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A Biallelic *ANTXR1* Variant Expands the Anthrax Toxin Receptor Associated Phenotype to Tooth Agenesis

Nuriye Dinckan^{1,10}, Renqian Du², Zeynep Coban Akdemir², Yavuz Bayram², Shalini Jhiangiani³, Harsha Doddapaneni³, Jianhong Hu³, Donna M. Muzny³, Yeliz Guven⁴, Oya Aktoren⁴, Hulya Kayserili⁵, Eric Boerwinkle^{3,6}, Richard A. Gibbs^{2,3}, Jennifer E. Posey², James R. Lupski^{2,3,7,8}, Zehra Oya Uyguner¹, and Ariadne Letra^{9,10,11}

¹Department of Medical Genetics, Istanbul Medical Faculty, Istanbul University, Istanbul, 34093, Turkey

²Department of Molecular and Human Genetics, Baylor College of Medicine, Houston, TX 77030, USA

³Human Genome Sequencing Center, Baylor College of Medicine, Houston, TX 77030, USA

⁴Department of Pedodontics, Faculty of Dentistry, Istanbul University, Capa, Istanbul, 34390, Turkey

⁵Department of Medical Genetics, Koc University, School of Medicine, Istanbul, 34010, Turkey

⁶Human Genetics Center, University of Texas Health Science Center at Houston School of Public Health, Houston, TX 77030, USA

⁷Department of Pediatrics, Baylor College of Medicine, Houston, TX 77030, USA

⁸Texas Children's Hospital, Houston, TX 77030, USA

⁹Department of Diagnostic and Biomedical Sciences, University of Texas Health Science Center at Houston School of Dentistry, Houston, TX 77054, USA

¹⁰Center for Craniofacial Research, University of Texas Health Science Center at Houston School of Dentistry, Houston, TX 77054, USA

Corresponding author: Ariadne Letra, DDS, MS, PhD Associate Professor Department of Diagnostic and Biomedical Sciences Craniofacial Research Center Adjunct and Director of Research, Department of Endodontics School of Dentistry University of Texas Health Science Center at Houston 7500 Cambridge Street| Room 5359 | Houston, Texas 77054 | 713 486-4228 Tel | 713-486-0402 Fax ariadne.m.letra@uth.tmc.edu.

CONFLICT OF INTEREST

J.R.L. has stock ownership in 23andMe and Lasergen, is a paid consultant for Regeneron, and a co-inventor on multiple United States and European patents related to molecular diagnostics for inherited neuropathies, eye diseases and bacterial genomic fingerprinting. The other authors declare no conflict of interest.

AUTHORS' CONTRIBUTIONS

N. Dinckan, R. Du, contributed to data acquisition, analysis, and interpretation, drafted the manuscript; Z. Coban- Akdemir, Y. Bayram contributed to data acquisition, analysis, and interpretation, critically revised the manuscript; S.N. Jhiangiani, H. Doddapaneni, J. Hu, D.M. Muzny, Y. Guven, O. Aktoren, H. Kayserili, E. Boerwinkle, R.A. Gibbs, contributed to data acquisition, revised the manuscript; J.E. Posey critically reviewed the manuscript; Z.O. Uyguner contributed to data acquisition, analysis, and interpretation, drafted the manuscript; J.R. Lupski, A. Letra contributed to conception, design, data acquisition, analysis, and interpretation, manuscript preparation and critically revised the manuscript. All authors gave final approval and agree to be accountable for all aspects of the work.

¹¹Pediatric Research Center, University of Texas Health Science Center at Houston McGovern Medical School, Houston, TX 77030, USA

Abstract

Tooth development is regulated by multiple genetic pathways, which ultimately drive the complex interactions between the oral epithelium and mesenchyme. Disruptions at any time point during this process may lead to failure of tooth development, also known as tooth agenesis (TA). TA is a common craniofacial abnormality in humans and represents the failure to develop one or more permanent teeth. Many genes and potentially subtle variants in these genes contribute to the TA phenotype. We report the clinical and genetic impact of a rare homozygous *ANTXR1* variant (c. 1312C>T), identified by whole exome sequencing (WES), in a consanguineous Turkish family with tooth agenesis. Mutations in *ANTXR1* have been associated with GAPO (growth retardation, alopecia, pseudoanodontia, and optic atrophy) syndrome and infantile hemangioma, however no clinical characteristics associated with these conditions were observed in our study family. We detected the expression of Antxr1 in oral and dental tissues of developing mouse embryos, further supporting a role for this gene in tooth development. Our findings implicate *ANTXR1* as a candidate gene for isolated TA, suggest the involvement of specific hypomorphic alleles, and expand the previously known *ANTXR1*-associated phenotypes.

