Molecular Characterization of Novel *FOXN1* Mutations Causal to Thymic Aplasias in Human Patients

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ABSTRACT

The Forkhead Box N1 (FOXN1) transcription factor plays a crucial role in thymic epithelial cell development. Humans and mice harboring FOXN1 mutations have a profound T-cell deficiency caused by their thymic aplasia. They also present with alopecia and nail dystrophy. Recently, two patients were identified with T-cell immunodeficiency. Both patients have normal hair and nailbed development. Genetic workup revealed that each patient carried distinct compound heterozygous mutations in FOXN1 not previously reported. Molecular characterization of these FOXN1 mutations will provide new insight into how this transcription factor functions in thymus development.

To characterize these mutations, CRISPR/Cas9 technologies were used to create similar compound heterozygous mutations in mouse models. The mice are currently being intercrossed to determine the impact of these novel FOXN1 mutations on thymus development. To determine how these mutations impact FOXN1 function, we undertook transcriptional reporter assays. Preliminary results suggest only one of these mutations led to loss of transcriptional activity. Western blot analysis indicated that this mutation led to a truncation of the protein. Further experiments including co-immunoprecipitation assays, transcriptome analyses, and functional studies will reveal how these compound heterozygous mutations impact the functions of FOXN1.

Findings from this study may lay the foundation for novel therapeutic strategies at restoring thymopoiesis in a number of distinct clinical settings. These can include patients undergoing radiation treatment, chemotherapy, and in any other conditions that can lead to a thymic aplasia.

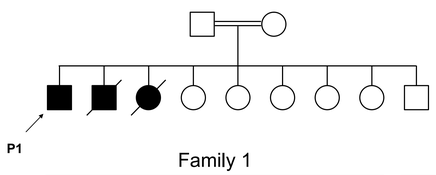
INTRODUCTION

Primary immunodeficiencies (PIDs) form a diverse group of genetic disorders that lead to altered immune function. They range from deficiencies of T cells that develop in the thymus, B cells responsible for antibody production, or a combination of the two. Most of these PIDs result from mutations in genes key to the development of the T and/or B cells. In the current study, we identified two unrelated children who presented with a T-cell lymphopenia. Exome sequencing revealed that these two patients harbored distinct compound heterozygous mutations in a transcription factor termed FOXN1. FOXN1 is expressed in epithelial cells and not hematopoietic cells. As a consequence, bone marrow transplantation is not a clinical option for the patients.

FOXN1 plays an essential role in thymic epithelial cell (TEC) development. TECs are the cell population that support T cell development. *Foxn1* was first identified as causal to the nude mouse phenotype (lack of hair), and these mice were immunocompromised because of an absence of T cells due to the loss of TECs. Humans carrying *FOXN1* deletions phenocopy the nude mice.

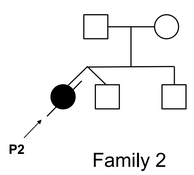
Unlike most patients characterized to date who have a homozygous mutations in *FOXN1*, the two patients in the current study carry compound heterozygous mutations in *FOXN1*. This leads to a unique phenotype of T-cell lymphopenia with normal hair follicle development and nail beds. To test if these mutations cause the immunodeficiency, mammalian expression vectors containing the mouse *Foxn1* gene with analogous mutations were created to assess the effect of the mutations on transcriptional activation and final protein product size. Additionally, CRISPR/Cas9 technologies were utilized to create mouse models with similar compound heterozygous mutations in *Foxn1* as in the patients.

Figure 1. Clinical Presentation



Family 1

- Middle Eastern ancestry
- Consanguinity: parents are 1st cousins
- Proband: T-cell deficiency, no alopecia, no nail dystrophy

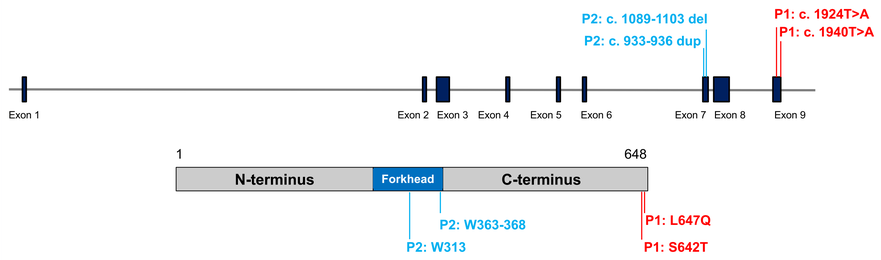


Family 2

- African American ancestry
- Proband: T-cell deficiency, no alopecia, no nail dystrophy
  - Died of respiratory infection
- Twin: no alopecia, nail dystrophy, or T-cell deficiency

