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Whole exome sequencing in an Italian family with isolated maxillary canine agenesis and canine eruption anomalies

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ABSTRACT

Objective: The aim of this study was the clinical and molecular characterization of a family segregating a trait consisting of a phenotype specifically involving the maxillary canines, including agenesis, impaction and ectopic eruption, characterized by incomplete penetrance and variable expressivity.

Design: Clinical standardized assessment of 14 family members and a whole-exome sequencing (WES) of three affected subjects were performed. WES data analyses (sequence alignment, variant calling, annotation and prioritization) were carried out using an in-house implemented pipeline. Variant filtering retained coding and splice-site high quality private and rare variants. Variant prioritization was performed taking into account both the disruptive impact and the biological relevance of individual variants and genes. Sanger sequencing was performed to validate the variants of interest and to carry out segregation analysis.

Results: Prioritization of variants "by function" allowed the identification of multiple variants contributing to the trait, including two concomitant heterozygous variants in *EDARADD* (c.308C > T, p.Ser103Phe) and *COL5A1* (c.1588G > A, p.Gly530Ser), specifically associated with a more severe phenotype (*i.e.* canine agenesis). Differently, heterozygous variants in genes encoding proteins with a role in the WNT pathway were shared by subjects showing a phenotype of impacted/ectopic erupted canines.

Conclusions: This study characterized the genetic contribution underlying a complex trait consisting of isolated canine anomalies in a medium-sized family, highlighting the role of WNT and EDA cell signaling pathways in tooth development.

1. Introduction

Dental agenesis is one of the most common human dental abnormalities, with a prevalence, excluding third molars, ranging between 0.15% and 16.2% (Rakhshan, 2015). This condition may be classified as "oligodontia", referring to the absence of more than six teeth (excluding third molars), or "hypodontia", referring to the absence of one to six teeth. Tooth agenesis may occur either as an isolated condition or in syndromic phenotypes, and has been reported in both familiar and sporadic cases (Nieminen, 2009). The etiology of tooth agenesis is still largely unknown, with both genetic and environmental factors supposed to contribute significantly to the pathogenesis.

Isolated hypodontia has been documented as being transmitted as a dominant or recessive trait, and has been associated with mutations in several genes: *PAX9* (Stockton, Das, Goldenberg, D'Souza, & Patel, 2000), *EDA* (Tao et al., 2006), *MSX1* (Vastardis, Karimbux, Guthua, Seidman, & Seidman, 1996), *AXIN2*, *EDARADD* (Bergendal, Klar, Stecksén-Blicks, Norderyd, & Dahl, 2011), *LRP6* (Massink et al., 2015), *WNT10A* (Kantaputra & Sripathomsawat, 2011), *GREM2* (Kantaputra et al., 2015), *BMP4*, *BMP2* (Mu et al., 2012), *WNT10 B* (Yu et al., 2016),

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PTH1R (Decker et al., 2008), *EDAR* (Arte, Parmanen, Pirinen, Alaluusua, & Nieminen, 2013) and *SMOC2* (Alfawaz et al., 2013). Recently, mutations in the *WNT10A* gene have been reported to underlie the isolated agenesis of the permanent maxillary canines (Kantaputra, Kaewgahya, & Kantaputra, 2014).

Tooth eruption is the movement of a tooth from its position within the jaw towards its functional position at the occlusal plane. Perturbation of the eruption path during the development of the craniofacial structures can cause tooth impaction, positional anomalies and malocclusions. The maxillary permanent canine is the second most frequent impacted tooth after the third molar, with a reported prevalence of 1–2% (Sajnani, 2015). The etiology of canine impaction is still largely unknown, but several contributing factors have been suggested, including localized, systemic and genetic causes (Becker & Chaushu, 2015; Leonardi, Barbato, Vichi, & Caltabiano, 2009; Leonardi, Peck, Caltabiano, & Barbato, 2003; Lombardo, Barbato, & Leonardi, 2007; Mercuri et al., 2013; Peck, Peck, & Kataja, 1994).

Recently, multiple lines of evidence highlighted the role of a few specific cell signaling pathways, e.g. the NF-κB and the WNT, as crucial mediators of tooth development and tooth agenesis and inclusion (Yin & Bian, 2015).

We report on a medium-sized family with maxillary canine anomalies, including agenesis, either monolateral or bilateral, impaction and ectopic eruption. Since tooth morphogenesis is regulated by a complex regulatory network, we used a whole exome sequencing (WES) approach on selected family members to characterize the molecular bases of these phenotypes in this family.

