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ASSOCIAÇÃO DE POLIMORFISMOS NOS GENES MSX1 E PAX9 COM AGENESIA DENTÁRIA

BRENO RAMOS BOEIRA JUNIOR

CAXIAS DO SUL, NOVEMBRO DE 2011.

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ORIENTADOR: PROF. DR. SERGIO ECHEVERRIGARAY

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Família Nuclear – Formada por pai, mãe e filho (s)

GenBank – *Genetic Sequence Data Bank* / Repositório de Sequências Nucleotídicas Online

IRF – *Interferon Regulatory Factor* / Fator Regulador de Interferon

MSX – Gene MSX: *Muscle Segment Homeobox*

NCBI – National Center for Biotechnology Information

OMIM – *Online Mendelian Inheritance in Man* / Banco de Dados Online de Herança Mendeliana

PAX – Gene PAX: *Paired Box*

PCR – *Polymerase Chain Reaction* / Reação em Cadeia da Polimerase

RPM – Rotações por Minuto

SNP – *Single Nucleotide Polymorphism* / Polimorfismo de Único Nucleotídeo

STRING – *Search Tool for the Retrieval of Interacting Genes* / *Proteins*

TMJ – *Temporomandibular Joint* / Articulação Temporomandibular

UTR – *Untranslated Region* / Região Não Traduzida

RESUMO

A agenesia dentária é a falha do desenvolvimento do germe dentário causando a ausência definitiva do dente. Constitui–se na anomalia dentária mais comum, afetando até um quarto da população em geral. A principal causa está relacionada com a função anormal de genes específicos que desempenham um papel chave durante a odontogênese, especialmente o MSX1 e o PAX9. Apesar da alta frequência dessa anomalia, apenas algumas mutações no MSX1 e no PAX9 foram associadas com a agenesia não–sindrômica até o momento. Uma vez que um número maior de indivíduos afetados é esperado nos próximos anos, uma análise mais profunda dos defeitos genéticos que levam à agenesia torna–se fundamental. O objetivo desta pesquisa foi estudar seis famílias segregando oligodontia/hipodontia não–sindrômica, além de investigar a presença de mutações nos genes MSX1 e PAX9 com a finalidade de associar fenótipo e genótipo. As famílias, que apresentam mais de um integrante com agenesia, foram selecionadas a partir de uma clínica particular. Amostras de células epiteliais bucais foram coletadas de todos os indivíduos por meio de escovas citológicas. O DNA foi isolado, amplificado e sequenciado. Todas as sequências foram comparadas com as existentes no GenBank. A homologia das sequências mutantes também foi comparada utilizando–se o software online BLAST. Os genes MSX1 e PAX9 de dez indivíduos controle não afetados e sem grau de parentesco também foram sequenciados. Foi identificada uma nova mutação missense no exon 2 próximo ao final do domínio pareado do PAX9 em três famílias. A mutação heterozigótica C503G resulta em uma troca de aminoácido de alanina para glicina no resíduo 168 (Ala168Gly), o qual é invariavelmente conservado entre várias espécies. A mudança alanina–glicina pode determinar uma alteração da estrutura proteica devido às propriedades únicas de flexibilidade da glicina, levando à agenesia dentária. Tal mutação não encontra–se registrada em nenhum banco de dados conhecido ou na literatura sendo, portanto, inédita. As mutações intrônicas IVS2–109G>C, IVS2–54A>G e IVS2–41A>G também foram identificadas no PAX9. Essas variantes polimórficas podem estar envolvidas no fenótipo uma vez que um probando, o qual apresentou todas as três mutações intrônicas em homozigose, foi afetado com a mais severa forma de oligodontia dentro da amostra. A transição * 6C>T foi identificada no exon 2 do MSX1, em apenas uma família. Devido ao fato de estar localizada seis bases após o stop codon, essa mutação homozigótica pode dificultar/alterar o término da tradução contribuindo, assim, para o fenótipo. Novos estudos acerca da expressão gênica em um número maior de famílias afetadas irá aumentar o conhecimento sobre agenesia dentária. Tal entendimento permitirá alcançar melhores opções de tratamento e, talvez, uma ferramenta de diagnóstico precoce que, possivelmente, envolverá o exame de DNA baseado em variantes polimórficas. Todos esses dados podem auxiliar a clínica odontológica em um futuro próximo.

Palavras–chave: agenesia dentária; MSX1; PAX9; mutação; biologia molecular.

Tooth agenesis, the failure of development of tooth bud causing definitive absence of the tooth, is the most common dental anomaly affecting up to one quarter of the general population. The main cause is related to abnormal function of specific genes which play key roles during odontogenesis, particularly MSX1 and PAX9. Despite the high frequency of this anomaly, there are only a restricted number of mutations in MSX1 and PAX9 that have been associated with non–syndromic tooth agenesis so far. Since a greater number of affected subjects is expected over the coming years, a deeper analysis of the gene networks underlying tooth agenesis is critical. The aim of this research was to investigate six families segregating non–syndromic oligodontia/hypodontia as well as to screen for mutations in their MSX1 and PAX9 genes, attempting to associate phenotype and genotype. Families were selected from a private office. They should present more than one relative affected with agenesis. All subjects had a sample of buccal epithelial cells collected with cytology brushes. DNA was isolated, amplificated and sequenced. All sequences were compared to those in GenBank. Homology of the mutant sequences was also compared using BLAST online software. MSX1 and PAX9 genes from ten unrelated unaffected control subjects were also sequenced. A novel missense mutation lying in the exon 2 close to the end of the paired domain of PAX9 was identified in three families. Heterozygous mutation C503G is expected to result in an alanine–to–glycine amino acid change in residue 168 (Ala168Gly), which is invariably conserved among several species. The alanine–glycine change might lead to protein structural alteration due to the unique flexibility properties of glycine, leading to tooth agenesis. This is a novel mutation since it is not registered neither in any known database nor in literature. Intronic mutations IVS2–109G>C, IVS2–54A>G and IVS2–41A>G were also identified in PAX9. These polymorphic variants may be involved in the phenotype as one proband, showing all three intronic mutations in homozygosis, was affected with the most severe oligodontia within the sample. Transition *6C>T lying in the exon 2 six base pairs after the stop codon was detected in MSX1 gene in one family. Due to its proximity to the stop codon, this homozygous mutation can hinder a regular translation termination thus contributing to the phenotype. Further studies with gene expression in larger affected families will increase knowledge of tooth agenesis. Such understanding will allow to achieve better treatment options and, perhaps, an early diagnosis tool which would possibly lie on the DNA examination based on polymorphic variants. All this data may assist dental practice in a near future.

Keywords: tooth agenesis; MSX1; PAX9; mutation; molecular biology.

1. INTRODUÇÃO

Os elementos dentários, os ossos craniofaciais, a articulação temporomandibular, a musculatura orofacial, a inervação e a vascularização correspondentes fazem parte do complexo sistema estomatognático e precisam estar em equilíbrio para que o indivíduo encontre–se saudável. A ausência ou a alteração na função de quaisquer desses componentes pode produzir desde um desconforto, passando por sintomatologias dolorosas e alterações estéticas e psicológicas, até estados patológicos orgânicos referidos também na oclusão dentária/mastigação, na deglutição e na fonação. A agenesia dentária pode determinar um quadro clínico envolvendo um ou mais desses sinais e sintomas.

A agenesia dentária é a falha no desenvolvimento do germe dental levando a sua ausência definitiva. Trata–se da anomalia dentária mais comum afetando até 25% da população em geral. Clinicamente, subdivide–se em: hipodontia (ausência de um até seis dentes permanentes, exceto os terceiros molares), oligodontia (mais de seis dentes permanentes estão ausentes, exceto os terceiros molares) e anodontia (ausência total de dentes). A ausência de terceiros molares igualmente é considerada como agenesia.

Os dentes mais frequentemente ausentes são os terceiros molares, seguidos por segundos pré–molares e incisivos laterais superiores permanentes. Parece haver uma relação entre o número de dentes ausentes e agenesia dentária herdada, já que a maioria dos casos de famílias com indivíduos afetados tem apresentado maior prevalência de hipodontia entre os familiares desses indivíduos do que na população em geral.

A agenesia dentária pode ser sindrômica ou não–sindrômica e familiar ou esporádica. Na maioria dos casos herdáveis, a herança é autossômica dominante; no entanto, a herança autossômica recessiva e a ligada ao X também podem estar envolvidas.

Estudos de desenvolvimento dos dentes em ratos permitiram identificar, até o momento, mais de 200 genes envolvidos direta ou indiretamente na regulação da odontogênese. Entre estes, dois genes, MSX1 e PAX9, estão altamente correlacionados com a agenesia. Ambos codificam fatores de transcrição induzidos por sinais epiteliais e expressos no mesênquima dental. Assim, funções anormais em tais genes podem afetar o desenvolvimento dentário. Apesar da alta prevalência da agenesia, a investigação de variantes genéticas nos genes MSX1 e PAX9 retornou um número restrito de mutações associadas à anomalia até o momento, justificando a necessidade de realização de mais estudos no sentido de ampliar a amostra existente na busca de novos alelos mutantes. Além disso, atualmente não há um método de diagnóstico, baseado em exame de DNA, que permita a detecção precoce da agenesia dentária.

Neste contexto, o objetivo da presente pesquisa foi investigar seis famílias nucleares afetadas por hipodontia/oligodontia visando associar fenótipo e genótipo por meio de investigação clínica e sequenciamento genético. Assim, o presente trabalho igualmente contribui para aumentar o conhecimento de variantes genéticas associadas etiologicamente à anomalia possibilitando que, em um futuro próximo, essas informações auxiliem no desenvolvimento de exame de DNA para detecção precoce de agenesia dentária.

2. OBJETIVOS

Com base nas informações relatadas, o objetivo geral da presente pesquisa é identificar mutações nos genes MSX1 e PAX9 em uma amostra constituída por seis famílias afetadas por hipodontia/oligodontia, associando genótipo e fenótipo.

Os objetivos específicos são:

- identificar e tipificar os dentes ausentes na amostra;
- diagnosticar e confirmar a presença de agenesia nos familiares dos probandos
- determinar o modo de herança da anomalia nas famílias estudadas
- investigar a existência de anomalias dentárias associadas
- investigar mutações em todos os familiares (afetados ou não) dos probandos;
- evidenciar e correlacionar potenciais fatores de risco com a manifestação de agenesia dentária;
- avaliar a homologia das sequências mutantes encontradas na amostra por meio de BLAST;
- contribuir, com novas variantes genéticas, para o delineamento de futuro exame de especificidade de DNA para diagnóstico precoce de agenesia dentária.

3. REVISÃO BIBLIOGRÁFICA

Tooth agenesis: molecular genetic approach and state–of–the–art

Abstract

Tooth agenesis, the failure of development of tooth bud causing definitive absence of the dental piece, is the most common human dental anomaly affecting up to one quarter of the general population. In the majority of cases its inheritance is autosomal dominant, however, autosomal recessive and X–linked inheritance may also be involved. The main cause seems to be related to abnormal function of specific genes which play key roles during odontogenesis, particularly MSX1 and PAX9 genes. Despite the high frequency of tooth agenesis, there are only a restricted number of mutations in MSX1 and PAX9 that have been associated with hypodontia and/or non–syndromic oligodontia so far, suggesting that this anomaly may result from a given combination of genetic variants. Since gene networks underlying tooth agenesis are still unclear, the aim of this review is to assess several aspects concerning tooth agenesis by means of a molecular approach based on the interaction between MSX1 and PAX9 genes. Etiopathogenesis, mutational analysis, bioinformatics information and current and future treatment options are also discussed.

Introduction

Stomatognathic system in which are included dental pieces, craniofacial bones, temporomandibular joint (TMJ) and soft tissues among others, is expected to be balanced in order to allow a healthy status to an individual. Disorders in any of these components are able to produce discomfort, painful symptomatologies, esthetics and psychological disorders, injuries to dental occlusion and abnormal swallowing. When it comes to injuries particularly related to human tooth agenesis, there are many: inefficient chewing and alimentary system dysfunction; unstable occlusion; TMJ and orofacial pain; low self–esteem due to unpleasant facial esthetics; abnormal phonation. Tooth agenesis is capable of producing clinical features which involve one or more of the aforementioned signs and symptoms.

Tooth agenesis is the failure of development of tooth bud causing definitive absence of the dental piece (Nieminen, 2009). It is the most common human dental anomaly showing up to 25% (Bredy *et al.*, 1991) or even 30% of prevalence (Haavikko, 1971; Arte, 2001), in extreme cases. This anomaly can be either part of a syndrome or a non–syndromic familiar disturbance (Vieira, 2003). In the majority of cases its inheritance is autosomal dominant, however, autosomal recessive and X–linked inheritance may also be involved (Franzier–Bowers *et al.,* 2002–A). Since each illness, except for environmental causes (trauma, irradiation, deleterious oral habits among others), is supposed to present a genetic component (Chemale, 2004), it is possible to consider that tooth agenesis may follow the same prerogative. Thus, the main cause seems to be related to abnormal function of specific genes which play key roles during odontogenesis, particularly MSX1 and PAX9 genes. So far mutational screening for both genes has only returned a restricted number of mutations. Accordingly, since a cause and effect relationship of MSX1 and PAX9 with tooth agenesis is not completely clear, new studies need to be performed in order to enlarge existent samples as well as to find new mutant alleles. Potential risk factors, which might be involved on development of dental agenesis, also demand further investigation.

In recent days, treatment for this anomaly is only applicable whenever it is already established and, in most cases, whenever the patient notices missing teeth. Indeed, a standard method for tooth agenesis early detection is not available yet. DNA specificity might be used on early diagnosis of the referred abnormality which would allow patients to receive a more effective treatment, even including stem–cell therapy.

The aim of this review is to assess several aspects concerning the human tooth agenesis by means of a molecular approach based on the interaction between MSX1 and PAX9 genes. Etiopathogenesis, mutational analysis, bioinformatics information and current and future treatment options are also discussed.

