Whole-Exome Sequencing Identifies Novel Variants for Tooth Agenesis

Journal of Dental Research
2018, Vol. 97(1) 49–59
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for Dental Research 2017
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DOI: 10.1177/0022034517724149
journals.sagepub.com/home/jdr

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Abstract

Tooth agenesis is a common craniofacial abnormality in humans and represents failure to develop 1 or more permanent teeth. Tooth agenesis is complex, and variations in about a dozen genes have been reported as contributing to the etiology. Here, we combined whole-exome sequencing, array-based genotyping, and linkage analysis to identify putative pathogenic variants in candidate disease genes for tooth agenesis in 10 multiplex Turkish families. Novel homozygous and heterozygous variants in *LRP6*, *DKK1*, *LAMA3*, and *COL17A1* genes, as well as known variants in *WNT10A*, were identified as likely pathogenic in isolated tooth agenesis. Novel variants in *KREMEN1* were identified as likely pathogenic in 2 families with suspected syndromic tooth agenesis. Variants in more than 1 gene were identified segregating with tooth agenesis in 2 families, suggesting oligogenic inheritance. Structural modeling of missense variants suggests deleterious effects to the encoded proteins. Functional analysis of an indel variant (c.3607+3_6del) in *LRP6* suggested that the predicted resulting mRNA is subject to nonsense-mediated decay. Our results support a major role for WNT pathways genes in the etiology of tooth agenesis while revealing new candidate genes. Moreover, oligogenic cosegregation was suggestive for complex inheritance and potentially complex gene product interactions during development, contributing to improved understanding of the genetic etiology of familial tooth agenesis.

Keywords: oligodontia, hypodontia, gene, WNT signaling pathway, next generation sequencing, array genotyping

Introduction

Tooth agenesis is a common craniofacial anomaly with esthetic and functional consequences. More than 300 syndromes have been reported in association with tooth agenesis, although the majority of the cases reflect nonsyndromic forms, found as familial or sporadic traits, and with considerable phenotypic heterogeneity. In familial cases, autosomal dominant inheritance is frequently observed, while autosomal recessive and X-linked inheritance have also been reported (Hennekam et al. 2010). Tooth agenesis can be classified based on the number of missing teeth as hypodontia (<6 teeth missing) or oligodontia (≥6 teeth missing). Hypodontia is common, with a prevalence of 3% to 10% depending on the population, whereas oligodontia is rare, with a prevalence of <1% (Yin and Bian 2015).

Variations in about one dozen genes have been shown to contribute to tooth agenesis, with each gene discovery incrementally improving an understanding of tooth development (Yin and Bian 2015). The best-characterized genes include Msh Homeobox 1 (*MSX1*) and paired box 9 (*PAX9*) for which homozygous null alleles result in arrest of tooth development in mice (Chen et al. 1996; Peters et al. 1998). Additional genes such as axis inhibition protein 2 (*AXIN2*; Lammi et al. 2004), ectodysplasin A (*EDA*; Song et al. 2009), and wingless-type MMTV integration site family member 10A (*WNT10A*; Kantaputra and Sripathomsawat 2011) have also been shown

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A supplemental appendix to this article is available online.

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to contribute to tooth agenesis. Recently, loss-of-function mutations in LDL receptor-related protein 6 (*LRP6*; Massink et al. 2015; Ockeloen et al. 2016) and missense mutations in the wingless-type MMTV integration site family member 10B (*WNT10B*; Yu et al. 2016) have been reported to segregate with autosomal-dominant tooth agenesis.

Despite comprehensive efforts to define genetic susceptibility, the signaling pathways associated with human tooth agenesis have not been fully elucidated, and novel causal variants remain to be identified. Here, we applied a combined approach using array-based genotyping, linkage analysis, and whole-exome sequencing to identify novel putatively causal gene variants for tooth agenesis in multiplex families from Turkey.

