Novel mutations in LRP6 highlight the role of WNT signaling in tooth agenesis

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**Purpose:** We aimed to identify a novel genetic cause of tooth agenesis (TA) and/or orofacial clefting (OFC) by combining whole-exome sequencing (WES) and targeted resequencing in a large cohort of TA and OFC patients.

**Methods:** WES was performed in two unrelated patients: one with severe TA and OFC and another with severe TA only. After severe mutations were identified in a gene encoding low-density lipoprotein receptor-related protein 6 (LRP6), all its exons were resequenced with molecular inversion probes in 67 patients with TA, 1,072 patients with OFC, and 706 controls.

**Results:** We identified a frameshift (c.4594delG, p.Cys1532fs) and a canonical splice-site mutation (c.3398-2A>C, p.?) in LRP6, respectively, in the patient with TA and OFC and in the patient with severe TA only. The targeted resequencing showed significant enrichment of unique LRP6 variants in TA patients but not in nonsyndromic OFC patients. Of the five variants in patients with TA, two affected the canonical splice site and three were missense variants; all variants segregated with the dominant phenotype, and in one case the missense mutation occurred de novo.

**Conclusion:** Mutations in LRP6 cause TA in humans.

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**Key Words:** LRP6; molecular inversion probes; tooth agenesis; Wnt/β-catenin canonical signaling pathway

**INTRODUCTION**

Tooth agenesis (TA) and orofacial clefting (OFC) are distressing to families. Both are common congenital disorders and can occur as isolated entities or accompanied by other symptoms or as part of a syndrome.1,2 As an isolated condition, TA in the severe form involves six or more teeth failing to develop (oligodontia) and has a prevalence of 0.1–0.5% in populations worldwide; the frequency of the mild form of TA, which involves one
to five teeth missing (hypodontia), is 3–20% of the population. For isolated nonsyndromic OFC, a worldwide prevalence of 0.1–0.2% has been reported.

Although genetic heterogeneity is present for selective tooth agenesis (STHAG), including causal mutations in MSXI (STHAG1; MIM 106600), PAX9 (STHAG3; MIM 604625), WNT10A (STHAG4; MIM 150400), or EDA (STHAGX1; MIM 313500), mutations of WNT10A, ligand of the Frizzled (FZD) coreceptor in the canonical WNT/β-catenin signaling pathway, are most frequently associated with isolated TA. Of all genes in which mutations causing STHAG have been identified, only MSXI has the annotation “with or without orofacial cleft,” which refers to the rare combination of TA and different types of OFC in the affected members in two families identified to date.

Mutations in other genes, e.g., IRF6 (MIM 607199) and TP63 (MIM 603273), have also been shown to cause combined TA-OFC, in syndromic as well as nonsyndromic phenotypes.

Although individuals with OFC present with higher frequencies of dental anomalies, including TA in their maxillary primary and permanent dentitions, than controls, it has been suggested that the combined TA-OFC phenotype is only rarely due to genetic factors; rather, it is due to acquired disturbances of the physical environment surrounding the developing dentition. Only some families carry rare mutations in specific genes that influence both tooth development and palatogenesis, suggesting rare monogenic conditions in such cases.

Here, we report the discovery of truncating mutations in LRPI (MIM *603507) by whole-exome sequencing in a case with TA-OFC and a case with TA only, and we hypothesize that LRPI mutations can cause TA, OFC, or combined TA-OFC. Through targeted resequencing, we identified five additional patients with TA harboring LRPI mutations. To support our hypothesis, we analyzed the expression of LRPI in the mouse embryos using chromatin immunoprecipitation quantitative PCR analysis. We generated the LRPI transgenic and knockout mice, which were analyzed using chromatin immunoprecipitation and qPCR. The results showed that LRPI is expressed in the developing teeth and palate of the mouse embryos.

For the full phenotype description and medical family history of both index patients, we refer to Supplementary Materials and Methods online.

Whole-exome sequencing
Two protocols for whole-exome sequencing and subsequent data analysis were used for the two index patients. The first one was performed through the Baylor-Hopkins Center for Mendelian Genomics using the methods previously reported; the second was performed in a clinical setting at the Department of Human Genetics of the Radboud University Medical Center, similar to previous reports.

To identify additional patients with LRPI variants, 29 in-house diagnostic exomes sequenced in patients with a range of craniofacial disorders were queried for rare (<1% in dbSNP141), nonsynonymous, and canonical splice-site variants in LRPI.

