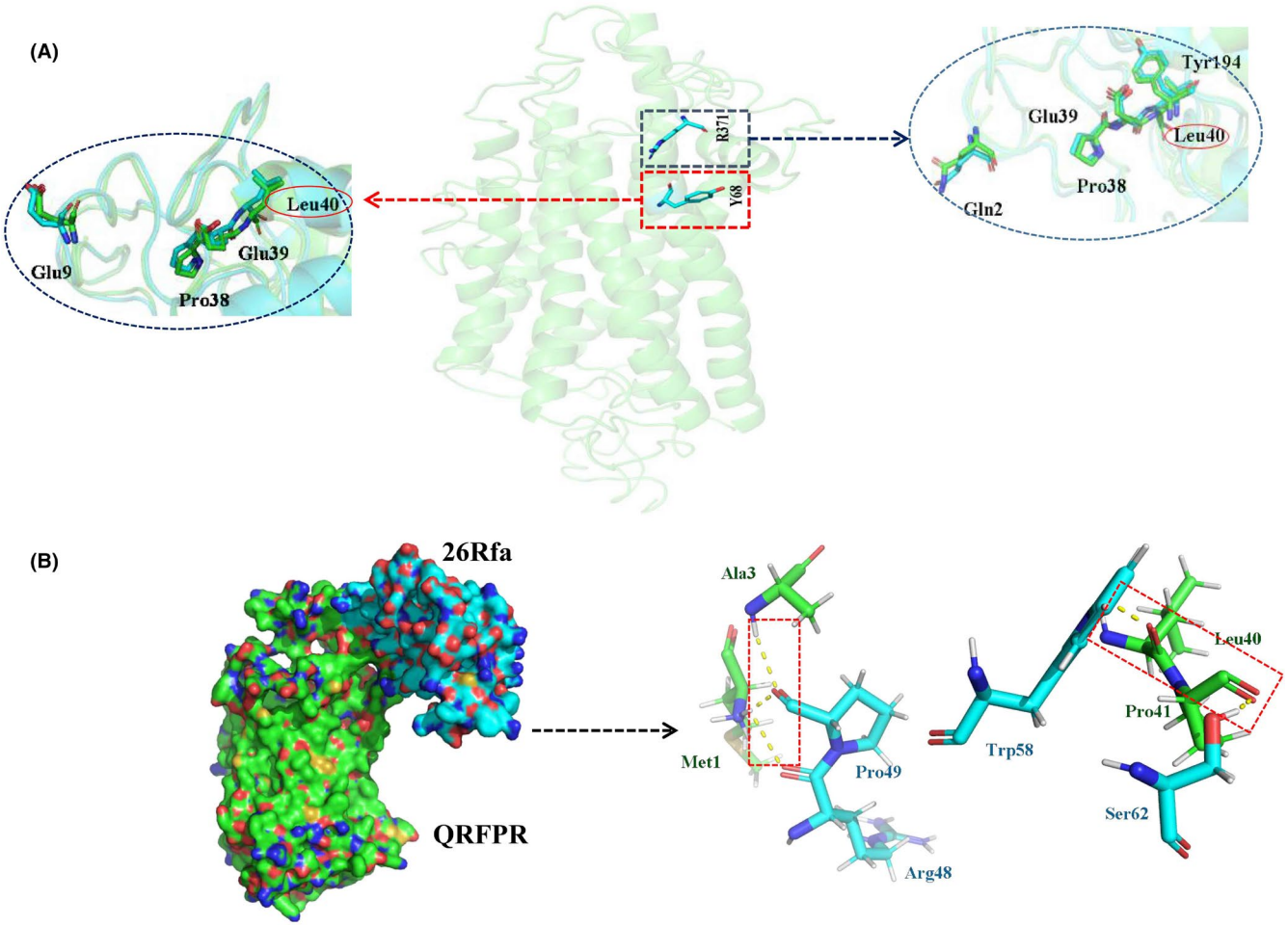

FIGURE 2 Structural consideration of the QRFPR variants at Y68 and R371. (A) Schematic presentation of the QRFPR proteins. The Y68H affects the spatial site barrier at amino acid sites 9, 38, 39, 40, and 371 of the QRFPR. R371W alters the spatial sites of the 2, 38, 39, and 40 amino acid sites of the QRFPR. The protein modeling is achieved by PyMOL Molecular Graphics System (Version 2.3.0) according to QRFPR. (B) QRPFR interaction with 26Rfa: Met1-Ala3-Leu40-Pro41 of the receptor and Arg48-Pro49-Trp58-Ser6 of the ligand generates hydrogen bonding to each other via molecular forces