Molecular Regulation

In spite of cells have the same genome, how each one will express its genes depends on a number of molecular interactions. These reactions will form a future normal or abnormal organ like tooth, or its absence. Different levels of the regulation of gene expression may even produce tumor. Gene expression capability is widely regulated by proteins named transcription factors, which can act like either an activator or inhibitor leading to a specific individual phenotype.

Cells interactions generally involve the action of signaling molecules known as signals which are usually proteins or peptides acting either on a surface specific receptor or inside the receiving cells (Wang & Thesleff, 2005). These signals are often delivered ranging neighboring cells, however, they can also reach far away cells according to their concentration (Gilbert, 2003; Nieminen, 2007). Peptide growth factors which belong to the evolutionarily conserved Wnt, Hedgehog and Fibroblast Growth Factor (FGF) families, Activins, Transforming Growth Factor–β (TGF–β) superfamily and Bone Morphogenetic Proteins (BMP) are the most important signals (Pires–da Silva & Sommer, 2003).

In order to be able of receiving signals cells must present receptors for each signaling protein family. When a ligand (protein) binds to its receptor the signal is mediated into the cell where protein interactions lead to the activation of certain transcription factors which will regulate gene expression (Bei & Maas, 1998; Gilbert, 2003). According to its competence cell may respond to this signal in many ways: delivering of antagonist signal; delivering of reciprocal signal; differentiation; apoptosis among others. For example, BMP4 is responsible for the activation of the homeodomain–containing transcription factor MSX1, whereas FGF8 accounts for activating the PAX9 in the mesenchyme at the prospective sites of odontogenesis. Thus, cells interactions, signaling, reactions and responses to the transcription factors depict how organs and tissues are formed during human evolution. Such events show that there are well conserved genetic structures in order to allow both variability and conservation to certain species (Nieminen, 2007). On the other hand, this system formed by complex genetic pathways may change due to evolutionary development. It has been hypothesized that man and other placental mammals interestingly will have their teeth number evolutionarily diminished in a reverse order of their eruption chronology. This might be explained by means of a reaction/diffusion model of morphogenesis in which repeated structures, such as teeth and vertebrae, are formed from the interaction of two molecules, an activator and an inhibitor (e.g. the aforementioned MSX1 and PAX9 interaction). In such a system, teeth most distant from the center of the morphogenetic field tend to disappear due to field attenuation (Koussoulakou *et al.*, 2009).

Odontogenesis

Vertebrates from class Reptilia, such as the alligator, have “replaceable” teeth in several dentitions (polyphyodonts) during their lifespan. Likewise, sharks are also able to replace their teeth hundreds of times. The mechanism underlying the tooth renewal appears

to be similar to that of the hair follicle regeneration, which contains cells showing stem cell–like properties (Nieminen, 2007). Since mammalians like man are diphyodonts, that is, they can replace some teeth only once, knowledge of the gene interactions involved in the human odontogenesis becomes primordial.

Reciprocal interactions between the epithelial and mesenchymal tissue components account for the origin of development of all ectoderm and mesoderm descendant organs like hair follicles, sweat glands, mammary glands and teeth (Kollar, 1970; Lammi *et al.*, 2003; Pispa & Thesleff, 2003). Thus, human odontogenesis can be depicted as a long term and complex process that starts in the cells of each trilaminar embryonic sheet (deciduous teeth along with permanent molars develop from surface epithelium, whereas other permanent teeth develop from dental lamina). These cells divide, migrate, aggregate and differentiate into specific patterns as they form the organic systems, beginning a process that gives rise to all tissues and embryonic organs derived from ectoderm, mesoderm and endoderm. There is a thickening of oral epithelium – ectoderm – protruding into the mesenchyme resulting in the dental lamina, which is the first sign of tooth development. After the formation of this placode, there is a proliferation of epithelial cells resulting in the tooth bud, which represents the primordium of the enamel organ of the deciduous teeth. In the fourth week of gestation begins tooth development (Moore & Persaud, 2004) which will include the eruption of the tooth and will be finished with the completion of root apex formation. Occurrence of any disturbance in the embryonic processes may produce tooth agenesis as well as anomalies that can present more severe, involving craniofacial structures (Mostowska *et al.*, 2003–B).

There are a number of gene interactions regulating teeth development through the distinct phases of initiation, morphogenesis, differentiation and mineralization, root formation and eruption. During initiation phase there is a thickening of the ectoderm forming a placode. The epithelium signals to the mesenchyme which then condenses around the epithelial bud on the beginning of the morphogenesis phase. Then the epithelium folds to surround the dental

papilla mesenchyme (cap stage). Tooth crown final shape is fixed during the bell stage where ameloblasts and odontoblasts differentiate producing enamel and dentin respectively.

Reciprocal signaling processes manage tooth development from initiation to eruption (Nieminen, 2007). Sonic Hedgehog (SHH) signaling pathway contributes on tooth root development, whereas FGF signaling effectively promotes the tooth root elongation as well as periodontal tissue formation (Nakatomi *et al.*, 2006; Ota *et al.*, 2007). Transcription Factors, Bone Morphogenetic Proteins (BMP), Wnt and Hedgehog families, Transforming Growth Factor–β (TGF–β), Fibroblast Growth Factor (FGF), Tumor Necrosis Factor (TNF) family and its receptor Edar constitute some of several signal molecules that allow cells to communicate with each other in order to form the human dentition (Thesleff, 2003). Furthermore, every tooth formation is also regulated by the enamel knot which appears at the bud–to–cap evolution. It is likely responsible for marking and stimulating the formation of tooth cusps and outlining tooth crown morphogenesis seemingly by regulating the formation of additional enamel knots (Bei & Maas, 1998; Jernvall & Thesleff, 2000; Thesleff, 2003; Nieminen, 2007).Thus, primary enamel knot signals along with additional enamel knots regulate where the epithelial sheet folds and a new cusp formation starts. Afterwards, when enamel knots are no longer needed, their cells are removed by apoptosis (Jernvall *et al.*, 1994).

Tooth Agenesis

Concepts and Clinical Classification

The most common craniofacial anomaly in man, tooth agenesis is the absence of one or more teeth (Shapiro & Farrington, 1983; Vastardis, 2000). Terms “missing tooth” and “congenitally missing tooth” (Muller *et al.*, 1970) have also been used to identify it although they can cause misunderstandings. Missing tooth does not properly depict tooth agenesis

since teeth might be lost in trauma events, by cavities and periodontal diseases, by extraction due to orthodontic treatment among others. Congenitally missing tooth refers to missing tooth at birth and it is known that some teeth begin their development only after birth suggesting that clinical diagnosis of tooth agenesis could only be considered during the first decade of life. Thus, it seems that tooth agenesis is a more suitable term to depict the failure to develop any of the 20 deciduous and 32 permanent teeth (Jorgenson, 1980; Vastardis *et al.*, 1996; Nieminen, 2007). Furthermore, an even more accurate concept should add either terms "selective" or "partial" to "tooth agenesis" (Vastardis *et al.*, 1996).

An inquiry that still needs enlightenment is the pathogenesis of the referred anomaly in order to classify the clinical situation of a given patient. Stages of teeth development are well known in spite of the related molecular regulation processes need further investigation. Accordingly, tooth agenesis may occur from either a bud stage arrest or a non formation of the tooth bud. During the gestational period human being usually develops all tooth buds that will give rise to the deciduous dentition besides some permanent teeth. Meanwhile, there can be an insufficient level of functional protein (haploinsufficiency) responsible for inducing the next stages of the dental development. Thus, one or a few teeth might not be formed and a tooth agenesis diagnosis would be made. On the other hand, another hypothetic subject fails to develop one or a few teeth. After deep investigation, the protein level is not problematic whatsoever. It might be concluded that this individual was indeed lacking the tooth bud (not formed) since there was not epithelial thickening when it was supposed to happen. After this brief hypothetic report, it is suggested that further studies are necessary to clear the actual etiology and pathogenesis of tooth agenesis.

Clinical classification of this anomaly regardless of being syndromic or non–syndromic, sporadic or familial (as referred by Online Mendelian Inheritance in Man database: OMIM #106600, #604625 accessed at http://www.ncbi.nlm.nih.gov/omim/, 2009), occurs according to the number of missing teeth (Cobourne, 2007). Until the seventies, third molars were not

included by a number of authors when they had to clinically classify dental agenesis (Hunstadbraten, 1973; Arya & Savara, 1974; Wisth *et al.*, 1974). From the early eighties onward clinical classification of dental agenesis changed. Hypodontia (hypo, hyp: Greek prefixes for less than, diminished) is usually used to depict the congenitally missing from one to six teeth except third molars. In recent years, it has been used to name common and mild forms of tooth agenesis (Stewart & Poole, 1982; Arte, 2001; Nieminen, 2007). Oligodontia (oligo, olig: Greek prefixes for abnormally few) indicates that more than six teeth, except third molars, are lacking and it is not associated with systemic disorders. This term is often indicated for more severe cases (Stockton *et al.*, 2000). These aforementioned terms should have their application carefully evaluated as they may mislead the clinician concerning the severity of some cases, particularly those in which third molars are excluded. Anodontia (a, an: Greek prefixes meaning absence of, lack of) constitutes an extreme case, the complete absence of teeth (Jorgenson, 1980; Arte, 2001). Dental agenesis may also be classified as syndromic/non–syndromic and familial/sporadic (Pawlowska *et al.*, 2009).

Epidemiology

From numerous studies it is possible to assume that the prevalence of hypodontia shows a rather large variation ranging from 1.6% (Graber, 1978; Dermaut *et al*., 1986) to 30% of the population (Haavikko, 1971). Higher rates of frequency usually include agenesis of third molars, whereas the lower ones do not include third molars.

Depending on the country and the ethnics (Arte, 2001; Polder *et al.*, 2004) of the studied subjects prevalence rates may vary: 2.8% in the United States, 3.4% in Switzerland, 7.4% in Canada, 6.3% in Australia and 6.6% in Japan. A little difference in incidence of hypodontia between white and black students was noticed in the United States (Muller et al., 1970; Arte, 2001). Hypodontia is more prevalent in the permanent dentition varying from 1.6%

to 10.1% (Dermaut *et al.*, 1986; Arte, 2001; Polder *et al.*, 2004; Londhe *et al.*, 2008). Although in lower rates (0.1% to 0.9%), deciduous dentition can also present the anomaly (Dermaut *et al.*, 1986). Although the majority of the studies have found tooth agenesis more prevalent among females (Muller *et al.*, 1970; Haavikko, 1971; Rune & Sarnäs, 1974; Davis, 1987, Polder *et al.*, 2004) than among males even at a ratio of 3:2 (Egermark–Eriksson & Lind, 1971) data do not lead to a statistically significant difference between genders, except in a few studies (Brook, 1974; Bergström, 1977). Regarding oligodontia, prevalence rates are much lower ranging from 0.1% to 0.3% (Haavikko, 1971; Dermaut *et al*., 1986), although Asians, particularly Chinese, show a higher frequency (Xuan *et al.*, 2008).

Interestingly, it seems that there is an increasing trend in agenesis during 20th century even though the available data are not sufficient to support this assumption (Polder *et al.*, 2004; Mattheeuws *et al.*, 2004; Nieminen, 2007). Even so, further research is necessary to unveil whether this trend is due to more accurate techniques and patient's awareness or whether human being are dealing with real tendency toward increased frequency of tooth agenesis (Vastardis, 2000). On the other hand, since general tooth size is under genetic control (Osborne *et al.*, 1958; Lundström, 1948) and during human evolution tooth size has been importantly reduced, particularly in the front portions of the jaws (Hooton, 1947), it has also been suggested that tooth agenesis is a phylogenic degeneration phenomenon (Bolk, 1914; Dalbergh, 1945; Baba–Kawano *et al.*, 2002). Since archeologists found tooth agenesis (with a deciduous molar remaining) as well as a great number of erupted third molars to be present in humans dated from 600 A.D., that hypothesis needs further studies (Salo, 2005).

Most cases of families with affected subjects have presented higher frequencies of peg–shaped lateral incisors and hypodontia in parents and siblings of the probands than in the general population (Brook, 1984; Arte, 2001). Likewise, in a Swedish survey hypodontia was found in 41% of parents and 26% of the siblings of the probands (Grahnen, 1956). Moreover,

relatives of probands with oligodontia are more likely to lack more teeth than do those with hypodontia (Brook, 1984).

Some authors support that a subject who lacks a third molar would have an overall congenital tendency of agenesis (Brekhus *et al.*, 1944; Garn *et al.*, 1962; Keene, 1964). Furthermore, likelihood of congenitally missing other teeth would be 13 times greater in an individual who lacks a lower third molar (Bailit, 1975) or any third molar (Garn & Lewis, 1962). In another study, agenesis of third molars was found in 50% of the subjects with agenesis of other teeth (Grahnen, 1956). That is, since agenesis of third molar is associated with teeth number reduction, factors that control third molar agenesis and agenesis of other teeth may be the same (Baum & Cohen, 1971).

Commonly Affected Teeth

Except for third molars, which are common sense among researchers as the most frequently missing teeth, absences of other teeth have shown some variation in which the lower second premolar and the permanent upper lateral incisor are the most often affected even though there is not an agreement regarding to the order of frequency. Many studies among European subjects have scored the lower second premolars most commonly affected followed by an upper lateral incisor or second premolar, whereas other studies have found the upper lateral incisors as the most often absent dental pieces (Grahnen, 1956; Haavikko, 1971; Hunstadbraten, 1973; Graber, 1978; Bredy *et al.*, 1991; Arte, 2001; Lidral & Reising, 2002; Nieminen, 2007). Whether third molar tooth agenesis is not considered, both upper lateral incisor and lower second premolar account for 85% of all missing teeth (Nieminen, 2007).

In a meta–analysis study, Polder *et al.* (2004) evaluated data for tooth agenesis in permanent dentition among Caucasian populations in North America, Australia and Europe. Prevalence of dental agenesis differed by continent since it was higher in Europe (males 4.6%;

females 6.3%) and Australia (males 5.5%; females 7.6%) than in North America (males 3.2%; females 4.6%). Authors divided the occurrence of dental agenesis into three main groups: common (lower second premolar > upper lateral incisor > upper second premolar), less common (lower central incisor > lower lateral incisor & upper first premolar > upper canine & lower second molar) and rare (upper second molar & upper first molar > lower canine > lower first molar & upper central incisor).

