Phenotype Characterization And Candidate Genotyping Of Hypodontia In Ectodermal Dysplasia (ED) And Non-Syndromic Groups

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Abstract

Matthew John Olmsted, DDS: Phenotype Characterization And Candidate Genotyping Of Hypodontia In Ectodermal Dysplasia (ED) And Non-Syndromic Groups (Under the direction of Sylvia A. Frazier-Bowers, DDS, PhD)

Hypodontia is a common clinical challenge in orthodontics and dentistry. It occurs as a syndrome—ectodermal dysplasia (ED) and as non-syndromic tooth agenesis (NSTA). This study quantifies patterns of agenesis and tooth morphology between ED (n=37) and NSTA (n=81) groups. 118 radiographs and 58 dental casts were measured for presence/absence and mesiodistal width of permanent teeth. In addition, the conicity of the maxillary incisors was determined using a novel index. 88 affected (ED or NSTA) individuals were genotyped using 1474 SNPs of candidate genes (AXIN2, BMP4, EDA, MSX1, and PAX9). Multivariate analyses determined that specific SNPs in BMP4 and MSX1 best explain the genetic differences between the two groups. When compared to NSTA, ED had reduced tooth widths, more conical maxillary incisors, and a higher occurrence of missing teeth (p<0.05), excluding mandibular second premolars. The new measure of conicity detected differences between control, ED, and NSTA groups.
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<table>
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<th>Abbreviation</th>
<th>Description</th>
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<tbody>
<tr>
<td>AXIN2</td>
<td>axin 2 gene</td>
</tr>
<tr>
<td>BMP4</td>
<td>bone morphogenetic protein 4</td>
</tr>
<tr>
<td>DHC</td>
<td>dental health component</td>
</tr>
<tr>
<td>ED</td>
<td>ectodermal dysplasia</td>
</tr>
<tr>
<td>EDA</td>
<td>ectodysplasin A gene</td>
</tr>
<tr>
<td>DiProPerm</td>
<td>Distance-Projection-Permutation</td>
</tr>
<tr>
<td>DWD</td>
<td>Distance Weighted Direction</td>
</tr>
<tr>
<td>ICCC</td>
<td>Inter Class Correlation Coefficient</td>
</tr>
<tr>
<td>IOTN</td>
<td>index of orthodontic treatment need</td>
</tr>
<tr>
<td>MSX1</td>
<td>muscle segment homeobox-1 gene</td>
</tr>
<tr>
<td>NEMO</td>
<td>NF-κB Essential Modulator gene</td>
</tr>
<tr>
<td>NFED</td>
<td>National Foundation of Ectodermal Dysplasia</td>
</tr>
<tr>
<td>NSTA</td>
<td>non syndromic tooth agenesis</td>
</tr>
<tr>
<td>NHP</td>
<td>non-human primates</td>
</tr>
<tr>
<td>PAX9</td>
<td>paired box 9 gene</td>
</tr>
<tr>
<td>SAO</td>
<td>Southern Association of Orthodontists</td>
</tr>
<tr>
<td>SNPs</td>
<td>Single Nucleotide Polymorphisms</td>
</tr>
<tr>
<td>XLHED</td>
<td>human x-linked hypohidrotic ectodermal dysplasia</td>
</tr>
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</table>
Chapter 1: Review of the literature

In this review tooth agenesis associated with the various ectodermal dysplasia syndromes and the non-syndromic presentation of tooth agenesis will be examined. Current genetic discoveries from mammalian and human tooth agenesis will be explored. Specifically, this review will take a look at the prevalence and pattern of hypodontia in two groups, non-syndromic tooth agenesis (NSTA) and ectodermal dysplasias (ED), and consider the implications of determining the genetic basis of tooth agenesis and its impact on diagnosis and treatment planning.

Hypodontia: Definition and Prevalence

Hypodontia (the congenital absence of teeth) is the most common dental developmental anomaly in man (1, 2). This phenomenon occurs when an individual fails to develop all 20 deciduous and 32 permanent teeth (2). Its phenotypic presentation is varied in terms of severity (Figure 1A-C) as observed by its many names: anodontia, congenitally missing teeth, dental agenesis, hypodontia, oligodontia, and tooth agenesis. While some individuals present with only a single missing tooth, others have complete absence of the entire dentition (anodontia) (3). Oligodontia (Figure 1B) is defined as a more severe form of tooth agenesis, in which six or more teeth are missing—not including missing third molars (“wisdom teeth”) (3). However, it is somewhat controversial whether the terminology should include both hypodontia and oligodontia (2), thus this discussion will attempt to limit the terminology to that of hypodontia, which will include both severe and mild forms of tooth agenesis.
The prevalence of permanent tooth agenesis has ranged widely from as little as 0.3\% (4) to as high as 36.5\% (5) in isolated populations. Over 20\% of the general population has been reported to fail in the development of at least one of the third molars, and 3-10\% of the population has failed to develop at least one of the other permanent teeth (2, 6, 7).

A meta-analysis, including 33 papers of human populations from North America, Australia and Europe, examined the prevalence of tooth agenesis, excluding patients with craniofacial syndromes or developmental disorders (8). The authors’ findings indicate:

1. Tooth agenesis has a higher prevalence in Europe and Australia than in North America.
2. Tooth agenesis in females appears to be 1.37 times higher than in males for all three continents.
3. Following 3\textsuperscript{rd} molars, the most common missing teeth are the mandibular second premolars, the maxillary lateral incisors, and the maxillary second premolars.
4. Agenesis of maxillary central incisors, maxillary and mandibular first molars, and mandibular cuspids are very rare.

Polder (8) also noted that 83\% of individuals affected with dental agenesis are missing one or two permanent teeth, while the absence of more than six permanent teeth is very rare in only 0.14\% of individuals with congenitally missing teeth.

Tooth agenesis is considered rare in the deciduous dentition with a prevalence of less than 1\% in Caucasian populations (2). The deciduous maxillary lateral incisors and together with the deciduous mandibular incisors account for 50\% and 90\%, respectively, of affected deciduous teeth (9). Agenesis of a primary incisor most often occurred with agenesis of the corresponding permanent tooth. While, in all but one case the agenesis of a
primary molar tooth was followed by agenesis of the corresponding permanent tooth (10). One can speculate that the phenomenon of ‘gene dosage’ explains the decreased occurrence of missing deciduous teeth. Specifically, the reduced gene dosage resulting from a mutation in any gene that leads to tooth agenesis (i.e. *PAX9* or *MSX1*) of permanent teeth is not sufficient to cause agenesis of deciduous teeth, which require less gene product to form normally.

**Ectodermal Dysplasia – Definition and Clinical Presentation**

ED is defined as a group of congenital disorders characterized by alterations in two or more structures derived from the ectoderm layer with at least one of these alterations involving the hair, teeth, nails, or sweat glands of the affected individual (11). The ectoderm is the outermost layer of the three primordial layers of cells during embryonic development. Surface ectoderm gives rise to the outer layer of skin, dermal appendages (hair, nails, sweat glands), teeth, and parts of the eye and inner ear. The abnormal organization of these cells into tissue and organs during development gives rise to mature structures, which are deficient in number (e.g. hypodontia of the dentition), size (e.g. hypoplasia of tooth size), character, or function. Ectodermal dysplasias are not caused by external, environmental factors (e.g. carious lesions leading to missing, extracted teeth) nor aging (e.g. physiologic hair loss) (12). Various EDs may involve other structures of ectodermal origin including: mammary glands, thyroid glands, thymus, anterior pituitary, adrenal medulla, central nervous system, external ear, melanocytes, cornea, conjunctiva, salivary gland, lacrimal gland, and lacrimal duct (13).
(A) Radiograph of 12 year-old, Caucasian female with congenitally missing maxillary and mandibular 2nd premolars, congenitally missing maxillary left first premolar, “peg-shaped” maxillary left lateral incisor, and no apparent development of maxillary right 3rd molar and mandibular 3rd molars. (B) Radiograph of 14 year-old black female with congenitally missing maxillary and mandibular 2nd premolars, maxillary lateral incisors, maxillary right 1st premolar, mandibular central incisor, and no development of mandibular 3rd molars. (C) Radiograph of 8 year-old male with Ectodermal Dysplasia, complete anodontia of mandible, and exhibiting development of only the maxillary first molars, maxillary canines, and maxillary central incisors. (D) Intraoral photograph of patient with Ectodermal Dysplasia and conical shaped maxillary and mandibular incisors. Courtesy of University of North Carolina School of Dentistry.
Visinoni et al. reviewed 157 different entities of EDs, of which 62 clinically different EDs have an associated gene or chromosome region (14). The modes of inheritance for various EDs include: autosomal dominant, autosomal recessive, x-linked dominant, and x-linked recessive. The most common identified form of ED includes hypohidrotic ectodermal dysplasia (HED), which most severely affects the hair, nails, teeth, and skin (15). In HED, all modes of inheritance are possible with the X-linked hypohidrotic ectodermal dysplasia (XLHED) being the most common (16). Men are more often and more severely affected than women with transmission from a female carrier, who usually presents unaffected since men are homozygous with one copy of the X chromosome (15).