docking model, and we predicted that Met1-Ala3-Leu40-Pro41 of the receptor and Arg48-Pro49-Trp58-Ser6 of the ligand generates hydrogen bonding to each other via molecular forces (Figure 2B). Our model predicts that both c.202T>C (p.Y68H) and c.1111C>T (p.R371W) variations change the polarity of amino acids, resulting in an altered spatial potential barrier of leucine at the 40th amino acid site of the QRFPR, affecting hydrogen bond formation between receptor and ligand, leading to reduced binding efficiency.

3.3 | Variations in the *QRFPR* c.202 T>C (p.Y68H) and c.1111C>T (p.R371 W) affect the regulation of intracellular calcium signaling

During neurodevelopment, calcium ions conduct neural signals by promoting neurotransmitter secretion. QRFPR, an orphan receptor for the neuropeptide 26RFa, can stimulate a transient elevation of intracellular calcium ions upon activation of QRFPR by receptor agonists. To detect the effect of QRFPR on the intracellular calcium ion concentration, we established a shRNA expression system for the QRFPR gene in HEK293 cells (Figure 3A). Further, we detected downregulation of QRFPR expression (Figure 3A) and noticed a significant decrease in intracellular calcium ion levels (Figure 3B). We then transfected c.202 T>C (p.Y68H) and c.1111C>T (p.R371W) mutant expression vectors and observed that the intracellular calcium concentration in HEK293 cells transfected with the mutant vector was significantly lower than that in the control group (Figure 3B).

3.4 | Variations in the *QRFPR* c.202 T>C (p.Y68H) and c.1111C>T (p.R371 W) affect the PI3 K-AKT-mTOR signaling pathway

It has been shown that GPCR acts through coupling with G proteins to control important physiological processes such as cell cycle, growth, and development through multiple signaling pathways, especially

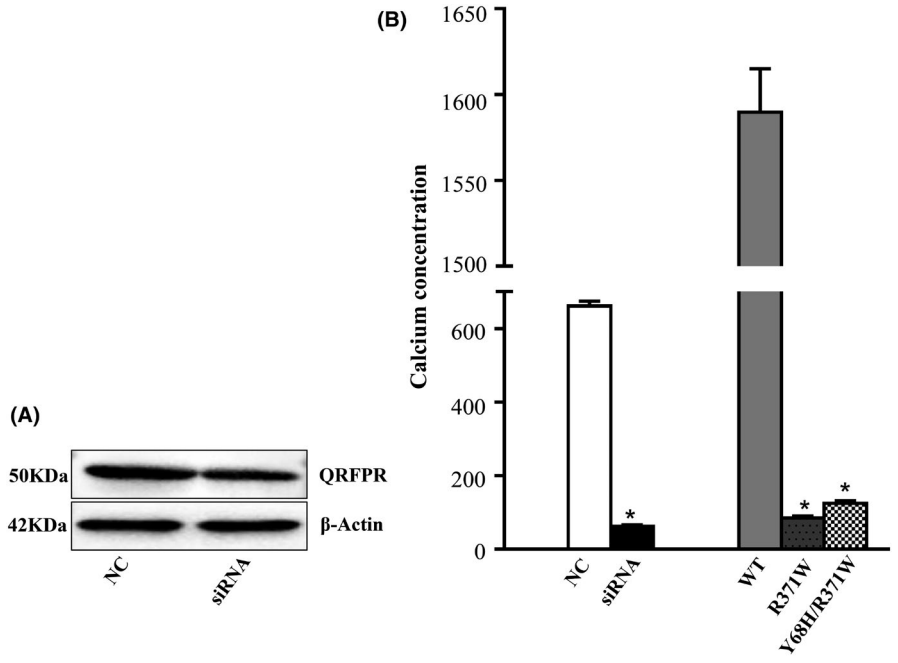

FIGURE 3 The effect of QRFPR on the intracellular calcium ion concentration (A) QRFPR expression was silenced using shRNA; (B) Wild-type and mutant QRFPR (R371W, Y68H) protein silenced with similar efficiency on the intracellular calcium ion concentrationn