A Finnish study reported the prevalence of missing teeth in the following order: 42% of lower second premolars; 29% of upper second premolars; 19% of upper lateral incisors; 4% of lower first premolars; 3% of lower central incisors; 1% of lower lateral incisors. Agenesis of first and second molars as well as lower canines was found exceptional (Haavikko, 1971). On the other hand, in American individuals who lacked one or two teeth the most commonly missing tooth was the upper lateral incisor, whereas the second premolar was most frequently missing in those subjects who had more than two absent teeth (Muller *et al.*, 1970).

Agenesis of the first tooth of each dental class (upper central incisor, lower first molar and canines) was found rather rare reaching about 0.016%, 0.03% and 0.03% respectively. This might be caused by molecular quantitative mechanisms affecting especially the teeth that are initiated and develop latest in their respective tooth class (Arte, 2001; Nieminen, 2007).

Tooth agenesis has shown no statistically significant difference for prevalence between maxilla and mandible (Grahnen, 1956; Haavikko, 1971; Rune & Sarnäs, 1974; Bergström, 1977, Polder *et al.*, 2004). In spite of some studies have found no significant difference between the left and right sides of the jaws (Rune & Sarnäs, 1974; Magnusson, 1977) tooth agenesis was found more prevalent on the left side in Scandinavian studies (Grahnen, 1956; Wisth *et al.*, 1974; Bergström, 1977). Nonetheless, bilateral agenesis of upper lateral incisors appears to be more common than unilateral agenesis. Even so, tooth agenesis usually shows a higher unilateral incidence than bilateral incidence (Polder *et al.*, 2004). According to some reports, the majority of affected individuals (up to 83%) have shown the absence of one or two

permanent teeth (Muller *et al.*, 1970; Bergström, 1977; Polder *et al.*, 2004). When it comes to families, the frequency of agenesis affecting one tooth class among relatives is significantly higher than affecting different tooth classes (Arte *et al.*, 2001).

As assessed in the permanent dentition, the deciduous dentition is also affected by tooth agenesis although it is found less prevalent. Agenesis occurs more frequently in the maxilla and the upper lateral incisor accounts for over 50% of the affected teeth. Whether the upper lateral incisor and the lower lateral incisor are considered together they both account for 90% of the missing teeth in the deciduous dentition. There is no significant gender distribution. Peg–shaped teeth were reported among Japanese children. Despite having been found, agenesis of first or second deciduous molar or deciduous canines is extremely rare. Correlation between agenesis of a deciduous tooth and its permanent successor does exist since agenesis of a given deciduous tooth is mostly followed by agenesis of the corresponding permanent tooth (Jorgenson, 1980; Arte, 2001 and Nieminen, 2007). Nonetheless, Ooshima *et al.* (1988) depicted a case of a child with normal permanent tooth germs and oligodontia in the deciduous dentition (eight teeth lacking). Remaining deciduous teeth had anomalies in size and morphology.

Etiology

The underlying genetic mechanism leading to isolated sporadic tooth agenesis – hypodontia – has not been clarified yet, whereas the etiology of oligodontia has lately been linked to some genetic mutations evaluated in human families studies. Meanwhile, some environmental factors have also been shown to be involved (Peres *et al.*, 2004; Pawlowska *et al.*, 2009). All this features will be discussed in the following.

Although both genetic and environmental factors may contribute, it has been found scientific proofs for the major role played by genetic factors in the etiology (Graber, 1978;

Woolf, 1971; Vastardis, 2000). Indeed, molecular genetics era has presented a number of studies correlating tooth agenesis and anomalies in size/morphology to defects in several genes (Vastardis *et al.*, 1996; Stockton *et al*., 2000; Lammi *et al.*, 2004; Klein *et al.*, 2005; Peres *et al.*, 2004; Chishti *et al.*, 2006; Vieira *et al.*, 2007; Küchler *et al.*, 2008; Menezes *et al*., 2009).

Hypodontia matches the criteria for a genetic disease as it is found higher prevalent among subjects related to hypodontia patients than in the general population (Brook, 1984). This statement is also suggested by studies of families where a concordance in intra–familial phenotypes was found (van den Boogaard *et al.*, 2000; Pardo *et al.*, 2006; Vieira *et al.*, 2007; Xuan *et al.*, 2008). Likewise, Woolf (1971) reported, among 103 probands and their affected family members, a high degree of concordance (69%) regarding to missing or peg–shaped upper lateral incisors supporting the existence of a genetic component underlying this trait. Twin studies have shown different findings (Vastardis, 2000) even though in most cases monozygotic show a more concordant phenotype than dizygotic twins (Burzynski & Escobar, 1983). According to data found by Markovic (1982) regarding the empirical risk of hypodontia occurring in twins, whether one of a pair of monozygotic twins is affected, the other will also be in 89% of the cases. On the other hand, whether the twins are dizygotic, the risk of the other has the anomaly is nearly zero. Reported results showed a high genetic component in the trait.

Although different patterns of inheritance have been suggested in literature, in most cases tooth agenesis is transmitted as an autosomal dominant trait with reduced penetrance and variable expressivity (Grahnen, 1956; Woolf, 1971; Burzynski & Escobar, 1983). Autosomal recessive (Ahmad *et al*., 1998; Pirinen *et al.*, 2001; Chishti *et al.*, 2006) and X–linked as well as a polygenic or a multifactorial model of inheritance (Brook, 1984; Peck *et al*., 1993; Mostowska *et al.*, 2006–C; Vieira *et al*., 2007) may also be involved. Concerning the latter, diverse phenotypes (variable expressivity) might be related to several independent defective genes which could act alone or in combination with other genes (Vieira *et al.*, 2007). Evaluating 171

probands who presented with tooth agenesis, Grahnen (1956) found a higher penetrance in those families whose probands were lacking more than six teeth suggesting that a more severe phenotype might indicate a greater tendency to segregate the trait.

Despite the underlying cause leading to tooth agenesis is genetic factor/inheritance, environmental factors must also be considered as etiological components. Gestational period and childhood are obviously the most important phases for odontogenesis. Thus, any sort of external agent might be capable of modifying chemical interactions as well as genetic pathways which primarily account for a regular teeth formation.

Maternal systemic diseases such as diabetes, rubella (Kraus *et al.*, 1969) and hypothyroidism during pregnancy have been found to be related to developmental dental anomalies and tooth size reduction. Findings from a sample with 870 white boys and girls reported by Garn *et al.* (1979) showed that children of hypothyroid mothers and diabetic mothers presented with greater crown sizes, whereas low birth length and low birth weight children as well as those of hypertensive mothers were associated with reduced odontometric dimensions.

Chemotherapy, radiotherapy and, during pregnancy, application of Thalidomide® may contribute to the pathogenesis of tooth agenesis (Arya & Savara, 1974; Näsman *et al.*, 1997; Hölttä, 2005). In mice, irradiation effects in tooth morphogenesis have shown to be dose–dependent. Mild radiation results only in temporary damages that may not be macroscopically evident, whereas high radiation may cause injuries during the formation of the dental hard tissues such as altered tooth shape and size, ankylosis (Burstone, 1950) or, in severe cases, tooth agenesis (Hölttä, 2005). The same injuries, except ankylosis, were reported by Bruce & Stafne (1950) during a follow–up study with five patients presented with oral or facial cancer. They were treated with irradiation during childhood having permanent teeth affected by the aforementioned anomalies. Level of damage on teeth depends on the age of patient and dosage (Näsman *et al.*, 1997; Hölttä, 2005).

Dental anomalies in recipient children were evaluated by Hölttä (2005) who concluded that teeth on last stages of morphogenesis may be protected from agenesis due to advanced mineralization even though they are, in a minimal rate (except third molars), subject to microdontia. Data also showed that the younger was the patient at the time of anticancer therapy (under five years old) the higher was the risk for developmental dental defects to be present. Under two years old the risk of the patient to be affected with tooth agenesis was nearly 100%.

During gestational period or childhood, developing teeth and other organ can be affected by nutritional disturbances along with inherited gene defects, thus leading to tooth agenesis in association with other anomalies (Graber, 1978). Environmental pollutants such as dioxin, polychlorinated biphenyl and dibenzofuran have also been etiologically associated with human dental anomalies (Wang *et al.*, 2003). Dioxin, a halogenated aromatic hydrocarbon found in food, has been studied due to likelihood of developing human teeth being arrested. Pathogenesis may include affected developing teeth in children via mother's milk. Even at later stages of development teeth may be affected, thus having mineralization defects and diminished tooth size. In mice, high level of dioxin exposure through maternal milk caused molar teeth to have morphogenesis arrested or retarded (Kattainen *et al.*, 2001; Kiukkonen, 2006). Effects in humans are controversial since available data do not support this assumption so far (Kiukkonen, 2006). However, a chemical disaster involving massive exposure to dioxin led a group of people to be affected with isolated tooth agenesis and enamel defects (Alaluusua *et al.*, 2004).

Any sort of trauma in the region of the face and jaws, such as surgical procedures on the jaws, extraction of deciduous teeth and fractures from any sort of accident, has been considered as a tooth agenesis etiological factor as well (Arya & Savara, 1974; Schalk–van der Weide, 1992; Näsman *et al.*, 1997).

Association between maternal smoking and tooth size has been investigated. A study with 2159 smoking pregnancies and their children was carried out in order to evaluate the effects of maternal smoking on permanent tooth crown dimensions. Statistically significant reduction of tooth crown was found. Still, authors suggested that smoking during pregnancy may produce modifications in craniofacial growth as well as in teeth developmental processes resulting on reduced tooth crowns that may be formed even during the postnatal development (Heikkinen *et al.*, 1994).

Associated Dental Anomalies

A number of dental anomalies have been associated with isolated tooth agenesis (Schalk–van der Weide, 1992; Ooshima *et al.*, 1988; Arte, 2001). Microdontia, impacted canines (Boeira Júnior *et al.*, 2000) and peg–shaped crowns (Grahnen, 1956) can be effortlessly identified by clinicians in affected patients. Although in lower rates, root anomalies, transpositions (Peck *et al.*, 2002) and enamel hypoplasia have also been shown to be linked (Ahmad *et al.*, 1998). Asymmetric morphology of tooth whose homologous is absent has been suggested as a milder form of hypodontia (Ranta, 1986).

Even though the permanent dentition is frequently more severely affected, dental anomalies can be found both in deciduous and permanent dentitions (Arte, 2001). Baum & Cohen (1971) found a direct relationship between agenesis and decreased mesiodistal tooth size. Even in the permanent canines, early appointed as morphologically stable, statistically significant variations in their dimension, both mesiodistally and buccolingually, were reported. From a longitudinal study, Baba–Kawano *et al.* (2002) found a direct correlation between missing lower third molars and significant delay of other teeth formation. Family members affected with reduced tooth size have been associated with identified mutations in MSX1 and PAX9 genes suggesting similar pathogenesis acting on both reduced crown diameter and tooth

agenesis (Nieminen, 2007). Evidence of association with several developmental dental anomalies has been suggested in literature: enlarged freeway space, interdental diastema, diminished alveolar growth, retained deciduous teeth (Rune & Sarnäs, 1974; Schalk–van der Weide *et al.*, 1993). Diminished number of cusps in premolars (Schalk–van der Weide, 1992) as well as ectopic eruption and infraposition of deciduous molars have been also suggested to be associated with tooth agenesis (Bailleul–Forestier *et al.*, 2008).

Literature has shown controversial results regarding the relationship with taurodontism. There are studies appointing negative association (Küchler *et al*., 2008), positive association (Schalk–van der Weide, 1992; Arte *et al.*, 2001) and other ones suggesting that correlation is more likely in cases of oligodontia (Schalk–van der Weide *et al.*, 1993). Even so, a study depicted families with known gene mutations segregating tooth agenesis without associated taurodontism (Vieira, 2003).

All the aforementioned dental anomalies seem to be somehow related to tooth agenesis. Thus, Alvesalo & Portin (1969) reported that agenesis and peg–shaping of upper lateral incisors are different expressions of the same dominant autosomal gene (Arte, 2001). Although development of alveolar bone has been suggested as reliant on entirely completion of teeth formation (Wisth *et al.*, 1974), there is not a consensus among authors regarding association between tooth agenesis and craniofacial structure. Possible influences of tooth agenesis on craniofacial form were investigated by Tavajohi–Kermani *et al.* (2002) who found little but significant correlation between tooth agenesis and changes in cephalometric measurements particularly regarding to the maxilla. Significant decreases in maxillary jaw size were associated with tooth agenesis. Authors also concluded that missing upper teeth had a greater influence on craniofacial form than did missing lower teeth.

Clefts and Syndromes

Earlier named cleft–palate syndrome (Fukuhara, 1965), orofacial clefts are congenital anomalies commonly associated with tooth agenesis. Genetically heterogeneous, it has also environmental factors involved on its etiology. The most prevalent form, non–syndromic clefts are likely due to secondary gene–environment interactions (Schutte & Murray, 1999). The most affected tooth in the area of cleft in both deciduous and permanent dentitions is the upper lateral incisor and its rotation, irregular morphology or occasional absence should be accepted as a microform of cleft (Meskin *et al.*, 1965; Tolarová, 1969; Fukuhara, 1965). Other visible associated congenital defects are deviation of nasal shelf, eccentric shape of nostrils and malformations of the ear (Fukuhara, 1965). That is, tooth agenesis, among other dental abnormalities, seems to share the same etiologic factors as those for the cleft itself. Thus, mutations in MSX1 gene have been reported in literature associated with both cleft and hypodontia patients (van den Boogard et al., 2000; De Muynk et al., 2004; Vieira *et al.*, 2004). Interaction between MSX1 and TGFA genes and oral clefts has also been found (Vieira *et al.*, 2004).