A brief focus on the molecular etiology of HED reveals mutations of the Ectodysplasin (EDA)-NF-κB pathway. Autosomal-dominant and autosomal-recessive forms of HED involve both EDA Receptor (EDAR) and EDA Receptor-associated Death-Domain genes (EDARADD). EDA protein, which is a member of the Tumor Necrosis Factor (TNF) family, is implicated in ectodermal structure, morphogenesis, and osteogenesis. Interactions between EDA with Wnt or BMP-Msx (factors involved with migratory neural crest differentiation) demonstrate the role of EDA signaling pathway in craniofacial patterning and growth (16). NF-κB Essential Modulator gene (NEMO) mutations are associated with syndromic HED with immunodeficiency and osteopetrosis. The genetic and protein interactions are complex and interrelated. Clauss et al. (2008) reviewed the main molecular interactions between the EDA pathway and the BMP-Msx, RANK-TRAF6- NF-κB, FGF, Wnt, and Activin-B pathways, which are all implicated in dental, craniofacial, and bone phenotypes associated with HED. These investigators
postulate that EDA protein activates NF-κB factor via its Receptor EDAR interacting with TRAF6, TAK1 Binding Protein 2 (TAB2), TGF-B Activating Kinase (TAK1), adapter molecules, and NEMO- IKKα/IKKβ. The downstream cascade of events includes phosphorylation of the inhibitor factor IκB by IKKα/IKKβ followed by a proteasomic degradation and the nuclear translocation of the dimeric NF-κB factor. NF-κB interactions with EDA and BMP-Msx are mediated by a specific growth factor—Ccn2-Connective Tissue Growth Factor (CTGF) and Follistatin BMP inhibitors].

Finally, the transcription of EDA and EDAR transcriptions are up-regulated by related pathways, Wnt-β-catenin-Lef1 and Activin-β-smad, respectively (16). The recent finding of only four genes (EDAI, EDAR, EDARADD, and WNT10A) accounting for 90% of hypohidrotic ectodermal dysplasia cases underscores the dynamic cellular interactions and signaling involved with formation of ectodermal structures (17).

Dental agenesis in the permanent dentition of HED patients was reported to range between 11.2 and 16.4 absent teeth (18, 19). In a sample of 30 persons with HED, phenotype varied with 83% experiencing oligodontia and 17% with hypodontia (19), and another study showed 12% to have the most severe phenotype of anodontia (20). The frequency of missing teeth in order of frequency for HED are as follows: maxillary lateral incisors (86%), mandibular central incisors (83%), mandibular lateral incisors (73%), mandibular second premolars (70%), maxillary second premolars (66%), mandibular second molars (53%), and maxillary second molars (53%) (19). The above findings suggest that in HED populations tooth agenesis appears higher for mandibular teeth than maxillary teeth and the most posterior teeth of each tooth class (incisor, premolar, molar) is more affected by the EDA mutation with the exception of the
mandibular central incisors. In HED the most permanent teeth most likely to be present include maxillary central incisor (42%), maxillary first molars (41%), mandibular first molars (39%), and maxillary canines (22%) (21).

Individuals without an associated syndrome or isolated tooth agenesis (non-syndromic tooth agenesis) share the mutual trait of congenitally missing teeth, which is found in many forms of ED. An individual with isolated NSTA may present with a single missing tooth or multiple missing teeth. Likewise, individuals with ED may present with multiple missing teeth to complete anodontia. This shared dental trait between ED and NSTA can present a potential diagnostic challenge for the dental or medical team of distinguishing an individual affected by tooth agenesis as having either an undiagnosed form of ectodermal dysplasia or an isolated form of tooth agenesis. Specifically, there is a phenotypic overlap between individuals with NSTA and individuals with ED that confounds diagnostic certainty that many patients seek. Three studies demonstrated some dental agenesis observed in HED are also encountered with non-syndromic oligodontia related to mutations of homeobox genes, Muscle Segment Homeobox-1 (Msx-1) and Paired Homeobox-9 (Pax-9) (22-25). Similarities of dental agenesis phenotypes between ED and NSTA patient groups may indicate possible interactions between genes of the Ectodysplasin (EDA)-NF-κB pathway and those of BMP4, EDA, MSX1 and PAX9 (26-29). Often patients find reassurance in having a definitive diagnosis that may also facilitate medical insurance coverage of dental and craniofacial features. The common thread between these two groups, hypodontia, is also a source for great variation between the groups. It is not uncommon for individuals with mutations in EDA to only have missing teeth (30, 31). This forms the natural question of
whether individuals who present with a severe form of tooth agenesis without an obvious affection of another ectodermal derivative might have ED. Hence, although the argument may appear tautological we hypothesize that there is specific quantifiable morphologic and genetic variation in tooth development between two groups: ED and NSTA.

**Molecular Advances in Tooth Formation**

Human studies of tooth agenesis have risen to the current level of understanding based upon mouse studies that revealed the development of dentition as a complex and intricate sequence of epithelial-mesenchymal interactions, similar to the formation of other ectodermal structures. These epithelial-mesenchymal interactions involve growth factors, transcription factors, signal receptors and other soluble morphogens. Disturbances within these complex processes may result in tooth agenesis. Studies of mutant mice and cultured tissue explants have examined the expression of more than 200 genes involved with tooth development, and provided insight into inductive signaling and hierarchies of downstream transcription factors necessary for tooth development (32).

Moreover, many genes have been identified as critical to the normal development of human teeth (2, 33, 34). Among those genes, *PAX9, MSX1, AXIN2* and *EDA1* are more commonly associated with human tooth agenesis (2, 22, 24, 25, 28, 31, 34-44). *AXIN2, PAX9* and *MSX1* genes have been identified as genes causative of NSTA, in which defects of these genes cause tooth agenesis as sole developmental malformation of humans (2). However, among the genes implicated in human tooth agenesis, several have not been positively identified as causing human tooth agenesis although they appear to cause the same in a mouse model. Bone morphogenetic proteins are among genes that
are involved in tooth development (27, 32) but have not yet been associated with the human tooth agenesis. This gene family, nonetheless, provides an excellent choice for further interrogation in the development of syndromic or non-syndromic tooth agenesis.

Specifically, bone morphogenetic protein 4 (BMP4) is a regulatory protein associated with induction of mesoderm and cartilage, endochondral bone formation, tooth development, limb formation, and bone fracture repair (45). In mouse models of tooth formation, Bmp4 gene codes for Bmp4 protein—a signaling molecule required for the formation of an epithelial signaling center, the enamel knot. For odontogenesis to progress beyond the bud stage, Bmp4 expression is required to be maintained by activation of both Msx1 and Pax9 (33).

Mutations in the AXIN2 gene have been implicated to cause tooth agenesis, intestinal polyposis, and a predisposition to colon cancer in a large Finnish family (2, 43). Axis Inhibitor 2 protein (AXIN2) is a regulator in the Wnt signaling pathway, which has widespread expression throughout the body during development (33) and operates early in tooth placode formation (33). The deregulation beta-catenin within Wnt signaling pathway by a mutation of AXIN2 has been associated with the formation of malignancies with the loss of heterozygosity being linked to breast cancer, neuroblastoma, colorectal cancer, and severe tooth agenesis.

Mutations in the EDA gene have been well associated with syndromic tooth agenesis (46) as previously mentioned in the discussion of ED and HED. However, an isolated form of tooth agenesis (especially associated with incisor agenesis) was found to be associated with EDA mutations in a large family (33). Ectodysplasin-A protein (EDA)—a type of tumor necrosis factor-like protein—is released as a signaling molecule
from its cell of origin. EDA binds to its target cell receptor, EDAR protein, and activates the NFκB pathway with its complex arrangement of intracellular proteins (16). In cases of isolated tooth agenesis caused by mutation in the EDA gene, it was suggested that tooth development requires a higher dosage of EDA signaling than hair and gland development (33).

The first genes identified with an association to non-syndromic tooth agenesis included the mesenchymally-expressed transcription factor genes of MSX1 (msh homeobox 1 gene with autosomal dominant inheritance located on chromosome 4p) (22) and PAX9 (paired-box 9 protein of chromosome 14q) (37). Different mutations (deletions, nonsense, missense, and frameshift mutations) associated with severe dominant inherited tooth agenesis in PAX9 and MSX1 can cause loss of function of one of the alleles (42, 47). The resulting altered proteins are not able to bind to known DNA target sequences nor activate transcription (24), leading to a reduction in the amount of functional protein for dental development.