Methods

Study Families

This study was approved by the Institutional Review Boards at Istanbul University, The University of Texas Health Science Center at Houston (HSC-DB-12-0255), and Baylor College of Medicine (H-29697). Families were enrolled through written informed consent and ascertained through the CRANIRARE2 Project, a European Union-funded collaborative ERA-net Project on craniofacial malformations, at the Medical Genetics Department, Istanbul Medical Faculty, Istanbul University, Turkey. Proband was selected based on radiographic records showing absence of 1 or more permanent teeth excluding third molars. Fifty individuals from 10 multiplex Turkish Caucasian families were recruited, 28 affected with TA (14 with oligodontia; 14 with hypodontia) and 22 unaffected. Demographic information, medical and dental history, and DNA samples from peripheral blood were obtained. Diagnosis of tooth agenesis for all probands and available relatives was confirmed by a dentist (N.D.), while certified clinical geneticists assessed for syndromic features. In 2 families, probands presented with mild features of ectodermal dysplasia while additional relatives did not. No syndromic features were identified in the remaining 8 families. Pedigree data revealed consanguinity in 8 families, and suggested autosomal dominant inheritance in 2 families, autosomal recessive inheritance in 1 family, and complex inheritance in 7 families. Clinical features and pedigrees of study families are presented in Table 1 and in the Appendix Figs. 1 to 10.

Whole-Exome Sequencing

Whole-exome sequencing was performed on peripheral blood DNA from 12 affected individuals, at the Baylor College of Medicine Human Genome Sequencing Center through the Baylor-Hopkins Center for Mendelian Genomics (BHCMG) initiative. Using 0.5 µg of DNA, an Illumina paired-end precapture library was constructed according to the manufacturer's protocol (Illumina) with slight modifications (<https://www.hgsc.bcm.edu/content/protocols-sequencing-library-construction>). Six precaptured libraries were pooled and then hybridized in solution to the HGSC VCRome 2.1 design (Bainbridge et al.

2011) according to the manufacturer's protocol (Nimblegen). The sequencing run was performed in paired-end mode using the Illumina HiSeq 2000 platform. With a sequencing yield of 7.7 Gb, the samples achieved 97% of the targeted exome bases covered to a depth of 20× or greater. Illumina sequence analysis was performed using the HGSC Mercury analysis pipeline (Challis et al. 2012; Reid et al. 2014), which moves data through various analysis tools from initial sequence generation on the instrument to annotated variant calls.

Variant Prioritization

In families informative for linkage, filtering was restricted to high-quality variants in regions of the genome showing evidence of cosegregation with tooth agenesis, which were further prioritized in downstream filtering/annotation. Variants were filtered based on a minor allele frequency ≤0.01 in the HapMap Europeans (<http://hapmap.org>), in-house BHCMG database, Exome Aggregation Consortium (ExAC; <http://exac.broadinstitute.org>), Exome Variant Server (ESP; <http://evs.gs.washington.edu/>) (Lek et al. 2016), and the Atherosclerosis Risk in Communities Study (ARIC) databases (ARIC Investigators 1989). Next, variants were prioritized if they met at least 1 of the following criteria: 1) known variants/genes, 2) located within a pathway previously linked to tooth agenesis, 3) evidence of interaction with known genes, 4) found in paralogues of known genes, 5) variant location with respect to functional protein domains, 6) predicted deleterious using PhyloP (Pollard et al. 2010) and LRT (Chun and Fay 2009), Sorting Intolerant from Tolerant (SIFT; Ng and Henikoff 2001), and MutationTaster (Schwarz et al. 2014) algorithms. Variants prioritized as potentially causative were further analyzed in terms of gene function and phenotype in Online Mendelian Inheritance in Man (OMIM) and PubMed databases, animal models, and tissue expression of the encoded protein. Variants that were considered potentially pathogenic were further experimentally confirmed by Sanger sequencing, as described in the Appendix.

Functional Analysis of LRP6 Variant

Quantitative real-time polymerase chain reaction analysis was performed to determine the impact of the *LRP6* c.3607+3_6del variation on gene expression. Details are described in the Appendix.

Additional Methods

Details of array-based genotyping, linkage analysis, and structural protein modeling methods are described in the Appendix.

Results

Array-Based Genotyping and Linkage Analysis

Array-based genotyping and linkage analysis were performed in 24 individuals from 4 families (TF-2, TF-4, TF-6, and TF-7), and all shared, rare, functional candidate variants consistent with potential linkage were recorded. Although linkage approaches

were often underpowered to reach genome-wide significance given the size of the pedigrees, linkage analysis remained informative for segregation, thus assisting candidate gene prioritization by localizing haplotypes cosegregating with the phenotype for the assumed inheritance model. Details of linkage analysis results are described in the Appendix.