Immunohistochemistry for LRPI in mouse heads
To check the expression of LRPI in developing teeth and palate of mice, we generated paraffin sections of E13 and E15 mouse embryo heads and stained them with anti-LRPI polyclonal antibody (cat. nr.orb18907, Progen, Sanbio, The Netherlands) counterstained with hematoxylin.

Regulation of LRPI by Trp63
We checked for Trp63 binding sites in LRPI using chromatin immunoprecipitation quantitative PCR analysis. To generate additional evidence for regulation of LRPI by Trp63, the expression of LRPI in the facio processes of Trp63−/− embryos was compared with that of their wild-type littermates. The breeding of the genetically modified mice was performed after ethical review and in accordance with the UK Animals (Scientific Procedures) Act of 1986.

Resequencing with molecular inversion probes
To genetically test our hypothesis regarding the contribution of rare LRPI coding variants to the etiology of TA and common OFC, we aimed to resequence all exons of LRPI in a large patient cohort and in controls. Molecular inversion probes were used to resequence LRPI in 67 patients with TA, 11 1,073 patients with OFC, and 706 controls (CO). The cases and controls were identified as part of the sample-collection efforts from Bonn (OFC, CO), Nijmegen (OFC), and Leuven (OFC, TA) (Supplementary Table S1 online).

Approval from the institutional review boards involving human subjects and the ethical committees of the respective university hospitals was obtained, as well as written informed consent from all individuals.

A total of 69 molecular inversion probes were designed for the coding exons of LRPI and used for targeted enrichment as previously described, with minor modifications. All coding exons of LRPI were covered, on average, 1,045 ± 480-fold (average ± SD) per sample. This was comparable for cases (1,040-fold) and controls (1,051-fold) (Supplementary Materials and Methods online).
Methods online). The identified variants were filtered using the same filter steps for cases and controls (Table 1).

RESULTS

Exome sequencing of index patients 1 and 2

After filtering for rare coding variants found with whole-exome sequencing (Supplementary Table S2 online), we identified a deleterious frameshift (c.4594delG; p.Cys1532fs) and a canonical splice-site mutation (c.3398-2A>C; p.?) in LRP6 in index patient 1 with severe TA and OFC and in the unrelated index patient 2 at 9 years of age showing mild facial dysmorphic features including a narrow nasal ridge, posteriorly rotated ears with a thin helix, small earlobes, and a long superior crus antihelix. (d) He has unaffected parents and two unaffected brothers. (i) The OPT shows TA of two deciduous teeth (52 and 62) and TA of nine permanent teeth (17, 15, 14, 13, 12, 22, 25, 27, 35, and 45). (I and m) There is an ectopic molar germ (tooth 24) in the left upper quadrant. (n) The occlusal photograph of the mandibular dental arch in the mixed dentition shows malposition of tooth 32. (o) The shapes of the palatal cusps of teeth 16 and 26 are abnormal, making them resemble a second molar on the occlusal photograph of the maxillary dental arch. (p and q) Clinodactyly of the fifth fingers. DT, deciduous teeth; PT, permanent teeth; OFC, orofacial clefting; STHAG, selective tooth agenesis.

Immunohistochemistry for Lrp6 in mouse heads

Immunohistochemistry staining of Lrp6 on sections of the embryonic mouse heads (Supplementary Figure S2a,b online) showed that Lrp6 is expressed in areas of bone formation at E13 (Supplementary Figure S2a online) but more clearly expressed and including palatal bone at E15 (Supplementary Figure S2b online). Interestingly, Lrp6 is expressed in the tooth follicle, especially in the inner enamel epithelium (Supplementary Figure S2b online), suggesting a role for Lrp6 in early tooth development.

Regulation of Lrp6 by Trp63

The presence of a functional Trp63 binding site in intron 7 of Lrp6 was identified using chromatin immunoprecipitation quantitative PCR analysis (Supplementary Figure S2c online). Although Lrp6 expression is downregulated in the facial processes of Trp63+/− embryos compared with their wild-type littermates, this reduction was not significant (Supplementary Figure S2d,e online). Furthermore, Lrp6 displayed reduced expression within the mesenchyme of E11.5 medial nasal processes between Trp63+/− and Trp63−/− embryos (data not shown), but this effect was subtle.
Resequencing with molecular inversion probes

Significant enrichment of rare variants was found only in cases with TA, as compared with controls (7/67 vs. 13/706; Fisher’s exact test after Bonferroni correction $P = 0.0056022$ (Table 1)), with a similar significant enrichment found when considering unique variants (5/67 vs. 3/706; $P = 0.0012354$) or predicted damaging variants (CADD PHRED-like score >20) (4/67 vs. 1/706; $P = 0.001515$). There was no difference between cases and controls for rare or unique synonymous variants. There was no statistically significant enrichment for unique LRP6 variants in OFC cases compared with controls.