In mice if either Pax9 or Msx1 are completely missing, Bmp4 levels cannot be maintained and then arrested development of the tooth occurs at bud stage (30). MSX1 mutations have been associated with a higher frequency of agenesis found for second premolars and maxillary first premolars than in mutations with PAX9, which is more commonly associated with agenesis of maxillary first and second molars, mandibular second molars, and with maxillary lateral or mandibular incisors also affected (2). MSX1 and PAX9 mutations have also been reported to exhibit tooth phenotypes with reduced dimensions, shortened roots, and a more simplified tooth form (40-42).
Despite dramatic advancement in the understanding of tooth formation and its underlying genetic component, only a fraction of tooth agenesis is explained by these four genes: AXIN2, EDA, MSX1, and PAX9. Moreover, the genetic determinants of most common types of tooth agenesis (premolar, incisor and third molar) have not been identified (2). More candidate genes remain to be discovered that best explain the pathogenesis of hypodontia. The developmental and regulatory processes necessary for odontogenesis provides an attractive model for embryogenesis in general. Normal tooth development with its timing of development, location, and species-specific morphology represents a series of highly coordinated events with well-conserved mechanisms (27, 48), which are highly applicable to basic developmental biological process. Further research into the genetic basis of tooth agenesis can both improve the understanding of tooth development and provide insight into the critical pathways involved with the formation of other complex tissues and organs systems, which if disrupted can lead to birth defects during embryogenesis.

Use of a non-human primate (NHP) model has also demonstrated the genetic basis of tooth agenesis. Several studies by Hlusko and Mahaney have led to an understanding of how genes influence anatomical variation of the NHP dentition, which represents the first quantification of the genetic architecture of the primate dentition. Their studies have demonstrated heritability of the following phenotypic traits: linear size metrics, 2-D areas, presence and degree of expression of cingular remnants, 2-D cusp orientation, and enamel thickness (49-55). The identification of a heritable component of tooth morphology both demonstrates that phenotype can be influenced by genetic variation and can lead to understanding the genetic etiology of pathological phenotypes.
The aforementioned primate models may improve our understanding of the genetic factors involved with the development of size and form of human teeth. A significant finding in human tooth agenesis studies is the marked variation seen in the patterns of missing teeth. Individuals having identical mutations in a specific gene such as MSX1 or EDA can vary in the severity of hypodontia ranging from having only a few missing teeth to complete anodontia (18, 56, 57). Teeth that are present can be smaller in size, and the lateral incisor frequently appears peg-shaped or conical (58-60). These findings may suggest that hypomorphic dentition falls within the same phenotypic spectrum as completely absent teeth. Moreover, the recent association of the EDAR and EDA genes with shovel shaped incisors and NSTA (30, 61, 62) supports the theory that different signaling pathways have overlapping functions (i.e. genetic variation of the same gene that leads to a syndromic form of tooth agenesis can also lead solely to shovel-shaped incisors). Thus, improved understanding of the observed phenotype can assist with identification of novel genetic associations. This refined phenotyping may in turn help in the identification of genetic alterations that predict the severity of the phenotype and improve the possibility of developing novel translational therapeutic approaches to treat this common developmental disorder.

**Clinical Presentation, Implications, and Challenges**

Hypodontia whether part of ED or NSTA carries an esthetic, functional, psychosocial, and financial burden for affected patients. In a retrospective study of 451 patients with hypodontia, the most common patient complaints included spacing between the teeth, poor esthetics, and awareness of missing teeth (63). Psychosocial status was found to be similar between orthodontic patients with hypodontia and those patients
without hypodontia but malocclusion of a similar treatment need as classified by index of orthodontic treatment need (IOTN), dental health component (DHC) 4 or 5 (64). In the otolaryngologic literature, the most common complaint of ED patients during childhood and adolescence was the concern about dental abnormalities and facial appearance (65). One study evaluated the psychosocial stress and adaptive functioning of 14 children and adolescents with varying degrees of ED. The authors noted that an individual’s ability to cope with ED was dependent on the severity of symptoms, which also had some influence on the child's intellectual potential and personality (66). Patients with hypodontia experience functional problems, especially with more difficulty in chewing when the deciduous teeth associated with the missing permanent teeth had been exfoliated (64) and reduced the surface area of the occlusal table (63). Complex, interdisciplinary, orthodontic and restorative treatment plans are often required to replace the missing teeth of hypodontia patients (67-75) at a financial expense to both the patient and his or her family.

The use of conventional restorative regimens (fixed partial dentures and complete removable dentures) for severe hypodontia and anodontia presents several clinical concerns. The use of conventional dentures to replace missing teeth of patients with an anodontia phenotype related to ED has been documented to have difficulty with patient compliance (76, 77). Moreover, irregular distribution and abnormal shape of retained teeth often limit the integration of bridges or crowns in patients with ED (73). The underdevelopment of the alveolar ridges, due to an absent permanent dentition in patients with hypodontia (78, 79), creates difficulty in obtaining adequate retention and support for conventional denture prostheses (80).
Implant-supported prostheses have been documented to benefit the oral rehabilitation of persons affected with severe phenotypes of oligodontia or anodontia resulting from ED or NSTA (81, 82). This treatment modality involves the placement of one or several implants into the alveolar bone of either the maxilla or mandible. The implants are restored with either a single prosthetic tooth, a fixed partial denture connecting two or more implants, a removable partial denture supported by two or more implants, or a removable complete denture supported by two or more implants. In a group of adult patients unaffected by ED or NSTA, one study demonstrated implants supporting a complete-arch fixed prostheses to have a 99% survival rate (implant retained in bone) and a 96% success rate (with less than 2mm of crestal bone loss) over a 24 to 94 month period (83). The 24-month implant survival rate in adults with ED was reported to be at 95%, with a consistently higher survival rate for implants placed in the mandible than for implants placed in the maxilla. However, implants are not a cure-all for hypodontia as 27% of patients in the same study experienced a failed implant, regardless of patient age or anatomical location of the implant placement (82).

Implants have additional limitations relating to bone morphology and age of ED patients with severe hypodontia. The lack of supporting alveolar bone previously mentioned in hypodontia often requires bone grafting in conjunction with implant placement to obtain stability in the adult patient (78). In a case report, Guckes et al. (1997) demonstrated endosseous implants could be placed into an ectodermal dysplasia patient as young as 3-years old (84). However, the placement of implants is often contraindicated for patients who have not yet completed craniofacial growth. The implant behaves similar to an ankylosed tooth, which does not participate in surrounding
growth and results in infraocclusion and multidimensional dislocation relative to the surrounding natural teeth, resulting in long term functional and esthetic disadvantages to the patient (85, 86). An improperly placed implant in the growing patient can also interfere with the position and eruption of adjacent tooth germs (87, 88). Implant placement in children with ectodermal dysplasia was also reviewed to have some clinical difficulty linked to alveolar bone hypotrophy (72).

Craniofacial skeletal growth of the child must also be considered with timing of implants in children with severe hypodontia. The dynamic growth of the maxilla and mandible during childhood and adolescence (89-91) in contrast to an implant with ankylosis behavior can result in an unpredictable implant dislocation during growth from the resorptive aspects of maxillary growth at the nasal floor and anterior surface of the maxilla (72). Transverse growth of the maxilla can also be restricted at the midpalatal suture if fixed implant prostheses cross the midpalatal suture in the growing patient (72, 92). In the mandible, transverse skeletal and alveolar changes occur early in childhood are less substantial than in the maxilla. Much growth occurs in the posterior mandible predominantly in late childhood with large amounts of anteroposterior, transverse and vertical growth (89). When teeth are present, the rotational growth of the mandible results in vertical increase of dental height and an anteroposterior compensatory change in the dentition (89). Although implants placed in the growing hypodontia patient at the anterior mandible would likely remain in an infraocclusal position and would probably be displaced in the anteroposterior direction, they may be the only viable location in a severe hypodontia patient with functional and psychosocial concerns (72, 80). The survival rate of implants placed in the anterior mandible of pediatric patients with ED was reported at
Despite these aforementioned growth concerns, craniofacial morphology did not differ significantly between implant-treated and non-treated ED children. This would suggest that treatment with intraosseous dental implants neither rescued nor interrupted normal craniofacial growth and development of the affected ED patients (93).

While implants have provided a contemporary solution to replacement of congenitally missing teeth that is comparable or superior to either tissue supported dentures or coping with the absence of teeth. The negative clinical outcomes of implant therapy in ED patients remain due to a lack of development of the alveolar ridge in the maxilla, bone structural hypermineralization, and thickened mandibular cortex, which can create thermal trauma as the denser bone is more difficult to drill (16). Moreover, complex orthodontic treatment may be required to optimize dental position, tooth axis, and alter the distribution of teeth and the spacing between the teeth prior to definitive restorative options (75). Debate remains as to the ideal timing of placing the implants, as some argue that the safest time to place implants in a hypodontia child is during the lower portion of the declining adolescent growth curve at or near adulthood, which can be confirmed by cephalogram radiographs, serial measure of stature, or hand-wrist radiographs (80). Ultimately the individual status of the existing dentition, functional status of mastication and phonetics, esthetics, and emotional and psychosocial well-being of the patient must all be considered in the timing of implant placement for hypodontia patients (73).

