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LADD syndrome with glaucoma is caused by a novel gene

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Purpose: Lacrimo-auriculo-dento-digital (LADD) syndrome is an autosomal dominant disorder displaying variable expression of multiple congenital anomalies including hypoplasia or aplasia of the lacrimal and salivary systems causing abnormal tearing and dry mouth. Mutations in the *FGF10*, *FGFR2*, and *FGFR3* genes were found to cause some cases of LADD syndrome in prior genetic studies. The goal of this study is to identify the genetic basis of a case of LADD syndrome with glaucoma and thin central corneal thickness (CCT).

Methods: Whole exome sequencing was performed, and previously described disease-causing genes (*FGF10, FGFR2,* and *FGFR3*) were first evaluated for mutations. Fifty-eight additional prioritized candidate genes were identified by searching gene annotations for features of LADD syndrome. The potential pathogenicity of the identified mutations was assessed by determining their frequency in large public exome databases; through sequence analysis using the Blosum62 matrix, PolyPhen2, and SIFT algorithms; and through homology analyses. A structural analysis of the effects of the top candidate mutation in tumor protein 63 (TP63) was also conducted by superimposing the mutation over the solved crystal structure.

Results: No mutations were detected in *FGF10*, *FGFR2*, or *FGFR3*. The LADD syndrome patient's exome data was searched for mutations in the 58 candidate genes and only one mutation was detected, an Arg343Trp mutation in the tumor protein 63 (*TP63*) gene. This *TP63* mutation is absent from the gnomAD sequence database. Analysis of the Arg343Trp mutation with Blosum62, PolyPhen2, and SIFT all suggest it is pathogenic. This arginine residue is highly conserved in orthologous genes. Finally, crystal structure analysis showed that the Arg343Trp mutation causes a significant alteration in the structure of TP63's DNA binding domain.

Conclusions: We report a patient with no mutations in known LADD syndrome genes (*FGF10*, *FGFR2*, and *FGFR3*). Our analysis provides strong evidence that the Arg343Trp mutation in *TP63* caused LADD syndrome in our patient and that *TP63* is a fourth gene contributing to this condition. *TP63* encodes a transcription factor involved in the development and differentiation of tissues affected by LADD syndrome. These data suggest that *TP63* is a novel LADD syndrome gene and may also influence corneal thickness and risk for open-angle glaucoma.

Lacrimo-auriculo-dento-digital (LADD) syndrome (OMIM 149730), also known as Levy-Hollister syndrome, is an autosomal dominant disorder displaying variable expression of multiple congenital anomalies. Levy first described LADD syndrome in 1967 as an isolated case with an absent lacrimal system, dry mouth, malformed ears, and dental, arm, and digital abnormalities [1]. Tearing is a central feature in LADD syndrome due to abnormalities of the lacrimal system that may range from hypoplasia to aplasia of puncta, ducts, and glands. Hypoplasia or aplasia of the salivary glands and ducts may also cause dry mouth. Patients with LADD syndrome have external ear abnormalities that include low-set, cup-shaped auricles. Sensorineural, conductive, and mixed hearing deficits may also be present [2]. Dental anomalies in LADD syndrome include microdontia, hypodontia, and enamel dysplasia. Finally, patients with LADD syndrome may have a variety of distal limb and digit abnormalities, such as hypoplastic thumbs and radii, lateral or medial bending of the digits (clinodactyly), and webbing or fusion of the digits (syndactyly) [3,4]. Most LADD cases have ocular involvement. Lacrimal system defects are present in 71% of patients with LADD syndrome and subsequent tear deficiency and dry eye may cause chronic keratoconjunctivitis or corneal ulcerations in 64% of cases [5]. Limbal stem cell deficiency has also been associated with LADD syndrome, which may predispose the patient to corneal epithelial erosions, neovascularization, and hypoesthesia [5,6].

We recently described a 30-year-old male patient that had typical features of LADD syndrome as well as thin corneas and open angle glaucoma [7]. He had classic abnormalities of his lacrimal system, ears, teeth, and fingers. He also had limbal corneal stem cell deficiency as has been previously

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Table 1. Analysis of the *TP63* mutation Arg343Trp.