All unique variants in TA cases were validated by Sanger sequencing, and segregation analysis showed segregation in all families (Supplementary Figure S3a–e online); hence all five variants were considered likely pathogenic (Supplementary Table S3a online). These contained two canonical splice-site variants as well as three missense variants. Of note, the sporadic case, TA2, showed de novo mutation. Based on the gene-specific mutation rates of Samocha et al., this chance of identifying a de novo missense mutation in LRP6 in 67 TA cases is extremely low ($P = 0.006076$ exact Poisson test). The ultimate proof of pathogenicity of the presented missense variants will require additional functional evidence.

Careful inspection of all medical records of the seven described cases harboring unique LRP6 mutations (Supplementary Figure S4 and Table S3b) revealed additional dental anomalies, including tooth ankylosis (n = 2), abnormal tooth shape (n = 2), enamel defects (n = 1), and other symptoms, such as clinodactyly (n = 3). Furthermore, growth hormone supplementation therapy had been considered for four of these seven patients.

Severe TA was also present in a patient carrying a rare LRP6 variant (Supplementary Figures S3f and S4j,k online). This suggests that several of the rare variants may still be involved in the phenotype.

DISCUSSION

We report that loss of function of LRP6 causes TA. LRP6 is a transmembrane cell surface protein that is a member of the low-density lipoprotein receptor gene family. LRP6 acts as a coreceptor for WNT together with Frizzled (FZD), transmitting signals in the canonical WNT/β-catenin-TCF signaling cascade, which is well known for its role in differentiation, proliferation, and migration processes during dental and orofacial development. Two genes of this pathway have already been implicated in human TA. Mutations in AXIN2 (MIM 608615) were associated with TA—colorectal cancer predisposition, and mutations in WNT10A can cause either isolated4 or syndromic TA (WNT10A; MIM 606268). Lrp6 has been described as positively regulating WNT/β-catenin signaling by Wnt receptor internalization (GO:0038013) in LRP6 signalosomes.14

Our findings regarding TA further expand the spectrum of LRP6 phenotypes. Although loss- or gain-of-function mutations in LRP6 and in another closely resembling Wnt coreceptor, LRP5, had already placed the Wnt pathway central in bone biology, Wnt has emerged as an important regulator of skeletal modeling and remodeling.15 LRP6 is also key to parathyroid hormone (PTH) signaling, which regulates osteoblast activity; PTH binds to its receptor PTH1R and thereby induces the PTH-PTH1R-LRP6 protein complex, leading to increased bone formation in rats.16 Because LRP6 mutations may disrupt this PTH-PTH1R-LRP6 interaction, effects on growth could result. This could explain why growth hormone supplementation therapy was being considered for four of our seven patients with unique LRP6 mutations. Other human diseases related to LRP6 variants include atherosclerosis,17 osteoporosis,15 and metabolic syndrome.18 Recently, rare LRP6 mutations were reported in spina bifida.19 Although genetic inactivation of Lrp6 was also reported to lead to cleft lip with cleft palate in a mouse model,20 our genetic data do not support a role for LRP6 mutations in nonsyndromic

Table 1 | Overview of LRP6 variants identified with MIP screen in OFC and TA patients versus controls

| Total cases with variant (n = 1,139) | TA cases with variant (n = 67) | OFC cases with variant (n = 1,072) | Controls with variant (n = 706) |
|---------------------------------------|-------------------------------|-----------------------------------|--------------------------|
| Rare (<0.1%) coding and SS variants   | 30                            | 8                                 | 22                       | 16                       |
| Synonymous                            | 3                             | 1                                 | 2                        | 3                        |
| Nonsynonymous                         | 27                            | 7\(^b\)                           | 20\(^c\)                 | 1\(^{3,c}\)              |
| Unique/private variants               | 13                            | 5                                 | 8                        | 5                        |
| Synonymous                            | 1                             | 0                                 | 1                        | 2                        |
| Nonsynonymous                         | 12                            | 5\(^d\)                           | 7\(^e\)                  | 3\(^{4,e}\)              |
| CADD PHRED >20                        | 7                             | 4\(^f\)                           | 3\(^{4}\)                | 1\(^{4}\)                |

All variants were coding or canonical splice-site variants with a population frequency of <0.1% (based on dbSNP142 and an in-house database with >5,000 samples). Three or fewer samples in this study had the same variant and a “GATK quality by depth” of >1,000; the latter was based on previous MIP data and extensive validations by Sanger sequencing showing low false-positive rates and high sensitivity. Minimal average coverage over all MIPs of included samples was 100-fold. Most unique and rare nonsynonymous variants reported here in cases have been validated by Sanger sequencing.