Keywords

Tooth agenesis; whole exome sequencing; *ANTXR1*; expression

INTRODUCTION

Tooth agenesis (TA) is the developmental absence of permanent teeth and the most common craniofacial abnormality affecting human dentition [Hennekam et al. 2010]. It may occur as an isolated clinical entity (nonsyndromic TA) or as a part of a syndrome (syndromic TA), it can also be familial or sporadic, in a spectrum of phenotypic heterogeneity with various inheritance patterns [Chhabra et al. 2014]. TA is most frequently observed as an autosomal dominant (AD) trait while autosomal recessive (AR) and X-linked (XL) families have been less frequently described [Vastardis 2000]. Oligodontia is a severe form of TA, in which six or more permanent teeth, excluding third molars, are absent [Nieminen 2009; Symons et al. 1993]. The prevalence of TA has been reported to range from 1.6% to 9.6% depending on the population studied [Vastardis 2000]. Limited studies of Turkish cohorts report a prevalence of TA ranging from 4.6% to 4.84%, which includes hypodontia 3.67% to 4.3%, and oligodontia 0.21% to 0.3% [Celikoglu et al. 2010; Karadas et al. 2014].

Four conserved signaling pathways, Bmp, Fgf, Shh, and Wnt, are critical for tooth development, and inactivation of any of these pathways resulted in arrest of tooth development in mice [Liu et al. 2015a]. Mutations identified in patients with tooth agenesis also affect genes in these pathways [Liu et al. 2015a]. Large families with clinically well-defined TA phenotypes have been valuable for the identification of novel loci and genes contributing to TA and may help elucidate the potentially complex genetic network underlying tooth development. Genes associated with nonsyndromic TA can display one of

several modes of inheritance: AD with or without reduced penetrance such as *MSX1* [Vastardis et al. 1996], *PAX9* [Frazier-Bowers et al. 2002], *WNT10B* [Yu et al. 2016], and *GREM2* [Kantaputra et al. 2015]; mixed AD and AR inheritance such as that observed with *WNT10A* [Kantaputra and Sripathomsawat 2011]; and XL inheritance observed with *EDA* [Song et al. 2009; Yin and Bian 2015]. More recently, heterozygous loss-of-function variants in *LRP6* have been associated with TA [Dinckan et al. 2017; Massink et al. 2015; Ockeloen et al. 2016]. Genes associated with syndromic TA include *EDAR* and *EDARADD*, observed in individuals with additional minor ectodermal findings [Arte et al. 2013]. *AXIN2*, encoding a negative regulator of the Wnt signaling pathway, has been observed in association with both nonsyndromic TA as well as syndromic TA, which is related to mild ectodermal dysplasia phenotype, with familial colon cancer [Lammi et al. 2004; Marvin et al. 2011; Yue et al. 2016].

Biallelic variants in *ANTXR1* (anthrax toxin receptor 1) have been reported recently to be associated with GAPO syndrome (MIM#230740), which is characterized by growth retardation, alopecia, pseudoanodontia, and optic atrophy segregating as an AR trait [Bayram et al. 2014; Stranecky et al. 2013]. In this study, we report a novel homozygous missense variant in *ANTXR1* identified by whole exome sequencing (WES) in a Turkish family with tooth agenesis. Further, we demonstrate expression of Antxr1 in the oral and dental tissues of mouse embryos, implicating a role for this protein in tooth development. Our findings expand the previously known *ANTXR1*-associated phenotypes to include isolated TA.

MATERIALS AND METHODS

Study Family

A 7-year-old boy (III-2) born to consanguineous parents was referred to the Department of Medical Genetics at Istanbul Medical Faculty due to the absence of his lower anterior teeth. His medical records revealed previous surgery for anal atresia on day 2 of life, and for ankyloglossia at 1.5 years. Physical examination at 7 years revealed mild brachycephaly, a low hanging columella, hypoplastic alae nasi, and retrognathia, and no significant findings on the examination of other systems (Fig. 1a). Anthropometric measurements showed the height/weight/occipitofrontal circumference of the proband to be 123 cm (50~75 percentile), 22 kg (25~50 percentile), and 52 cm (close to 25 percentile), respectively. At 10 years, oral examination revealed the absence of lower incisors and canines, as well as two upper and all four lower premolars, from the oral cavity (Fig. 1b). Radiographic examination revealed the congenital absence of the lower incisors and canines bilaterally, consistent with a diagnosis of oligodontia (Fig. 1c). Clinical examination of the probands' parents and 7-year-old sibling, and evaluation of their dental records revealed no TA, or any additional dental anomalies (Fig. 2a).