2. Materials and methods

2.1. Study subjects

The index patient (III:6, Fig. 1) was a 16-year-old girl from a small town in central Italy, who was referred to the Department of Oral and Maxillo Facial Sciences of Sapienza University of Rome. The patient showed a bilateral absence of permanent maxillary canines and anamnestic analysis suggested the presence of several family members also affected by canine anomalies.

Clinical standardized assessment, including panoramic radiographs, oral photographs and anamnestic data, was performed on fourteen members of the family by a trained orthodontist. The dentition status of all subjects was assessed by a dental specialist. A detailed clinical evaluation of all family members included in this study allowed us to exclude any syndromic features. After clinical evaluation, blood samples were collected from all available family members and genomic DNA was extracted from leukocytes by using the Gentra Puregene Blood kit (Qiagen, Hilden, Germany), as per manufacturer's protocol. All clinical and genetic studies were approved by the regional Ethical Review Board of the "Umberto I" General Hospital of Rome (Ref. 3781) and were conducted according to the ethical principles defined in the Declaration of Helsinki. All subjects enrolled in this study signed an informed consent form.

2.2. Whole exome sequencing

Exome enrichment and massively parallel sequencing were performed on 2 µg of genomic DNA of three family members with canine anomalies (III:1, III:4, and III:6). WES was carried out by Complete Genomics (Mountain View, CA) (III:6) and BGI (Shenzen, China) (III:1 and III:4). The exome data of subject III:6 were analyzed by means of Complete Genomics-BGI proprietary instruments and software. For III:1 and III:4 samples, exome capture was carried out using SeqCap EZ Human Exome Kit V.3.0 (Roche, Basel, Switzerland) and data analysis was performed using an in-house implemented pipeline (Kortüm et al., 2015). Paired-end reads were aligned to the human genome (UCSC GRCh37/hg19) with the Burrows-Wheeler Aligner (BWA V.0.7.12). Single Nucleotide Variants (SNVs) and small INDELs were identified by means of the GATK's HaplotypeCaller (V.3.5), according to GATK's best practices.

High-quality variants were filtered against a public database (ExAC V.0.3.1) to retain novel and annotated changes with an unknown frequency or having a minor allele frequency (MAF) ≤5% and occurring with a frequency ≤10% in an in-house database including approximately 600 exomes. SnpEff toolbox (V.4.2) was used to predict the functional impact of variants, which were filtered to retain only those located in exons with an effect on the coding sequence and splice site regions. Functional annotation of variants was performed using SnpEff V.4.2 and dbNSFP V.2.9. The functional impact of variants was analyzed by Combined Annotation Dependent Depletion (CADD) V.1.3, a tool for scoring the deleteriousness of DNA variants, using as threshold a value of 10 (Kircher et al., 2014).

2.3. Variant prioritization and validation

Although canine anomalies are considered complex, multifactorial defects, segregation of the trait in the first two branches of this family suggested autosomal dominant transmission of a phenotype characterized by impacted/ectopic erupted canines (Fig. 1). These canine