MIP, molecular inversion probe; OFC, orofacial clefting; TA, tooth agenesis.

\(^{4}\)Splice-site canonical dinucleotide; CADD PHRED-like score.\(^{2,1}\)

\(^{1}\)Five unique, nonsynonymous variants in LRP6 in 67 cases with OD were highly significant when compared with the variant load in 706 controls (Fisher’s exact test after Bonferroni correction $P = 0.0012354$), whereas seven unique, nonsynonymous variants in LRP6 in 1,072 cases with OFC do not show significance when compared with the variant load in 706 controls.\(^{1}\)Four unique, predicted damaging variants in LRP6 in 67 cases with OD were highly significant when compared with one such variant in 706 controls (Fisher’s exact test after Bonferroni correction $P = 0.001515$), whereas seven unique, nonsynonymous variants in LRP6 in 1,073 cases with OFC do not show significance when compared with the variant load in 706 controls.

\(^{2}\)Significant enrichment of rare variants was found only in cases with TA, as compared with controls (7/67 vs. 13/706; Fisher’s exact test after Bonferroni correction $P = 0.0056022$ (Table 1)), with a similar significant enrichment found when considering unique variants (5/67 vs. 3/706; $P = 0.0012354$) or predicted damaging variants (CADD PHRED-like score >20) (4/67 vs. 1/706; $P = 0.001515$). There was no difference between cases and controls for rare or unique synonymous variants. There was no statistically significant enrichment for unique LRP6 variants in OFC cases compared with controls.

\(^{3}\)Additional additional dental anomalies, including tooth ankylosis (n = 2), abnormal tooth shape (n = 2), enamel defects (n = 1), and other symptoms, such as clinodactyly (n = 3). Furthermore, growth hormone supplementation therapy had been considered for four of these seven patients.

\(^{4}\)Severe TA was also present in a patient carrying a rare LRP6 variant (Supplementary Figures S3f and S4j,k online). This suggests that several of the rare variants may still be involved in the phenotype.

\(^{5}\)Careful inspection of all medical records of the seven described cases harboring unique LRP6 mutations (Supplementary Figure S4 and Table S3b) revealed additional dental anomalies, including tooth ankylosis (n = 2), abnormal tooth shape (n = 2), enamel defects (n = 1), and other symptoms, such as clinodactyly (n = 3). Furthermore, growth hormone supplementation therapy had been considered for four of these seven patients.

\(^{6}\)Severe TA was also present in a patient carrying a rare LRP6 variant (Supplementary Figures S3f and S4j,k online). This suggests that several of the rare variants may still be involved in the phenotype.

\(^{7}\)Recently, rare LRP6 mutations were reported in spina bifida.19

\(^{8}\)Although genetic inactivation of Lrp6 was also reported to lead to cleft lip with cleft palate in a mouse model,20 our genetic data do not support a role for LRP6 mutations in nonsyndromic
OFC in humans. Moreover, our functional data fail to show significant regulation of Lrp6 by Trp63. Because we only found evidence for molecular underpinning of TA by LRP6 mutations, it remains unsolved whether the LRP6 mutation in our index patient with combined TA-OFC also contributed to the OFC development in this patient.

Based on our results, an estimate can be made regarding the frequency of LRP6 mutations in the normal population because frequencies of TA in general populations are known. In our cohort, 3 of 706 control individuals (0.4%) were carriers of unique LRP6 variants. Although this frequency matches population frequencies for severe TA ranging between 0.1 and 0.5%, it remains unclear whether those variants are pathogenic. We hypothesize that LRP6 is a major cause of severe TA but not a cause of common TA (3 to 20% in populations worldwide).

In conclusion, we show that LRP6 mutations cause severe TA with or without other dental anomalies such as ankylosis, enamel defects, and tooth-shape anomalies. However, our genetic data do not provide evidence that rare monogenic LRP6 mutations underlie nonsyndromic Orofacial clefts.

Note: during the preparation of this article, the following paper was published: MP Massink et al., Loss-of-function mutations in the WNT coreceptor LRP6 cause autosomal-dominant oligodontia, Am J Hum Genet; 2015;97:621–626. This study provides additional independent evidence for the causative role of LRP6 mutations in TA.

SUPPLEMENTARY MATERIAL

Supplementary material is linked to the online version of the paper at http://www.nature.com/gim

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DISCLOSURE

The authors declare no conflict of interest.

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