

Two novel mutations in Gli-similar 3 in patients with congenital hypothyroidism and thyroid dysgenesis

Jie Lan

The Affiliated Hospital of Qingdao University

Chunhui Sun

The Third People's Hospital of Qingdao

Xinping Liang

Qingdao Women and Children's Hospital

Ruixin Ma

The Affiliated Hospital of Qingdao University

Yuhua Ji

Yantaishan Hospital

Miaomiao Li

The Affiliated Hospital of Qingdao University

Shiguo Liu

The Affiliated Hospital of Qingdao University

Fang Wang (✉ 18700840614@126.com)

The Affiliated Hospital of Qingdao University

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Abstract

Background: Thyroid dysgenesis (TD) is the main cause of congenital hypothyroidism (CH). As variants of the transcription factor Gli-similar 3 (*GLIS3*) have been associated with CH and *GLIS3* is one of candidate genes of TD, we screened and characterized *GLIS3* mutations in Chinese patients with CH and TD.

Methods: To detect mutations, we sequenced all *GLIS3* exons in the peripheral blood genomic DNA isolated from 50 patients with TD and 100 healthy individuals. Wild-type and mutant expression vectors of Glis3 were constructed. Quantitative real-time PCR, western blotting, and double luciferase assay were performed to investigate the effect of the mutations on *GLIS3* protein function and transcriptional activation.

Results: Two novel heterozygous missense mutations, c.2710G>A (p.G904R) and c.2507C>A (p.P836Q), were detected in two unrelated patients. Functional studies revealed that p.G904R expression was 59.95% lower and p.P836Q was 31.23% lower than wild-type *GLIS3* mRNA expression. The p.G904R mutation also resulted in lower *GLIS3* protein expression compared with that encoded by wild-type *GLIS3*. Additionally, the luciferase reporter assay revealed that p.G904R mediated impaired transcriptional activation compared with the wild-type protein ($p < 0.05$) but did not have a dominant-negative effect on the wild-type protein.

Conclusions: We for the first time screened and characterized the function of *GLIS3* mutations in Chinese individuals with CH and TD. Our study not only broadens the *GLIS3* mutation spectrum, but also provides further evidence that *GLIS3* defects cause TD.

Introduction

Congenital hypothyroidism (CH) is the most common endocrine disease in infants, with a recent reported incidence of 1 in 1,400–2,800 and a sex ratio of approximately 1:2 (male:female)[1; 2]. Although its clinical features are not obvious at birth, if not diagnosed and treated in a timely manner, severe CH can lead to growth failure and permanent intellectual disability. CH is divided into permanent and temporary forms, which are in turn divided into disorders with primary, secondary, and peripheral causes [3]. Two pathophysiological mechanisms are responsible for primary, permanent CH: thyroid dysgenesis (TD) and thyroid hormone (TH) synthesis disorders. TD accounts for 80–85% of CH cases, including thyroid dysplasia, ectopic thyroid, or the absence of thyroid tissue [4], and approximately 10–15% of CH is caused by TH dyshormonogenesis [5]. Although TD usually occurs sporadically, the rate of asymptomatic thyroid developmental anomaly among first-degree relatives of people with TD was significantly higher than that of normal people [6]. Furthermore, genetic factors have been described in about 5% of cases. Previous studies have shown that mutations in one of several genes involved in thyroid formation may be related to TD, including thyroid stimulating hormone receptor (TSHR) or transcription factors PAX8, NKX2-1 or

FOXE1 [7-9]. More recently, some additional genes have been associated with TD, including NKX2-5, JAG1, CDCA8, TUBB1, NTN1, GLIS3 [10].