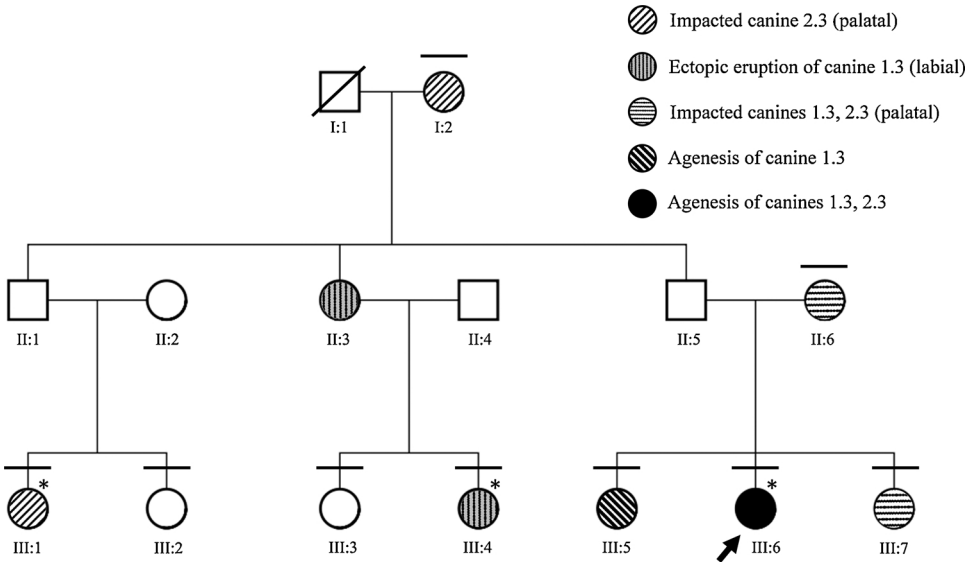

Fig. 1. Pedigree of the family with canine anomalies, object of this study. The arrow indicates the index patient. Black lines indicate individuals for whom DNA was available for the molecular analyses. The asterisks indicate individuals who underwent whole exome sequencing (WES). Affection status is shown: subjects I:2 and III:1 show monolateral upper left palatal impacted canine. Subjects II:3 and III:4 show monolateral upper right ectopic labial eruption of canine. Subject III:5 shows monolateral agenesis of the right upper permanent canine. The index patient III:6 has a bilateral agenesis of maxillary permanent canines. Subject II:6 and subject III:7 show bilateral, palatal impacted maxillary canines.

anomalies can be regarded as different manifestations of the same trait, as the labial ectopic eruption is considered a possible manifestation of the facial impaction (Peck et al., 1994). Incomplete penetrance and variable expressivity were postulated. In the third branch of the family, a different and more complex segregation model could be suggested, with subjects affected by a more severe phenotype (*i.e.* canine agenesis) possibly resulting from the contribution of paternally and maternally transmitted DNA variants affecting the same or different genes. The WES data of three selected family members, one for each branch of the family, were therefore analyzed in order to identify and prioritize variants segregating according to different inheritance patterns and matching at least one of the following criteria: known causative variants, variants in known genes (Supplementary Table 1), variants in genes functionally related to teeth development and variants predicted deleterious using CADD scoring system (Kircher et al., 2014). Potentially causative variants were further analyzed in terms of gene function, gene expression, animal models and phenotype, retrieving information from several databases, *i.e.* OMIM-Online Mendelian Inheritance in Man (https://www.omim.org), HPO-Human Phenotype Ontology (http://human-phenotype-ontology.github.io), MGI-Mouse Genome Informatics (http://www.informatics.jax.org), ZFIN-Zebrafish Information Network (https://zfin.org) and literature (PubMed).

Selected candidate variants were further analyzed by Sanger sequencing. Variants were PCR-amplified by using GoTaq G2 Flexi DNA polymerase (Promega, Madison, WI, USA) and custom primers (Supplementary Table 2). Sanger sequencing was performed by using the ABI BigDye Terminator Sequencing Kit V.3.1 as per the manufacturer's protocol and an ABI Prism 3500 Genetic Analyzers (Applied Biosystems, Foster City, CA, USA). Sequence electropherograms were analyzed by using ChromasPro V.1.7.5 (Technelysium Pty Ltd, Brisbane, Australia). Segregation analysis was performed on all family members for whom DNA was available.

3. Results

3.1. Study subjects

Overall, 14 family members were enrolled in this study (Fig. 1), 11 females and 3 males, with an age range of 13–83 years. Eight subjects were affected by canine anomalies: 4 by canine palatal impaction, either monolateral (I:2, III:1) or bilateral (II:6, III:7); 2 by canine agenesis, either monolateral (III:5) or bilateral (III:6); 2 by canine ectopic eruption, both monolateral (II:3; III:4). The phenotypes showed exclusive female expression and no other dental (e.g. agenesis of the third molars, agenesis, microdontia or peg-shaped maxillary lateral incisor) or extra-dental associated feature was detected, besides the presence in patient III:1 of a microdontic lateral incisor (1.2), placed on the opposite side of the impacted maxillary canine (2.3). The collected data, including detailed clinical and radiographic features of the affected members, are reported in Table 1. Oral photographs and panoramic radiographs of selected affected members are reported in Fig. 2.

3.2. WES data analysis

Exome sequencing was performed on three affected subjects (III:6, III:1 and III:4, Fig. 1). Detailed WES data output is reported in Supplementary Table 3.