Genetic analysis

The study was approved by the Istanbul University Institutional Ethical Review Board, and The University of Texas Health Science Center at Houston Committee for Protection of Human Subjects (HSC-DB-12-0255). After obtaining written informed consent from the

parents, DNA was isolated from peripheral blood samples of the proband, unaffected brother and parents, using standard extraction procedures. Whole exome sequencing (WES) was performed on the proband's sample, at the Baylor College of Medicine Human Genome Sequencing Center (BCM-HGSC) through the Baylor Hopkins Center for Mendelian Genomics (BHCMG) initiative. Briefly, an Illumina paired-end pre-capture library was constructed according to the manufacturer's protocol (Illumina) with slight modifications (<https://www.hgsc.bcm.edu>), and then was hybridized in solution to HGSC VCRome 2.1 exome capture design [Bainbridge et al. 2011]. The raw data were processed using the Mercury pipeline, which includes a set of bioinformatics tools to perform read mapping, variant calling, and variant annotation [Reid et al. 2014].

The variants called from WES were then filtered based on the minor allele frequency reported in several databases such as the Atherosclerosis Risk in Communities Study (ARIC) databases [Investigators 1989], the Exome Variant Server database (<http://evs.gs.washington.edu>), the 1000 Genomes Project database [Consortium et al. 2010], the Exome Aggregation Consortium (ExAC) database [Lek et al. 2016], and the internal BCHMG database. Variants were prioritized based on their consequences across transcripts, inter- and intra- species conservation, amino acid conservation, *in silico* functional algorithms predicting the possible impact of an amino acid substitution on the structure and/or function of protein, known association with human or model organism phenotype, and protein function, as previously described [Dinckan et al. 2017]. Variants prioritized as potentially associated with the phenotype were further analyzed using collective information from OMIM, PubMed, gene-associated animal models, and tissue expression of the encoded protein. To confirm the identified variant and its segregation within the family, conventional PCR and Sanger sequencing were conducted.

Expression analysis

We sought to determine the relevance of ANT XR1 during tooth development by evaluating the expression of its homologue Antxr1 in murine tooth sections at different developmental stages. Immunofluorescence was performed using tooth sections of wild type C57BL/6 mouse embryos at embryonic days (E) 12.5, 14.5, 16.5, 18.5 and postnatal day 0 (P0). Sections were deparaffinized in xylene, rehydrated in gradient alcohol baths and rinsed with deionized H₂O at room temperature. Antigen retrieval was performed using sodium citrate buffer (Dako, Santa Clara, CA) at 100°C for 30 minutes and allowed to cool down for 30 minutes at room temperature. To avoid nonspecific binding of the antibodies, sections were immersed in blocking solution (1% bovine serum albumin and 10% goat serum diluted in PBS) and incubated with AffiniPure Fab fragment goat anti-mouse (Jackson ImmunoResearch Laboratories, West Grove, PA), for 1 hour at room temperature. Sections were then washed and incubated with rabbit polyclonal anti-Antxr1 primary antibody (Abcam, Cambridge, MA) at 4°C overnight, then washed and incubated with Alexa Fluor® 555 goat anti-rabbit IgG secondary antibody (Invitrogen, Carlsbad, CA) for 2 hours in a dark chamber at room temperature. Lastly, sections were washed, counterstained with DAPI (Invitrogen, Carlsbad, CA) and mounted with ProLong Gold Antifade Reagent (Invitrogen, Carlsbad, CA). Imaging was performed after 48 hours in a Nikon Eclipse fluorescence

microscope (Nikon, Germany) equipped with a Zyla 5.5 sCMOS camera (Andor Technology, UK).