The GLIS3 gene is located on chromosome 9p24.2, containing 11 exons, encoding for a 90 KD - size protein. It is highly expressed in the kidney, thyroid gland, endocrine pancreas, thymus, testis, and uterus. Lower levels of expression were also described in brain, lung, ovary, and liver [11]. GLIS3 is a member of the GLI-similar 1-3 (GLIS1-3) subfamily of Krüppel-like zinc finger protein transcription factors which play a key regulatory role in embryo-genesis and many biological processes, such as thyroid hormone biosynthesis, pancreatic β cell generation and insulin expression, and spermatogenesis [12]. Since Taha et al first described a new syndrome including CH, neonatal diabetes mellitus, and variable other abnormalities as hepatic fibrosis, congenital glaucoma, and polycystic kidneys in 2003 [13], Senee et al regarded this rare syndrome involving neonatal diabetes mellitus and hypothyroidism (NDH) as caused by the mutations in GLIS3 and identified a repressor and activator of transcription in 2006 [14]. A compound heterozygous GLIS3 variant p.[Gly727Arg];[Gln1347Lys] in two siblings with thyroid hemiagenesis was described in Poland [15]. Thyroid hemiagenesis is a rare type of TD, but it does not associated with reduced thyroid function [16]. Additionally, rare heterozygous GLIS3 missense variants were identified in a recent study of a large cohort (18/177) of Caucasian patients with isolated CH. Investigation of the thyroid phenotype of these affected cases shows that TD and in-situ thyroid gland are accounted for halves [17]. However, to date, the relationship between the GLIS3 mutations and its associated CH phenotype remains unclear. Therefore, we screened GLIS3 exons in the peripheral blood genomic DNA of 50 Chinese patients with CH and TD to detect mutations and tried to elucidate the functions of the identified mutations.

Materials And Methods

Patients

We selected 50 children diagnosed with CH and TD (23 with athyreosis, 21 with ectopy, and 6 with hypogenesis; male: 22, female: 28; average age: 2.5 ± 0.5 years) from Shandong province, including Jinan, Qingdao, and Weifang, between 2007 and 2016, via a neonatal screening program. There were three criteria for inclusion. First, the child was diagnosed with CH. Heel blood samples were collected from the newborn and analyzed to determine serum thyroid-stimulating hormone (TSH) levels. Children with TSH levels ≥ 10 $\mu\text{IU/mL}$ were recalled, and their serum TSH, free thyroxine (FT4), and free triiodothyronine (FT3) levels were further evaluated. CH was diagnosed based on a high level of TSH (>4.2 $\mu\text{IU/mL}$) and low level of FT4 (<12 pmol/L). Second, the children were diagnosed with TD after thyroid ultrasound or thyroid nucleus scanning. Third, other congenital diseases, such as blood and immune system diseases, malignant tumors, and mental disorders, were excluded. This study was approved by the ethics committee of the Affiliated Hospital of Qingdao University, and blood samples were collected from 50 subjects and 100 healthy controls (male: female ratio, 1:1.2, age range, 1-40 years) after obtaining their written informed consent.

Genetic analysis

DNA samples were extracted from the peripheral blood leukocytes of 50 patients with TD using a QIAamp Blood DNA Mini Kit (QIAGEN, Hilden, Germany). Primers for the 13 exons covering the coding sequence, flanking intronic sequence, 5'untranslated region, and 3' untranslated region of *GLIS3* were designed using Primer 5.0. Polymerase chain reaction (PCR) was performed in a total volume of 25 μ L, which contained 250 nM dNTPs, 100 ng template DNA, 0.5 μ M each of the forward and reverse primers, and 1.25 U AmpliTaq Gold DNA polymerase in 1 \times reaction buffer (10 mM Tris-HCl [pH 8.3], 50 mM KCl, and 2.5 mM MgCl₂). PCR amplifications were performed under the following reaction conditions: pre-denaturation at 95°C for 5 min; 35 cycles of 95°C for 30 s, 55°C for 30 s, and 72°C for 30 s, and 72°C for a final extension of 7 min.

The PCR products were identified by agarose gel electrophoresis and analyzed using the Bio-Rad Gel Doc™ XR+ imaging system (Hercules, CA, USA). Products appearing as distinct, single bands were subjected to Sanger sequencing and compared with the *GLIS3* reference sequence (NC-000009.12) to identify mutations.

Vectors and plasmids

The human *GLIS3*-tag pcDNA3.1 vector and control vector pcDNA3.1 were purchased from Synbio Technologies (Suzhou, China). Mutant *GLIS3*-G904R and *GLIS3*-P836Q were generated using a PCR-based site-directed mutagenesis method with the Fast Mutagenesis System (TransGen, Beijing, China). Plasmid DNA was extracted from cells cultured overnight using an EndoFree Plasmid Mini Kit (CWBI0, Jiangsu, China) according to the manufacturer's instructions at 37°C with shaking at 250 rpm.