Gene	Detected Coding Sequence Variations	gnomAD Allele Frequency	Blosum62 Score	PolyPhen Score	SIFT Score
TP63	Arg343Trp	0%	−3	Probably Damaging	Deleterious

The Arg343Trp mutation was not observed in the gnomAD database of over 126,000 human exomes suggesting that it is essentially absent from unselected populations. Mutation analyses with Blosum62, PolyPhen2, and SIFT all suggest that the Arg343Trp mutation is likely pathogenic.

reported [5]. This patient had additional corneal abnormalities and open angle glaucoma, which have not been previously identified as part of LADD syndrome. His central corneas were very thin at 387 µm right eye (OD) and 334 µm left eye (OS) and he also had severe irregular astigmatism and corneal neovascularization. Moreover, this LADD syndrome patient had classic signs of open angle glaucoma including glaucomatous optic neuropathy and corresponding visual field defects [7].

Genetic studies of several LADD syndrome pedigrees led to the identification of disease-causing mutations in the fibroblast growth factor receptor 2 (*FGFR2*) gene [2]. Linkage analysis of these families mapped a LADD syndrome gene to chromosome 10q26, which encompassed the *FGFR2* gene. DNA sequencing subsequently detected *FGFR2* mutations in three LADD syndrome pedigrees and in one sporadic LADD syndrome case. However, no *FGFR2* mutations were identified with genetic studies of two additional LADD syndrome pedigrees, indicating that this condition is heterogeneous and may be caused by more than one gene. These pedigrees were consequently tested for mutations in other genes in the *FGFR* gene family that are related to *FGFR2*. A mutation in the fibroblast growth factor receptor 3 (*FGFR3*) gene was discovered in one pedigree, while a mutation in the fibroblast growth factor 10 (*FGF10*) gene was detected in the other pedigree [2]. Mutations in *FGFR2*, *FGFR3*, and *FGF10* are plausible causes of LADD syndrome because defects in these genes have been previously reported in humans and transgenic mice with similar abnormalities of the lacrimal and salivary glands as well as limb and digit anomalies [8-10]. These data indicate that the cluster of mutations in three members of the fibroblast growth factor (FGF) signaling pathway have central importance in the pathogenesis of LADD syndrome. In this report, we describe research to identify the genetic cause of our patient's case of LADD syndrome that includes thin corneas and open angle glaucoma among its clinical features.

METHODS

Patient enrollment: The study was approved by the Institutional Review Board at the University of Iowa and adhered to the tenets of the Declaration of Helsinki and the ARVO statement on human subjects. Clinical assessments of this patient were previously reported [7]. After the patient was enrolled in the study, a blood sample was drawn and a skin biopsy was obtained as previously described [11,12]. DNA was isolated from a blood sample using the QIAamp Blood Maxi Kit (Qiagen, Valencia, CA) and fibroblast cells were isolated and cultured from the skin biopsy using standard techniques [13].

DNA sequencing, filtering algorithms, and mutation analyses: Whole exome sequencing was performed on the LADD syndrome patient's DNA with the Agilent v5 kit (Santa Clara, CA) and an Illumina Hiseq2500 next-generation DNA sequencer (San Diego, CA) using the manufacturers' protocols. The Burrows-Wheeler aligner (BWA) was used to align DNA sequencing reads to the human reference genome [14]. DNA sequence variations were identified using the Genome Analysis Toolkit (GATK) [15], CoNIFER [16], and a custom sequence analysis and annotation pipeline (Wynn Institute for Vision Research, Iowa City, IA).