3.3. Candidate variant prioritization and selection

A total of 2205, 1429, and 1381 private and rare variants with predicted functional impact were identified in subjects III:6, III:1, and III:4, respectively. According to a hypothesis of a common genetic cause of the observed phenotypes, we analyzed variants shared by all three sequenced subjects, but no candidate gene with a plausible role in tooth morphogenesis was identified. Assuming a different etiopathogenesis for canine agenesis and canine impaction/ectopic eruption, we focused on variants of subject III:6 (bilateral canine agenesis) and on variants shared only by subjects III:1 and III:4 (impacted/ectopic eruption). In subject III:6, the prioritization of candidate variants led to the identification of a heterozygous missense variant in *EDARADD* (NM_145861, c.308C > T, p.Ser103Phe, rs114632254), previously associated with isolated tooth agenesis (Table 2, Supplementary Figs. 1 and 2) (Arte et al., 2013; Bergendal et al., 2011; Salvi et al., 2016). Segregation analysis disclosed the presence of the *EDARADD* variant also in her sister (III:5; monolateral canine agenesis) and her mother (II:6; bilateral canine maxillary inclusion). The variant was annotated in ExAC with a frequency of 2.1% and in an in-house database with a frequency of 8.5%. The CADD scoring system predicted a high functional impact (27.8).

Data analysis also disclosed in the same subject (III:6) a heterozygous missense *COL5A1* variant (NM_000093, c.1588G > A, p.Gly530Ser, rs61735045), which had previously been associated with a syndromic phenotype, including dental anomalies, in the homozygous state (Giunta et al., 2002). The variant was shared by affected subjects III:5 and III:6, and unaffected subject III:2, and segregated from the affected grandmother (I:2; Table 2, Supplementary Figs. 1 and 2). The variant was annotated in ExAC (3.6%) and in-house database (8.7%), with a high CADD score (24.9).

Subjects III:1 and III:4, who showed a phenotype of impacted/ectopic erupted canines, shared missense heterozygous variants in *RSPO4, T*, and *NELL1* genes (Table 2, Supplementary Figs. 1 and 2). Segregation analysis disclosed the presence of the *RSPO4* variant (NM_001029871, c.317G > A, p.Arg106Gln, rs6140807) in affected subjects (III:1, III:4, and I:2), and in subject III:3, who did not manifest any dental anomaly. The missense variant in *T* (NM_003181, c.1013C > T, p.Ala338Val, rs117097130) was identified in affected subjects (III:1, III:4 and I:2) and in unaffected subjects (III:2 and III:3). Finally, the *NELL1* variant (NM_001288713, c.1244G > A, p.Arg415His, rs141323787) was identified in affected subjects (III:1, III:4 and I:2) and in subjects who did not exhibit any dental anomaly (III:2 and III:3).

The above mentioned candidate variants were annotated with a frequency $\leq 0.9\%$ in ExAC and $\leq 2.2\%$ in our in-house database. All these variants were predicted to be deleterious by CADD scoring

Table 1
Clinical and radiographic characteristics of the affected members.

Subject	Sex	Agenesis	Impaction	Type of inclusion	Side of inclusion	Ectopic eruption	Root resorption of permanent teeth	Third molar	Persistence of deciduous canines	Lateral tooth anomalies
III:6	F	1.3, 2.3	–	–	–	–	No	Present	6.3	–
III:7	F	–	1.3, 2.3	Bilateral	Palatal	–	No	Present	5.3, 6.3	–
III:5	F	1.3	–	–	–	–	No	Present	5.3	–
III:1	F	–	2.3	Monolateral	Palatal	–	No	Present	6.3	Microdontia 1.2
III:4	F	–	–	–	–	1.3	No	Present	–	–
II:6	F	–	1.3, 2.3	Bilateral	Palatal	–	No	Present	5.3, 6.3	–
II:3	F	–	–	–	–	1.3	No	Present	–	–
I:2	F	–	2.3	Monolateral	Palatal	–	No	Present	–	–