GlisBS was inserted into the PGL3-basic luciferase reporter construct by homologous recombination. The 10- μ L sample contained 1 μ L PGL3-basic, 1 μ L GlisBS, 5 μ L 2 \times SoSoo Mix (TSINGKE, Beijing, China), and 3 μ L ddH₂O. The recombination reaction was performed at 50°C for 15 min. After gel extraction and translation according to the manufacturer's specifications, positive clones were sending for sequencing (Qingke Zi Xi Biological Company, Qingdao, China).

Cell culture and quantitative reverse transcription PCR

For *in vitro* studies, we used 293T cells (passage 4) cultured in Dulbecco's modified Eagle's medium (Biological Industries, Beit HaEmek, Israel) containing 10% fetal bovine serum, 100 IU/mL penicillin, and 100 μ g/mL streptomycin. The cells were incubated at 37°C in 5% CO₂ in a humidified atmosphere. Total RNA was isolated with TRIzol™ reagent (Ambion, Austin, TX, USA) from 293T cells transfected for 24 h. Reverse transcription (RT) was performed in a reaction mixture comprised of 1 μ g total RNA, 1 μ L random primer, 10 μ L 2 \times TS Reaction Mix, 1 μ L TransScript® RT/RI Enzyme Mix, 1 μ L gDNA Remover, and RNase-free water to a final volume of 20 μ L (all reagents from TransGen Biotech). The reaction mixtures were incubated at 25°C for 10 min and then at 42°C for 30 min. Next, the samples were heated to 85°C for 5 s to inactivate TransScript RT/RI and gDNA Remover. Real-time PCR for each cDNA was performed in

triplicate in a 20- μ L reaction mixture containing 5 ng cDNA, 0.4 μ L each forward and reverse primer (both 10 μ M), and 10 μ L 2 \times TransStart[®] Green qPCR SuperMix (TransGen Biotech, Beijing, China); the final volumes were achieved by adding an appropriate volume of water. The samples were incubated in 96-well plates on an Applied Biosystems 7900HT Fast Real-Time PCR system (Applied Biosystems, Foster City, CA, USA) at 95°C for 15 min, followed by 40 cycles at 95°C for 10 s and 56°C for 32 s. The $2^{-\Delta\Delta Ct}$ method was used to determine the relative quantitative levels of individual cDNA expression. Transcript levels were normalized to the level of *β -actin*, and values were expressed as relative differences compared to those in their corresponding controls. The GLIS3 primers used were forward, TTCAACGCCCGCTATAAACTG, and reverse, ATACGGCTTCTCGCCTGTGT.

Western blot analysis

After 48h of transfection, the cells were lysed and collected with radioimmunoprecipitation assay buffer and phenylmethylsulfonyl fluoride at 100:1 ratio (Beyotime, Shanghai, China). Protein concentrations were determined using a BCA kit (Thermo Scientific, Waltham, MA, USA). We separated 40 μ g of each sample by 12% sodium dodecyl sulfate-polyacrylamide gel electrophoresis. Western blotting was performed according to a routine process as previously described [18] with a rabbit anti-GLIS3 primary antibody (1:200; Proteintech, Rosemont, IL, USA) and secondary goat anti-rabbit antibody conjugated to horseradish peroxidase (1:5,000). The results were analyzed on a FluorChem Q (ProteinSimple, San Jose, CA, USA) with a chemiluminescent horseradish peroxidase substrate (Immobilon[®] Western, Millipore, Billerica, MA, USA).

Dual-luciferase reporting system

The luciferase reporter system uses luciferin as a substrate to detect firefly luciferase activity. We transfected 293T cells with 250 ng wild-type (WT) or mutant GLIS3 expression vector, with the *Renilla* luciferase expression vector as an internal reference and pcDNA3.1 as a control vector. We detected luciferase activity 48 h after transfection with the Dual-Luciferase[®] Reporter Assay System kit (Promega, Madison, WI, USA). Firefly luciferase activity was recorded and calculated as a ratio to *Renilla* luciferase activity.