First, DNA variations in the coding sequences or the canonical splicing sites of known LADD genes (*FGFR2*, *FGFR3*, and *FGF10*) were identified and analyzed. Next, variations present in the entire exome were filtered and prioritized using previously described methods [17,18]. Variants were filtered to identify those that altered the coding sequences or canonical splicing sites of known genes. Variants that were previously reported in the public database of the Exome Sequencing Project (ESP) [19] were judged to be too common to cause LADD syndrome and were excluded. Candidate mutations and genes were further filtered based on their annotation and reported function using an advanced search of the GeneCards Human Gene Database [20] with input keywords "lacrimal," "auricular," "dental," "digital," "salivary," and "morphogenesis." Top candidate mutations were further evaluated by investigating their prevalence in

TP63											
Human:	R	C	F	E	A	**R**	I	C	A	C	P
Rhesus:	R	C	F	E	A	**R**	I	C	A	C	P
Mouse:	R	C	F	E	A	**R**	I	C	A	C	P
Dog:	R	C	F	E	A	**R**	I	C	A	C	P
Elephant:	R	C	F	E	A	**R**	I	C	A	C	P
Chicken:	R	C	F	E	A	**R**	I	C	A	C	P
Frog:	R	C	F	E	A	**R**	I	C	A	C	P
Zebrafish:	R	C	F	E	A	**R**	I	C	A	C	P
Lamprey:	R	C	F	E	A	**R**	I	C	A	C	P

Figure 1. TP63 amino acid sequence conservation. The arginine amino acid that is altered by the Arg343Trp mutation (indicated with a bold "R") is highly conserved in the TP63 transcription factor across many species (indicated by the gray shading), which suggests that it may have a conserved role in the protein's function.

the public database curated by the Genome Aggregation Database (gnomAD) [21]. The pathogenicity of these candidate mutations was also estimated using the BLOSUM62 matrix [22], PolyPhen2 [23], and SIFT [24] analyses. For the top candidate, *TP63*, the Arg343Trp mutation was assessed for alteration of conserved protein sequences by constructing homology tables using the UCSC browser (genome.ucsc.edu) [25]. The likely effects of the Arg343Trp mutation on TP63 protein structure were investigated based on a wild-type structure determined by X-ray crystallography (3US2) [26]. The analysis began by mutating the Arg343 residue of the crystal structure to Trp, followed by repacking of nearby residues using a rotamer optimization algorithm [27] and a potential energy function defined by the polarizable atomic multipole AMOEBA force field [28] in the program Force Field X [29].

RESULTS

We recently reported a patient with LADD syndrome that included early-onset open angle glaucoma and remarkably thin corneas (central corneal thickness of 387 µm OD and 334 µm OS) [7]. This patient was of non-Hispanic Caucasian and Asian heritage. Reportedly, neither the patient's parents nor other family members are affected by LADD syndrome, but were unavailable for examination or inclusion in our genetic study.

In this report, we searched for the genetic cause of our patient's LADD syndrome using whole exome sequencing. In the first stage of our analysis, we searched our patient's exome data for mutations in the three known LADD syndrome genes, *FGFR2*, *FGFR3*, and *FGF10*. A heterozygous, synonymous variant, Leu703Leu, was discovered in *FGFR2*. However, no plausible disease-causing mutations were detected in these genes.

As a second stage, we identified genes previously annotated to influence the structures altered in LADD syndrome using the keywords "lacrimal," "auricular," "dental," "digital," "salivary," and "morphogenesis" as potential causes of this disease using the online resource GeneCards [20]. A total of 58 candidate genes were prioritized for analysis (Appendix 1). Three of the top four candidates on this gene list were the previously identified LADD syndrome genes—*FGFR2*, *FGFR3*, and *FGF10*—providing some validation for our prioritization approach. The fourth top candidate on the list was tumor protein 63 (*TP63*).

When we examined our patient's exome, we detected a plausible disease-causing mutation in only one of the 58 candidate genes. A missense mutation, Arg343Trp, was identified in *TP63*. This mutation is not present in the exomes of more than 126,000 humans (gnomAD database) [21], including over 24,000 Asian and 56,000 Non-Finnish Europeans who have the most relevant heritage for use as a control data set for this study's LADD syndrome patient. These data suggest that the *TP63* Arg343Trp mutation is extremely rare, as would be expected for a mutation thought to cause LADD syndrome. The potential pathogenicity of the *TP63* mutation was further assessed with several mutation analysis algorithms (Table 1). The Arg343Trp mutation generated a Blosum62 score of −3, a PolyPhen2 score of "probably damaging," and a SIFT score of "deleterious." Moreover, the Arg343Trp mutation alters an amino acid that has been highly conserved in the encoded TP63 protein across many diverse species (Figure 1). Finally, molecular modeling of the TP63 protein based on its solved crystal structure was conducted both with and without the Arg343Trp mutation using structural refinement algorithms based on the polarizable AMOEBA force field. These analyses showed that the Arg343Trp mutation caused a dramatic conformational alteration of TP63 that would likely be deleterious to its DNA-binding domain (Figure 2).

