

ORIGINAL ARTICLE

Functional characterization of the FOXP4 variants support the loss of function as a Mechanism of *FOXP4*-associated disease

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ABSTRACT

Background: We recently reported 10 heterozygous missense and loss-of-function *FOXP4* variants that cause neurodevelopmental disease and multiple congenital abnormalities in 13 patients. However, the functional impact of the reported variants is not yet characterized.

Methods: The variants were constructed and introduced into expression vectors and then transfected into cell lines along with an empty control. Western blotting was used to assess mutant FOXP4 protein expression.

Results: Western blot analysis confirmed that all heterozygous *FOXP4* variants resulted in very low or undetectable levels of the overexpressed mutant FOXP4. Similarly, homozygous *FOXP4* variants resulted in very low or undetectable levels of the overexpressed mutant proteins, consistent with the predicted impact of the frameshift variants. These data support the pathogenicity of these variants by causing a loss-of-function effect on protein expression.

Conclusion: This study provides a functional characterization supporting that autosomal recessive forms of FOXP4-related disease are likely caused by loss of function. These data warrant a future study to investigate how the same disease mechanism produces different inheritance patterns.

Keywords: *FOXP4*, heterozygous, homozygous, recessive, dominant, loss of function, neurodevelopmental.

Introduction

The *FOXP4* gene encodes a DNA-binding transcription factor that primarily regulates gene expression by modulating the transcription machinery in the cell nucleus. During embryonic development, this protein plays a vital role in directing tissue- and cell-type-specific programs, particularly in the normal formation of the heart, lungs, gut, and central nervous system (1). Heterozygous *FOXP4* variants in humans have been reported in association with autosomal dominant neurodevelopmental disorders with variable abnormalities, including diaphragmatic hernia, skeletal abnormalities, and other features (2–4). The reported variants include missense, frameshift, and nonsense variants (4,5). Functional characterization of these variants confirmed that loss of function is the disease mechanism (5).

Recently, we reported 11 families with dominant and novel recessive forms of FOXP4-associated disease presenting with short stature, failure to thrive, dysmorphic features, neurodevelopmental disease, congenital heart defects, pulmonary, and gastrointestinal abnormalities

(6). Recessive FOXP4 disease was far more severe than the dominant form. All the reported patients presented with failure to thrive, short stature, and dysmorphic features. In those patients, we identified 10 heterozygous and homozygous *FOXP4* variants, including missense, frameshift, nonsense, and splicing variants. All heterozygous and homozygous variants recruited in this study were absent or had low allele frequency in controls, were confirmed as *de novo*/inherited from affected parents if heterozygous, and were predicted to undergo

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nonsense-mediated decay or disrupt canonical splicing. However, the functional impact of the reported variants was not characterized.

In this study, we functionally characterized the predicted impact of the reported *FOXP4* variants by Western blotting. We show that all heterozygous and homozygous *FOXP4* variants resulted in very low or undetectable levels of the overexpressed mutant FOXP4.

Materials and Methods

Variant selection for expression studies

Out of the 10 reported variants, we only characterized 6 variants (Table 1), excluding four variants from the characterization. One of these four variants (p.Ala514Thr) was previously characterized (5). The other three variants included two splicing variants and one missense variant (p.Arg527Cys) that were not characterized in this study

due to our inability to characterize these variants without patients' samples.

Plasmids construction and mutagenesis

The Flag-tagged FOXP4 (Flag-FOXP4) plasmid was obtained from Addgene (Cat# 153148). To create site-specific mutants of the FOXP4 gene, we used GenScript's custom mutagenesis service to construct six plasmids expressing the *FOXP4* variants. The constructed plasmids were Sanger sequenced to verify them.

Plasmids transfection

HEK-293 cells were cultured in Dulbecco's modified Eagle medium (DMEM) supplemented with 10% fetal bovine serum (FBS) and maintained at 37°C in a humidified atmosphere with 5% CO₂. HEK-293 cells seeded in 6-well plates were transfected using Lipofectamine 2000 reagent (Thermo Fisher Scientific, USA) according to

Table 1. List of the selected *FOXP4* variants to be tested by Western blot.