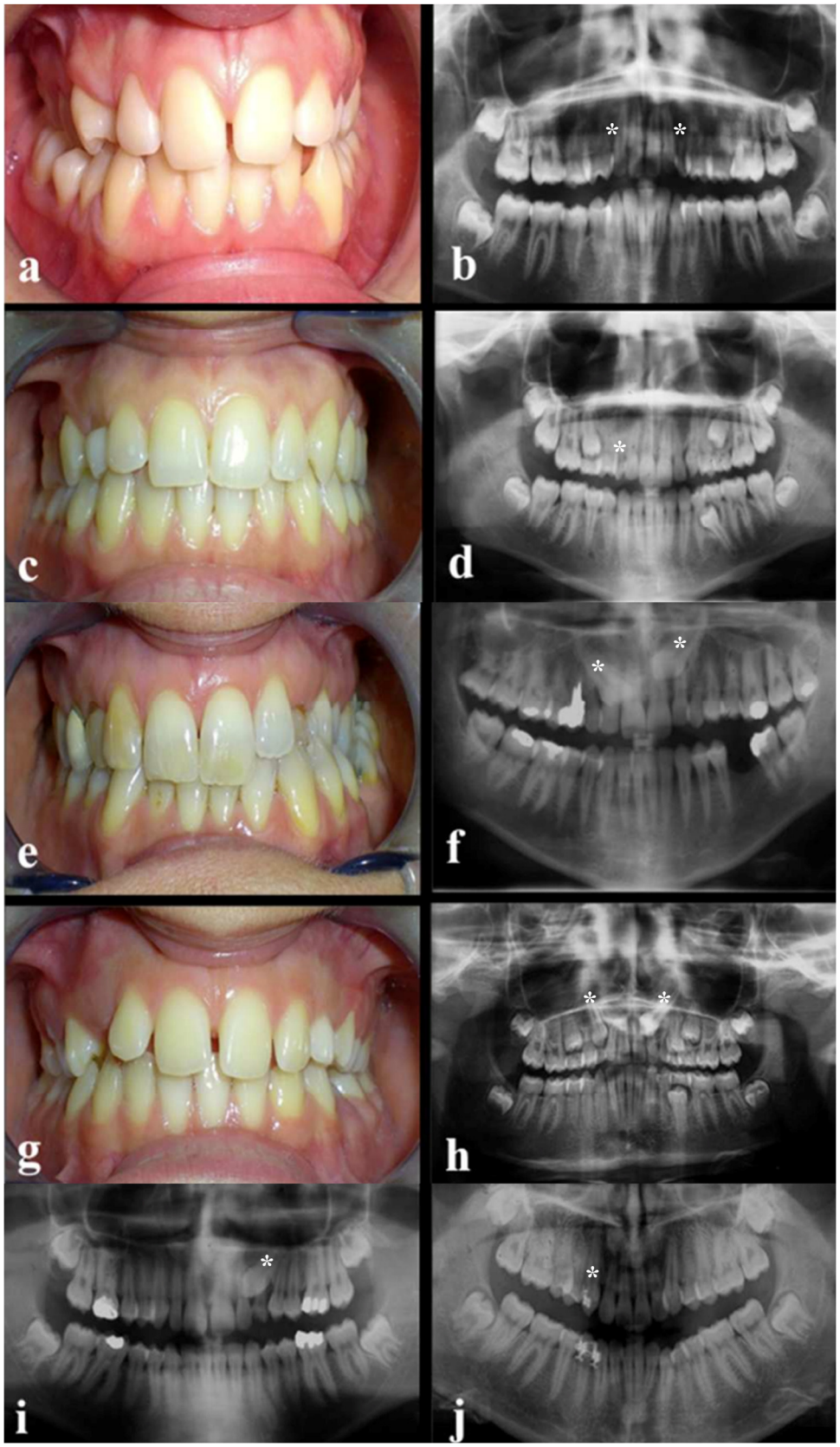

Fig. 2. Clinical photographs and panoramic radiographs of dentitions of six affected family members.
(A–B) Subject III:6. The index patient shows congenital absence of 1.3 and 2.3 and the persistence of the left upper deciduous canine. Panoramic radiograph shows the agenesis of permanent maxillary canines and root resorption of left deciduous canine. All third molars are present.
(C–D) Subject III:5. Agenesis of 1.3. Panoramic radiograph shows the persistence of the right upper deciduous canine with root resorption; 3.5 anomalous radicular distal tip is observed.
(E–F) Subject II:6. Palatal bilateral maxillary impacted canines and persistence of the upper deciduous canines are observed.
(G–H) Subject III:7. Palatal bilateral maxillary impacted canines and persistence of the upper deciduous canines are observed.
(I) Subject III:1. Impacted canine 2.3; persistence of the left upper deciduous canine and 1.2 microdontic lateral incisor are shown.
(J) Subject III:4. Ectopic eruption 1.3. Panoramic radiograph shows the orthodontic treatment. Mild crowding of maxillary arch can also be observed.
The asterisks indicate the missing, impacted or ectopically erupting permanent maxillary canines.