Whole-Exome Sequencing

For each individual sequenced, whole-exome sequencing analysis produced approximately 200,000 raw variants, which were prioritized to a few (1 to ~10) based on the analyses parameters. Rare and potentially pathogenic novel variants were identified in Dickkopf WNT Signaling Pathway Inhibitor 1 (*DKK1*), Kringle Containing Transmembrane Protein 1 (*KREMEN1*), Laminin Subunit Alpha 3 (*LAMA3*), and *LRP6* genes, as well as known variants in *WNT10A*.

Details on the variants identified are presented in Tables 1 and 2 and Figures 1 and 2. Additional pedigrees and sequencing results are presented in Appendix Figures 1 to 10.

Variants Identified in Families with Isolated Tooth Agenesis

WNT10A Variants. Three previously reported pathogenic *WNT10A* variants were identified in 6 families with isolated tooth agenesis. In 1 family (TF-1), a rare homozygous stop-gain variation c.697G>T (p.E233*) was identified as pathogenic. This variant is predicted to result in a prematurely terminated protein of 232 amino acids instead of 417 amino acids (Tables 1 and 2, Fig. 1A).

In 2 families (TF-2 and TF-3), a heterozygous missense variant c.682T>A was identified as pathogenic (Fig. 1A, 2). In TF-3, in addition to the *WNT10A* c.682T>A variant, a heterozygous missense variant in *LAMA3* c.1097G>A was also identified segregating with tooth agenesis, thus suggesting digenic inheritance in this family and *LAMA3* as a potential novel candidate gene (Tables 1 and 2, Fig. 2). While the *WNT10A* c.682T>A variant was predicted as damaging for resulting in a phenylalanine to isoleucine change (p.F228I) with the potential to disrupt nearby disulfide bridges in the protein structure, *LAMA3* c.1097G>A is predicted to result in an arginine to histidine (p.R366H) substitution, although its function appears as potentially deleterious or neutral, depending on the prediction algorithm used (Table 2, Fig. 3).

In the 3 remaining families (TF-4, TF-5, and TF-6), a rare homozygous missense variant c.433G>A was identified as pathogenic in the probands, although segregation patterns in TF-4 and TF-5 could not confirm an autosomal recessive inheritance pattern. For TF-4, affected individuals presented homozygous or heterozygous genotypes, while the unaffected mother was a heterozygote carrier (Appendix Fig. 4). Similarly, in TF-5, heterozygous genotypes were found in affected and unaffected individuals (Appendix Fig. 5). In TF-6, affected individuals were all homozygotes, whereas unaffected relatives were heterozygotes (Tables 1 and 2, Fig. 1A). This variant

results in a valine to methionine change at position 145 (p.V145M), classified as potentially damaging or neutral by different function prediction algorithms (Table 2, Fig. 3).

LRP6 Splice Variant. In 1 family (TF-7), a heterozygous variant predicted to affect splicing in *LRP6* (c.3607+3_6del) was identified in all affected individuals, while no *LRP6* variation was identified in unaffected individuals (Tables 1 and 2, Fig. 1A). Of note, affected family members presented periocular hyperpigmentation and hypoplastic alae nasi, although very mild and considered unrelated to the phenotype.

To determine if the *LRP6* c.3607+3_6del variant would cause an unstable mRNA subject to nonsense-mediated decay, we performed *LRP6* expression analysis using RNA from peripheral blood of all affected individuals and 1 unrelated control individual. Decreased *LRP6* mRNA levels were noted in the affected individuals, consistent with degradation of the variant message (Fig. 1A). Sanger sequencing confirmed the presence of only reference sequence in all 3 individuals (and no presence of inclusion of intronic sequence or exclusion of exonic sequence), supporting a degraded variant message (Fig. 1A).