Variant (NM_001012426.2)	Protein Consequence	Variant Type	Zygoty in patient
c.10G>T	p.Glu4*	Nonsense	Heterozygous
c.618del	p.Gln206Hisfs*161	Frameshift	Heterozygous
c.511-558_1483del	p.Ala171Thrfs*57	Frameshift	Heterozygous
c.1672G>A	p.Val558Met	Missense	Heterozygous
c.229del	p.Leu77Cysfs*8	Frameshift	Homozygous
c.815del	p.Leu272Profs*95	Frameshift	Homozygous

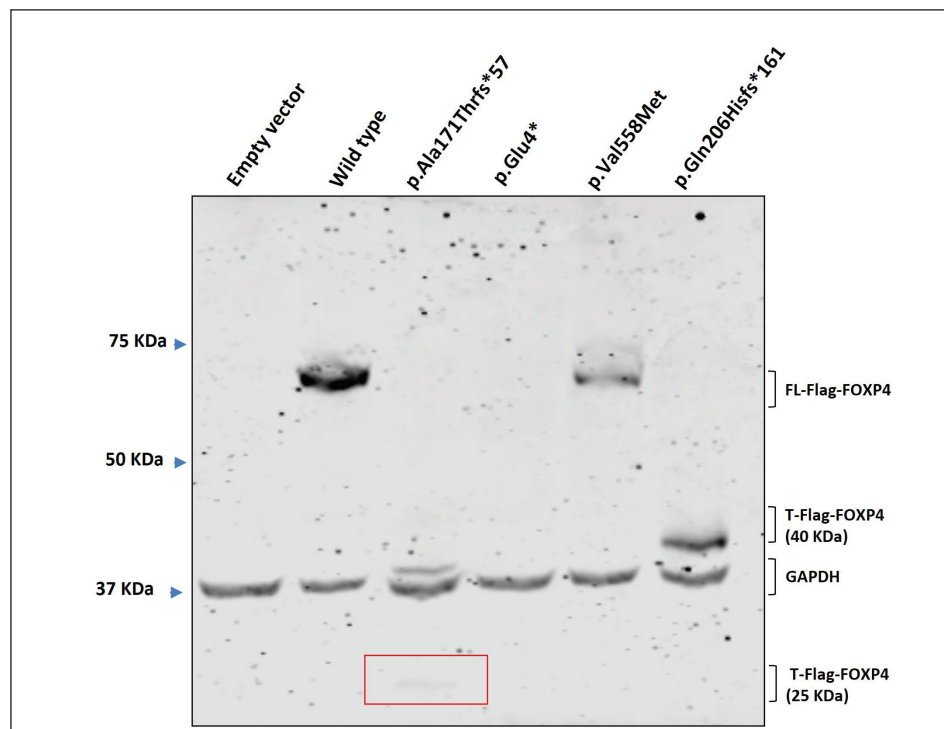


Figure 1. The impact of the heterozygous variants on protein expression.

Western blot analysis assessing the effects of the detected heterozygous missense and LoF variants on FOXP4 expression using a Flag-tagged expression vector. GAPDH was used as a loading control. WT: wild type; NC: negative control; FL: full-length Flag-tag FOXP4; T: truncated Flag-tagged FOXP4. The experiments were performed in triplicate.

the manufacturer's protocol. The transfection mixture was added dropwise to the cells in serum-free DMEM and then incubated for 4-6 hours. We then replaced the medium with complete DMEM containing 10% FBS and cultured cells for 72 hours before harvesting.

Results

Expression studies of the heterozygous *FOXP4* variants

To test the variants' effect on *FOXP4* expression, we generated plasmid constructs expressing the missense and loss-of-function (LoF) variants (Table 1). All tested variants, including the missense variant, resulted in very low or undetectable levels of mutant Flag-tagged *FOXP4* protein, consistent with a LoF effect (Figure 1). The p.Gln206Hisfs*161 and p.Ala171Thrfs*57 variants produced truncated forms of the Flag-tagged *FOXP4* at ~40 and 25 kDa, respectively, as expected based on the position of the premature stop codons.

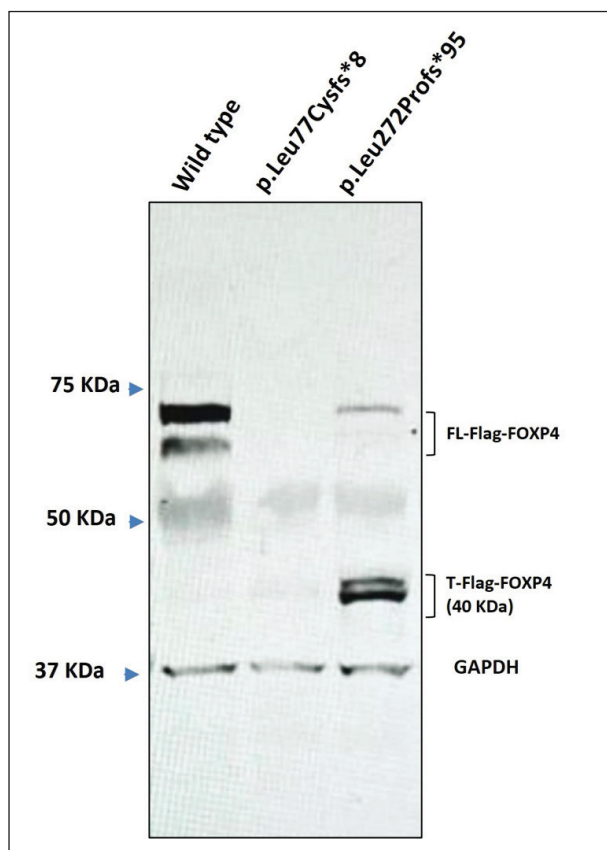


Figure 2. The impact of the identified homozygous frameshift variants on protein expression.