Postnatal environmental factors (including surgical treatment) are likely to be linked to enamel defects and, in some cases, to agenesis of permanent teeth (Ranta, 1986). Moreover, some authors have suggested that the absence of teeth adjacent to a cleft site is likely the consequence of local developmental anomalies at the cleft site (Lidral & Reising, 2002). The larger the cleft, the greater the number of missing teeth (Larson *et al*., 1998). Children with cleft palate showed a higher prevalence of hypodontia outside the area of the cleft when compared to children affected with cleft lip (Ranta, 1986; Larson *et al*., 1998). Individuals with isolated cleft palate with and without a positive family history of clefts have shown similar prevalence rates for hypodontia.

Timing of permanent teeth formation is delayed approximately six months in cleft children when compared to non–cleft children. Whether tooth agenesis is associated, delay increases. Clefts and dental anomalies such as tooth agenesis, microdontia, metric asymmetry

of the crown or root size, amelogenesis imperfecta, delayed formation and eruption on both deciduous and permanent teeth are suggested to have a common etiology in the majority of cases. Aforementioned dental anomalies show a more common occurrence in cleft children than in non–affected subjects (Ranta, 1986). Still, families segregating lip and/or palate cleft may show increased susceptibility to cancer, particularly colon cancer. Evidences supported by genetic studies have shown that some genes, such as the AXIN2 (AXIS inhibition protein 2), may be concurrently correlated to tumor development and tooth agenesis (Menezes *et al*., 2009).

Since inheritance is the main cause to tooth agenesis, its association to genetic syndromes is obvious. Association between hypodontia and 36 syndromes was depicted by Jorgenson (1980). Best known are Down syndrome; Wolf–Hirschhorn syndrome which includes features such as the "Greek warrior helmet" facial appearance (hypertelorism and prominent glabella) and orofacial clefts; Rieger syndrome; Witkop tooth–nail syndrome whose cause was found to be related to a nonsense mutation in MSX1 gene; Van der Woude syndrome and Hemifacial microsomia (Gorlin *et al.*, 1990; Arte *et al.*, 1996; Jumlongras *et al*., 2001; Nieminen *et al.*, 2003). Oligodontia, anodontia, gene mutations and deletions of critical portions of some chromosomes are also involved. Nowadays, more than 70 syndromes categorized in OMIM database are reported to have tooth agenesis among their clinical features suggesting common etiology. Due to a more severe phenotype, oligodontia is more likely to be related to specific syndromes such as ectodermal dysplasias (Ruprecht *et al.*, 1986; Vastardis, 2000; Bailleul–Forestier *et al.*, 2008) which may also present with hypertrophic labial frenulae, peg–shaped incisors, malformation of the ears and polidactily (Freire, 1996). Some authors have claimed that oligodontia should not be considered just an isolated phenomenon, but rather a set of dental anomalies in which the lack of the tooth bud is only one of the features (Rune & Sarnäs, 1974; Wisth *et al.*, 1974; Ooshima *et al.*, 1988). Anodontia, an extreme phenotype, without associated abnormalities is quite unlikely (Arte, 2001). Anodontia is usually part of

syndromes such as anhidrotic ectodermal dysplasia which has also sparse or absent scalp hair and absence of sweat glands as associated features (Lowry *et al.*, 1966).

Roles of the MSX1 and PAX9 Genes

Studies of tooth development in mice have shown more than 200 genes to be involved in odontogenesis regulation (Thesleff & Nieminen, 1996; Nieminen *et al.*, 1998), which is a highly complex phenomenon regulated at the molecular level. Knowledge of the expression of such genes is of high significance for the understanding of non–hereditary and hereditary diseases that affect the dental development, since tooth agenesis is an anomaly which may result from different mutations in different genes (Chemale, 2004). Genes transcription in the nucleus is regulated by proteins named transcription factors which are clustered regarding the molecules responsible for mediating their binding to DNA. To date, literature has reported that transcription factors such as MSX1 (muscle segment homeobox 1) and PAX9 (paired box 9) play important roles in tooth development showing sequential and reciprocal signaling processes instead of one–way pathways (Nieminen, 2007).

MSX1 contains the homeobox – a 180 base–pair sequence with three α–helical regions – and it has also been called master regulatory gene as well as other homeobox–containing transcription factors. They are expressed in a spatially and temporally restricted manner. Showing a strong evolutionary conservation, the genes that contain this homeobox are specific regulators of position during embryogenesis regulating their own transcription according to their order in the genome. Theoretically, any mutation in such genes may cause cells to misread their position forming organs in different regions than those which they should originally do. A specific cluster of homeobox genes also regulates the patterning along the antero–posterior axis of human embryos (Kissinger *et al.*, 1990; Thesleff, 1998; Arte, 2001). In humans, MSX1 (earlier named HOX7) is located on chromosome 4 and it is found highly

expressed in the mesenchyme of developing tooth germs (Kim *et al.,* 2006), particularly during the early stages (bud and cap). Expression of Msx1 has been surveyed in null mutant mice showing actual interaction to missing teeth rather causing anodontia (complete absence of teeth), since teeth formation was arrested at the bud stage. Middle third of the face, cranial shape, overall head size and mandible length fail to develop normally as well (Satokata & Maas, 1994). Other authors have suggested that similar craniofacial phenotype changes in individuals with tooth agenesis may occur (Tavajohi–Kermani *et al.*, 2002). On the other hand, it is remarkable that Msx1 null mutant mice may have rescued the tooth germ epithelial development from the bud to the cap stage by either external Bmp4 addition (refer to Bmp4 function below) or transgenically activated Bmp4 expression (Chen *et al.*, 1996). Low expression of Msx1 may underlie the Dumbo rat phenotype which has hypoplasia of the maxilla and mandible among other abnormal craniofacial features (Katerji *et al.*, 2009).

PAX9 is a developmental control gene that belongs to the PAX gene family containing the paired domain (paired–box), which is compounded by a 128 base–pair sequence with two distinct helix–turn–helix motifs capable of mediating sequence specific interaction with DNA. Located on chromosome 14, it encodes for transcription factors that act in the organogenesis regulation during early embryonic development.

The PAX9 is expressed in the mesenchyme derived from neural crest of the maxillary and mandibular arches, showing a direct relationship with the craniofacial development, especially in the formation of the palate and teeth. This gene also establishes the place and time of organ initiation or morphogenesis. Furthermore, it has been suggested that PAX9 would act marking mesenchymal specific sites where future teeth will form (Peters & Balling, 1999; Klein *et al.,* 2005). Likewise Msx1–deficient mice, it was observed that mice lacking transcription factor Pax9 had tooth development arrested at the bud stage (Peters *et al.*, 1998).

Defects in the two aforementioned genes are suggested to cause selective tooth agenesis in humans: MSX1 is particularly associated with second premolars and third molars agenesis, whereas PAX9 is mostly associated with permanent molars (van den Boogaard et al., 2000). Concerning oligodontia phenotype, MSX1 is frequently associated with the absence of maxillary first premolars, whereas the PAX9 is most frequently associated with the absence of the maxillary and mandibular second molars (Kim *et al.*, 2006).

Understanding gene interactions will lead to unveil the mechanism underlying tooth agenesis. Bone morphogenetic proteins (BMPs) play critical role in the morphogenesis of skeletal elements by means of transcription factors regulation. Bmp4 is deeply involved in molecular regulation of the Msx1 gene. Evidence from mice studies showed that the mesenchymal transcription factors Msx1 and Pax9 are regulated by epithelial signals, such as BMPs and FGFs, and involved in reciprocal signaling from mesenchyme to epithelium (Peters & Balling, 1999). Indeed, the expression of Msx1 in mesenchyme during tooth morphogenesis is initially activated by epithelial Bmp4 signals and it is Pax9–dependent. Interaction between Msx1 and Pax9 through the paired domain of Pax9 also regulates Bmp4 expression (Ogawa *et al.*, 2006) – involved on enamel knot formation – thus inducing the transition from the bud to cap stage of tooth development.

Enamel knot is a morphologically distinct cluster of epithelial cells in the center of the tooth germ containing densely packed and non–dividing cells from the inner enamel epithelium. It has been suggested that enamel knot may stimulate cusp growing by means of FGF–4 synthesis and by controlling folding of cusp slopes. Interestingly, it does not proliferate itself and it is only associated with the primary cusp playing no role in the formation of other cusps later in tooth morphogenesis. Thus, it has been assumed that enamel knot would be a control center for tooth formation (Jernvall *et al.*, 1994). Since the Bmp4 signal to the epithelium is imperative for the epithelial signaling center formation – the enamel knot – and the enamel knot is needed for the bud–to–cap–stage transition, any mutation may be able to

unbalance the gene cascade causing tooth bud to be arrested which may lead to tooth agenesis (Satokata & Maas, 1994; Peters *et al.*, 1998; Peters & Balling, 1999). In short, Msx1 and Bmp4 form an auto–regulatory gene network in which Pax9 is included mainly at an early stage, although Pax9 activation is required for completion of later stages of tooth development (Nieminen, 2007). Evidence from human population studies has also shown that there is a similar molecular mechanism regulating odontogenesis (Vieira *et al.*, 2004). Accordingly, pathogenesis of inherited tooth agenesis involves any failure in tissue interactions and genetic networks caused by gene defects.

Interactions among other Genes

Other genes potentially involved in tooth agenesis pathogenesis have been reported by literature and their likely functions will be briefly showed as it follows. It is suggested that the first signal for tooth development is sent by oral epithelium. However, both epithelial and mesenchymal signals – such as transcription factor genes Msx2 and Lef2 and signaling genes Bmp2 and Edar – are required for the dental placodes formation. Among these signals, FGFs and BMPs as well as Wnt and Shh antagonically interact in order to define tooth positions and it is hypothesized that signaling genes Shh and FGFs also contribute promoting tooth bud growing (Wang & Thesleff, 2005). Moreover, there is support that Wnt signaling plays a key role in the initiation of the ectodermal placodes. Once initiated, ectodermal placodes formation would be modulated by Eda signaling. In order to avoid formation of other ectodermal placodes, it seems that BMPs and TGF–β antagonize signaling pathways responsible for dental placodes initiation.

Studies with mice have provided support for the hypothesis that cusp development depends on normal amounts of Bmp and Wnt as well as on Eda signaling. Still, epithelium

would act as a guide for the mesenchyme to define the number of enamel knots that will be formed thus establishing how many cusps tooth will have in last instance.

To date, inexistence of a particular genetic program for each tooth has been suggested even though some genes are expressed only in one tooth class. For example, homeobox transcription factor Barx1 is specific for the future molar mesenchyme and Islet1 transcription factor expression appears to occur only in the developing incisors epithelium (Nieminen, 2007). Obviously, further studies are necessary in order to clarify whether expression of specific tooth class–genes does occur.

MSX1, PAX9, IRF6 (interferon regulatory factor 6) and TGFA (transforming growth factor α) have been evaluated regarding how they interact among themselves and with tooth agenesis. Mice show Irf6 highly expressed in the palatal rugae, hair follicles and tooth germs (Kondo *et al.*, 2002), which are structures strongly related to the Msx1 expression. Studies with families have shown findings supporting that MSX1 and IRF6 may share a common genetic pathway, since two IRF binding sites in the promoter of MSX1 and one in the intron were found. Moreover, it was reported positive association between genetic variation in the IRF6 locus and humans lacking premolars (Vieira *et al*., 2007) and lacking lateral incisors and second premolars (Barbosa, 2005).

Interaction between PAX9 and IRF6 related to tooth agenesis was not found (Barbosa, 2005). There is no consensus among authors concerning interaction between MSX1 and TGFA. Some studies show no statistically significant evidence of interaction between these genes in human tooth agenesis (Vieira *et al.*, 2004), whereas others indicate strong correlation (Jugessur *et al*., 2003) among cleft subjects. Wnt signaling has also been investigated since it is implicated in regulation of embryonic patterning and morphogenesis of a large amount of organs, potentially including the development of the human dentition. Indeed, a nonsense mutation in the Wnt–signaling regulator AXIN2 was identified in a study with a Finnish family segregating oligodontia as an autosomal dominant trait. It was observed that oligodontia

would be a risk factor for developing colorectal neoplasia in that family, suggesting a potential correlation between these two distinct phenotypes. Interestingly, both stimulation and inhibition of Wnt signaling may lead to tooth agenesis (Lammi *et al.*, 2004). Similar results were found in another study, in which AXIN2 polymorphic variants were suggested to be associated with both hypodontia and oligodontia (Mostowska *et al.*, 2006–A).

Mutations Associated With Tooth Agenesis

The key role of molecular genetics regarding the identification of the genetic causes underlying tooth agenesis is being comproved. The better understanding lies in the tooth agenesis associated with syndromes and in the non–syndromic (isolated) inherited severe agenesis (oligodontia). Results achieved so far encourage further research, since there is a long way to go until a deep knowledge of pathogenesis of tooth agenesis is reached.

Several studies (Nieminen *et al.*, 1995; Scarel *et al.*, 2000; Frazier–Bowers *et al.*, 2002–B; Frazier–Bowers *et al.*, 2003; Vieira *et al.*, 2004; Costa, 2005; Gerits *et al.*, 2006; Swinnen *et al.*, 2008) failed to detect mutations in MSX1 and PAX9 suggesting that other genes, among the more than 200 reported to be involved in tooth development, are probably implicated in non–syndromic hypodontia and/or oligodontia affecting those individuals and families. Indeed, only a restricted number of mutations in MSX1 and PAX9 genes have been associated with hypodontia and/or oligodontia in humans so far. In a general manner, these mutations appear to affect all major signaling pathways and the MSX1, PAX9 and other transcription factors that mediate these signals during odontogenesis. A loss of function in MSX1 and PAX9 is expected thus leading to haploinsufficiency (reduced amount of the functional protein) of these genes. Accordingly, abnormalities in the odontogenesis may occur including the arrest of tooth bud. The etiology of oligodontia has been linked to the haploinsufficiency of the MSX1 and PAX9, whereas hypodontia has been more correlated to point mutations in either gene.