**Advances in Molecular Studies and Novel Therapeutic Approaches**

In seeking a systemic treatment of HED based upon advances in the fields of genetics and molecular biology, recent experimental approaches with recombinant EDA
or transgenesis of EDA-A1 have been developed. Gaide and Schneider (2003) provided the first example of short-term recombinant protein therapy to permanently correct a developmental genetic defect. They administered recombinant proteins containing the receptor-binding domain of EDA fused to the C terminus of an IgG1 Fc domain (Fc:EDA1) to pregnant Tabby mice, which shared many symptoms with human x-linked hypohidrotic ED (XLHED) patients. The mice had mutations of the ectodysplasin A gene (Eda) on the X chromosome, analogous to the human EDA gene. The two main splice variants of Eda, encode for proteins EDA1 and EDA2, which engage the tumor necrosis factor (TNF) family receptors EDAR and XEDAR, respectively. The EDA1 recombinant proteins were engineered to cross the placental barrier. This recombinant treatment permanently rescued the Tabby phenotype in the offspring, as the jaw and molars of the mice regained both their normal sizes and the classic wild-type pattern of sharp cusps. Use of EDA1 recombinant protein after birth restored the sweat glands of the mice offspring. However, the offspring of the Fc:EDA1-treated mice did not correct in terms of the hypodontia phenotype (94).

Another more recent study has demonstrated both reversion of oligodontia and dental dysmorphologies can be obtained by post-natal single or multiple EDA-A1 intravenous administrations in XLHED dogs (95). Casal et al. (2007) demonstrated that all teeth of the treated dogs showed normal development, which was confirmed by clinical and radiographic examinations (95). If this present model of using intravenous recombinant proteins has been successful in both mice and dogs, then future therapeutic use of recombinant EDA-A1 in humans could show the ability of recombinant EDA-A1 to correct the pathological features of XLHED. Rather than offering premature
placement of implants to rehabilitate the dentition and psychosocial distress of a child affected by hypodontia, intravenous recombinant therapy could be the new treatment of choice. With this new technology, the understanding of the genetic basis on both ED and NSTA becomes paramount as the use of recombinant protein therapy could offer a systemic cure of restoring one’s phenotype. Early detection of these traits in parents and patients becomes equally important to enable a more successful phenotypic rescue.

**Significance of Thesis**

In order to improve the treatment offerings and diagnosis for the hypodontia phenotype whether affected with a form of ED or NSTA, the nuances of the phenotypic presentation (pattern of agenesis, size of teeth, and shape of teeth) and the underlying genetic basis of this phenotype must be more fully understood. The improved understanding of the function of *AXIN2*, *BMP4*, *EDA*, *MSX1*, and *PAX9* genes and their encoded proteins have enabled further study of these candidate genes and potential as use in recombinant protein therapy for affected patients. Distilling the phenotypic overlap of severely affected hypodontia patients with either ED or NSTA will also help in the selection of candidate genes for future study. Using a sample from the database of the NFED and UNC School of Dentistry, our study tests the hypothesis that specific phenotypic and genetic variation exists that best explains the differences observed between ED and NSTA. The improved understanding of this variation will lead to improve diagnosis and potential treatments.
Chapter 2: Phenotype characterization of patients with hypodontia in ectodermal dysplasia and non-syndromic groups

Abstract

Introduction: Hypodontia is the most common craniofacial anomaly in humans (5-15%). It can present as an isolated feature, i.e. non-syndromic tooth agenesis (NSTA) or as part of a syndrome, such as ectodermal dysplasia (ED). Hypodontia carries an esthetic, functional, psychosocial, and financial burden for affected patients. Improved understanding of the overlap of phenotypes between these groups may lead to a refined clinical diagnosis of traits and lead towards an improved understanding of underlying genetic modifiers that lead to ED versus NSTA phenotypes.

Objective: This study seeks to identify phenotypic differences of hypodontia between two groups: ED and NSTA, to test the hypothesis that specific phenotype variants explain differences between syndromic and non-syndromic groups.

Methods: 230 ED and NSTA patients were recruited from UNC Dental School or the National Foundation of ED. Using panoramic radiographs and available dental casts, permanent teeth were classified: 1) normal, 2) morphologic anomaly (reduced width or altered shape), 3) missing. Dental casts were used to measure mesiodistal widths and conical index angle (facial slope) of permanent, erupted teeth. Multivariate analyses—Distance-Weighted Discrimination (DWD) and DiProPerm statistical tests (p-value<0.05) were utilized.
Results: 118 individuals (37 ED, 81 NSTA, 67 Male, 51 Female) had radiographs and 58 had plaster models (10 ED, 48 NSTA). The ED group had a higher occurrence of missing teeth for all teeth (p<0.05) except for mandibular 2nd premolars (p>0.05). Tooth widths were reduced in ED group compared to the NSTA group. We defined a new parameter, conical index angle, to quantify the extent of morphological differences between ED and NSTA at the maxillary incisors. All four maxillary incisors of the ED group were more conical than matched control teeth; however, only the lateral incisors of the NSTA group were more conical than the controls.

Conclusions: ED has a higher occurrence of hypodontia and tooth anomalies for all teeth, except the mandibular 2nd premolars. Conical index is an objective method to identify conical teeth.

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Introduction

Tooth agenesis (congenitally missing teeth or hypodontia) is the most common human craniofacial anomaly (2) with 2-10% of the general population affected (8). Hypodontia can occur as an isolated feature or as part of a syndrome, such as ectodermal dysplasia (ED). Esthetic, functional (63), and psychosocial (64) concerns related to hypodontia can require complex orthodontic and restorative treatments (67-71) with a heavy financial burden to the patient. An improved understanding of hypodontia phenotypes and the genetic modifiers that lead to these phenotypes may help refine phenotypic differentiation between ED and non-syndromic tooth agenesis (NSTA), improve clinical diagnostics, and provide the ability to deliver risk assessments as part of the health care experience.
This study seeks to identify morphologic variation of hypodontia between two groups: ED and non-syndromic tooth agenesis (NSTA). We will clinically characterize syndromic and non-syndromic hypodontia samples from individuals with ED and NSTA, which explain observed differences of hypodontia or hypomorphology between ED and NSTA groups. We hypothesize that there is morphologic variation in tooth agenesis associated with two groups: ED and NSTA. Our hypothesis includes that the morphologic variation will be observed between the ED and NSTA groups in the following: severity of hypodontia (number of congenitally missing teeth), size of teeth (width of teeth), and shape of teeth (conical shape of the maxillary incisors).

Materials and Methods

Recruitment

Study participants (N=118) were recruited by referral or identification from the UNC School of Dentistry and National Foundation Ectodermal Dysplasia (NFED). The study was approved by the Biomedical Institutional Review Board, IRB study #: 04-1711 and CRTC #: 2215. The recruited individuals were previously identified as having congenitally missing teeth or ectodermal dysplasia, and were assigned to ED (N=37) or NSTA (N=81) groups based on this prior diagnosis. Study participants met the following inclusion criteria: 1) Previous diagnosis of either ED or congenitally missing teeth; 2) panoramic or full mouth series radiographs with date of radiograph exam; 3) when available, dental casts without obvious visual distortion. Study participants were excluded from the study if they presented with another known genetic syndrome (e.g. Down’s Syndrome, Cleft Lip and Palate). Participant demographics (age, gender) and
previous dental diagnosis (ED, NSTA, previous extractions) were recorded when accessible from the participants’ dental chart or UNC School of Dentistry database.

An unaffected control group (N=20) was sampled for comparison between the ED and NSTA groups. The control group was randomly selected among a subset of initial dental records at the UNC Orthodontic Graduate Clinic that met the following criteria: 1) Initial dental casts and panoramic radiograph had been taken between June 2007-June 2010, 2) age was at least 12 years-old at time of records, 3) all permanent teeth erupted except third molars, 4) no diagnosis of missing teeth, nor malformed teeth.

**Phenotypic Analysis and Characterization**

Two independent examiners were calibrated for measurement technique. The examiners reviewed dental casts and radiographs for all permanent teeth #1-#32 (maxillary right to maxillary left to mandibular left to mandibular right) of the ED, NSTA, and unaffected control sample. Primary teeth and permanent teeth charted as previously extracted were excluded from the study. For the permanent teeth evaluated in the study, the two examiners: 1) recorded the presence or absence of each permanent tooth within a given participant’s dentition, 2) measured the mesiodistal width of each erupted, permanent tooth from the available dental casts with digital calipers (#500-159-2, Mitutoyo Aurora, IL, USA) (Figure 2), 3) assigned teeth an ordinal tooth agenesis score (0-3) based on the following descriptions:

1. present and normal (0), permanent tooth is present on cast or is developing on radiograph with no visible shape or size abnormality
2. present and having size or shape anomalies (1), permanent tooth is present on cast or is developing on radiograph and has either a visible reduction in size or altered shape (e.g. conical shape crown)

3. present and having size and shape anomalies (2), permanent tooth is present on cast or is developing on radiograph and has both visible reduction in size and altered shape (e.g. conical shape crown)

4. congenitally missing (3), permanent tooth is neither present on cast nor radiograph

Figure 2 - Measurement of mesiodistal width of mandibular dentition. Dental cast of 16 year-old male with agenesis of teeth #20 and #29.