Statistical analysis

Statistical analysis was performed using the paired Student's *t*-test. A *p*-value less than 0.05 was considered as statistically significant; ** represents *p* < 0.01 and * represents *p* < 0.05. Graphs were prepared using GraphPad Prism 7.0 software (GraphPad, Inc., La Jolla, CA, USA).

Results

Screening of *GLIS3* mutations in a cohort of patients with CH and TD

Among the 50 unrelated patients with CH and TD, we identified two variants of *GLIS3*. The variant c.2710G>A is located on exon 11, resulting in a glycine-to-arginine substitution at codon 904 of the protein (p.G904R) (Figure 1A). The variant c.2507C>A is located on exon 10 and causes a proline-to-glutamine substitution at codon 836 (p.P836Q) (Figure 1B). The mutations were not detected in 100 healthy individuals.

Multiple sequence alignment of *GLIS3* from different species (*Homo sapiens*, *Cricetulus griseus*, *Mus musculus*, *Aotus nancymaae*, *Sus scrofa*, *Camelus ferus*, and *Pan troglodytes*) indicated that G904 and P836 were highly conserved (Figure 2). The values predicted by PolyPhen-2 (<http://genetics.bwh.harvard.edu/pph2>) and SIFT (<https://sift.bii.a-star.edu.sg>) for the p.G904R mutations were 0.946 and 0.007, respectively. The PolyPhen-2 and SIFT predicted values of the p.P836Q mutation were 0.976 and 0.002, suggesting that both mutations were deleterious.

Clinical characteristics of patients with *GLIS3* mutations

Patient 1 (P1), a 2-year-old boy with the G904R mutation, was born by vaginal delivery after full-term gestation with a birth weight of 3,000 g. He was first diagnosed with CH at 20 days of age with a serum TSH level >110 μ IU/mL, FT3 level of 5.8 pmol/L, and FT4 level of 2.67 pmol/L. The patient had no family history of thyroid disease. B-ultrasound inspection indicated athyreosis, suggesting a diagnosis of CH. He was initially prescribed 25 μ g/d levothyroxine (L-T4), and his current dose is 50 μ g/d at 5 years old. P2, a 3-year-old girl harboring the P836Q mutation, was a full-term infant from unrelated parents with no family history of thyroid disease. Fourteen days after birth, she was diagnosed with CH based on a serum TSH level >100 μ IU/mL, FT3 level of 4.1 pmol/L, and FT4 level of 5.31 pmol/L. Her B-ultrasound results, similar to those of P1, showed athyreosis. After diagnosis, L-T4 replacement therapy was initially administered at a dose of 25 μ g, and her current dose is 83 μ g/d at 7 years of age. Both patients exhibit normal growth and intelligence.

Effects of G904R and P836Q mutations on *GLIS3* mRNA and protein levels

We successfully expressed WT and mutant *GLIS3* in 293T cells. As shown in Figure 3A, quantitative RT-PCR revealed that the mRNA expression levels of G904R and P836Q were 59.95% and 31.23% lower than that of WT *GLIS3*, respectively. We also examined the expression of the proteins generated by 293T cells after transfection with the control and mutant vectors. As shown in Figure 3B, the protein expression level of the G904R mutant was significantly lower than that of the WT protein, and the protein expression level of the P836Q mutant did not significantly differ from that of the WT protein.

Effect of mutations on *GLIS3*-mediated transcriptional activation

After co-transfection of *GlisBS* and G904R, luciferase activity was significantly lower than that in cells transfected with WT *GLIS3* ($p < 0.05$) and it was partially recovered by additional co-transfection with WT *GLIS3* (Figure 4). However, the ability of P836Q to promote reporter gene expression was intact.

Discussion

The GLIS3 protein has a relatively large N-terminus, the zinc finger domain (ZFD) which including ZF1–ZF5, and C-terminal transactivation domain (TAD). Targeted deletion of the N-terminus increases GLIS3 transcriptional activity suggesting the N-terminal domain may act as a repressor [19]. The ZFD and TAD play important roles in GLIS3-mediated transcriptional activation, and ZF2–5 are required for GLIS3 DNA-binding site sequence recognition [20]. In-vitro studies have determined the optimal sequence for GLIS3 binding known as the Glis binding site (GlisBS), 5'-(G/C)TGGGGGG(A/C) [21]. The GlisBS are located within the regulatory regions of target genes. Once bound to the DNA, GLIS3 can repress or enhance the expression of target genes [22].