through the mTOR signaling pathway. To investigate the transduction pathway of the *QRFPR* in the mTOR signaling pathway, we overexpressed QRFPR in HEK293 cells, which activated PI3K and upregulated phosphorylated p85 expression in the presence of a receptor agonist (Figure 4A). At the same time, the mTOR upstream signaling molecule AKT was activated, and expression of p-AKT and p-mTOR was significantly upregulated, confirming that *QRFPR* activates the mTOR signaling pathway through the PI3K pathway (Figure 4A).

To further examine the role of *QRFPR* in the PI3K-AKT-mTOR signaling pathway, we used shRNA to interfere with the expression of QRFPR in HEK293 cells. When *QRFPR* was silenced, the activity of mTOR signaling pathway was significantly inhibited, and the expression of p-p85, p-AKT and p-mTOR was downregulated in HEK293 cell (Figure 4B), further indicating that *QRFPR* mediates the activation of PI3K-AKT-mTOR pathway through the regulation of downstream signaling molecules.

To investigate whether the c.202T>C (p.Y68H) and c.1111C>T (p.R371W) variations affect the QRFPR-mediated PI3K-AKT-mTOR signaling pathway, we transfected wild-type *QRFPR* or the c.202T>C (p.Y68H) and c.1111C>T (p.R371W) variations, and in comparison with the wild type, we found that c.202T>C (p.Y68H) and c.1111C>T (p.R371W) variations inhibit the activity of the mTOR signaling