Additionally, the shape of erupted, maxillary incisors were classified as (0) normal, (1) peg-shape, (2) cone-shape. Lines of best fit were constructed onto the incisal edge, long axis of the tooth, mesiofacial line angle, and distofacial line angle of the maxillary incisors (Figure 3, Figure 4). The slopes (m) of the lines of best fit on the
mesiofacial line angle and distofacial line angle were calculated by the following equation:

\[ m = \frac{(y_{(1/3)} - y_{(0)})}{(x_{(1/3)} - x_{(0)})} \]

\( m = \) (height of tooth measured from incisal edge to 1/3 height of crown) – (distance from midline to line angle at incisal edge) – (distance from midline to line angle at 1/3 crown height)

**Figure 3 - Conical Incisor Index of ED**

**Figure 4 - Conical Incisor Index of NSTA**

Dental casts of ED (Figure 3) NSTA (Figure 4) participants show lines of best fit drawn onto the maxillary right central incisor. Lines of best fit shown include: the
mesiofacial line angle (green), incisal edge (yellow), parallel line to incisal edge at 1/3 height of clinical crown (yellow), and height of clinical crown (red) drawn perpendicular to incisal edge. The slope (m) of the line of best fit at mesiofacial line angle was calculated by the following equation: 

\[ m = \frac{y_{(1/3)} - y_{(0)}}{x_{(1/3)} - x_{(0)}} = \frac{\text{height of tooth measured from incisal edge to 1/3 height of crown}}{\text{(horizontal distance from midline tooth to line angle at incisal edge)} - \text{(horizontal distance from midline tooth to line angle at 1/3 height of crown)}}. \]

**Intraexaminer Reliability**

Ten casts and radiographs of ten subjects were randomly selected. The measurement procedures were repeated with at least two weeks between the initial and replicate measurements. Intra-class correlation coefficients (ICCC) were calculated to assess intra- and inter-operator reliability.

**Statistical Analyses**

To compare the differences between patients with ED and NSTA for the following variables: hypodontia pattern, mesiodistal tooth widths, and conical index of maxillary incisors, a simple t-test and its nonparametric alternative, the Mann Whitney test were initially utilized. Level of significance was set at p=0.05. With 32 tests, Bonferroni’s correction indicates the p-value be set to 0.0016\(^{19}\).

Furthermore, a multivariate analysis of Distance Weighted Discrimination (DWD) was made to determine if there exists a significant difference between the groups in respect to ordinal tooth agenesis score. DWD is a modern computationally intensive optimization method designed for high dimension low sample size for data, in which the data vectors are larger than the sample size (96). DWD is a modification of Support
Vector Machines (SVM) (97), a non-probabilistic binary linear classifier. In both DWD and SVM, given inputs within a set of data points are mapped onto an infinite dimensional space. A hyperplane or set of hyperplanes is constructed onto the infinite dimensional space of data points such that the data is separated or classified into categories, which are divided by a clear gap (i.e. functional margin) as wide as possible. Good separation is achieved by the hyperplane that provides the largest functional margin and thereby the lowest potential error of the classifier (96).

Direction-Permutation-Projection (DiProPerm) is a technique utilized for hypothesis testing with DWD analyses (96). DiProPerm first utilizes DWD to find the appropriate directional vector or hyperplane best separating the data. This vector is then projected onto a one-dimensional subspace, on which a one dimensional test statistic (e.g. two sample t-test) can be constructed. To yield a p-value the true statistic is compared to a population of permutated test statistics, which are constructed from several random DWD vectors again projected onto a one-dimensional subspace, which has a computed permutation test statistic (96). The teeth were then ranked in order of those best explaining the SNP differences between the ED and NSTA groups based on the DWD and DiProPerm analyses.

Results

Table 1 illustrates participant demographics from our recruited sample and control group. Of the 250 patients within our database, we were able to collect 118 radiographs of patients with either ED (n=37) or NSTA (n=81), and an additional 20 radiographs from our control group. The majority of the ED group (59.5%) was male and the majority of the NSTA group (64.2%) was female. Of the 118 participants (not
including control group) with radiographs, 66 (15 ED, 51 NSTA) participants had available dental casts. Patient age appeared to vary more in the ED group compared to the NSTA or control groups.

In the radiographic evaluation, a total of 4416 potential tooth locations (1184 ED, 2592 NSTA, 640 control) were assessed. For statistical analyses and calculation of the radiographic tooth score we excluded 16 participants due to incomplete radiographic information for all 32 permanent teeth. The reduced sample set consisted of 102 subjects (29 ED, 73 NSTA) had 3028 potential tooth locations included within the statistical analyses (928 ED, 1460 NSTA, 640 control). For the measurement of mesiodistal widths, 2752 potential tooth locations were measured (480 ED, 1632 NSTA, 640 control), and for measurement of the conical index angle 344 potential tooth locations were measured (60 ED, 204 NSTA, 80 control). Our inter-examiner and intra-examiner reliability appeared good to excellent according to our ICCC for: ordinal radiographic score (mean r > 0.82), mesiodistal-tooth width (mean r>0.91) and conical index angle (mean r>0.86).

Comparisons were made between ED and NSTA groups for each tooth individually for the mean ordinal radiographic tooth score (Table 2, Figure 5, Figure 6). Using the t-test, a significant difference was detected between the ED and NSTA groups for all teeth but maxillary first molars and mandibular second premolars (T3, T14, T20 and T29). For our non-normally distributed data set, the Mann-Whitney test appears more appropriate for analyses (Figure 10). The Mann-Whitney test showed a significant difference for all teeth, but maxillary left third molar, mandibular right third molar, and mandibular second premolars (T16, T20 and T29 and T32). For those teeth where we
noted a significant difference (Table 2, Figure 5, Figure 6), the mean and median of the score are higher for patients with ED, indicating that patients with NSTA have fewer teeth anomalies or missing teeth problems compared to those with the ED syndrome. All members of the control group were found to all have all permanent teeth present and appear normal on the radiograph, yielding an ordinal radiographic tooth score of 0 for all teeth across all controls. The Mann-Whitney test indicated a statistically significant difference existed between the control with both ED and NSTA for ordinal radiographic tooth score.

The mean mesiodistal width of each tooth was narrower in the ED group when compared to the NSTA group. These differences were statistically significant for all teeth, except #5 (p=0.0678), #12 (p=0.0712), #19 (p=0.0567), #20 (p=0.0619), #29 (p=0.0599), and #30 (p=0.0681). Third molars and permanent, mandibular incisors were either absent or unerupted (unable to be measured) for all casts measured within the ED group. The NSTA group had no measurable maxillary, left third molars in the sample.

We compared the facial slopes of the maxillary incisors between ED, NSTA, and an unaffected control group (Figure 9). A two-sided t-test indicated that the control group and NSTA had a statistically significant (p<0.05) greater facial slope than the ED group for all maxillary incisors. The results of our study defined a new term, conical index angle of the incisor, which is calculated by subtracting the inverse tangent of the facial slope from 180 degrees (Table 3). The control group had a statistically significant (p<0.05) greater conical index for the maxillary lateral incisors. No statistical difference was noted between the control group and NSTA group for the maxillary central incisors.
Through DWD analyses, gender does not appear to be an important variable for explaining differences in hypodontia pattern between ED and NSTA groups. Figure 11 shows a DWD Loadings Plot indicating which teeth have the greatest importance in explaining differences between ED and NSTA. The mandibular canines and mandibular lateral incisors (#22, 23, 26, 27) were best at separating ED from NSTA with ED having the higher ordinal tooth agenesis score for those teeth. The mandibular second premolars (#20 and 29) were best at separating ED from NSTA with NSTA having the higher ordinal tooth agenesis score (Figure 11).

Table 1 - Participant demographics of ED, NSTA, and control groups for radiograph, dental cast, and DNA analyses.