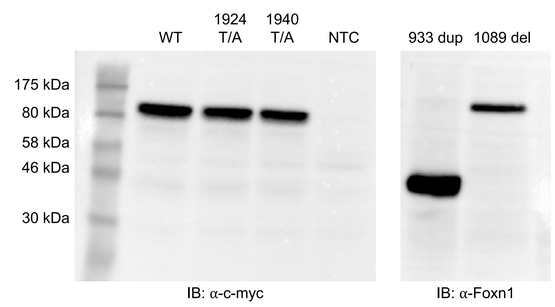
RESULTS

Figure 2. Mapping of Compound Heterozygous *FOXN1* Mutations in Two SCID Patients



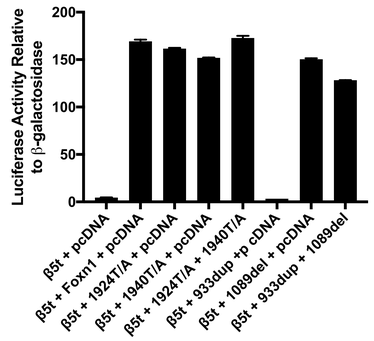
- Patient 1 (P1) harbors two single nucleotide substitutions(T/A) near the C-terminus of Foxn1
- Patient 2 (P2) carries a 15-nucleotide deletion and a 4-nucleotide duplication in the Forkhead domain of Foxn1

Figure 3. Western Blot of HEK 293T Cells Transfected with FOXN1 Constructs



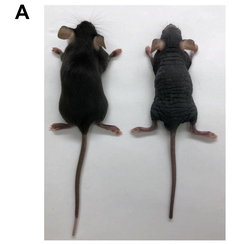
- The four-nucleotide repeat from the 933 dup created a frameshift that led to a truncation of the final protein product
- All other mutations did not alter the size of the Foxn1 protein compared to wild-type (WT)

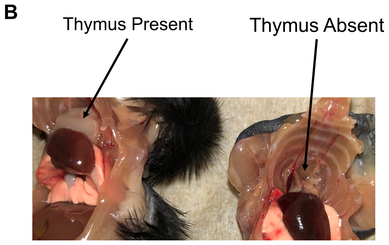
Figure 4. Luciferase Reporter Assay of *FOXN1* Constructs



- The 933dup mutation completely abrogated the ability of Foxn1 to bind to the β5t promoter and activate transcription
- The other mutations did not appear to significantly alter the ability of Foxn1 to induce transcription
- n = 1; error bars represent SD of duplicate well values

Figure 5. 933 dup/933 dup Mice Exhibit Partial Nudity and Athymia





- Figure 3A. The 933dup/933dup mouse exhibits impaired fur growth compared to its wild-type littermate.
- Figure 3B. The 933dup/933dup mouse has no thymus compared to its wild-type littermate.
- Compound het mice with the 1924/1940 equivalent mutations have normal thymopoiesis (not shown)

CONCLUSIONS

- The 933-936 duplication in the Forkhead domain of Foxn1 creates a frameshift that introduces a new codon sequence that prematurely truncates the protein
- The 933-936 nucleotide duplication is sufficient to nullify Foxn1's ability to activate transcription.
- Mice homozygous for the 933-936 duplication are athymic and have an impaired ability to grow fur.
- The 933-936 duplication is a promising candidate for explaining the SCID phenotype in Patients 1 and 2
- The 1924/1940 mutations do not significantly alter the transcriptional activation ability of Foxn1 and may not be causal to the T-cell deficiency seen in the identified patients
- The 1089-1103 deletion may still cause athymia through a mechanism other than directly impairing transcriptional activation—will determine in mouse crosses

FUTURE DIRECTIONS

- Complete the mouse crosses to replicate the compound heterozygous genotypes found in the patients
- Re-analyze patient and family genomes to find other mutations that may alter *FOXN1* transcription or protein structure
- Explore what role *FOXN1* plays in hair follicle and nailbed development and why these processes were preserved in the patients with the compound heterozygous mutations near the DNA binding domain
- Co-immunoprecipitation studies with compound heterozygous FOXN1 mutations

ACKNOWLEDGEMENTS

- van Oers Lab
- UT Southwestern Medical Student Summer Research Program
- The patients and their families
- Georg Hollander group for providing the β5t luciferase plasmid