DKK1, LAMA3, and COL17A1 Variants. Heterozygous variants in 3 novel candidate genes were identified segregating with hypodontia in 1 family (TF-10). A heterozygous splicing variant in *DKK1* (c.548-4G>T), a missense heterozygous variant in *LAMA3* (c.2798G>T), and a heterozygous potential splicing variant in Collagen Type XVII Alpha 1 (*COL17A1*) (c.3277+3G>C) were identified in the proband and his affected mother but not in the unaffected father (Tables 1 and 2, Fig. 2). The *LAMA3* (c.2798G>T) variant results in a glycine to valine change (p.G933V) and is predicted as deleterious for causing a destabilized disulfide interfering with protein packing and conformation (Fig. 3).

Variants Identified in Families with Suspected Syndromic Tooth Agenesis

KREMEN1 Variants. Novel variants in *KREMEN1* were identified as pathogenic in 2 families with suspected syndromic tooth agenesis (TF-8, TF-9). Of note, while both family probands presented mild clinical findings of ectodermal dysplasia (sparse hair, dry skin, sparse eyebrows and eyelashes, protruded lips, and heat intolerance), other family members showed no signs of ectodermal dysplasia. In TF-8, a homozygous missense variant (c.146C>G), resulting in a threonine to arginine substitution (p.T49R), was identified in the proband, while heterozygous genotypes were found in both affected and unaffected relatives. In TF-9, a homozygous in-frame deletion (c.773_778del) was identified in the proband but not in the affected mother (Tables 1 and 2, Fig. 1B, Fig. 3).

Discussion

This study identified novel gene variants in known genes as well as novel candidate genes for familial tooth agenesis and further

Table 1. Pathogenic Variants Identified in Tooth Agenesis Families.

Family	Individual	Relationship	Missing Teeth No.	Missing Teeth Types ^a	Chromosome(s)	Gene(s)	cDNA change(s)	Amino Acid Change(s)	Inheritance
Families with isolated tooth agenesis									
TF-1	III-2	Proband	25	I, PM, M	2	WNT10A	c.697G>T	p.E233*	Autosomal recessive
	II-5	Mother	0						
	II-6	Father	0						
TF-2	III-3	Sister	28	I, C, PM, M					Complex
	III-2	Proband	12	I, PM	2	WNT10A	c.682T>A	p.F228I	
	II-12	Mother	4						
	II-13	Father	0						
TF-3	III-2	Brother	4						Complex
	III-1	Proband	10	I, PM	2; 18	WNT10A; LAMA3	c.682T>A; c.1097G>A	p.F228I; p.R366H	
	II-8	Mother	2	I (UL)					
TF-4	II-9	Father	0						Autosomal recessive
	V-5	Proband	19	I, PM, M	2	WNT10A	c.433G>A ^b	p.V145M	
	IV-9	Mother	0						
	IV-10	Father	Unknown						
	V-1	Sister	Unknown						
	V-2	Sister	0						
	V-3	Brother	Unknown						
	V-4	Sister	17						
	V-9	Cousin	Unknown						
TF-5	III-3	Proband	21	I, PM, M	2	WNT10A	c.433G>A ^b	p.V145M	Complex
	II-11	Mother	2	I (UL)					
	II-12	Father	0						
	III-5	Brother	0						
TF-6	V-2	Proband	15	I, PM, M	2	WNT10A	c.433G>A	p.V145M	Autosomal recessive
	IV-10	Mother	0						
	III-3	Father	Unknown						
	V-1	Brother	Unknown						
	V-3	Brother	Unknown						
	V-4	Sister	0						
	V-5	Brother	0						
	V-6	Sister	0						
TF-7	III-2	Proband	16	I, PM	12	LRP6	c.3607+3_6del	?	Autosomal dominant
	II-2	Mother	Unknown						
	II-3	Father	0						
	III-1	Brother	0						
TF-10	II-1	Uncle	12						Complex
	III-1	Proband	5	I	10; 18; 10	DKK1; LAMA3; COL17A1	c.548-4G>T; c.2798G>T; c.3277+3G>C	?; p.G933V; ?	
	II-7	Mother	2	I (UL)					
	II-6	Father	0						
Families with suspected syndromic tooth agenesis									
TF-8 ^a	III-2	Proband	22	I, C, PM, M	22	KREMEN1	c.146C>G ^b	p.T49R	Complex
	II-11	Mother	2						
	II-12	Father	0						
	III-1	Brother	0						
	III-3	Brother	0						
TF-9 ^c	III-2	Proband	14	I, C, PM, M	22	KREMEN1	c.773_778del	F258_ P259del	Complex
	II-4	Mother	2	I (UL)					
	II-5	Father	0						
	III-1	Sister	0						
	I-1	Maternal grandmother ^d	0						
	I-2	Maternal grandfather ^d	0						