<table>
<thead>
<tr>
<th></th>
<th>ED n (%)</th>
<th>NSTA n (%)</th>
<th>CONTROL n (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Radiographs (n=118)</td>
<td>37 (31.4)</td>
<td>81 (68.6)</td>
<td>20</td>
</tr>
<tr>
<td>Male (n=51)</td>
<td>22 (59.5)</td>
<td>29 (35.8)</td>
<td>10</td>
</tr>
<tr>
<td>Female (n=67)</td>
<td>15 (40.5)</td>
<td>52 (64.2)</td>
<td>10</td>
</tr>
<tr>
<td>Age (Mean +/- SD)</td>
<td>21.32 +/- 12.02</td>
<td>17.86 +/- 12.62</td>
<td>14.06 +/- 2.20</td>
</tr>
<tr>
<td>Dental Casts (n=66)</td>
<td>15</td>
<td>51</td>
<td>20</td>
</tr>
<tr>
<td>Male (n=)</td>
<td>9</td>
<td>16</td>
<td>10</td>
</tr>
<tr>
<td>Female (n=)</td>
<td>6</td>
<td>35</td>
<td>10</td>
</tr>
<tr>
<td>Age (Mean, SD)</td>
<td>25.32 +/- 7.73</td>
<td>16.32 +/- 4.02</td>
<td>14.06 +/- 2.20</td>
</tr>
</tbody>
</table>
Table 2 - T-test and Mann-Whitney test comparing patients with ED and NSTA. For $\alpha = 0.05$, Bonferroni’s correction gives us significance level of 0.0016 for each test. The t-test is significant for all teeth but T3, T14, T20 and T29. Mann-Whitney is significant for all teeth but T16, T20 and T29 and T32. For a non-significant result, p-values are presented in bold and red font.
<table>
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<tr>
<th>Tooth</th>
<th>Mean Group E</th>
<th>Median Group E</th>
<th>StD Group E</th>
<th>p-value t-test</th>
<th>p-value Mann-Whitney</th>
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<tr>
<td>T1</td>
<td>1.4658</td>
<td>2.5862</td>
<td>1.5008</td>
<td>5.95E-05</td>
<td>4.81E-04</td>
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<td>0.1644</td>
<td>2.2069</td>
<td>0.5531</td>
<td>3.36E-09</td>
<td>3.41E-12</td>
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<td>0.1370</td>
<td>0.9310</td>
<td>0.4188</td>
<td>1.80E-03</td>
<td>5.64E-05</td>
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<tr>
<td>T4</td>
<td>0.7534</td>
<td>1.9310</td>
<td>1.2336</td>
<td>3.36E-04</td>
<td>3.72E-04</td>
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<tr>
<td>T5</td>
<td>0.5068</td>
<td>2.1034</td>
<td>1.0425</td>
<td>1.30E-06</td>
<td>3.68E-07</td>
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<tr>
<td>T6</td>
<td>0.3288</td>
<td>1.7931</td>
<td>0.6883</td>
<td>1.01E-07</td>
<td>2.13E-09</td>
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<tr>
<td>T7</td>
<td>1.5205</td>
<td>2.8966</td>
<td>1.3029</td>
<td>5.22E-13</td>
<td>7.33E-07</td>
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<td>T8</td>
<td>0.4384</td>
<td>2.1379</td>
<td>0.6006</td>
<td>1.30E-09</td>
<td>1.38E-10</td>
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<td>T9</td>
<td>0.4795</td>
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<td>0.6689</td>
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<td>1.5205</td>
<td>2.0690</td>
<td>1.4824</td>
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<td>0.7902</td>
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<td>0.1096</td>
<td>2.1034</td>
<td>0.4269</td>
<td>1.32E-10</td>
<td>7.68E-15</td>
</tr>
<tr>
<td>T28</td>
<td>0.2192</td>
<td>2.1379</td>
<td>0.7119</td>
<td>2.13E-08</td>
<td>8.10E-11</td>
</tr>
<tr>
<td>T29</td>
<td>1.4110</td>
<td>2.0000</td>
<td>1.4985</td>
<td>6.75E-02</td>
<td>6.83E-02</td>
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<tr>
<td>T30</td>
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<td>1.2759</td>
<td>0.6291</td>
<td>4.79E-04</td>
<td>1.59E-05</td>
</tr>
<tr>
<td>T31</td>
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<td>2.0000</td>
<td>0.7173</td>
<td>2.59E-07</td>
<td>1.33E-09</td>
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<tr>
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<td>2.6897</td>
<td>1.4782</td>
<td>5.36E-04</td>
<td>4.16E-03</td>
</tr>
</tbody>
</table>
Figure 5 - Mean radiographic tooth score between ED and NSTA groups for teeth 1-16

Figure 6 - Mean radiographic tooth score between ED and NSTA groups for teeth 17-32
Figure 7 - Mean mesiodistal tooth widths (mm) for teeth 1-16 between NSTA and ED groups

Figure 8 - Mean mesiodistal tooth widths (mm) for teeth 17-32 between NSTA and ED groups
Figure 9 - Conical index by group for teeth 7-10.

Table 3. Conical Incisal Angle (degrees) of NSTA, ED, and control groups.

<table>
<thead>
<tr>
<th>Tooth Number</th>
<th>NSTA (n=48)</th>
<th>ED (n=15)</th>
<th>Control (n=20)</th>
<th>p-value</th>
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</thead>
<tbody>
<tr>
<td>7</td>
<td>97.77</td>
<td>112.62</td>
<td>94.59</td>
<td>0.001</td>
</tr>
<tr>
<td>8</td>
<td>94.12</td>
<td>102.38</td>
<td>93.70</td>
<td>0.021</td>
</tr>
<tr>
<td>9</td>
<td>94.44</td>
<td>104.08</td>
<td>93.62</td>
<td>0.033</td>
</tr>
<tr>
<td>10</td>
<td>97.32</td>
<td>107.76</td>
<td>94.57</td>
<td>0.001</td>
</tr>
</tbody>
</table>
Figure 10 - Histograms for each tooth (e.g. Tooth #1) display a non-normal distribution of the scores.
Figure 11 - DWD Loading values, with universal tooth number given for each of the variables in decreasing magnitude order. Variables with larger magnitude are more important in separating the data between ED and NSTA groups. A positive value indicates patients with NSTA have higher levels of the ordinal tooth score. Negative values indicate patients with ED have higher levels of ordinal tooth score.

**Discussion**

Phenotypic analysis of two groups with syndromic and NSTA respectively reveals that there are quantifiable differences in shape, size and pattern of teeth. This quantifiable difference can be most systematically detected in a new parameter, defined here, termed conical incisal index (or angle). We applied a novel statistical approach, DWD, (Figure 11), to determine the most important teeth for separating the phenotypic
tooth patterns between ED and NSTA groups based on the radiographic index. Accordingly we determined that teeth #27, 26, 2, 23, 22, 29, 10, 20, 7, 15 were the ten most important teeth for separating the data between ED and NSTA groups in regards to ordinal tooth score. Teeth #27, 26, 2, 23, 22, 10, 7, 15 all had higher levels of ordinal tooth score for ED, while teeth #29 and #20 had higher ordinal tooth score for NSTA.

Additionally, we found that 64.2% of the NSTA group was female and 59.5% of the ED group was male. Our findings are consistent with the literature, as Polder (8) found tooth agenesis in females to be 1.37 times higher than in males. The higher number of males in our ED sample could be a reflection of an increased sampling of X-linked hypohidrotic ectodermal dysplasia, which accounts for 95% of randomly selected hypohidrotic ectodermal dysplasias (46). However, our available database did not differentiate among the sundry diagnoses of ectodermal dysplasias to evaluate if our sample had an increased sampling of X-linked hypohidrotic ectodermal dysplasias.

The finding that no statistical difference existed between maxillary first molars and mandibular second molars (Table 2, Figure 5, Figure 6) is consistent with the literature. Numerous publications have noted mandibular second premolars to be the most frequent congenitally missing teeth in non-syndromic groups, while maxillary molars are often highly conserved (8). The higher frequency of missing mandibular second premolars in our sample of NSTA and ED was both high, while the frequency of missing maxillary first molars was low.

Ordinal tooth score differences between ED and NSTA are best explained by both contralateral teeth: mandibular canines, mandibular lateral incisors, maxillary 2nd molars, maxillary lateral incisors, and mandibular 2nd premolars. These findings seem consistent
with Butler’s (1995) field theory in which the dentition is divided into three morphologic fields corresponding to incisors, canines, and premolars/molars. The tooth closest to the midline within each field is presumed most stable, while each adjacent distal tooth within the same field to become less stable (98). In our study the more distal tooth among the classes of mandibular incisors, maxillary molars, maxillary lateral incisors, and mandibular premolars experienced a higher ordinal tooth score and best differentiated ED from NSTA. Tooth agenesis in HED population appears to follow a similar pattern with agenesis being higher for mandibular teeth than maxillary teeth, and the most posterior teeth of each tooth class (incisor, premolar, molar) being more affected with the exception of the mandibular central incisors, which are the most affected in HED (19).

The ordinal radiographic score of the third molars did not distinguish ED from NSTA. Given third molars are the most common congenitally missing teeth (2, 7, 8, 99), it appears much overlap of phenotype between ED and NSTA can occur at the third molars. In our sample ordinal radiographic tooth score for mandibular 2nd molars did not appear to be significantly different (Figure 8). Following third molars, mandibular second premolars, maxillary lateral incisors, and maxillary second premolars are the most common congenitally missing teeth (1, 8). Again overlap of phenotype between ED and NSTA appears to occur at the mandibular second premolars in our sample. However, DWD analysis (Figure 11) indicates mandibular second premolars to be one of the tooth classes best differentiating ED from NSTA with a higher ordinal value for the NSTA group.