The search for gene defects such as those affecting the MSX1 and PAX9 genes used to take months or even years before the Human Genome Project in 2001. Nowadays, it is possible to be accomplished within weeks or months. Venous blood samples have been normally used in order to obtain DNA for later genetic analysis, however, modern techniques for DNA isolation and purification have shown that buccal epithelial cells are a reliable source of DNA for the direct sequencing of PCR products (polymerase chain reaction), that is, the screening for mutations. Theoretically, an easiest, fastest, non–invasive and painless human material collection method will lead a larger amount of subjects to agree to participate on the studies for the present issue since when blood samples are required, a significant smaller amount of individuals agree to donate (Hansen *et al.*, 2007–B). Accordingly, it is assumed that novel mutations will be found in the next years shedding more light into the genetic pathways that underlie tooth agenesis. In order to substantially increase this understanding, the genetic evaluation of a great number of families segregating tooth agenesis will be required.

MSX1 and PAX9 Gene Pathways by means of Bioinformatics

Investigation and sequencing of genomes around the world, as in the Human Genome Project, are providing massive amounts of data which require new technologies to handle with. Also, new research fields are being created or enhanced in order to further study these information sources. Thus, bioinformatics (computational molecular biology) is a bright field that uses computers to handle biological information such as the investigation of the genetic code, protein interactions, related functions and clinical outcomes. The experimental knowledge coming from dozens of scientific journals around the world needs to be organized and correlated, whether applicable. Since there are a number of proteins whose interactions still need to be enlightened, STRING version 8.2 software (Search Tool for the Retrieval of Interacting Genes/Proteins) can be a useful tool. MSX1 and PAX9 proteins form a genetic

network where other proteins are also included. But how strong is MSX1 and PAX9 relationship and what are the other proteins that they interact with? MSX1 and PAX9 present an interaction scored 0.995 according to the String tool. At the time of the analysis, they correlated with each other in a stronger way than among others. This sort of information is possible from crossing multiple data as provided by specialized publications. Many other softwares are available through world wide web and they are changing the way information is being processed.

Concerning dentistry and other health sciences, it seems that the capability of achieving new levels for early diagnosis and approaches to treatment will involve further studies on molecular genetics as well as powerful tools to process all these new data. Nonetheless, the way clinicians will deal with all this information in their offices is an imperative question that was already raised (Wright & Hart, 2002). How to effectively apply this knowledge in treatment planning? Perhaps the answer comes across a specific training on molecular genetics given the new demands of clinical dentistry. Still, clinicians will be given new tools for diagnosis and treatment evaluation in order to complement the current therapeutic approach, which will personalize even more the dental treatment.

Current and Future Therapeutic Approach

Missing teeth compromise human health both physically and psychiatrically and usually require a multidisciplinary treatment. Orthodontic space closure, deciduous teeth maintenance, implant therapy, adhesive bridgework among other prosthetic resources and esthetic dentistry are the most common current approaches (Furquim *et al.*, 1997; Ith–Hansen & Kjaer, 2000; Arvystas, 2003; Sabri, 2004; Fiorentino & Vecchione, 2007). However, the human health care in the 21st century demands for treatment options which are more biologically compatible. At the same time, the clinical dentistry is being reformed in order to

support clinicians in this all new horizon by training them for the new genomics tools (Diehl, 2006). Molecular genetics and bioengineering play key roles for the development of new technologies such as the use of BMP2 synthetic protein for assisting alveolar bone growing, which is available for use already. Another promising field is the craniofacial genetics whose research is suggested to provide a better insight regarding the phenotypic correlation between facial morphology and tooth agenesis (Tavajohi–Kermani *et al.*, 2002). Bioengineered teeth are also underway and they will be a valuable clinical tool in the future (Duailibi *et al.*, 2004).

Although current dentistry has achieved a high level of accuracy, a biological replica of the missing tooth is the natural evolution for a therapeutic approach that is entirely compatible with the human organism. A regenerative dentistry is on its way since the regeneration of tissues and organs has become a feasible aim. Adult stem cells isolated from human dental tissues such as the dental pulp, dental follicle and periodontal ligament theoretically match the purpose of differentiation into tooth–related cells that will produce dental tissues. Nonetheless, there are still some hindrances to overcome such as the correct number of stem cells and the growth factors to be combined, an accurate spatial arrangement and how are the tooth size and morphology managed. Still remains unsolved an accessible source of epithelial, tooth–related stem cells which will form the enamel since the only source so far known is the tooth germ of young children (Koussoulakou *et al.*, 2009). Even if all of these items had been worked out, the formation process of this "bio–tooth" until its completion would require a long–term course, what is commercially unfeasible. However, cutting edge technology along with the growing knowledge basis on the bioengineering field will allow this aim to be accomplished.

Regarding tooth agenesis, it has been suggested that its frequency increased during the 20th century. Since a greater number of affected subjects is expected over the coming years, a deeper analysis of the gene networks underlying tooth agenesis is critical in order to achieve better treatment options and, perhaps, an early diagnosis tool. This early diagnosis

would possibly lie on the DNA examination, based on polymorphic variants, which could be performed at any age including newborns. However, a minimum casuistic of affected individuals is required for establishment of such diagnostic tool. Clinicians are able to contribute by referring affected families for investigative research thus assisting on the development of new therapeutic resources.

Conclusions

The most common craniofacial anomaly in man, tooth agenesis is still a challenge to be overcome. An etiology strongly conditioned by genetic factors, the small number of linked mutations found so far, the right time and local for each involved gene to be expressed and the complexity of the genetic networks regulating the odontogenesis are some of the factors that need enlightenment. The last decade brought a faster and more accurate method of processing and analyzing genetic data increasing the knowledge concerning the pathogenesis of tooth agenesis. Nonetheless, as this understanding grows new queries are built demanding new responses. Since one of the major hindrances for disease modern investigation is still the recruitment of subjects, any survey on the present issue even involving a few cases may contribute. Likewise, clinicians are able to cooperate by referring families segregating tooth agenesis for genetic research. Such behaviors may assist in finding polymorphic variants and mutational hotspots for tooth agenesis since genetic pathways underlying most cases are still unknown. Moreover, an early diagnosis based on the DNA specificity may be performed by means of a comparative analysis among these genetic variants.

Based on affected cases/reported causative mutations rate it is possible to assume that both non–syndromic hypodontia and oligodontia seem to show polygenic inheritance or multifactorial pathogenic mechanism, instead of earlier thoughts referring a monogenic inheritance pattern. The screening for mutations in subjects with isolated oligodontia returned

positive in a few cases also supporting a heterogeneous trait. However, some genes play key roles during development of dentition such as MSX1 and PAX9. Thus, genetic networks that they are involved in need more enlightenment as well as their level of association with other genes. Given the complexity of development of the human dentition, there is a long way to go.

A remarkable point when performing mutational analysis is the way results are discussed. Genes are not directly responsible for the formation of organs and tissues as they compound large and complex genetic pathways where many other molecules interact with each other. Indeed, a mutated gene can contribute to some specific phenotype but there can also be several other defective genes as well as faulty cellular elements involved. Therefore, the key for the development of resources for tooth agenesis prevention and treatment rather involves clarifying processes regulating the initiation and morphogenesis of teeth. Advances in technology and further studies evaluating gene expression in larger samples may be valuable in order to shed more light into the pathogenesis of tooth agenesis.

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4. RESULTADOS E DISCUSSÃO

Essa tese será apresentada em forma de capítulos contendo os seguintes artigos: 1º) artigo de revisão entitulado "Dentistry and molecular biology: a promising field for tooth agenesis management"; 2º) artigo original entitulado "Polymorphism in MSX1 gene in a family with autosomal dominant hypodontia"; 3º) artigo original entitulado "A novel missense mutation in the PAX9 gene associated with familial tooth agenesis".

CAPÍTULO I

1°) Artigo de Revisão submetido ao Journal of Oral Pathology & Medicine – Fator de Impacto 2.1

Dentistry and molecular biology: a promising field for tooth agenesis management

Dentistry and molecular biology: a promising field for tooth agenesis management

Authors: Boeira Junior, B.R.; Echeverrigaray, S.

Abstract

Tooth agenesis is the most common dental anomaly affecting up to one quarter of the general population. The main cause is related to abnormal function of specific genes which play key roles during odontogenesis, particularly MSX1 and PAX9. Despite the high frequency of tooth agenesis, there are only a restricted number of mutations in MSX1 and PAX9 that have been associated with non–syndromic hypodontia and/or oligodontia so far. Since a greater number of affected subjects is expected over the coming years, a deeper analysis of the gene networks underlying tooth agenesis is critical. By means of a literature review based on Medline, PubMed, Lilacs, NCBI and String, performed between 1991 and 2010 and focused on mutations etiologically associated with tooth agenesis, this work aimed to assess latest advances on the genetic etiology of tooth agenesis and to offer an insight on how they can assist dental practice in a near future. A better knowledge of the genetic networks underlying tooth agenesis will allow to achieve better treatment options and, perhaps, an early diagnosis tool which would possibly lie on the DNA examination based on polymorphic variants. Such test based on DNA analysis may be available and reachable for clinicians, resulting in a more accurate diagnosis and allowing a better approach for this anomaly.

Keywords: dental agenesis; mutation; MSX1 transcription factor; PAX9 transcription factor; molecular biology.

Introduction

Tooth agenesis is the most common dental anomaly showing up to 25% of prevalence[1]. It can be either part of a syndrome or a non–syndromic familial disturbance[2]. Although environmental factors can contribute to the phenotype of agenesis, in the majority of cases it is inherited as an autosomal dominant trait. Autosomal recessive and X–linked inheritance have also been reported in a few subjects[3]. Since there are 3 billion nucleotides in human genome and 99.9% are the same among persons, "only" 0.1% (or 3 million nucleotides) is what makes people different, and here is where genomic analysis for tooth agenesis takes place. Its main cause is related to abnormal function of specific genes which play key roles during odontogenesis, particularly MSX1 and PAX9. Once there is a gene defect, i.e. a mutation, there may be arrest of tooth bud development leading to agenesis. So far mutational screening for both genes has only returned a restricted number of mutations (Figure 1). Thus, further research by using molecular biology methods is necessary.

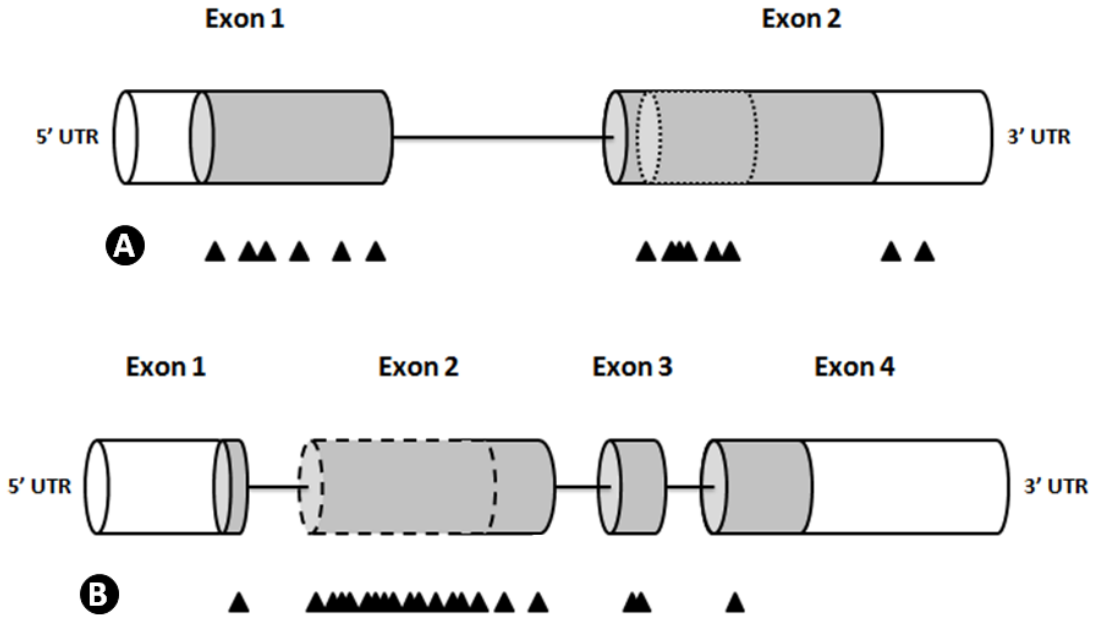

Figure 1 Representative structure of (A) MSX1 and (B) PAX9 genes and their mutations associated with hypodontia and/or oligodontia. Short–dotted line: homeobox domain; large–dotted line: paired domain; black triangles: approximate location of each identified mutation to date.

Tooth agenesis approach is still a challenge in day–to–day practice involving a multidisciplinary treatment. Currently, diagnosis can only be performed whenever it is already established and, in most cases, whenever the patient notices missing teeth. Indeed, a standard method for tooth agenesis early detection is not available yet. DNA specificity might be used on early diagnosis of the referred abnormality which would allow patients to receive a more effective treatment.

The aim of this review is to assess latest advances on genetic etiology of tooth agenesis and to offer an insight on how they can assist dental practice in a near future.

Clinical Features of Tooth Agenesis

Clinically, hypodontia (Figure 2) depicts congenitally missing from one to six teeth except third molars and it has been used to name common and mild forms of agenesis[4]; oligodontia indicates that more than six teeth, except third molars, are lacking and it is often applied for more severe cases[5]. These terms should have their application carefully evaluated as they may mislead clinicians regarding the severity of some cases, particularly those in which third molars are excluded. Anodontia constitutes an extreme case, the complete absence of teeth, and it is usually part of syndromes[4].