DISCUSSION

Our genetic studies of a patient with LADD syndrome, glaucoma, and thin corneas detected no mutations in the previously reported LADD syndrome genes, *FGF10*, *FGFR2*, and *FGFR3*. Subsequently, whole exome sequencing identified a novel Arg343Trp mutation in the *TP63* gene as a potential cause of LADD syndrome. This *TP63* mutation is exceedingly rare and was not detected in over 126,000 individuals in the gnomAD database. Moreover, a range of mutation analysis approaches (Blosum62, SIFT, PolyPhen2), homology studies, and protein modeling all suggest that the Arg343Trp mutation is likely pathogenic. Additional support for the pathogenicity of the *TP63* mutation might be obtained by examining our patient's family with genetic tests. Since both of his parents are reportedly unaffected by LADD syndrome, one might expect them to test negative for the Arg343Trp mutation. Such test results would indicate that our patient carries a *de novo TP63* mutation and would be further evidence that the Arg343Trp mutation is disease-causing. Unfortunately, neither parents were available for examination or enrollment in our study.

TP63 encodes a transcription factor that is expressed in ectodermal tissue during development, and mutation of this gene is a highly plausible cause of the congenital features of LADD syndrome. Mutation of *TP63* has been previously associated with the spectrum of developmental diseases