Our analysis also revealed that mandibular canines were some of the best teeth in differentiating ED from NSTA. Since tooth agenesis of maxillary central incisors,
maxillary and mandibular first molars, and mandibular canines is very rare (8), and mandibular teeth have a higher incidence of agenesis in HED (19), mandibular canine agenesis might be a potential phenotypic indicator of ED. However, future studies will be needed to confirm the role of mandibular canines in diagnosis. In HED the permanent teeth most likely to be present include maxillary central incisor, maxillary first molars, mandibular first molars, and maxillary canines (21), none of which appeared to be within the top ten teeth for distinguishing ED from NSTA for the DWD analysis.

The decreased average mesiodistal width of ED subjects (Figure 7, Figure 8) compared to NSTA indicates ED subjects have a smaller tooth size. Studies have confirmed smaller tooth sizes in both ED and NSTA patients (59, 100-103). In our sample these differences were statistically significant for all teeth, except #5 (p=0.0678), #12 (p=0.0712), #19 (p=0.0567), #20 (p=0.0619), #29 (p=0.0599), and #30 (p=0.0681). Teeth #20 and #29 (mandibular second premolars), as discussed before are the most common missing teeth after third molars and it is likely that the overlap between ED and NSTA observed in regards to tooth agenesis would again be observed to overlap between groups in regards to tooth size. Teeth #19 and #30 (mandibular first molars) are highly conserved teeth in both ED and NSTA populations (8, 19, 82, 93, 99). We were unable to measure third molars and mandibular incisors in the ED group, which were either absent or unerupted. This is plausible as the most commonly missing teeth in HED following third molars are maxillary lateral incisors (86%), mandibular central incisors (83%), and mandibular lateral incisors (73%) (19). Thus, teeth being on average 1.5-2.0mm narrower than published averages could be a potential distinguishing indicator of
ED from NSTA, however additional studies of a larger sample size are need to confirm this finding.

Our newly developed conical index angle to assess the slopes of the maxillary incisor tooth shape (Figure 3, Figure 4, Figure 9, Table 3) represents a valid method to assess conical shape of teeth and by extension associate the clinical diagnosis with ED versus NSTA. The higher the conical index angle (Table 3) the more conical shaped an incisor appears, or inversely stated the lower the conical index angle the more a tooth appears cone shaped. Given conical shaped teeth are often associated with ED, our results of ED having a statistically significant lower conical index than NSTA and unaffected control groups would be expected. With the diversity of crown morphology at the maxillary lateral incisors (normal shape, peg shape, cone shape), our NSTA group had a statistically significant lower conical index than the unaffected control group, which was selected based on criteria of normally shaped teeth. The conical index developed provides a relatively inexpensive and valid, objective gradient to assess the cone shape of a maxillary incisor, rather than a subjective, arbitrary classification of a tooth as being “cone shaped.” While our sample size is small, future studies utilizing the conical index should confirm its reliability across other samples.

Conclusions

1. ED has a higher occurrence of hypodontia and tooth anomalies than NSTA groups for all teeth except the maxillary left third molar, mandibular right third molar, and mandibular 2\textsuperscript{nd} premolars, which did not have a significant difference.
2. Mesiodistal tooth width was greater for NSTA compared to ED for all teeth except mandibular 2\textsuperscript{nd} premolars, mandibular 1\textsuperscript{st} molars, and maxillary first premolars.

3. Conical index angle and facial slope are both objective methods to identify conical shaped teeth and delineate the severity of phenotype in regards to cone shaped incisors.
Chapter 3: Candidate Genotyping of Ectodermal Dysplasia and non-syndromic tooth agenesis

Abstract

Background: Tooth agenesis or hypodontia occurs in as much as 15-20% of the population, excluding third molars. It can present as an isolated feature as in non-syndromic tooth agenesis (NSTA), or as part of a syndrome, such as ectodermal dysplasia (ED). The co-morbidities associated with either clinical classification include functional and psychosocial concerns, complex orthodontic and restorative treatments and a significant financial burden. The phenotypic and genetic overlap between ED and NSTA underscores the gap in the knowledge of the developmental processes that lead to hypodontia phenotypes. Hence, understanding the genetic modifiers that lead to these phenotypes may help refine clinical diagnosis and risk assessment.

Objective: This study therefore seeks to identify the differences that separate the genetic spectrum of hypodontia in two groups: ED and NSTA, to test the hypothesis that specific genetic variants explain differences between syndromic and non-syndromic tooth agenesis.

Methods: DNA was extracted and genotyped using an Affymetrix 6.0, 500K SNP chip platform for a total of 88 affected individuals with ED or NSTA. To determine which SNPs best explain the genetic differences between the two cohorts, a subset of 1474 SNPs within candidate genes (EDA, MSX1, PAX9, BMP4 and AXIN2) and a chromosomal region associated with tooth shape were selected to run multivariate
statistical analyses including Distance-Weighted Discrimination (DWD) and DiProPerm.

**Results:** We found that specific SNPs in BMP4 and MSX1 best explained differences between the 2 groups. Additional SNPs that lie in a chromosomal region associated with tooth shape in the non-human primate were also identified as explanatory of genetic differences between groups.

**Conclusions:** Results from these studies suggest that candidate SNPs may act as genetic modifiers that account for genetic and phenotypic differences between ED and NSTA.

**Funding:** This work was supported by the National Foundation for Ectodermal Dysplasia and UL1RR025747 (NCRR) to Dr. Sylvia Frazier-Bowers.

**Introduction**

Tooth agenesis or hypodontia occurs in as much as 15-20% of the population, excluding third molars (2, 8, 99). The co-morbidities associated with all severities of tooth agenesis include functional and psychosocial concerns (63-65), complex orthodontic and restorative treatments and a significant financial burden to patients. Tooth agenesis can present as an isolated feature as in non-syndromic tooth agenesis (NSTA), or as part of a syndrome, such as ectodermal dysplasia (ED)—a classification of over 150 different syndromes affecting derivates of the ectoderm like hair, teeth, nails, skin, and sweat glands.

Previous studies investigating the underlying genetics of tooth agenesis in both mouse models and humans have linked tooth agenesis to various mutations found in the *PAX9, MSX1, AXIN2,* and *EDA* genes (2, 22, 25, 26, 28, 30, 33, 39, 40, 43, 57, 61, 104-109). Although not identified in human tooth agenesis, other candidate genes have been
validated as important in normal tooth development and tooth agenesis in the murine model (27, 32, 48, 110). For instance, bone morphogenetic protein, BMP4 has been identified as a critical factor in multiple pathways of murine tooth development including: signaling with Msx1 and Pax9, transitioning from the cap stage to bell stage, and induction the enamel knot (2, 27, 30, 33, 48). Along with other candidate genes, BMP4 is a high-priority candidate for interrogation in human studies of tooth agenesis. Moreover, evidence of a genetic basis of tooth shape has been corroborated in non-human primate (NHP) studies, which have led to an understanding of how genes influence anatomical variation of the NHP dentition. These studies have demonstrated heritability of the following phenotypic traits: linear size metrics, 2-D areas, presence and degree of expression of cingular remnants, 2-D cusp orientation, and enamel thickness (49-55). The identification of a heritable component of tooth morphology both demonstrates that phenotype can be influenced by genetic variation and can lead to understanding the genetic etiology of pathological phenotypes.

Studies on non-syndromic tooth agenesis have found similarity in hypodontia pattern to those of patients with ED when mutations were observed in the PAX9 or MSX1 genes. This phenotypic and genetic overlap between various forms of ED and NSTA underscores the gap in the knowledge of the developmental processes that lead to various hypodontia phenotypes. Further evidence of the genetic link underscoring subtle phenotypic traits such as tooth shape can be found in the recent study that identified specific EDA polymorphisms in a Japanese cohort are responsible for shovel-shaped incisors (62). This finding is in stark contrast to the known literature that has established alterations in EDA as causative for a severe form of tooth agenesis including defects in
other ectodermal structures such as sweat glands, skin and hair (12, 14, 17, 30, 33, 61, 111, 112). Taken together, these parallel research findings underscore the fact that the molecular basis of tooth development is a complex and highly regulated process. Over 200 transcriptional factors, signaling molecules, and receptors are known to be expressed during the development of teeth (odontogenesis) (32) as multiple interactions between mesenchymal and epithelial cells occur. Because teeth are ectodermal derivatives, they are also subject to genetic alterations with similar transcriptional factors involved with ectoderm and mesoderm interactions. The overlap of these distinct processes and signaling pathways is still poorly understood, but advances in our knowledge over the past two decades hold great promise to improve our current therapeutic regimen (16).