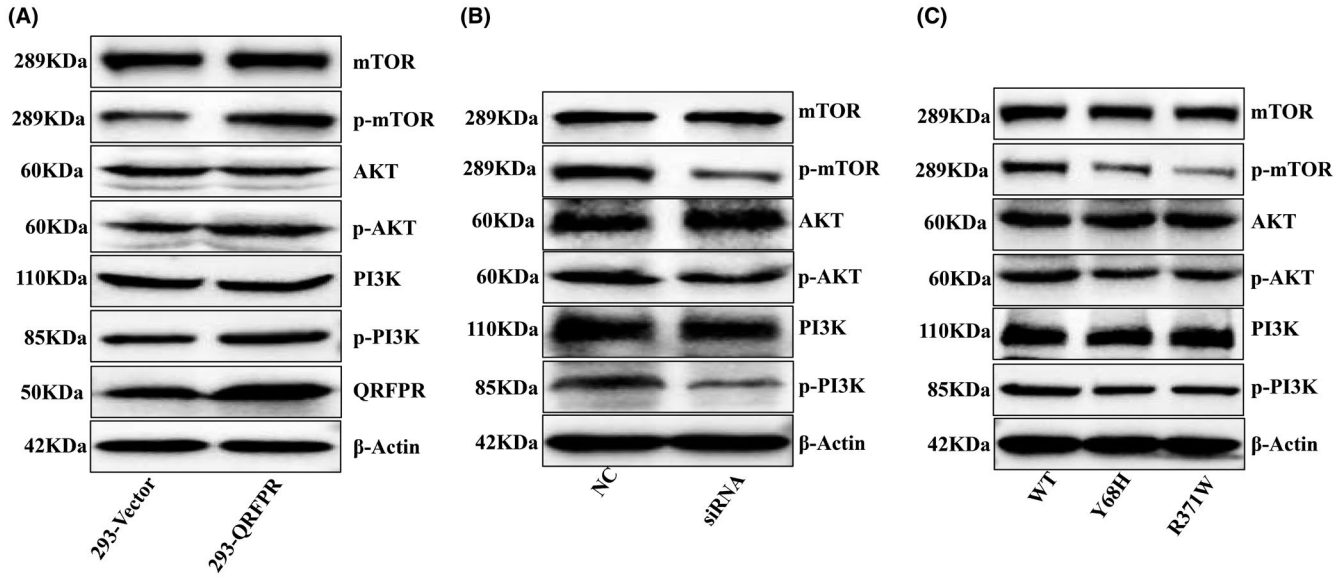

FIGURE 4 Representative immunoblots of lysates, showing the levels of p-mTOR, mTOR, p- AKT, AKT, p- PI3K, PI3K, and QRFPR protein levels together with normalizer β-Actin (A) QRFPR overexpression in HEK293 cells can activate PI3K, AKT, and mTOR; (B) the expression of p-PI3K, p-AKT and p-mTOR were inhibited by QRFPR silencing; (C) the *QRFPR* variations (R371W, Y68H) can inhibit the expression of p-PI3K, p-AKT and p-mTOR

pathway in HEK293 cells, and expression of phosphorylated PI3K (p-p85), p-AKT, and p-mTOR was downregulated (Figure 4C).

4 | DISCUSSION

Intellectual disability (ID) is a common morbidity, affecting at least 1% of the population, and genetic causes were identified in up to 42% of the affected individuals.[1] Progression of ID is usually a stable process of cognitive impairment that manifests itself in early childhood as delays in language and other cognitive domains, and persists into adulthood with varying degrees of limited intellectual functioning.[14] Despite the diversity of ID clinical phenotypes and the complexity of pathogenic mechanisms, ID genes are clustered, as observed by gene function and molecular information network distribution. ID genes are generally associated with neuronal growth and development, neuronal differentiation and migration, synaptic function, and functional gene transcription and translation.[15,16] Greenwood Genetic Center has investigated ID patients and found that genetic variation is the main reason for the occurrence of severe ID.[17,18] For families with ID patients, a specific diagnosis can translate into useful clinical information, and genetic etiology is an essential factor. Many diagnostic methods have been utilized to determine the pathogenesis of ID, but in more than half of patients with ID, the etiology is unknown. A distinct advantage of high-throughput sequencing is its ability to identify new disease genes and establish a true link between the candidate genes and the human disease.

Based on whole exome sequencing (WES), we found two variations in *QRFPR*. The *QRFPR* is involved in a variety of physiological and pathological regulation, but an association with human disease has not been demonstrated yet. The patients in this study were all mentally retarded, developmentally delayed, non-verbal, and had difficulty in swallowing and eating. So we considered c.202 T>C (p.Y68H) and c.1111C>T (p.R371W) as potentially pathogenic point variations.

QRFPR is highly expressed in the central nervous system, and upon ligand activation, it regulates intracellular Ca^{2+} and cAMP concentrations.[19] QRFPR directly affects neuronal growth, development, and hormone secretion and plays an important regulatory role in all stages of growth and development on sleep, mood, growth and development, and even differentiation and development of the nervous system. In 2017, Anazi S et al.[20]. applied WES directly on a cohort of patients with ID. A patient who was diagnosed with developmentally delayed and attention-deficit hyperactivity disorder was identified with *QRFPR* homozygous variants. Santoro ML et al. found that *QRFPR* is one of four genes significantly downregulated in the prefrontal cortex of rats in a spontaneously hypertensive rat model, which is used to assess schizophrenia and attention-deficit/hyperactivity disorder.[21] The strong association of *QRFPR* with brain development and function supports the *QRFPR* as a candidate gene for ID. As *QRFPR* is involved in a variety of physiological and pathological regulation, and the patients in our study diagnosed with ID and difficulty in swallowing and eating were identified with *QRFPR* variants. In connection with the *QRFPR* variants reported by Anazi S, we reconfirmed the role of *QRFPR* in neurodevelopment. This is the first time that we have analyzed the tertiary structure of QRFPR and preliminarily elucidated the role of QRFPR in the PI3K-AKT-mTOR signaling pathway, in attempt to explain the effect of QRFPR on neural development.