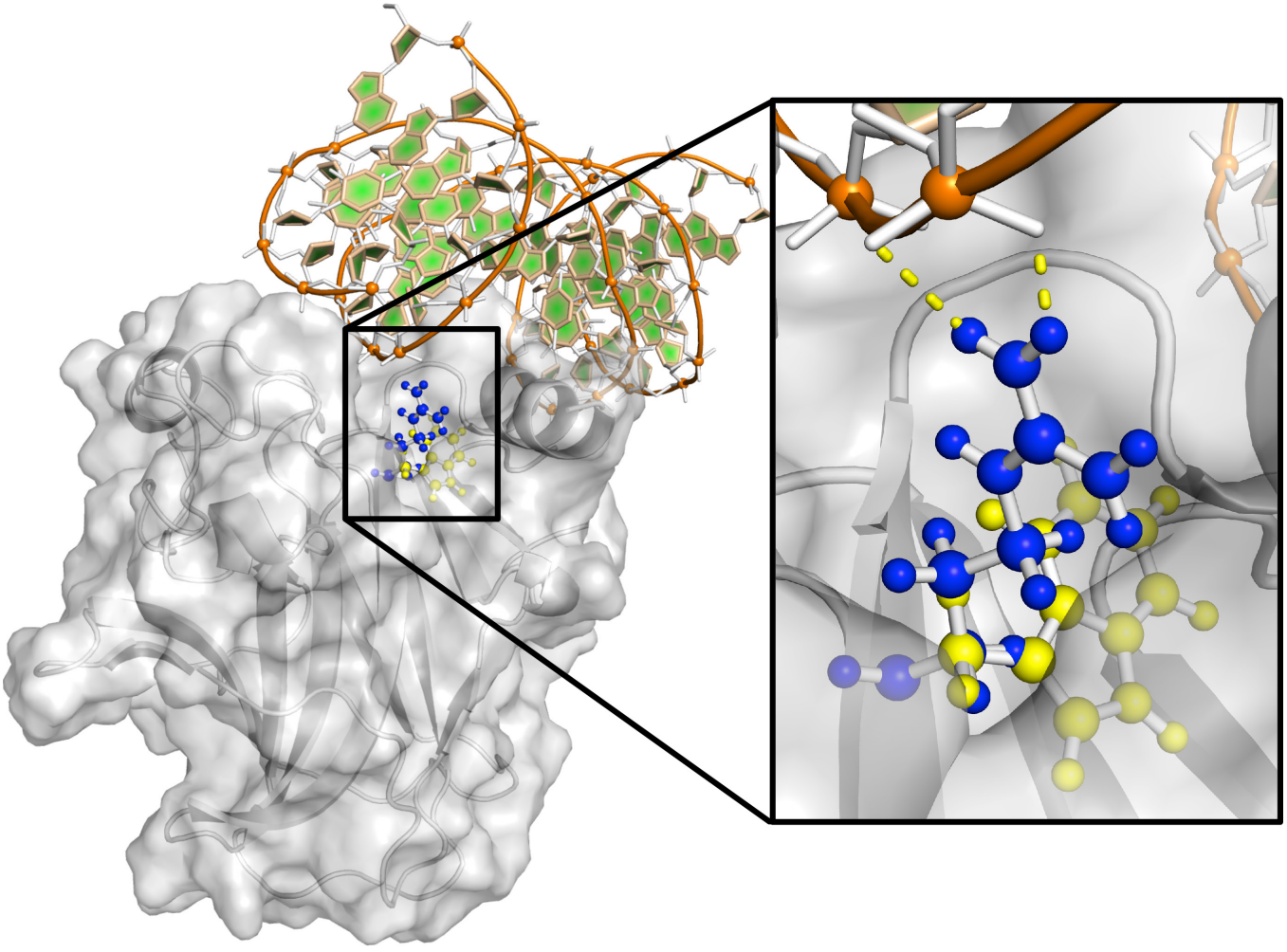

Figure 2. Modeling of the effects of the Arg343Trp mutation on TP63 structure. Beginning from a TP63 crystal structure, mutation of residue 343 from Arg to Trp followed by side-chain repacking demonstrates a loss of favorable electrostatic contacts between wild-type Arg and negatively charged phosphates of the accompanying double stranded DNA. This suggests that the functional consequence of Trp is diminished binding affinity and thereby a change (i.e., a reduction) in transcriptional control. The wild-type structure of TP63 (with an Arg residue at position 343) is shown in blue, the mutant structure with a Trp residue at position 343 is shown in yellow, and a DNA helix is shown in brown and green.

termed ectrodactyly, ectodermal dysplasia, and cleft lip/palate syndrome (EEC, OMIM 604292) [30]. The features of EEC overlap significantly with LADD syndrome. Both syndromes may include lacrimal duct abnormalities, hearing abnormalities, digital abnormalities (syndactyly), and dental abnormalities (hypodontia). There are even families in which some members have LADD syndrome while others have cleft lip and palate and were diagnosed with EEC [31]. The overlap of the clinical features of LADD syndrome and ECC may be due in part to common genetic bases, that is, that some mutations in *TP63* may be more likely to cause LADD syndrome while others may be more likely to cause ECC. Together these data and observations provide additional support for our hypothesis that mutation of *TP63* may cause LADD syndrome. We further suggest the possibility that *TP63* may also be associated with risk for thin central corneal thickness and for glaucoma. Overall, the evidence is compelling that mutation of *TP63* plays a role in the pathogenesis of LADD syndrome, however, further investigation with functional studies is warranted. Additional support for the pathogenicity of the *TP63* mutation might be obtained by examining our patient's family with genetic tests. Since both of his parents are reportedly unaffected by LADD syndrome, one might expect them to test negative for the Arg343Trp mutation. Such test results would indicate that our patient carries a de novo *TP63* mutation and would be further evidence that the Arg343Trp mutation is disease-causing. Unfortunately, neither parents were available for examination or enrollment in our study.

APPENDIX 1.

Fifty-eight candidate genes were identified by searching the annotated human genes in the GeneCards database using the keywords, "lacrimal," "auricular," "dental," "digital," and "morphogenesis." The candidate genes were prioritized based on the strength of the annotation for these terms. To access the data, click or select the words "Appendix 1."

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