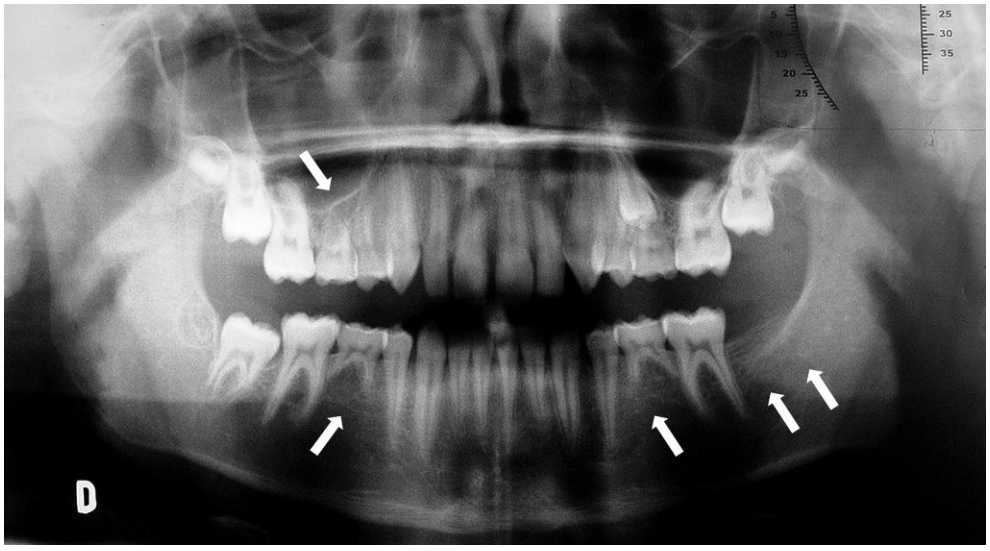

Figure 2
Pantomographic X–ray of a patient affected with hypodontia. Arrows indicate agenesis of five teeth.

Several studies have scored either upper lateral incisor or lower second premolar as the most often absent teeth, after third molars[1,4,6]. Whether third molar agenesis is not considered, both upper lateral incisor and lower second premolar account for 85% of all missing teeth. When it comes to families, the frequency of agenesis affecting one tooth class among relatives is significantly higher than affecting different tooth classes[4]. Deciduous dentition is also affected by agenesis although it is less prevalent. Correlation between agenesis of a deciduous tooth and its permanent successor does exist since agenesis of a given deciduous tooth is mostly followed by agenesis of the corresponding permanent tooth[4,6].

A number of dental anomalies have been associated with isolated tooth agenesis. Microdontia, impacted canines[7] and peg–shaped crowns can be effortlessly identified in affected patients. Although in lower rates, ectopic eruption, transpositions, enamel hypoplasia, enlarged freeway space and retained deciduous teeth have also been shown to be linked. Furthermore, it has been suggested that dental developmental anomalies and tooth agenesis would result from different expressions (defects) of the same genes, mostly MSX1 and PAX9[6,8].

Possible influences of tooth agenesis on craniofacial form were investigated by Tavajohi–Kermani *et al.*[9] who found little but significant correlation between agenesis and changes in cephalometric measurements particularly regarding the maxilla. Significant decreases in maxillary jaw size were associated with tooth agenesis. Authors also concluded that missing upper teeth had a greater influence on craniofacial form than did missing lower teeth.

Etiology

The underlying genetic mechanism leading to tooth agenesis has lately been linked to some gene mutations although some environmental factors, such as

chemotherapy/radiotherapy, trauma in jaws and maternal diabetes have also been shown to be involved[10]. Indeed, studies of families have shown a concordance in intra–familial phenotypes[11]. For example, in most cases of twins, monozygotic show a rather more concordant phenotype than dizygotic twins[12].

Interestingly, there seem to be an association between agenesis and cancer. Families segregating lip and/or palate cleft may show increased susceptibility to cancer, particularly colon cancer. Evidences have also shown that some genes, such as the AXIN2 (AXIS inhibition protein 2), may be concurrently correlated to tumor development and tooth agenesis[13].

Roles of the MSX1 and PAX9 Genes

Studies of tooth development in mice have shown more than 200 genes to be involved in odontogenesis regulation, which is a highly complex phenomenon regulated at the molecular level. To date, literature has reported that genes such as MSX1 (muscle segment homeobox 1) and PAX9 (paired box 9) play important roles in tooth development showing sequential and reciprocal signaling processes instead of one–way pathways[14]. That is, there are several ways for those processes to occur thus preserving tooth development in most cases.

MSX1 contains the homeobox (a specific sequence for DNA interaction) and it has also been called master regulatory gene since it participates in several organs development. MSX1 is expressed in a temporally restricted manner, from fourth gestational week until completion of root formation of all teeth. It is also a specific regulator of organs position during embryogenesis regulating position of several organs other than dentition. Theoretically, any mutation in such gene may cause cells to misread their position forming organs in different regions than those which they should originally do[4]. MSX1 is located on chromosome 4 and it is found highly expressed in the mesenchyme of developing tooth germs, particularly during the early stages (bud and cap)[15].

PAX9 is a developmental control gene containing the paired domain, which is a sequence capable of specific interaction with DNA. Located on chromosome 14, it encodes for transcription factors that act in organogenesis regulation during early embryonic development. It is expressed in the mesenchyme in the maxillary and mandibular arches, showing a direct relationship with the craniofacial development, especially in the formation of the palate and teeth. This gene also establishes the place and time of organ initiation or morphogenesis. Furthermore, it has been suggested that PAX9 would act marking mesenchymal specific sites where future teeth will form[16].

Defects in these two genes are suggested to cause selective tooth agenesis: MSX1 is particularly associated with second premolars and third molars agenesis, whereas PAX9 is mostly associated with permanent molars. Concerning oligodontia phenotype, MSX1 is frequently associated with the absence of upper first premolars, whereas the PAX9 is most frequently associated with the absence of the upper and lower second molars[15].

Interactions Among Other Genes

Other genes potentially involved in tooth agenesis pathogenesis have been reported by literature. In short, MSX1 and BMP4 (bone morphogenetic protein 4) form an auto–regulatory gene network in which PAX9 is included mainly at an early stage, although PAX9 activation is required for completion of later stages of tooth[17]. Accordingly, any mutation may be able to unbalance the gene network causing tooth bud to be arrested, which may lead to tooth agenesis. Wnt signaling has also been investigated since it is implicated in regulation of embryonic patterning and morphogenesis of a large amount of organs including development of human dentition. Indeed, a mutation in the Wnt–signaling regulator AXIN2 was identified in a Finnish family affected with oligodontia. Moreover, oligodontia would be a risk factor for

developing colorectal neoplasia in that family, suggesting a potential correlation between these two distinct phenotypes[18].

Mutations Associated With Tooth Agenesis and How This Knowledge Can Assist

Molecular genetics plays a key role in the identification of the genetic causes underlying tooth agenesis is being comproved. Whether several studies[17,19–22] failed to detect mutations in MSX1 and PAX9 suggesting that other genes, among the more than 200 involved in tooth development, are probably implicated, indeed only a restricted number of mutations in MSX1 and PAX9 genes have been associated with hypodontia and/or oligodontia so far. In a general manner, these mutations cause loss of function in MSX1 and PAX9 thus leading to haploinsufficiency. Accordingly, amount of the functional protein to keep tooth development going is reduced and abnormalities in the odontogenesis may occur including the arrest of tooth bud. Etiology of oligodontia has been linked to the haploinsufficiency of the MSX1 and PAX9, whereas hypodontia has been more correlated to mutations in PAX9. The first mutation associating MSX1 and tooth agenesis was depicted in 1996 based on a family with autosomal dominant agenesis of second premolars and third molars[23]. The first mutation in PAX9 causally related to tooth agenesis was found in 2000 from a family segregating oligodontia lacking most permanent molars[5].

Venous blood samples have been normally used to obtain DNA for later genetic analysis, however, modern techniques for DNA isolation and purification have shown that buccal epithelial cells are a reliable source of DNA to search for mutations. Clinicians are well positioned to collect these buccal cells by using cytology brushes. Theoretically, an easiest, fastest, non–invasive and painless human material collection method will lead a larger amount of subjects to agree to participate in studies for the present issue since when blood samples are required, a significant smaller amount of individuals agree to donate[24]. Accordingly, it is

assumed that novel mutations associated with tooth agenesis will be found in the next years substantially increasing the understanding regarding its genetic etiology.

MSX1, PAX9, Bioinformatics and Dentistry

Investigation and sequencing of genomes around the world, as in the Human Genome Project, are providing massive amounts of data which require new technologies to handle with. Also, new research fields are being created or enhanced to further study these information sources. Thus, bioinformatics (computational molecular biology) is a bright field that uses computers to handle biological information such as the investigation of the genetic code, protein interactions and clinical outcomes. Experimental knowledge coming from dozens of scientific journals around the world needs to be organized and correlated, whether applicable. Since there are a number of proteins whose interactions still need to be enlightened, STRING version 8.2 software (Search Tool for the Retrieval of Interacting Genes/Proteins) can be a useful tool. MSX1 and PAX9 proteins form a genetic network where other proteins are also included. But how strong is MSX1 and PAX9 relationship and what are the other proteins that they interact with? MSX1 and PAX9 present an interaction scored 0.995 according to the String tool (Figure 3). At the time of this analysis[25], they correlated with each other in a stronger way than among others. Such information is possible from crossing multiple data as provided by specialized publications. Many other softwares are available through world wide web and they are changing the way information is being processed.

Regarding dentistry and other health sciences, it seems that the capability of achieving new levels for early diagnosis and approaches to treatment will involve further studies on molecular genetics as well as powerful tools to process all these new data. Nonetheless, the way clinicians will deal with all this information in their offices is an imperative question that has already been raised[26]. How to effectively apply this knowledge in treatment planning?

Perhaps the answer comes across a specific training on molecular genetics given the new demands of clinical dentistry. Furthermore, clinicians will be given new tools for diagnosis and treatment evaluation in order to complement the current therapeutic approach, which will personalize even more the dental treatment.

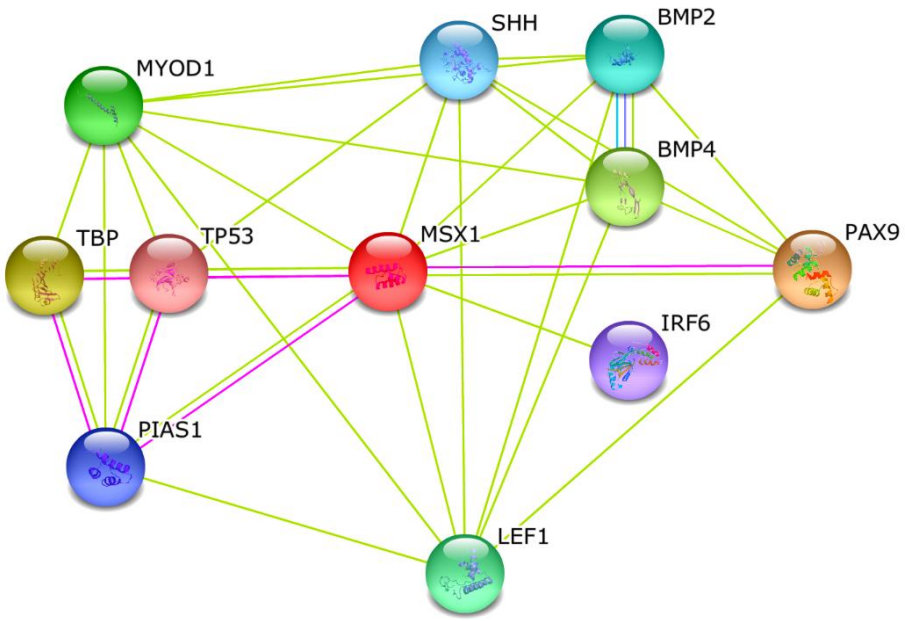

Figure 3 MSX1 protein network showing its predicted functional partners and interaction levels. MSX1 and PAX9 combined association scored 0.995, which is the highest score among presented proteins meaning they are highly correlated.

Current and Future Therapeutic Approach

Missing teeth compromise human health both physically and psychiatrically and usually require a multidisciplinary treatment. Orthodontic space closure, deciduous teeth maintenance, implant therapy, adhesive bridgework among other prosthetic resources and esthetic dentistry are the most common current approaches[27]. However, human health care in the 21st century demands for treatment options which are more biologically compatible. At the same time, clinical dentistry is being reformed in order to support clinicians in this all new horizon by training them for the new genomics tools. Molecular genetics and bioengineering

play key roles for the development of new technologies such as the use of BMP2 synthetic protein for assisting alveolar bone growing, which is available for use already. Another promising field is the craniofacial genetics whose research is suggested to provide a better insight regarding the phenotypic correlation between facial morphology and tooth agenesis[9].

Although current dentistry has achieved a high level of accuracy, a biological replica of the missing tooth is the natural evolution for a therapeutic approach that is entirely compatible with the human organism. A regenerative dentistry is on its way since the regeneration of tissues and organs has become a feasible aim. Adult stem cells isolated from human dental tissues such as the dental pulp, dental follicle and periodontal ligament theoretically match the purpose of differentiation into tooth–related cells that will produce dental tissues: a bioengineered tooth. Nonetheless, there are still some hindrances to overcome such as the correct number of stem cells and the growth factors to be combined, an accurate spatial arrangement and how the tooth size and morphology are managed. Still remains unsolved an accessible source of epithelial, tooth–related stem cells which will form the enamel since the only source so far known is the tooth germ of young children[28]. Even if all of these items had been worked out, the formation process of this "bio–tooth" until its completion would require a long–term course, what is commercially unfeasible. However, cutting edge technology along with the growing knowledge basis on the bioengineering field will allow this aim to be accomplished.

Regarding tooth agenesis, it has been suggested that its frequency has increased during 20th century[29]. Since a greater number of affected subjects is expected over the coming years, a deeper analysis of the gene networks underlying tooth agenesis is critical. Such effort will allow to achieve better treatment options and, perhaps, an early diagnosis tool which would possibly lie on the DNA examination based on polymorphic variants (mutations). Once a great number mutations are available, it will be possible to design such DNA test to be performed even in newborns and other subjects who have not completed dentition yet and

have risk of being affected. Thus, waiting for tooth agenesis manifestation will no longer be needed. This early diagnosis tool based on DNA analysis may be available and reachable for clinicians, resulting in a more accurate diagnosis and allowing a better approach for tooth agenesis. However, a minimum casuistic of affected individuals is required for establishment of such diagnostic tool. Clinicians are able to contribute by referring affected families for investigative research thus assisting on the development of new therapeutic resources.