^aI, incisors; C, canines; PM, premolars; M, molars; UL, upper laterals.^bThe heterozygous variant is not segregating with tooth agenesis.^cProbands displayed mild ectodermal dysplasia features.^dHistory of tooth agenesis was obtained by family report.

implicates a predominant role for Wnt/ β -catenin pathway genes (Appendix Fig. 11) in tooth agenesis phenotypes. In 8 families, coding or nonsense variants in a single gene belonging to the Wnt/ β -catenin signaling pathway were identified as putatively

causative, whereas in 2 families, variation in a Wnt pathway gene was found cosegregating with additional gene(s). Novel heterozygous variants in *LRP6*, *DKK1*, *LAMA3*, and *COL17A1* genes, as well as known variants in *WNT10A*, were identified as

Table 2. Details of the Variants Identified.

Gene	cDNA Change	Amino Acid Change	Variant Type	Variant Location	Protein Domain ^a	ExAC Frequency ^b	PhyloP ^c	Mutation Taster ^d	VIPUR ^e
<i>WNT10A</i>	c.697G>T ^f	p.E233*	Stop gain	Exon 3	wnt1	0.00004	2.47	A	—
	c.682T>A ^f	p.F228I	Missense	Exon 3	wnt1	0.01273	2.00	A	0.62
	c.433G>A	p.V145M	Missense	Exon 3	wnt1	0.00002	2.00	D	0.35
<i>LRP6</i>	c.3607+3_6del	Unknown	Splicing	Intron 16	Unknown	NA	NA	NA	—
<i>LAMA3</i>	c.1097G>A	p.R366H	Missense	Exon 10	Laminin G-like 2	0.00026	1.52	D	0.12
	c.2798G>T	p.G933V	Missense	Exon 21	Laminin G-like 8 and 9	NA	2.51	D	0.76
<i>DKK1</i>	c.548-4G>T	Unknown	Splicing	Intron 3	Unknown	0.00057	NA	NA	—
<i>COL17A1</i>	c.3277+3G>C	Unknown	Splicing	Intron 47	Unknown	0.00005	NA	NA	—
<i>KREMEN1</i>	c.146C>G	p.T49R	Missense	Exon 2	Kringle	NA	2.49	D	0.88
	c.773_778del	p.F258_P259del	Frameshift	Exon 6	CUB	NA	NA	D	—

NA, not available.

^aBased on UniProt knowledgebase (<http://www.uniprot.org>).

^bMinor allele frequency based on the ExAC database (Exome Aggregation Consortium, <http://exac.broadinstitute.org>).

^cPhyloP (PhyloP scores measure evolutionary conservation at individual alignment sites; the larger the score, the more conserved the site; minimal = -1, maximum = 3).

^dMutationTaster prediction (A, known to be deleterious; D, probably deleterious).

^eVIPUR scores greater than 0.5 are considered deleterious to >94% accuracy.

^fClinVar ID 4460 and 4462, based on ClinVar database (<https://www.ncbi.nlm.nih.gov/clinvar/>).

likely pathogenic in families with isolated tooth agenesis. Novel variants in *KREMEN1* were identified as likely pathogenic in families with suspected syndromic tooth agenesis.

The role of the Wnt/ β -catenin signaling pathway during tooth development has been extensively discussed (Yin and Bian 2015), and loss- and gain-of-function experiments have shown the importance of Wnt/ β -catenin signaling at multiple stages of tooth development (Sarkar and Sharpe 1999). Secretion of Wnt4, Wnt6, and Wnt10 from the dental epithelium is essential for tooth development, and disruption of this process results in the absence of Wnt/ β -catenin activity and formation of a dysfunctional enamel knot leading to arrest of tooth development (Zhu et al. 2013).