Currently the restorative techniques that exist to replace missing teeth of patients affected by tooth agenesis include use of conventional dentures, conventional tooth pontics, implant-supported dentures, implant-supported crowns, and implant-supported pontics. Among those options, implants may offer a more stable advantage over tissue supported dentures, however implants cannot replace the biocompatibility of a natural tooth’s periodontal ligament and often require bone grafting in patients with an atrophic alveolar ridge. Implants also are more advantageous in a non-growing patient (82, 84), as growth-related problems of the patient with an Osseo-integrated implant include infraocclusion and displacement of the implant.

The possibility of replacing a tooth with a natural alternative is becoming a reality. Recent studies involving a mouse and dog models utilized intravenous, recombinant protein therapy to restore the wild-type phenotype in the offspring of affected dogs and mice with ED. The ability to restore one’s phenotype has even greater
potential with refined understanding of the odontogenic process. Thus, the identification of novel genes contributing to tooth formation and variance can elucidate the requisite molecular controls and discover yet unidentified biological pathways that can be targeted in the development of novel drug/protein therapies or biologic tooth replacements.

Our study seeks to identify the differences that separate the genetic spectrum of hypodontia in two groups: ED and NSTA, to test the hypothesis that specific genetic variants explain differences between syndromic and non-syndromic tooth agenesis.

Materials and Methods

Recruitment

Human participants were recruited by referral or identification from the UNC School of Dentistry and National Foundation Ectodermal Dysplasia (NFED). The study has been approved by the Biomedical Institutional Review Board, IRB study #: 04-1711 and CRTC #: 2215. The recruited individuals had been previously identified as having congenitally missing teeth or ectodermal dysplasia, and were assigned to ED or NSTA groups based on this prior diagnosis. Study participants met the following inclusion criteria of previous diagnosis of either ED or congenitally missing teeth, and the following exclusion criteria: subject affected with another known genetic syndrome (e.g. Down’s Sydrome, Cleft Lip and Palate). Available participant demographics (age, gender, ethnicity) and previous dental diagnosis (ED, NSTA, previous extractions) were collected and recorded from the recruited participant’s dental provider (orthodontist and general dentist) and/or from the dental school database. Participants were asked to submit a saliva sample for DNA analyses.
**Genotyping**

DNA was harvested from saliva samples (cheek cells within saliva) of consenting subjects. Individuals not located near the UNC School of Dentistry provided buccal samples through self-collection and shipment of the sample using a pre-paid return envelope. High density 500K SNP-chip (Affymetrix, Santa Clara, CA) was utilized to detect regions with modest scale deletions within the entire genome for each participant. Our study focused primarily on identifying genetic variation (i.e. Single Nucleotide Polymorphisms—SNPs) for the following candidate genes: $AXIN2$, $BMP4$, $EDA$, $MSX1$, $PAX9$, within the entire genomic data set obtained from the 500K SNP-chip.

**Statistical Analyses**

Multivariate analyses and hypothesis tests, such as Distance Weighted Discrimination and the Direction Projected Permutation, were used to consider all data of the selected candidate genes within the genotype simultaneously to compare mean differences between ED and NSTA, and to determine if these differences are significant ($p<0.05$). DWD is a modern computationally intensive optimization method designed for high dimension low sample size for data, in which the data vectors are larger than the sample size (96). DWD is a modification of Support Vector Machines (SVM) (97), a non-probabilistic binary linear classifier. In both DWD and SVM, given inputs within a set of data points are mapped onto an infinite dimensional space. A hyperplane or set of hyperplanes is constructed onto the infinite dimensional space of data points such that the data is separated or classified into categories, which are divided by a clear gap (i.e. functional margin) as wide as possible. Good separation is achieved by the hyperplane
that provides the largest functional margin and thereby the lowest potential error of the classifier (96).

Direction-Permutation-Projection (DiProPerm) is a technique utilized for hypothesis testing with DWD analyses. DiProPerm first utilizes DWD to find the appropriate directional vector or hyperplane best separating the data. This vector is then projected onto a one-dimensional subspace, on which a one dimensional test statistic (e.g. two sample t-test) can be constructed. To yield a p-value the true statistic is compared to a population of permutated test statistics, which are constructed from several random DWD vectors again projected onto a one-dimensional subspace, which has a computed permutation test statistic (96). The genes were then ranked in order of those best explaining the SNP differences between the ED and NSTA groups based on the DWD and DiProPerm analyses.

Results

We were able to recruit 100 participants, who provided a saliva sample for genetic analysis. Our sample of 100 patients was reduced to 88 participants since 12 individuals did not have a full set of SNP data available (experimental error). Participant demographics of the 88 (54 ED, 34 NSTA) included participants are found in Table 4.

DWD analyses of the SNPs indicate SNPs on the MSX1, AXIN2, and BMP4 genes are most important in explaining the genetic differences observed between ED and NSTA groups within our sample (Figure 12). Moreover, the greater number of SNPs for each gene corresponded to the rank order of those genes best explaining to the genetic differences observed between ED and NSTA (Table 5, Figure 12). Overall, DiProPerm
analysis (Figure 13) indicates a marginally significant difference ($p=0.0533$) between patients with ED and patients with NSTA for the SNP data.

Using DWD, we also conducted candidate SNP analysis (individually) for the aforementioned candidate genes of (example of this analysis is provided in Figure 14) and an orthologous region in a non-human primate model that was linked to tooth shape (chromosome 19). We found that there is no single SNP in a given gene that explains most of the variation between ED and NSTA.

### Table 4. Participant demographics of ED and NSTA groups for genetic analyses.

<table>
<thead>
<tr>
<th></th>
<th>ED</th>
<th>NSTA</th>
</tr>
</thead>
<tbody>
<tr>
<td>DNA (n=88)</td>
<td>n, (%)</td>
<td>n, (%)</td>
</tr>
<tr>
<td>Male (n=)</td>
<td>54 (61.4)</td>
<td>34 (38.6)</td>
</tr>
<tr>
<td>Female (n=)</td>
<td>26 (70.3)</td>
<td>22 (46.8)</td>
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</table>

### Table 5. Number of SNPs associated with each candidate gene.

<table>
<thead>
<tr>
<th>Candidate Genes</th>
<th>Associated SNPs</th>
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</thead>
<tbody>
<tr>
<td>AXIN2</td>
<td>64</td>
</tr>
<tr>
<td>BMP4</td>
<td>170</td>
</tr>
<tr>
<td>EDA</td>
<td>31</td>
</tr>
<tr>
<td>MSXI</td>
<td>93</td>
</tr>
<tr>
<td>PAX9</td>
<td>28</td>
</tr>
</tbody>
</table>
Figure 12 - DWD Loading values, with SNPs given for each of the variables in decreasing magnitude order. SNPs with larger magnitude are more important in separating the data between ED and NSTA groups. A positive value (right side of the histogram) indicates patients with NSTA have higher levels. Negative values indicate patients with ED have higher levels.
Figure 13 - DiProPerm results indicate a marginal significant difference between patients with ED and NSTA. The left panel shows the projection of the data onto the DWD direction vector -symbols- and smoothing histograms -curves. We can observe a good separation of the data according to the group. The right panel shows the permutation test where each black dot is the value of the t-statistics for each permutation and the green line is the t-statistic obtained for the original data. The empirical p-value is less than $10^{-15}$ and is shown as 0, which means that there exists a marginal significant difference between patients with ED and NSTA.
Figure 14 - Allele variation representing AA, AB and BB shows how the ED versus NSTA the distribution of subjects carrying a given genotype

Discussion

Genotypic analysis of two groups with syndromic and NSTA respectively reveals that there are quantifiable differences in genes associated with tooth agenesis. We applied a novel statistical approach, DWD, (Figure 12), to determine the most important SNPs and genes for separating the genotypic variation between ED and NSTA groups. Accordingly we determined MSX1, AXIN2, and BMP4 are most important in differentiating NSTA from ED (Table 5, Figure 12). It was surprising that EDA and PAX9 genes did not have an effect between distinguishing the ED and NSTA groups, as EDA directly is associated with syndromes of ED, especially HED (113). However, the EDA gene has recently been linked to isolated NSTA (30, 31, 33). We found that there is no single SNP in a given gene that explains most of the variation between ED and NSTA,
indicating potential modifier genes not assessed in our limited study could exist that contribute to the regulation of tooth formation in both ED and NSTA groups. Perhaps additional studies can focus on odontogenic and embryogenic pathways involving the \textit{MSX1, AXIN2, and BMP4} genes. With recent experimental approaches of recombinant EDA or transgenesis of EDA-A1 in both mice and dog models, short-term recombinant protein therapy can be utilized to permanently correct a developmental genetic defect and restore the wild type phenotype (94, 95). Future use of recombinant protein therapy utilizing \textit{AXIN2, BMP4, EDA, MSX1, PAX9}, and other future discovered genes associated with tooth agenesis might lead to novel treatment options for patients burdened by the clinical presentation of hypodontia.

\textbf{Conclusions}

1. The genetic differences between ED and NSTA are best explained by \textit{BMP4} and \textit{MSX1}.

2. Further studies are needed to discover additional modifier genes involved in the complex processes of tooth development to develop novel treatment approaches to hypodontia.
References


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