In the current studies, patients with loss-of-GLIS3-function mutations most develop a syndrome as neonatal diabetes and congenital hypothyroidism (NDH). The patients showed decreased levels of T3 and T4 along with elevated TSH and thyroglobulin (TG). Furthermore, depending on the nature of the mutation, NDH patients may develop a wider range of abnormalities, such as polycystic kidney, hepatic fibrosis, glaucoma, osteopenia and mild mental retardation [14; 23]. Similar phenotypes have been observed in Glis3-deficient mice, however, histological examination of the thyroid gland suggested that Glis3 does not significantly affect thyroid gland development [24]. Three types of GLIS3 alterations associated with NDH have been described. Homozygous frameshift mutations, p.Arg780Profs*79, p.Gly311Alafs*15, and p.Pro772Leufs*35 resulting in an early termination codon and the loss of the GLIS3 transactivation domain. Deletion mutation encompassing exons 5–9, 3–4, 9–11, 10–11 and larger deletions covering regions >100 kb that include exons 1–2, 1–4, or 5–9 and several homozygous missense mutations, such as p.Arg589Trp, p.Cys536Trp and p.His561Tyr [25]. Notably, the individuals with NDH have widely ranging thyroid structures, including athyreosis, hypoplasia, perifollicular fibrosis, interstitial fibrosis, and normal thyroid anatomy [22], which may be attributed to the tissue-specific expression of variable-length transcripts derived from exons 11 of the GLIS3 gene [26]. Besides, in several patients with apparently normal thyroid morphology, elevated TSH and TG levels seem to be resistant to levothyroxine treatment [21].

As the inconsistent clinical features of CH caused by GLIS3 mutation, which has made it difficult to ascertain its causative mechanism. In the study of Kang et al, the development of hypothyroidism in Glis3KO mice seems to be related to dyshormonogenesis, its mechanism of action is that GLIS3 acts downstream of TSH/TSHR and is essential for the induction of TSH/TSHR-mediated TH biosynthesis and the proliferation of thyroid follicular cells [27]. In addition, despite a relevant role in thyroid cell proliferation, no significant thyroid developmental defects were observed in Glis3 knock-out mice [27]. These findings are different from those observed in patients with NDH, in which the phenotype of the thyroid gland varies from aplasia/ dysplasia to dyshormonogenesis. Therefore, it is possible that in a different genetic background, GLIS3 deficiency might also affect embryonic thyroid development [26]. More recent evidence demonstrates that the down-regulation of GLIS3 in zebrafish embryos leads to thyroid developmental defects. GLIS3 morphants showed a decreased expression of the early transcription factors nkx2.4 and pax2a in the thyroid primordium [28].

Nevertheless, in the study of Glis3KO mice, the expression of genes essential for thyroid development (including Pax8, Ttf1 (Nkx2.1) and Ttf2 (Foxe1)) was slightly increased or unchanged in the thyroid glands [27]. A reasonable explanation about the difference in thyroid phenotype between the mouse and zebrafish models may be due to the time difference in Glis3 expression during embryonic development [29]. As a result, the current studies suggested that Glis3 might regulate multiple levels of thyroid function.

In this study, we identified two novel heterozygous missense mutations of GLIS3 in two unrelated patients among 50 Chinese patients with CH and TD. They are both athyreosis, only patient carried G904R mutation presenting with abdominal distention and lethargy when diagnosed with CH. This is different from Fu C et al 's study, the patient with GLIS3 heterozygous missense variants (c.2159G >A /p.R720Q) has an increased size thyroid gland, and also carried a compound heterozygous DUOX2 variant inherited from parents [30]. However, due to the rejection of the children's parents, we did not conduct a family study. As both mutations we detected are located in the key C-terminal transcriptional activation region of GLIS3 and conserved across various species, we explored the effects of these mutations on the function of GLIS3 protein and its ability to activate transcription. We found that both mutant transcripts were expressed at a lower level than the WT mRNA, whereas only the G904R mutation caused lower expression of GLIS3 protein. In addition, G904R had impaired transactivation, indicating that this mutant failed to activate transcription in the GlisBS region. This may provide an explanation for why the patient carrying G904R mutation featured TD. We also found the mutation had no dominant-negative effect on the WT protein, which suggests that a nonfunctional dimer is not formed between the mutant protein and the WT protein, so that the function of the WT protein is not inhibited. Another mutation (P836Q) promotes the reporter gene expression unaffected, it may cause the phenotype of TD through other means. Our research also showed that different mutations of GLIS3 can cause CH with TD through different pathogenic mechanisms.