WNT10A has been the focus of many studies, and variations in this gene have been reported to contribute to more than 50% of tooth agenesis cases (Song et al. 2014; van den Boogaard et al. 2012). More than 50 *WNT10A* variants have been identified in 15.8% of tooth agenesis patients with 1 to 3 missing teeth and in ~52% of patients with >4 missing teeth. In the present study, 3 previously reported pathogenic *WNT10A* variants were identified in 6 families, in which a range of 2 to 28 missing teeth was observed. The stop-gain variation c.697G>T was identified segregating with tooth agenesis in 1 family. This variant is predicted to result in a prematurely terminated protein of 232 amino acids instead of 417 amino acids (p.E233*) and was first described in cases with odonto-onycho-dermal dysplasia syndrome, characterized by dystrophic nails, erythematous facial lesions, and oligodontia (Adaimy et al. 2007). To our knowledge, this is the first report of the c.697G>T variant in isolated tooth agenesis. The heterozygous c.682T>A (p.F228I) variant, identified in 2 families, has been widely reported in homozygous or heterozygous forms in affected individuals, often combined with additional variants in *WNT10A* or in other genes (He et al. 2013; Vink et al. 2014). Interestingly, heterozygous genotypes for c.682T>A have also

been found in ~2.3% of unaffected controls (van den Boogaard et al. 2012). In another family, we identified cosegregation of the c.682T>A variant, predicted to disrupt nearby disulfide bridges in the WNT10A protein, with a heterozygous missense variant in *LAMA3* (c.1097G>A; p.R366H) predicted as damaging. This suggests potential mutational burden in this family. Mutations in *LAMA3* cause junctional epidermolysis bullosa, an autosomal recessive skin disorder characterized by the presence of multiple blisters and erosions, dystrophic nails, enamel hypoplasia, and hypodontia (McGrath et al. 1995). Further, targeted disruption of *LAMA3* in mice resulted in defects of ameloblast differentiation (Ryan et al. 1999).

Lastly, the *WNT10A* c.433G>A (p.V145M) variant was found in homozygous and heterozygous forms in individuals with tooth agenesis, while heterozygous genotypes were also found in unaffected individuals. This variant was previously reported in a patient with ectodermal dysplasia and tooth agenesis (Cluzeau et al. 2011), although no distinctive features of ectodermal dysplasia were found in our families with *WNT10A* mutations. Collectively, our results are in agreement with previous *WNT10A* variants identified in individuals with and without tooth agenesis; hence, the genetics potentially contributing to tooth agenesis remain to be established and may be dependent on a variant allele. Furthermore, while secretion of Wnt10a from the dental epithelium was essential for tooth development in wild-type mice, *Wnt10a*-knockout mice showed abnormal tooth morphogenesis and supernumerary teeth, contrary to the tooth agenesis phenotype in humans (Yang et al. 2015). The 3 variants identified in our study fall within the wnt1 domain of the protein; however, very little is known about the structure of Wnts as they are relatively insoluble and yet are known to share characteristics of secretory proteins such as a signal peptide, several potential N-glycosylation sites, and 22 conserved cysteines that are probably involved in disulfide bonds (Logan and Nusse 2004).

Figure 1. Clinical and genetic findings in families in which 1 candidate gene was identified as pathogenic for isolated and suspected syndromic tooth agenesis. Probands' panoramic radiographs and schematics of missing teeth, familial segregation analyses, and corresponding Sanger sequencing chromatograms are shown. Missing teeth are indicated by red stars in radiographs and filled boxes in schematic maxillary (MAX) and mandibular (MAN) arches. Probands are indicated by arrows in each pedigree. **(A)** Families with isolated tooth agenesis. Previously reported mutations in *WNT10A* (c.697G>T, c.682T>A, and c.433G>A) were identified segregating with tooth agenesis in 5 families (TF-1, TF-2, and TF-6 presented here; TF-4 and TF-5 presented in the Appendix). A novel likely pathogenic heterozygous potential splicing mutation in *LRP6* (c.3607+3_6del: p.?) was identified in 1 family (TF-7). Quantitative polymerase chain reaction was performed to detect *LRP6* gene expression on 3 affected individuals (II-1, II-2, and III-2) and 1 unrelated unaffected control individual. *LRP6* expression was decreased in all affected individuals as compared with the control individual. Sanger sequencing confirmed the presence of only wild-type message between exons 16 and 17. **(B)** Families with suspected syndromic tooth agenesis (TF-8 and TF-9). Variants in *KREM1* (c.146C>G and c.773_778del) were identified in the probands with tooth agenesis and mild