Table 2
Candidate variants identified through WES approach. Potentially deleterious DNA variants identified in the *EDARADD*, *COL5A1*, *RSPO4*, *T*, and *NELL1* genes are reported.

Gene	Chr	Genomic position	Genbank accession number	Nucleotide substitution	Aminoacid substitution	Variant ID	ExAC	in-house database	CADD
EDARADD	1	236645609	NM_145861	c.308C > T	p.Ser103Phe	rs114632254	2.1%	8.5%	27.8
COL5A1	9	137642654	NM_000093	c.1588G > A	p.Gly530Ser	rs61735045	3.6%	8.7%	24.9
RSPO4	20	947909	NM_001029871	c.317G > A	p.Arg106Gln	rs6140807	0.9%	2.2%	22.8
T	6	166574346	NM_003181	c.1013C > T	p.Ala338Val	rs117097130	0.5%	0.8%	20.1
NELL1	11	20968970	NM_001288713	c.1244G > A	p.Arg415His	rs141323787	0.4%	0.6%	26.2

system, with a value of 22.8, 20.1 and 26.2, respectively (Table 2).

4. Discussion

The main objective of this study was to characterize a family with several subjects affected by isolated canine anomalies from a clinical and molecular perspective. The peculiar aspect of this family appeared in the occurrence of several members with a variable phenotype that specifically involved maxillary canines, including agenesis, inclusion, and ectopic eruption, without any other dental or extra-dental associated feature. The only exception was represented by one subject (III:1, Fig. 2 and Table 1), who showed a microdontic lateral incisor (1.2) placed on the opposite side of the impacted maxillary canine (2.3). Interesting to note, the ectopic labial eruption of maxillary canine observed in patients II:3 and III:4 was not due to inadequate arch space permitting the exclusion of the mechanical effect as the cause of eruption disorder.

The exclusively female expression of the phenotypes, which appears to be characterized by incomplete penetrance in males, is in keeping with several data which report a strong difference in the prevalence of maxillary canine impaction between females and males (Ericson & Kurol, 1986), which has been explained as being related to an earlier dentition development in the former (Rutledge & Hartsfield, 2010). However, this observation might be also caused by the skewed proportion of females in the family tree (11 females vs 4 males).

Canine anomalies are regarded as complex traits. However, this uncommon family suggests a significant genetic contribution to these phenotypes, that might either represent different manifestations of the same basic disorder or they might share, in part, a molecular basis. In the first two branches of the family, four members with impacted or ectopic erupted canines are present. A transmission of the trait according to an autosomal dominant segregation model, with incomplete penetrance and variable expressivity, is suggested by the evidence that ectopic eruption may be a manifestation of the impaction (Peck et al., 1994) and that both buccally and palatally displaced canines share similar etiologies (Sajnani & King, 2012). A more complex segregation pattern could occur in the third branch of the family, due to a possible concomitant paternal and maternal contribution. The sequencing data of further family members of this branch of the family could help to elucidate this aspect.