RESULTS

A total of 214,129 variants were identified by WES in the proband. After the application of our in-house developed computational pipeline that parses the data and filters the variant call files for rare variants, homozygous variants in *ANTXR1* (c.1312C>T, p.[Arg438Cys]), *MMP8* (c.929T>C, p.[Leu310Pro]), *DPH1* (c.746C>G, p.[Pro249Arg]), and *KDM2B* (c.83_84delCA, p.[Thr28Serfs*8]) were prioritized for follow-up based on their frequencies in our internal CMG database, the prediction of deleteriousness by bioinformatic tools, and a posited autosomal recessive inheritance model with biallelic variants. Of these four variants, *ANTXR1* was considered the strongest candidate for pathogenicity based on amino acid conservation, predictions of deleteriousness, and minor allele frequencies in the ExAC databases (in which only the *ANTXR1* variant was not observed in homozygosity). Prior reports of *ANTXR1* in association with pseudoanodontia provided additional evidence supporting the potential involvement of *ANTXR1* in tooth development [Bayram et al. 2014; Stranecky et al. 2013]. Segregation analysis was performed on the *ANTXR1* and *KDM2B* variants, and only the variant in *ANTXR1* followed the expected mode of inheritance (Fig. 2).

A homozygous missense variant NM_032208.2:c.1312C>T in exon 16 of *ANTXR1* (Fig. 2c), resulting in an arginine to cysteine substitution (p.Arg438Cys) in the encoded protein (NP_115584.1), was therefore considered pathogenic for the oligodontia phenotype in the proband (Fig. 2a). This variant is annotated as rs144438225 in the dbSNP database (<http://www.ncbi.nlm.gov/SNP/>). In the ExAC database, 44 heterozygous variants are reported out of 60,701 individuals (allele frequency <0.0004) from different populations, but no homozygous variants are reported [Lek et al. 2016]. In the ARIC database, this variant was detected 3 times out of 15,792 participants, yet the status of these variants was not provided [Investigators 1989]. This variant has not been reported in the 1000 Genomes Project, nor in the Greater Middle East Variome database [Scott et al. 2016]. The carrier frequency of this variant was 0.001 in the internal BHCMG database (>6,000 exomes) and 0.003 in the 952-individual Turkish cohort which is part of the internal BHCMG database. Computational analysis of variant function by Mutation Taster [Schwarz et al. 2014], SIFT [Ng and Henikoff 2003], and PolyPhen-2 algorithms [Adzhubei et al. 2013] predicted this variant as deleterious; PhyloP [Pollard et al. 2010] predicted the variant to be in a conserved residue (Fig. S1).

Segregation of the variant in the family showed that the unaffected parents are heterozygous for the *ANTXR1* variant allele, and the unaffected brother is wild type (Figs. 2a and S2). This suggests autosomal recessive inheritance passed on by the two parent carriers for the mutation. Further analysis of WES data and a calculation of B-allele frequency to deduce absence of heterozygosity (AOH) regions in the proband revealed that this variant mapped to a ~30 Mb region of AOH (Fig. 2b). The predicted consequence of the variant is shown to affect the C-terminus (Ant-C) domain of ANTXR1 (Fig. 2d).

Expression analyses revealed the localization of Antxr1 in the oral epithelium, dental epithelium and mesenchyme during mouse tooth development. Antxr1 was detected at E12.5 in oral and epithelial cells in the developing tongue (T), maxillary processes (MXP), mandibular process (MNP), and in the invagination of the dental lamina (DL) and subjacent condensed ectomesenchyme (Fig. 2e, A–C). At E14.5 cap stage of tooth development, Antxr1 expression was evident in the inner dental epithelium (IE) of the enamel organ (Fig. 2e, E and F) and in the enamel knot (EK) (Fig. 2e, F). At E16.5 and E18.5 bell stage of tooth development, Antxr1 was markedly evident in the inner dental epithelium (IE) and the stratum intermedium (SI) of mandibular molar (Fig. 2e, G–I), and similarly on maxillary molar (Fig. 2e, J). At P0, Antxr1 expression shifted to the cytoplasm of the polarized layer of ameloblasts and differentiating odontoblasts (arrow) (Fig. 2e, K).

DISCUSSION

In this study, we identified a homozygous missense variant c.1312C>T (p.Arg438Cys) in *ANTXR1* in association with oligodontia in a Turkish family. *ANTXR1* encodes a 564-amino acid protein that functions as an integrin-like membrane receptor protein, and with three functional domains: a von Willebrand factor type A domain at the N-terminal (VWA), an anthrax receptor extracellular domain (Anth_Ig), and an intracellular anthrax receptor C-terminus region domain (Ant_C) (Fig. 2d) [Punta et al. 2012].