Western blot analysis assessing the effects of the detected homozygous frameshift variants on *FOXP4* expression using a Flag-tagged expression vector. 2. The wild-type lane showed two distinct bands between 50 kDa and 75 kDa, likely representing alternative isoforms of *FOXP4*. GAPDH was used as a loading control. WT: wild type; NC: negative control; FL: full-length Flag-tagged *FOXP4*; T: truncated Flag-tagged *FOXP4*. The experiments were performed in triplicate.

Based on their absence in controls, *de novo* status, segregation with disease, conservation, and observed and predicted LoF impact on *FOXP4* expression, we classified all variants as likely pathogenic except the recurrent p.(Ala514Thr) variant, which we classified as pathogenic according to the standard guidelines by the American College of Medical Genetics.

Expression studies of the homozygous *FOXP4* variants

To test the effects of both variants on *FOXP4* expression, we generated plasmid constructs expressing the mutant *FOXP4* proteins. Both variants resulted in very low or undetectable levels of the mutant Flag-tagged *FOXP4* protein (Figure 2). The p.Leu272Profs*95 variant produced a truncated Flag-tagged *FOXP4* of ~40.4 kDa, as expected based on the position of the premature stop codon. Based on allele frequency, segregation with disease, conservation, and the observed and predicted LoF impact on *FOXP4* expression, we classified both variants as likely pathogenic.

Discussion

We recently reported 11 families with dominant and recessive forms of *FOXP4* disease presenting with short stature, dysmorphic features, neurodevelopmental, heart, and congenital abnormalities (6). In those patients, we identified 10 heterozygous and homozygous *FOXP4* variants, including missense, frameshift, nonsense, and splicing variants. In this study, we support the pathogenicity of six missense, nonsense, and frameshift variants reported in association with *FOXP4*-related dominant and recessive diseases. All the identified variants resulted in no or low protein expression, consistent with a loss-of-function effect.

Functional characterization of previously reported *FOXP4* variants associated with autosomal dominant disease showed that rare heterozygous variants disrupt transcriptional function via distinct mechanistic pathways (5). Through dual-luciferase reporter assays, the loss of function was confirmed in all mutations residing within the evolutionarily conserved forkhead box DNA-binding domain, which selectively abolished the protein's native target-gene transcriptional repressor activity. Furthermore, immunofluorescence studies identified aberrant subcellular localization patterns in a key subset of these forkhead domain missense variants, demonstrating significant nuclear exclusion where the mutant proteins failed to compartmentalize into the nucleus and instead aggregated within the cytoplasm. Most importantly, while variants positioned outside of the functional domains retained normal repressor profiles and wild-type cellular localization, the pathogenic domain variants maintained their intrinsic ability to homo- and heterodimerize with wild-type FOXP partners. This biochemically highlights a molecular cascade where haploinsufficiency or potential dominant-negative disruptions undermine normal downstream transcriptional networks and cellular pathways. Consistently, our data support loss of function as a disease mechanism associated with

FOXP4-related disease. A limitation of our analysis is that overexpression studies are not an appropriate approach to model nonsense-mediated decay in nonsense and frameshift variants. However, the overexpressed proteins carrying those variants were very low or undetectable, suggesting that the truncated proteins, even if they escape nonsense-mediated decay, are not stable.

In conclusion, our study supports the disease mechanism of the *FOXP4*-related dominant and recessive disease. Follow-up functional studies are warranted to further characterize the biological mechanisms by which heterozygous and homozygous variants in *FOXP4* can cause disease through loss of function.

Declaration of conflicting interests

The author of this article has no affiliations with or involvement in any organization or entity with any financial interest or nonfinancial interest in the subject matter or materials discussed in this manuscript.

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Author contributions

Naif A.M. Almontashiri conceived and coordinated the project, conducted and interpreted the experiments and data, and wrote the manuscript.

Data availability

All data generated or analyzed during this study are included in this published article and its supplementary information files.

Ethical approval

The study protocol was approved by the Medical Laboratory Institutional Review Board (IRB) at Taibah University (MLT-2019-07).

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