To date, only very few studies reported Next Generation Sequencing (NGS) strategies to study orodental pathologies, in particular canine anomalies (Massink et al., 2015; Prasad et al., 2016; Ockeloen et al., 2016; Salvi et al., 2016; Yamaguchi et al., 2017). To study the molecular basis of the phenotypes in this family, we used a WES approach on selected affected family members. We identified a heterozygous mutation in the *EDARADD* gene (c.308C > T, p.Ser103Phe) in two subjects with the most severe phenotype (*i.e.* canine agenesis). The variant was transmitted by the mother, who is not related to the family and who presents a phenotype of bilateral impacted canines. This variant has been previously associated with isolated oligodontia, including canine teeth, and is predicted to be functionally relevant (Arte et al., 2013; Bergendal et al., 2011; Salvi et al., 2016). *EDARADD* codes for a protein that interacts with EDAR, a death domain receptor required for hair, teeth and the development of other ectodermal derivatives. *EDARADD* is expressed in epithelial cells during the formation of hair follicles and teeth. Indeed, mutations in *EDARADD* have been associated with one of the ectodermal dysplasias, characterized by defective development of hair, teeth, and eccrine sweat glands (van der Hout et al., 2008). *EDARADD* mutation carriers in this family did not show symptoms of ectodermal origin other than teeth dysgenesis. This finding suggests the involvement of the EDA signaling pathway in isolated oligodontia and confirms the considerable variation in clinical expression of *EDARADD* mutations. The most severe phenotype of subjects III:5 and III:6 could be caused by the concurrent contribution of paternally inherited DNA variants at other loci. In fact, the association of the p.Ser103Phe *EDARADD* variant with other mutations in genes related to teeth development has been already reported in isolated oligodontia, suggesting addictive effects of other pathways, e.g. the WNT signaling pathway (Arte et al., 2013; Salvi et al., 2016). Consistent with this hypothesis, a *COL5A1* variant (c.1588G > A, p.Gly530Ser) was identified in subjects with canine agenesis (III:5 and III:6), segregating from the paternal affected grandmother (I:2). *COL5A1* codes for a component of type V collagen. The variant localizes to the “interrupted collagenous region” of the protein and is predicted to interfere with the correct folding of the COL2 domain (Symoens et al., 2011). Haploinsufficiency of *COL5A1* is a cause of Ehlers-Danlos syndrome, where dental pathology, including hypodontia of permanent teeth and delayed eruption, is a common feature. Previously described cases suggest that this substitution causes a recessive form of Ehlers-Danlos syndrome (Giunta et al., 2002). The Gly530Ser change was previously reported as a disease modifying variant in Ehlers-Danlos syndrome type 1 (Steinmann & Giunta, 2000) and also as a biallelic causative mutation, with heterozygous parents showing subtle clinical signs (Giunta et al., 2002). None of the mutation carriers in the analyzed family showed symptoms of connective disorder. However, it should be noted that the clinical significance of this variant has not been definitely established as there are conflicting interpretations of its pathogenicity (https://www.ncbi.nlm.nih.gov/clinvar/variation/38863).

According to a supposed dominant segregation model with incomplete penetrance and variable expressivity, several shared candidate variants in genes functionally related to tooth morphogenesis were identified in the two analyzed subjects who were affected by canine eruption anomalies (III:1 and III:4). Potential harmful missense variants were identified in *RSPO4, T,* and *NELL1* genes. *RSPO4* codes for a member of the R-spondin protein family that has essential roles in vertebrate development and is expressed in the dental papilla (Pemberton et al., 2007). *RSPO4* is a secreted protein in WNT signaling and is mutated in inherited anonychia, a rare autosomal recessive condition characterized by the absence or severe hypoplasia of nails. The p.Arg106Gln variant is positioned in the Furin-like repeat two domain of the protein, which is required for β-catenin stabilization (Ishii et al., 2008). The *T* gene codes for Brachyury, a transcription factor placed downstream of the WNT/b-catenin signaling pathway (Arnold et al., 2000). Murine models demonstrated that this gene is involved in establishing notochord cell identity and differentiation, and in the organization of the axial development (Herrmann, 1992). The p.Ala338Val substitution lies in the second transactivation domain

(Kispert, Koschorz, & Herrmann, 1995) and has been previously associated with variable vertebral phenotypes, including multiple regional vertebral segmentation defects (Ghebranious et al., 2008). Nel-like molecule-1 (Nell-1) is a secreted protein that plays an important role in osteoblast differentiation, bone formation and regeneration. It is strongly expressed in neural tissue, with epidermal growth factor-like domains suggesting specificity for craniofacial regions. The expression patterns during tooth development suggest that it plays an important role in tooth morphogenesis (Tang, Wang, Du, Yang, & Wang, 2013).

The WES data and segregation analyses of the family pointed to two different signaling pathways as responsible for the dental phenotypes, one of them (*i.e.* EDA) for the canine agenesis, and the other (*i.e.* WNT) for the less severe canine eruption anomalies. Further functional research on the mechanisms through which the EDA and WNT pathways regulate tooth morphogenesis and eruption is warranted to shed light on the pathogenesis of tooth agenesis. However, other genetic, epigenetic and environmental contributory factors may also be involved. Analysis of further family members might better elucidate the molecular bases of these phenotypes.

Conflicts of interest

The authors declare that they have no competing interests.

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Ethical approval

All clinical and genetic studies were approved by the regional Ethical Review Board of the “Umberto I” General Hospital of Rome (Rif. 3781).

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at https://doi.org/10.1016/j.archoralbio.2018.04.011.

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