The results we obtained in our protein structural and functional analyses suggested that the c.202 T>C (p.Y68H) and c.1111C>T (p.R371W) variations may have pathogenic effects on the function of *QRFPR*. QRFPR has seven transmembrane helix structures and loops linking transmembrane regions, including three intracellular and three extracellular loops. The transmembrane region has 35%-38% sequence homology to several neuropeptide receptors such as neuropeptide FF2, Y2, and glycopeptide GalR1 receptors and is a key region involved in cell signaling. c.202 T>C (p.Y68H) is located in exon 1 of the *QRFPR*, in the first transmembrane α-helix near the intracellular end, and is the major ligand-binding region. The c.1111C>T (p.R371W) is located in exon 6 of the *QRFPR*, near the cytosolic end at the C terminus, and is likely to be a G protein-binding region. The c.202T>C (p.Y68H) and c.1111C>T (p.R371W) variations are predicted to be deleterious in the *QRFPR* and are highly conserved in a variety of organisms, suggesting that these two sites may be essential for the normal function of the *QRFPR*. The inherent properties of QRFPRs, such as their hydrophobic nature, flexibility, and the membrane-like environment required to ensure proper folding, make crystal preparation particularly difficult. Therefore, computer-assisted molecular modeling is an alternative method to obtain information on the tertiary structure of the QRFPR.[22] Using the crystal structures of the human neuropeptide YY1 receptor with a 29% homologous sequence to QRFPR, and the human neurokinin 1 receptor with a 22% homologous sequence to QRFPR for modeling, we used a Monte Carlo algorithm. We obtained a molecular model of QRFPR, which was subsequently used for ligand docking studies. The docking model predicts that the neuropeptide 26RFa binds to QRFPR and can generate hydrogen bonds to each other via Met1-Ala3-Leu40-Pro41 in the first extracellular region of the receptor and Arg48-Pro49-Trp58-Ser62 of the ligand. According to our structural model observations, Y68 and R371 are located on the β-fold sheet and on the loop of the protein's tertiary structure, respectively. The substitution of Y68H and R371W leads to a change in the polarity of amino acids, altering the side chain conformation of amino acid residues and reducing the stability of the QRFPR structure. The model also predicts that after Y68H and R371W substitution, the surface area of the amino acid side chains becomes larger, which clashes spatially with the neighboring Leu40 residues and may alter the hydrogen bonding interaction between ligand and receptor, making the mutated amino acids no longer suitable as hydrogen-bonded receptors, resulting in a decrease in the ability of 26RFa to stimulate cellular signals and affecting downstream signal transduction.

During neurodevelopment, Ca^{2+} conducts neural signals by promoting neurotransmitter secretion. *QRFPR* can stimulate a transient elevation of intracellular calcium ions upon activation of QRFPR by receptor agonists. Increasing evidence suggests that

altered Ca^{2+} signaling may be relevant to the pathology of neurodevelopmental ID. Previous studies by Ramanjaneya M have demonstrated that both QRFPR and its ligand 26RFa are expressed in both human HEK293 and CHO cells and that *QRFPR* causes transient increases in intracellular Ca^{2+} levels when stimulated by 26RFa.[8] Our results indicated that downregulation of QRFPR expression or variation in Y68H and R371W resulted in a decrease in intracellular calcium levels, suggesting that *QRFPR* possesses calcium-regulatory capacity. When *QRFPR* dosage is insufficient or function was lost, both neurotransmission capacity and calcium levels decreased. Ca^{2+} is a key secondary messenger in most cells, including developing neurons. Ca^{2+} signaling plays a vital role in the formation of neural circuits by regulating cell viability, growth cone motility and turning.[23] In neural synapses, the signal of postsynaptic calcium ion concentration accurately reflects synaptic activity.[24] Studies have reported that some ID-related proteins can regulate the intracellular Ca^{2+} concentration and excitatory synaptic transmission by binding to calmodulin.[25] Based on the results of this experiment, we speculate that *QRFPR* is involved in the regulation of intracellular Ca^{2+} concentration, which may affect excitatory synaptic transmission.