Conclusion

In summary, our findings broaden the GLIS3 mutation spectrum and provide a possible explanation for how GLIS3 defects cause TD. These two mutations (p.G904R; p.P836Q) were reported for the first time in the Chinese population. Since only 50 CH children were selected in the case group in this study, the mutation rate of GLIS3 gene is not representative. We are currently using high-throughput sequencing technology to expand sample collection across the country, further screening GLIS3 gene mutation points to provide a more reliable GLIS3 gene mutation rate.

Declarations

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Compliance with Ethical Standards

Conflict of Interest Statement

The authors have no conflicts of interest relevant to this article.

Author Contributions

FW, ML, RM and SL conceived and designed the study; XL, YJ, CS contributed to the collection of subjects; JL, CS did the experiment and wrote the manuscript; FW, YJ and SL revised the manuscript and eventually approved it by all authors.

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Figures

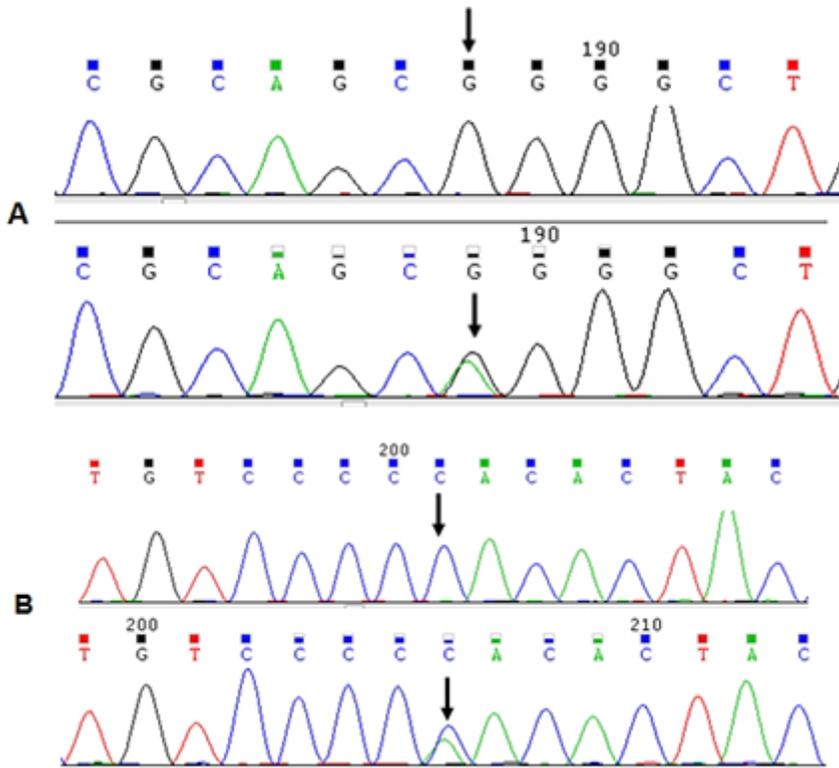


Figure 1

Partial GLIS3 sequences from patients with missense mutations and individuals with the WT sequence. A. Arrow indicates a heterozygous G>A transition at nucleotide 2710 of the coding sequence of GLIS3 (c.2710G>A). B. Arrow indicates a heterozygous C>A transition at nucleotide 2507 of the coding sequence of GLIS3 (c. 2507C>A).