Conclusions

The most common craniofacial anomaly, tooth agenesis is still a challenge to be overcome. An etiology strongly conditioned by genetic factors, the small number of linked mutations found so far, the right time and local for each involved gene to be expressed and the complexity of the genetic networks regulating odontogenesis are some of the factors that need enlightenment. On the other hand, the last decade brought a faster and more accurate method of processing and analyzing genetic data increasing the knowledge regarding the etiology of tooth agenesis. Thus, training clinicians for the new genomic tools seems important though, given the significant issues of data analysis, expertise of geneticists is also required. Since one of the major hindrances for disease modern investigation is still the recruitment of subjects, any survey on the present issue even involving a few cases may contribute. Likewise, clinicians from private offices and dentistry residency clinics are able to cooperate by referring families segregating tooth agenesis for genetic research. Such behaviors may assist in finding polymorphic variants and mutational hotspots for tooth agenesis since genetic pathways underlying most cases are still unknown. Moreover, an early diagnosis based on the DNA specificity may be performed by means of a comparative analysis between these genetic variants and DNA from unaffected subjects showing likelihood of a given individual presents with agenesis.

Regardless the restricted number of mutations associated with tooth agenesis to date, it is clear that some genes play key roles during development of dentition, such as MSX1 and PAX9. Thus, the key for the development of better resources for tooth agenesis diagnosis and treatment rather involves clarifying processes regulating the initiation and morphogenesis of teeth, including gene defects such as mutations. Advances in technology and further studies evaluating gene expression in larger samples may be valuable in order to develop an early diagnosis tool and a better approach for tooth agenesis.

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CAPÍTULO II

2°) Artigo Original submetido ao Journal of Dental Research – Fator de Impacto 4.1

Polymorphism in MSX1 gene in a family with autosomal dominant hypodontia

Polymorphism in MSX1 gene in a family with

autosomal dominant hypodontia

Abstract

Authors – Boeira Junior BR, Echeverrigaray S

*MSX1 codes for a transcription factor deeply involved in the gene networks that regulate odontogenesis, particularly during early stages. The few mutations identified in this gene so far are linked only with non–syndromic oligodontia. In order to evaluate milder phenotypes, a family segregating non–syndromic hypodontia was screened for mutations in MSX1 gene. Three out of the four family members were lacking permanent upper lateral incisors. Transition *6C>T lying in the exon 2, a known polymorphism, was identified and it is located six base pairs downstream the stop codon. All three affected family members were homozygous for the mutant allele, whereas unaffected father was heterozygous. Nonetheless, MSX1 gene of ten unrelated unaffected control subjects did not show this genetic variant. Due to its proximity to the stop codon, this homozygous mutation might hinder a regular translation termination thus contributing to the phenotype. Existence of additional mutations in the non–coding regulatory regions of MSX1, or even in another gene (multigenic trait), should also be considered requiring further studies in larger families affected with hypodontia involving permanent upper lateral incisors.*

KEYWORDS: hypodontia; MSX1 Transcription Factor; mutation; tooth agenesis.

Introduction

Tooth agenesis, the congenital absence of one or more teeth, is among the most common craniofacial anomalies. It affects 1 in 5 people but its rate is much lower ranging from 2% to 10% when third molars are not considered (1). Clinical classification of this anomaly occurs according to the number of missing teeth. Hypodontia depicts absence of one to six permanent teeth (excluding third molars) and its prevalence varies from 1.6% to 9.6%. Oligodontia occurs when more than six permanent teeth are lacking (excluding third molars) and it is found in 0.3% of general population (2). Tooth agenesis can be either a non–syndromic familial disturbance or an isolated form, although it is a constant feature associated with several syndromes. Anomaly inheritance is usually autosomal dominant even though a few autosomal recessive and X–linked inheritance cases have been reported (3).

The most often absent teeth are third molars followed by lower second premolars and permanent upper lateral incisors. Interestingly, bilateral agenesis of upper lateral incisors appears to be more common than unilateral agenesis (4). When it comes to families, the number of missing teeth seems to be correlated with hereditary tooth agenesis. Frequency of agenesis affecting one tooth class among relatives is significantly higher than affecting different tooth classes indicating a similar gene expression pattern. Furthermore, parents and siblings of probands usually show higher frequencies of hypodontia than the general population does (5).

Even though some environmental factors have been shown to be involved in the etiology, there is an underlying genetic mechanism leading to non–syndromic hypodontia that has not been clarified yet. Studies regarding tooth development in

knockout mice indicated that odontogenesis regulation depends on more than 200 genes. MSX1 and PAX9 are among those most associated with the anomaly (6, 7). Both genes are transcription factors coding for DNA–binding proteins which are involved in epithelial–mesenchymal interactions. This genetic network will give rise to teeth (8). Therefore, defects in either gene may affect odontogenesis. Located on chromosome 4, MSX1 is directly involved in craniofacial and members development. Additionally, its expression seems to be most critical during early tooth development (8, 9). Mutations in this gene are suggested to cause selective tooth agenesis. Subjects affected with oligodontia are usually lacking premolars and third molars (9, 10), whereas those presenting with hypodontia are mostly missing second premolars and/or upper lateral incisors. Associated dental anomalies such as microdontia, impacted canines and ectopic eruption have also been reported (2).

Gene mutations that result in tooth agenesis may also be associated with other abnormalities such as cancer. Relationship between MSX1 and p53 Tumor Suppressor has been investigated. Interestingly, the homeodomain of MSX1 acts mostly as a protein–protein interacting motif binding to p53 in the cell nucleus, thus stimulating apoptosis of cancer cells (11). This is a relatively novel role of MSX1 suggesting that tooth agenesis might be an indicator of tumor susceptibility.

The first identified mutation in MSX1 gene associated with tooth agenesis was described in 1996 on a family with autosomal dominant agenesis of second premolars and third molars (12). Even so, despite the high prevalence of tooth agenesis, mutational screening for MSX1 has returned only a few causative mutations so far (Table 1). Most of these mutations were found within the homeobox domain, which is a highly conserved sequence enabling MSX1 to bind to DNA specific sites as well as to

other proteins. Moreover, all these genetic variants have been identified from oligodontia phenotypes only. In a general manner, MSX1 gene would be affected by mutations that affect protein function. Moreover, these mutations would induce haploinsufficiency by diminishing the amount of functional protein to 50% (13), particularly when nonsense and frameshift mutations are involved (9, 10, 15, 17). Accordingly, the arrest of developing tooth bud might occur leading to definitive absence of the tooth.

In the present research, we analyzed a Brazilian nuclear family affected with non–syndromic hypodontia involving upper lateral incisors. They were screened for mutations in the MSX1 gene to verify relationship between genotype and phenotype.

Table 1. Identified mutations in MSX1 that have been associated with tooth agenesis.

Gene	Exon	Intron	Mutation		References	Phenotype	Type
			Nucleotide	Residue*			
MSX1			–	–	Nieminen *et al.*[13]	Oligodontia / Wolf–Hirschhorn syndrome	Gene deletions
	1		62–63insG	Gly22ArgfsX168	Kim *et al.*[9]	Oligodontia	Insertion
	1		182T>A	Met61Lys	Lidral *et al.*[14]	Oligodontia	Missense
	1		314C>A	Ser105X	van den Boogard *et al.*[10]	Oligodontia / Cleft lip–palate	Nonsense
		•	740–751del	–	Pawlowska *et al.*[8]	Oligodontia	Intronic deletion
	2		559C>T	Gln187X	De Muynck *et al.*[15]	Oligodontia / Cleft lip– palate	Nonsense
	2		581C>T	Ala194Val	Mostowska *et al.*[16]	Oligodontia	Missense
	2		587G>C	Arg196Pro	Vastardis *et al.*[12]	Oligodontia	Missense
	2		605C>A	Ser202X	Jumlongras *et al.*[17]	Oligodontia / Witkop syndrome	Nonsense
	2		655G>A	Ala219Thr	Chishti *et al.*[18]	Oligodontia / Dental anomalies	Missense
	2		662C>A	Ala221Glu	Xuan *et al.*[19]	Oligodontia	Missense

*change of residues referred by the authors or obtained from sequence alignment.

Materials and methods

Family selection and pedigree construction

The proband was 11 years old when he was referred by his general dentist to orthodontic consultation in a private office. The main complaint was a possible lack of the permanent upper lateral incisors as the patient had remaining deciduous upper lateral incisors. As his family presented dental history involving absence of teeth, the proband and relatives were invited to participate in this study. Family members are Caucasian and Italian descent from an immigrant community in the region of Veneto, Italy. Diagnosis of the anomaly was verified by anamnesis, clinical examination and panoramic radiograph of all family members allowing determining the pedigree (Fig. 1). Custom questionnaires were applied in order to better assess the familial medical history. Associated dental anomalies were also registered. The affected members of the family were reported to have had normal deciduous dentition. All four individuals of the family, including minors, signed consent form. The protocol for this research was approved by the Research Ethics Committee of the University of Caxias do Sul, Rio Grande do Sul, Brazil, according to the Resolution 196/96 of the National Health Council.

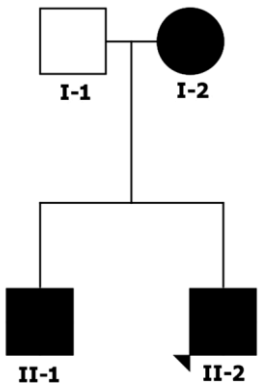

Figure 1. Pedigree showing phenotypes and autosomal dominant mode of inheritance: squares, males; circle, female; black, hypodontia; white, unaffected. Arrow indicates the proband.

DNA collection, screening and mutational analysis

All family members had a sample of buccal epithelial cells collected with cytology brushes. For DNA extraction, Wizard® Genomic DNA Purification Kit (Promega, Madison, WI, USA) was used according to the manufacturer's recommendations. The amplification process by polymerase chain reaction (PCR) was performed with two sets of primers covering the two exons and boundaries of the MSX1 gene, as follows: MSX1x1F 5'– GCTGGCCAGTGCTGC – 3'; MSX1x1R 5'– ACGGGGTCCTCTCGGGCTTC – 3'; MSX1x2F 5'– ACTTGGCGGCACTCAATATC – 3'; MSX1x2R 5'– AAGCTATGCAGGAGACATGG – 3'. All primers were obtained from Primer– BLAST software (www.ncbi.nlm.nih.gov/tools/primer– blast). PCR was carried out in an Eppendorf Mastercycler® Gradient Thermal Cycler (Eppendorf, Hamburg, Germany). PCR reactions contained: 1.5 U Platinum® Taq DNA Polymerase (Invitrogen, Karlsruhe, Germany); 1X PCR Buffer (Invitrogen, Karlsruhe, Germany); 2 mM $MgCl_2$; 0.2–0.8 mM dNTPs; 300 nM each primer; 10% DMSO and 20 ng of genomic DNA. Conditions for PCR were as follows: 94° C for 6 min; 94° C for 30 s; 63° C and 65° C for 30 s; 72° C for 30–45 s after 30 cycles. Soon after, the resulting amplicons were submitted to an ExoI–SAP enzymatic purification process to eliminate non–incorporated dNTPs and primers. Direct sequencing of both strands of each purified PCR product was performed on a model ABI Prism® 3130xl sequencer (Applied Biosystems, Foster City, CA, USA). All sequences were compared with GenBank accession number AF426432 by using BioEdit 7.0 software. Homology of mutant sequences was also compared by means of BLAST (http://blast.ncbi.nlm.nih.gov/Blast.cgi). Proband was first screened for mutations and,

once detected, his relatives were screened for that genetic variant by the same method. Sequencing of MSX1 genes from ten unrelated control subjects was also performed.

Results

Clinical and radiographic analysis of proband (II–2) confirmed the diagnosis of non–syndromic hypodontia (OMIM #167416) involving congenital absence of permanent upper lateral incisors and upper third molars (Figs. 2 and 3a). Furthermore, bilateral impaction of permanent upper canines was also observed as associated dental anomaly (Figs. 2 and 3b). Interestingly, affected mother (I–2) and brother (II–1) of the proband presented with the same missing teeth, upper lateral incisors, except for the lacking of upper third molars occurring only in the proband. On the other hand, associated dental anomalies were not identified in affected relatives of proband; their permanent upper canines had a normal eruption as can be seen in Figure 3 (d, e).

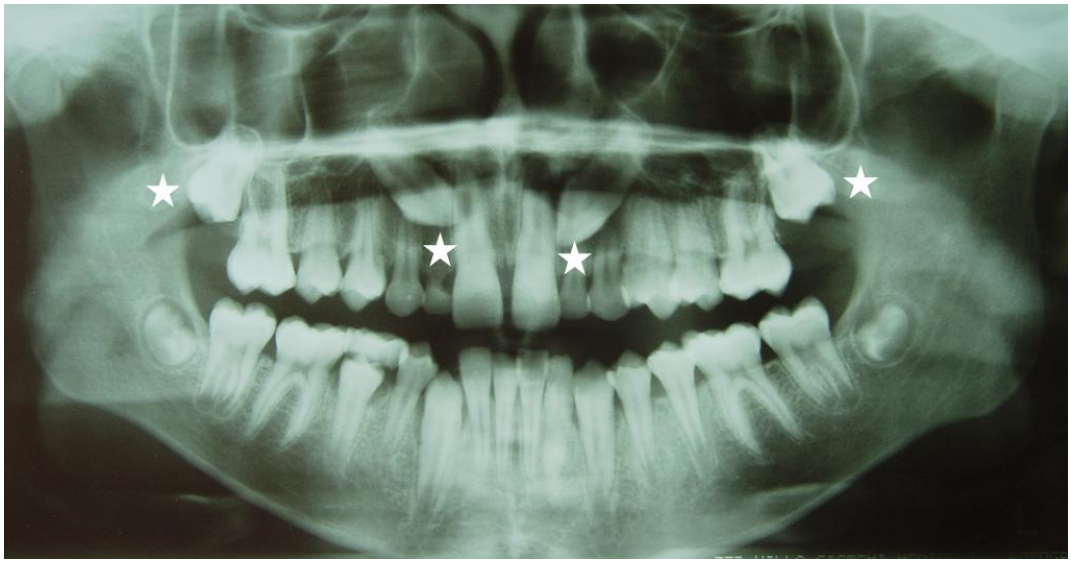

Figure 2. Panoramic X–ray of proband. Stars represent agenesis of permanent upper lateral incisors and upper third molars (hypodontia). Please note impaction of permanent upper canines concomitant with remaining deciduous upper lateral incisors and canines.