Figure 2. Clinical and genetic findings in families in which oligogenic inheritance was proposed in isolated tooth agenesis. Variants in *WNT10A* (c.682T>A) and *LAMA3* (c.1097G>A) were identified as potentially pathogenic in TF-3, whereas variants in *DKK1* (c.548-4G>T), *LAMA3* (c.2798G>T) and *COL17A1* (c.3277+3G>C) were identified in TF-10.

While the contribution of each *WNT10A* variant to tooth agenesis is yet to be determined, our findings suggest that the encoded protein appears to be stabilized significantly by disulfide bridges, and much of the protein may be flexible or relatively unstable without these.

LRP6 is a coreceptor in the Wnt/ β -catenin pathway and has been recently reported as a candidate gene for tooth agenesis. Five *LRP6* variants, including frameshift, missense, and splice-site variants, were reported in individuals with tooth agenesis (Massink et al. 2015; Ockeloen et al. 2016). Here, we identified a novel *LRP6* splice-site variant (c.3607+3_6del) segregating

with autosomal-dominant oligodontia in 1 family. Functional analysis showed that this variant affects RNA splicing and leads to an mRNA likely to undergo nonsense-mediated decay. In mice, *Lrp6* expression was noted in the tooth follicle and inner enamel epithelium (Ockeloen et al. 2016), while homozygous deletion of *Lrp6* led to severe skeletal abnormalities and lethality (Pinson et al. 2000). Our findings therefore expand the spectrum of *LRP6*-associated tooth agenesis phenotype.

In recent years, oligogenic inheritance and multilocus variation models have been proposed for a number of Mendelian diseases, further establishing the concept of mutational load in

human genetic disease (Posey et al. 2017). In this study, we identified 1 family in whom novel heterozygous potential splicing mutations in *DKK1* (c.548-4G>T) and *COL17A1* (c.3277+3G>C), and a heterozygous missense variant in *LAMA3* (c.2798G>T), were segregating with tooth agenesis, suggesting potential oligogenic inheritance. As described above, we identified another missense variant in *LAMA3* (c.1097G>A) segregating with tooth agenesis. The finding of the c.2798G>T variant in the present family further supports a role for this gene as a candidate for tooth agenesis. Structural modeling of the individual *LAMA3* variants predicts their location within laminin G-like domains of the protein, resulting in a destabilized disulfide due to poor packing of the protein and thereby affecting ligand binding and protein function.

DKK1, *COL17A1*, and *LAMA3* were considered as biologically plausible candidate genes because of their biological roles and/or disease-associated phenotypes. *Dkk1* encodes a high-affinity dickkopf homolog 1 transmembrane receptor that cooperates with *Lrp6* to block Wnt signaling during development and other cellular processes (Fedi et al. 1999). In mice, *Dkk1* is expressed in the dental mesenchyme, odontoblasts, and osteoblasts, and its ectopic expression in the oral epithelia of transgenic mouse embryos resulted in blocked epithelial and mesenchymal signaling, leading to arrest of tooth development at the early bud stage (Li et al. 2011). Variations in *COL17A1* have also been described in epidermolysis bullosa patients with enamel defects (Tasanen et al. 2000), and our findings of cosegregation of a splicing variant *COL17A1* (c.3277+3G>C) and a missense variant in *LAMA3* (c.2798G>T) in a family with tooth agenesis and no signs of epidermolysis bullosa are intriguing. The finding of likely pathogenic alleles at those loci suggest the potential for oligogenic inheritance and multilocus variation models in tooth agenesis, likely contributing to the variable phenotypes.