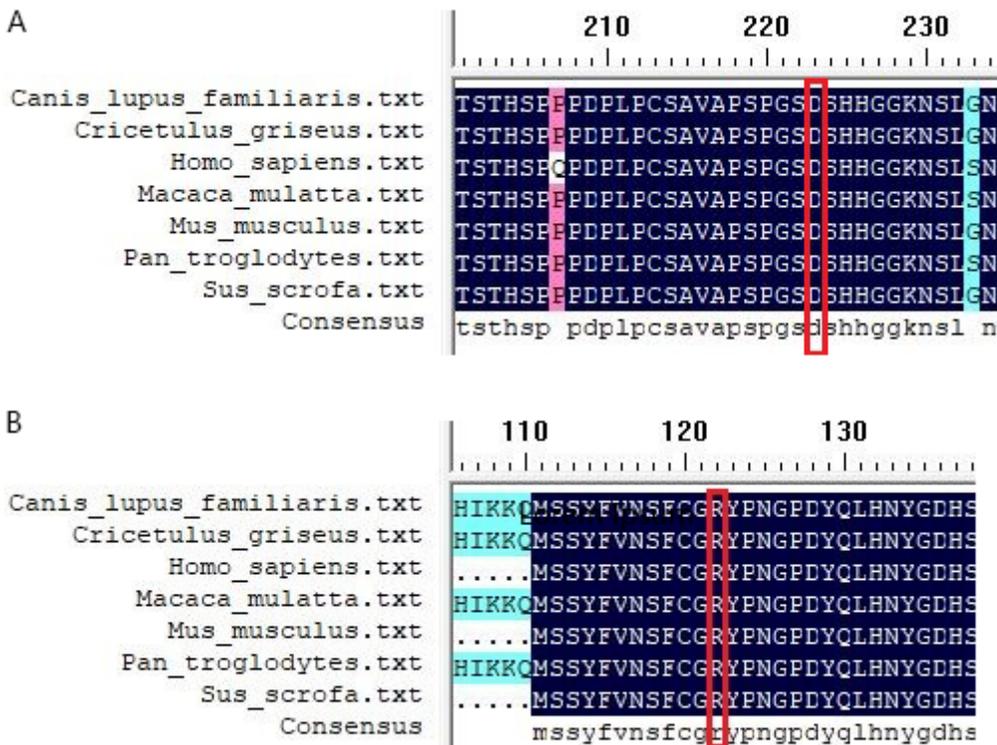


Figure 2

Comparison of the GLIS3 protein amino acid sequences across different species. Red rectangular frames indicate the locations of G904R and P836Q.