Homozygous variants in *ANTXR1* have been associated with GAPO syndrome, characterized by growth retardation, alopecia, failure of tooth eruption, and optic atrophy [Tipton and Gorlin 1984]. To date, nine different biallelic *ANTXR1* variants have been suggested as etiologic for GAPO syndrome [Bayram et al. 2014; Benetti-Pinto et al. 2016; Chattopadhyay et al. 2017; Salas-Alanis et al. 2016; Stranecky et al. 2013] (Fig. 2c). Two nonsense variants (c.262C>T [p.Arg88*] and c.505C>T [p.Arg169*]), and one splice site alteration (c.1435-12A>G, [p.Gly479Phefs*119]) in the *ANTXR1* gene were the first reported variants in four unrelated GAPO syndrome patients [Stranecky et al. 2013]. The nonsense variants reside within the VWA domain, whereas the splice site variant was predicted to abolish a splicing acceptor site of the *ANTXR1* transcript. Recently, WES studies of GAPO syndrome patients revealed additional frame-shift (c.1221dupT [p.Ala408Cysfs*2]), missense (c.1150G>A [p.G384S]), and synonymous (c.411A>G [p.G137=]) variants (the latter which is predicted to abolish a canonical donor site), as likely pathogenic [Bayram et al. 2014]. The c.411A>G and c.1221dupT variants reside within VWA and Ant_C domain respectively and c.1150G>A located at a highly conserved site [Bayram et al. 2014]. Two missense variants (c.410A>T [p.Gln137Leu] and c.265G>A [p.Gly89Arg]) located in the VWA domain were also identified in two families affected with GAPO syndrome, one of Hispanic and one of South Asian descent, respectively [Chattopadhyay et al. 2017; Salas-Alanis et al. 2016]. In addition, a splice site mutation c.378+3A>G (p.[Arg99_Arg126del]) was indicated to result in exon 4 skipping in a Brazilian GAPO syndrome patient also affected with premature ovarian insufficiency [Benetti-Pinto et al. 2016].

The missense variant c.1312C>T (p.Arg438Cys) identified in our study resides within the Ant_C domain (Fig. 2d) that functions at the cytoplasmic region of the anthrax receptor,

replacing the basic charged polar amino acid with an uncharged polar amino acid containing a sulfhydryl moiety. This semi-conserved residue substitution contrasts with other reported missense variants, which may explain why the patient in this study was not affected with GAPO syndrome, as no additional structural abnormalities were identified beyond the oligodontia trait. It should be noted that previous reports of GAPO syndrome patients describe a 'pseudoanodontia' phenotype referring to failure of tooth eruption. However, the lack of additional details or supporting panoramic radiographs in the majority of previous reports makes interpretation of their oral phenotypes difficult. Our findings of a biallelic *ANTXR1* variant in a family with oligodontia and parental consanguinity is intriguing and warrants additional investigation.

ANTXR1 has been proposed to play important roles in extracellular matrix (ECM) homeostasis, and its expression was detected in a number of different tissues, including the central nervous system, heart, lung, and lymphocytes [Cullen et al. 2009]. Targeted disruption of *Antxr1* in mice resulted in viable mice without major structural defects, although dental overgrowth, incisor misalignment, and dental dysplasia were observed [Cullen et al. 2009]. Histopathological analysis revealed an accumulation of ECM in various tissues including the ovaries, uterus, skin, and periodontal ligament of the incisors, and to a lesser extent around the molars. Such accumulation of ECM in the tooth-surrounding tissues was considered the reason for the incisor misalignment and their observed overgrowth, and the associated degeneration of the enamel organ and associated ameloblasts and odontoblasts resulting in dental dysplasia. Interestingly, despite the excess of ECM in affected tissues and overexpression of collagen types I and IV in *Antxr1*^{-/-} mice, no evidence for increased number of fibroblasts was found [Cullen et al. 2009]. Our expression assays showed that *Antxr1* was expressed in the developing mouse craniofacies in different temporal and spatial patterns. *Antxr1* was localized in the epithelium of developing tongue, maxillary and mandibular processes, as well as in the dental epithelium and mesenchyme at early stages of tooth development. At later stages, *Antxr1* expression was noted in the epithelium of the enamel organ and in the dental papilla, and then shifted to the polarized layer of ameloblasts and differentiating odontoblasts. These findings indicating that *Antxr1* is present during normal tooth morphogenesis. Yet the precise role of *ANTXR1* in TA phenotypes remains to be established.