Finally, novel variations in *KREMEN1* (c.146C>G and c.773_778del) were found as likely pathogenic in 2 families with suspected syndromic tooth agenesis. *KREMEN1* encodes a kringle domain-containing transmembrane protein that binds to *Dkk1*, creating a *Dkk1*-*Kremen*-*Lrp6* ligand-receptor complex critical for Wnt signaling (Ellwanger et al. 2008). While in this complex, *Kremen* triggers the internalization of *Lrp6* inhibiting Wnt signaling. In the absence of *Dkk1*, however, *Kremen* can increase Wnt signaling through *Lrp6* binding (Mao and Niehrs 2003). Targeted disruption of *Kremen1* in

Figure 3. Structural modeling of individual mutations. WNT10A is colored blue, and its binding partner is colored green. Disulfide bridges are highlighted in yellow, and modification sites are colored magenta. (A) WNT10A F228I variant is predicted to disrupt protein structure by destabilizing disulfide bridges. (B) WNT10A V145M is predicted to be probably deleterious by MutationTaster, or neutral by VIPUR. (C) KREMEN1 T49R is predicted to be deleterious by VIPUR due to an unfavorable backbone conformation. The domains of KREMEN1 are colored blue (Kringle domain), cyan (WSC domain), and green (CUB domain), with disulfide bridges colored in yellow and modification sites in magenta. (D) LAMA3 is colored in blue, and R366H falls into a region of LAMA3 that is missing in isoforms 3 and 4 (colored in cyan). (E) LAMA3 G933V is predicted to be deleterious by VIPUR because of poor packing. G933 occurs in the first laminin G-like domain of LAMA3 (laminin G-like domains are colored in cyan).

mice induces limb defects and high bone density but no other obvious phenotypes (Ellwanger et al. 2008). The identified c.146C>G variant is predicted to cause an unfavorable backbone conformation in the Kringle domain of KREMEN1. Meanwhile, the c.773_778del variant is predicted to interfere with a CUB domain found in mostly developmentally regulated proteins. Supporting our findings, a homozygous missense variant in *KREMEN1* (c.626T>C) was identified in 4 syndromic oligodontia families of Palestinian origin (Issa et al. 2016), suggesting that *KREMEN1* is more likely to be involved in syndromic tooth agenesis.

In conclusion, we have identified novel candidate genes and variants contributing to isolated and syndromic tooth agenesis and potential oligogenic inheritance models in some families. Further, our findings support the importance of Wnt/ β -catenin pathway genes as regulators of tooth development. Additional studies might elucidate the role of these genes individually or their additive effects in tooth agenesis.

Author Contributions

N. Dinckan, R. Du, L.E. Petty, contributed to data acquisition, analysis, and interpretation, drafted the manuscript; Z. Coban-Akdemir, I. Paine, contributed to data acquisition, analysis, and interpretation, critically revised the manuscript; S.N. Jhangiani, A.P. Erdem, H. Kayserili, H. Doddapaneni, J. Hu, D.M. Muzny, E. Boerwinkle, R.A. Gibbs, Z.O. Uygur, contributed to data acquisition, critically revised the manuscript; E.H. Baugh, contributed to data analysis and interpretation, critically revised the manuscript; J.R. Lupski, contributed to conception, design, data acquisition, analysis, and interpretation, critically revised the manuscript; J.E. Below, A. Letra, contributed to conception, design, data acquisition, analysis, and interpretation, drafted the manuscript. All authors gave final approval and agree to be accountable for all aspects of the work.

Acknowledgments

We would like to thank all participants in this study. Thanks to Claudia Biguetti and Leticia Souza for assistance with illustrations. We also thank Dr. Wu-Lin Charng for discussion on *LRP6* experiments. This work was supported by the Scientific and Technological Research Institution of Turkey, TUBITAK-ERA NET (CRANIRARE-2, grant SBAG-112S398); Istanbul University Research Fund (Project No. 48398); the Baylor-Hopkins Center for Mendelian Genomics (UM1 HG006542), jointly funded by the U.S. National Human Genome Research Institute (NHGRI) and National Heart, Lung, and Blood Institute (NHLBI); and U.S. National Institute of Dental and Craniofacial Research (NIDCR) R03-DE024596 (to A.L.). J.R.L. has stock ownership in 23andMe and Lasergen, is a paid consultant for Regeneron, and is a co-inventor on multiple U.S. and European patents related to molecular diagnostics for inherited neuropathies, eye diseases, and bacterial genomic fingerprinting. The other authors declare no potential conflicts of interest with respect to the authorship and/or publication of this article.

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