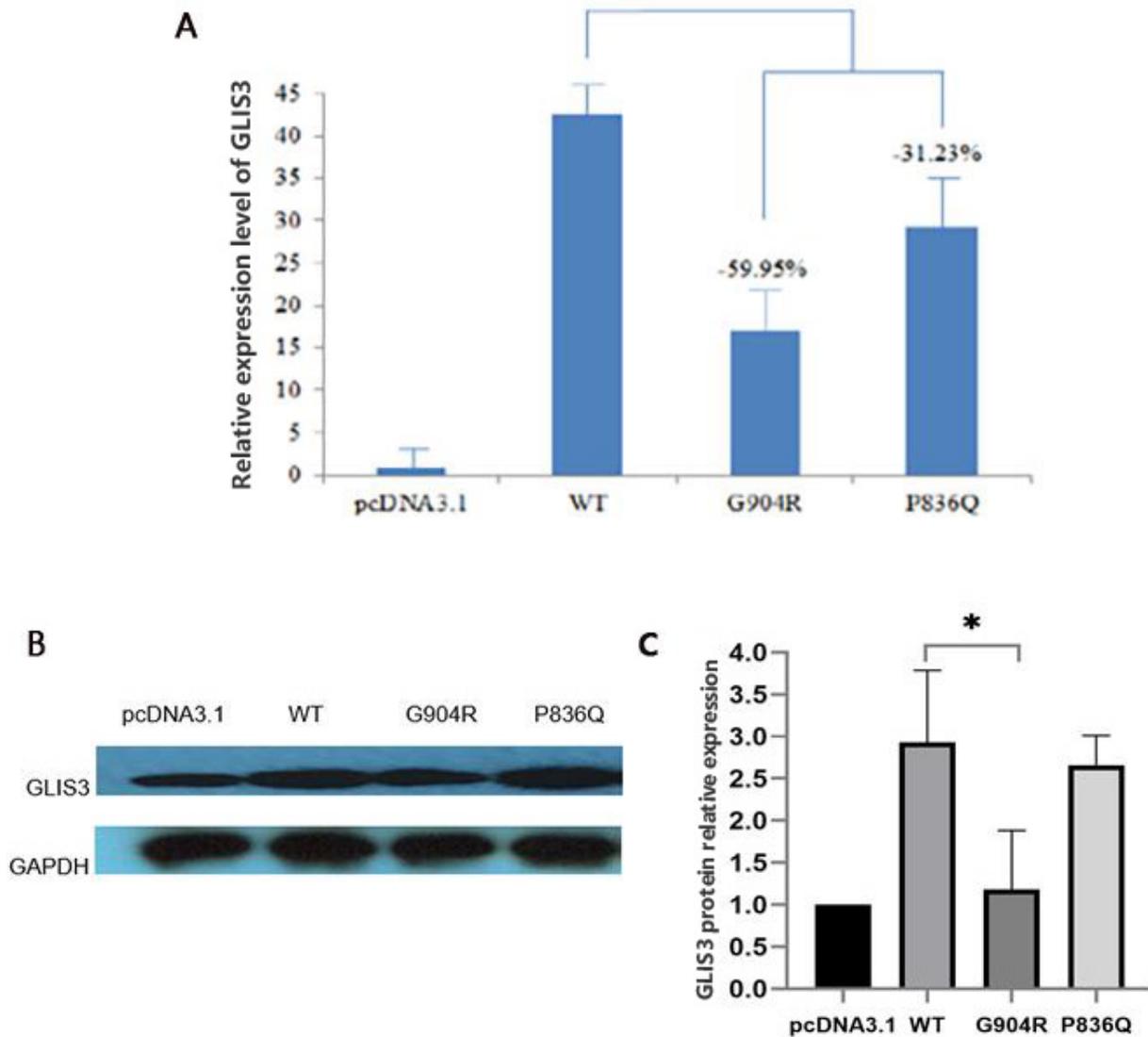


Figure 3

Expression of WT and mutant GLIS3 in transfected 293T cells. A. At 24 h after transfection, we observed the mRNA expression of WT and mutant GLIS3. B. G904R mutation, but not the P836Q mutation, reduced the expression of GLIS3 compared to that with WT GLIS3. C. Histograms represent the relative intensity of GLIS3. GAPDH was used as a quantitative protein control.

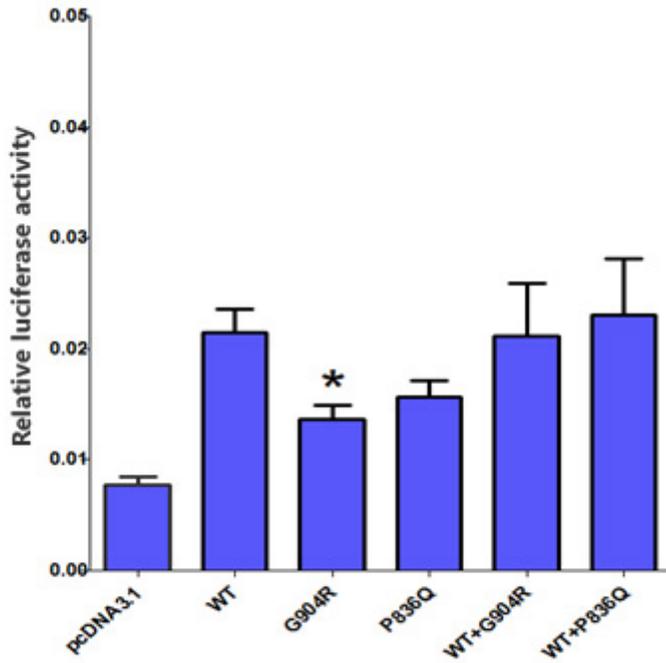


Figure 4

Luciferase activity driven by mutant G904R and P836Q GLIS3 in 293T cells co-transfected with the GLIS3 expression vector and GlisBS-Luciferase vector. * represents $p < 0.05$ compared with WT. The data are expressed as the mean \pm SD from at least three independent experiments.