Additional studies have reported *ANTXR1* as a tumor-specific endothelial marker implicated in colorectal cancer, and upregulated in tumor angiogenesis [Carson-Walter et al. 2001; Fernando and Fletcher 2009; Liu et al. 2015b]. Expression of *ANTXR1* was detected at basal levels in normal tissues, whereas it is increased in the vascular compartment of multiple primary tumors and metastases [Carson-Walter et al. 2001]. Previously, *ANTXR1* was suggested to modulate canonical Wnt signaling during normal vascular development [Abrami et al. 2008] and a direct interaction between *ANTXR1* and *LRP6* was proposed, as *ANTXR1* was shown to regulate the availability and function of *LRP6* favoring beta-catenin stabilization [Verma et al. 2011]. *LRP6* is a receptor for Wnt and thereby transmits the canonical Wnt/beta-catenin signaling cascade, playing a role in the regulation of cell differentiation, proliferation, and migration, during embryonic development and in tumor development as well. Recently, we and others have identified pathogenic variants in *LRP6* in individuals and families with tooth agenesis [Dinckan et al. 2017; Massink et al. 2015;

Ockeloen et al. 2016]. Interestingly, Lammi et al. showed that a nonsense variant in *AXIN2* was segregating in a family with oligodontia and colorectal cancer, and suggested that altered regulation of Wnt signaling caused oligodontia and predisposition to familial colon cancer [Lammi et al. 2004]. Numerous studies have since reported on mutations in *AXIN2* and other cancer-associated genes with TA [Yamaguchi et al. 2017; Yue et al. 2016]. These findings highlight the complex nature of TA and emphasize the need to consider modifier genes and/or gene-gene interactions in studies of this condition.

In summary, we identified a homozygous missense variant in *ANTXR1* as likely pathogenic for oligodontia, and further demonstrated expression of Antxr1 during stages of tooth development in mice. These findings support a role for this gene in tooth development. While these findings may reflect the presence of a private mutation in a particular family, *ANTXR1* may present a variety of functions in developmental processes and signaling pathways that are yet to be elucidated by additional investigations in humans and functional studies in model organisms.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

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Figure 1. Clinical findings of the proband. (a) Facial features of the proband include mild brachycephaly, low hanging columella, hypoplastic alae nasi and retrognathia. (b) Intraoral features of the proband include a mixed dentition stage and clinical absence of the permanent upper lateral incisors, canines and second premolars. (c) Radiographic examination reveals congenital absence of the permanent lower incisors and canines, as well as unerupted teeth. Missing teeth are indicated by stars in the panoramic radiograph and by filled boxes in the subjacent schematic of the dentition.

Figure 2.

Molecular findings in the family. (a) Pedigree and genotyping of available family members. The homozygous variant NM_032208.2:c.1312C>T (p.Arg438Cys) in *ANTXR1* was identified and confirmed in proband III-2, identified as heterozygous in the unaffected consanguineous parents (II-8 and II-9), and not detected in the unaffected brother (III-1). Chromatograms of Sanger sequencing of four available family members are indicated under each sequenced individual. The position of c.1312C>T is shaded in blue. (b) AOH plots for chromosome 2 of proband III-2. The blue horizontal line marks a region of AOH measuring

~30 Mb. The red vertical line indicates the position of the variant c.1312C>T which is located within the AOH region. (c) The distribution of previously reported variants and the presently described variant are indicated within the exonic structure of *ANTXR1*. The identified missense variant c.1312C>T (in red) is located in exon 16 of 18. (d) The predicted amino acid consequence of each variant is depicted along a schematic representation of the ANTXR1 protein structure with its different domains. The mutation p.Arg438Cys (in red) resides within Ant_C domain. (e) Immunofluorescence expression of Antxr1 in developing mouse embryos. Antxr1 was detected at E12.5 in oral and epithelial cells in the developing tongue (T), maxillary processes (MXP), mandibular process (MNP), and in the invagination of the dental lamina (DL) and subjacent condensed ectomesenchyme (A-C). At E14.5 cap stage of tooth development, Antxr1 expression was evident in the inner dental epithelium (IE) of the enamel organ (E and F) and in the enamel knot (EK) (F). At E16.5 and E18.5 bell stage of tooth development, Antxr1 was markedly evident in the inner dental epithelium (IE) and the stratum intermedium (SI) of mandibular molar (G-I), and similarly on maxillary molar (J). At P0, Antxr1 expression shifted to the cytoplasm of the polarized layer of ameloblasts and differentiating odontoblasts (arrow) (K).