Accordingly, the interaction of the 26RFa orphan receptor QRFPR with G proteins and downstream signaling remain controversial. Many GPCRs have been shown to simultaneously couple to multiple G protein subtypes, leading to different downstream signaling pathways.[26] It has been suggested that mTOR can be activated by the upstream PI3K-AKT pathway to regulate neuroprotein synthesis.[12] mTOR is closely related to neurodevelopment, and downregulation of mTOR expression can cause abnormal brain function.[11] It was originally hypothesized that GPCRs exert the balanced actions to transmit extracellular stimuli into intracellular signals through different G proteins and β-arrestins. 26RFa treatment resulted in increased levels of cytosolic Ca^{2+}, and the activation on Ca^{2+} mobilization could be partially suppressed by siRNA-mediated downregulation of QRFPR. Meanwhile, our study found that the phosphorylation levels of the PI3K, Akt, and mTOR proteins were significantly up- or downregulated by *QRFPR* gene overexpression or silencing, respectively, in HEK293 cells. The variation of Y68H and R371W in *QRFPR* also resulted in decreasing in phosphorylation levels of the PI3K, Akt, and mTOR proteins. Hence, we hypothesized that these signaling pathway proteins are the regulators downstream of *QRFPR*. The PI3K-AKT-mTOR signaling pathway always plays a critical role in cell proliferation, differentiation, and neurosynaptic development. Taken together, in addition to the involvement of intracellular Ca^{2+} mobilization, *QRFPR* has been shown to activate PI3K-AKT-mTOR signaling pathway. Intracellular Ca^{2+} is a known messenger involved in cell proliferation and neurodevelopment. Aberrant Ca^{2+} signaling and abnormal synaptic activity are associated with the pathophysiology of autism spectrum disorder and attention-deficit/hyperactivity disorder.[27] The new study suggests that PI3K-AKT-mTOR are probably downstream of Ca^{2+}.[28] *QRFPR* can directly affect intracellular Ca^{2+} concentration by binding to ligand 26RFa. Hence, we hypothesized that the regulation of *QRFPR* function on neurodevelopment through PI3K-AKT-mTOR signal pathways may depend on Ca^{2+} cascades transduction. But the regulatory relationship of PI3K-AKT-mTOR and QRFPR requires further investigation.

In conclusion, we provide evidence that the QRFPR is a G protein-coupled receptor, upon activation, triggering Ca^{2+} mobilization and PI3K-AKT-mTOR phosphorylation. The variation of Y68H and R371W in *QRFPR* altered PI3K-AKT-mTOR signaling activity, and this is the first report to establish that a variation in *QRFPR* altered PI3K-AKT-mTOR signaling pathway activity in neurodevelopmental ID. Therefore, it is vital to investigate how alterations in the PI3K-AKT-mTOR signaling pathway induced by *QRFPR* variations affect cellular processes in brain development. Future in-depth clinical, functional, and molecular structural studies will help to further elucidate the role of *QRFPR* variations in neurodevelopmental ID and difficulty in feeding, as well as the preference of different molecular structures for Gi and Gq signaling pathways. A conditional gene knockout model may be developed in the subsequent studies to investigate the function of *QRFPR* in neurodevelopment. Identification of a potential CaSR-binding site of *QRFPR* and the upstream molecules of *QRFPR* requires further investigation.

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CONFLICTS OF INTEREST

The authors declare no conflict of interest regarding the publication of this paper.

AUTHOR CONTRIBUTIONS

All authors contributed to the study conception and design. Huanzheng Li and Ran Lou contributed to conception and design of the study. Xueqin Xu, Chenyang Xu, Yunzhi Xu, Lin Hu, Yanbao Xiang collected samples, genotyped the cases, and finished the follow-up. Shaohua Tang performed genetic counseling. Yuan Yu and Xuan Lin contributed to preparing the figure. Huanzheng Li, Ran Lou, and Shaohua Tang helped in the statistical analysis. All authors read and approved the final manuscript.

DATA AVAILABILITY STATEMENT

The data that provided the evidence for the study are available from the corresponding author upon reasonable request.

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