Pedigree construction indicated that phenotypes in this family showed an autosomal dominant segregation pattern, affecting both siblings (Fig. 1). Expressivity was found to be variable. Medical records analysis did not disclose health problems or disorders related to nails, hair follicles or sweat glands. The particular case of this family points to upper lateral incisors as the most absent teeth (six occurrences) followed by upper third molars (two occurrences).

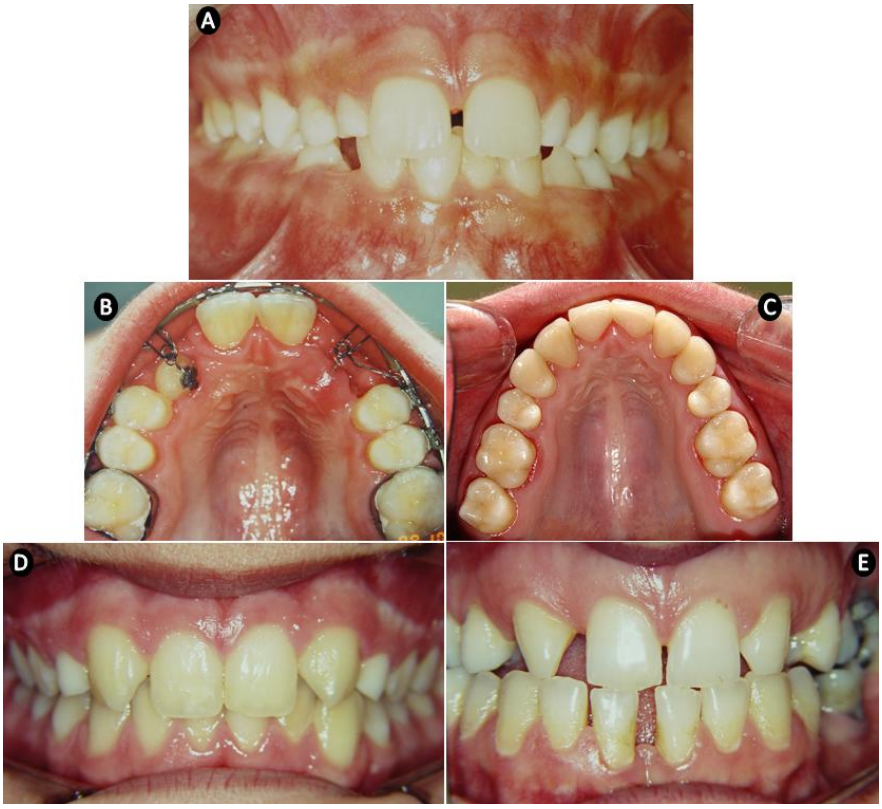

Figure 3. Intraoral views from affected family members. (A) Situation of the proband before orthodontic treatment and agenesis of permanent upper lateral incisors. (B) Closed eruption procedure followed by traction of impacted upper canines. (C) Post–treatment occlusal view with orthodontic space closure and 13, 23 replacing the lacking 12, 22. (D) Intraoral image from proband's affected brother and (E) mother, showing absence of the same permanent upper lateral incisors.

Mutational analysis of MSX1 gene of proband revealed one mutation in exon 2 (Fig. 4). Transition *6C>T was detected in homozygous state in all three affected members of the family, whereas unaffected father of proband also presented it but as

a heterozygosis (Table 2). Nonetheless, sequencing of ten unrelated control subjects did not reveal this genetic variant. No mutation was identified in exon 1 of MSX1 gene. Moreover, sequencing of PAX9 gene exons 1 to 4 of proband and relatives allowed identification of three known polymorphisms (data not shown) that, however, could not be associated with the phenotype.

Table 2. Mutations, phenotypes and missing teeth observed in the studied family.

Relatives	Phenotype	Missing Teeth[b]	MSX1 – *6C>T[c]
I–1	Unaffected		CT
I–2	Hypodontia	12,22	TT
II–1	Hypodontia	12,22	TT
II–2[a]	Hypodontia	12,18,22,28	TT

[a] indicates proband.
[b] missing teeth numeration follows FDI standards.
[c] same letters represent homozygous mutations, whereas different letters represent heterozygous mutations.

Discussion

Tooth development is a long and complex phenomenon regulated at the molecular level. Thus, gene mutations can be capable of causing failure in this genetic network leading to the definitive absence of one or more teeth (20). In this study, proband and his affected brother and mother presented the same genotype and phenotype, except for the upper third molars missing in proband only. Transition *6C>T is a known polymorphism and it is listed in dbSNP database (http://www.ncbi.nlm.nih.gov/snp) under accession number #rs8670. Interestingly, all three affected family members presented this polymorphism in homozygous state. The same variant, in heterozygous state however, was identified in the unaffected father,

whereas ten unrelated control subjects did not present this mutation. Accordingly, it can be hypothesized that this homozygotic variant might have somehow contributed to the phenotype. Likelihood of additional mutations in the non–coding regulatory regions or even in another gene should also be considered (21). Moreover, it is remarkable that the original sample, from which polymorphism #rs8670 was first identified, consisted of 15 unrelated Caucasians parents of children with cleft. There are evidences that cleft and hypodontia phenotypes are associated with mutations in the MSX1 gene (10, 15, 22).

Located 6 base pairs downstream the stop codon in exon 2, homozygous transition *6C>T might produce alterations in translational process. Due to its proximity to the stop codon, this mutation can hinder an ordinary translation termination by affecting interaction among ribosome, mRNA, release factors eRF1/eRF3 and associated proteins. This genetic variant was previously reported in a Polish individual affected with sporadic non–syndromic oligodontia (8). It should be noted that all three affected family members in this study present with hypodontia, a milder phenotype, suggesting that mutations outside the coding regions could also contribute either to less or more severe tooth agenesis forms.

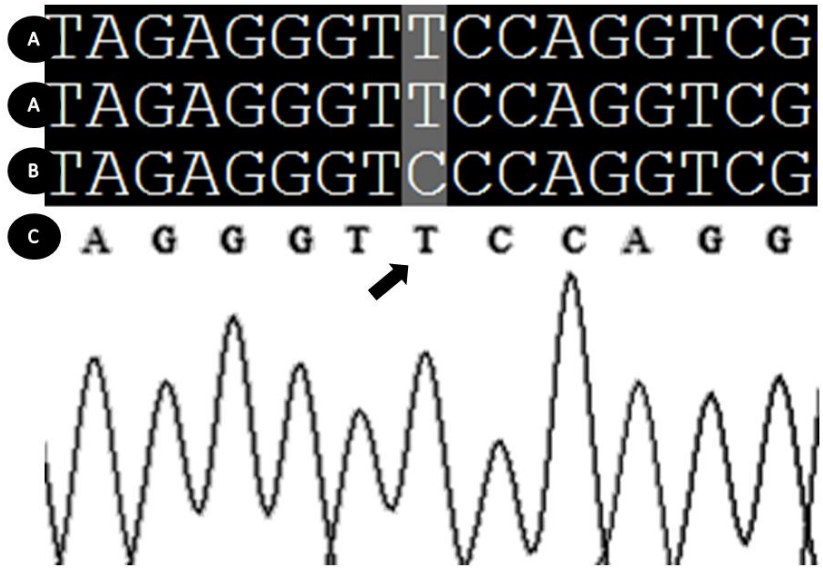

Figure 4. Sequencing and chromatogram of proband's MSX1 exon 2. (A) MSX1 sequences of proband. (B) Wild–type sequence from GenBank. (C) Arrow indicates homozygous polymorphism *6C>T.

Since one of the most common forms of hypodontia is agenesis of upper lateral incisors (ULIA), it has been investigated in a particular manner. Recently, Pinho *et al.* (23) suggested that ULIA is a distinct kind of hypodontia. From a survey with 62 probands and first–degree relatives from Portugal, the results indicated that proband's relatives showing ULIA have a 15 fold greater risk of developing the same type of agenesis than general population. Furthermore, they found that ULIA almost never segregates with other forms of agenesis, supporting the almost identical phenotype of the affected family members in the present research.

Oligodontia caused by mutations in MSX1 gene involves absence of first premolars in three out of four cases (9). Regarding hypodontia, upper lateral incisors and lower second premolars are usually the most affected teeth. In this study, all three affected family members are lacking upper lateral incisors supporting the results indicating that this is a common phenotype for hypodontia cases (2). On the other hand, mutations have not been found in MSX1 coding regions in patients with non–syndromic hypodontia to date, thus suggesting that a multigenic inheritance may be considered as a likely etiology for this anomaly (2, 24–26). That is why there has been a growing interest in investigating even small families segregating tooth agenesis; these data may contribute to increase knowledge of the anomaly (8).

Among affected family members, only the proband presented with impacted canines as associated dental anomaly. At the time of first consultation, he still had upper deciduous canines and lateral incisors (Fig. 3a), whereas his affected brother and mother showed both permanent upper canines normally erupted (Fig. 3d, e). Such cases usually require a multidisciplinary approach (27, 28). Thus, a treatment based on closed eruption surgical technique and orthodontic traction of the palatally impacted

canines was employed followed by their tooth–supported restorations for better esthetic result (Fig. 3b, c). Findings from Arte *et al.* (2) support that occurrence of hypodontia together with ectopic canines is not an uncommon condition, showing a higher frequency in relatives of patients affected with hypodontia.

It has been suggested that incidence of tooth agenesis has increased during the 20th century (29). Since a greater number of affected subjects is expected over the coming years, it should be considered a deeper analysis of the gene networks underlying this anomaly as well as the likelihood of a multigenic trait (simultaneous alteration in different genes). Either way, further research is necessary to better understand how genetic alterations lead to non–syndromic hypodontia.

Conclusions

This study depicts a family segregating non–syndromic hypodontia as an autosomal dominant trait. Three out of the four family members were affected and, interestingly, presented the same phenotype involving absence of upper lateral incisors. Proband also lacked upper third molars and had impacted upper canines as associated dental anomaly. Mutation *6C>T was identified in exon 2 of the MSX1 gene 6 nucleotides after the stop codon. It is a known polymorphism that was found homozygous in proband and his affected brother and mother, whereas his unaffected father presented a heterozygous condition. Nonetheless, MSX1 gene of ten unrelated unaffected control subjects did not show this genetic variant. Due to its proximity to the stop codon, this homozygous mutation could hinder an ordinary translation termination thus contributing to the phenotype. Further studies searching for

mutations in MSX1 and other genes in larger families affected with hypodontia involving permanent upper lateral incisors may elucidate the genetic mechanisms leading to this particular form of tooth agenesis.

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CAPÍTULO III

3°) Artigo Original submetido ao Journal of Dental

Research – Fator de Impacto 4.1

A novel missense mutation in the PAX9 gene associated with familial tooth agenesis

A novel missense mutation in the PAX9 gene associated

with familial tooth agenesis

Abstract

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PAX9 is a transcription factor deeply involved in the gene networks that regulate odontogenesis. To date only a restricted number of mutations in this gene have been associated with non–syndromic tooth agenesis. Six families segregating non–syndromic oligodontia/hypodontia were screened for mutations in PAX9 gene. A novel missense mutation lying in the exon 2 close to the end of the paired domain in three families was identified. Heterozygous mutation C503G is expected to result in an alanine-to-glycine amino acid change in residue 168 (Ala168Gly), which is invariably conserved among several species. The alanine–glycine change might lead to protein structural alteration due to the unique flexibility properties of glycine. Three mutations in intron 2 were also detected. Variations IVS2–109G>C, IVS2–54A>G and IVS2–41A>G were identified in both affected and unaffected members of the sample, however, these polymorphic variants may be involved in the phenotype as one proband showing all three intronic mutations in homozygosis was affected with the most severe oligodontia within the sample.

KEYWORDS: anodontia; dental informatics; molecular biology; odontogenesis; oligodontia; PAX9 Transcription Factor.

Introduction

Tooth agenesis is the failure of development of tooth bud leading to its definitive absence. It is the most common dental anomaly affecting up to 20% of the population, when the absence of third molars is considered (1). The frequency of hypodontia (lack of one to six permanent teeth, except third molars) varies from 1.6% to 9.6%, whereas oligodontia (more than six permanent teeth are missing, except third molars) prevalence rates are much lower ranging from 0.1% to 0.3% (2). This anomaly can be either part of a syndrome or a non–syndromic familial disturbance but sporadic forms are reported as well. In most cases, the agenesis inheritance is autosomal dominant, however, autosomal recessive and X–linked inheritance may also be involved (3).

The most frequently missing teeth are third molars, followed by lower second premolars and permanent upper lateral incisors (4). There seems to be a relationship between the number of lacking teeth and familial tooth agenesis. Most cases of families with affected subjects showed higher frequencies of hypodontia in parents and siblings of the probands than in the general population. Moreover, relatives of persons with oligodontia are more likely to lack more teeth than do those with hypodontia (5). It has also been reported that the frequency of agenesis affecting one tooth class among relatives is significantly higher than affecting different tooth classes (2).

Although both genetic and environmental factors may contribute in the etiology of tooth agenesis, scientific data accumulated in the last decades indicate the major role played by genetic factors in this anomaly (1). Studies of tooth development

in mice allowed identifying more than 200 genes directly or indirectly involved in odontogenesis regulation. Among these, two genes, MSX1 and PAX9, are highly correlated with agenesis (6, 7). Both genes code for transcription factors induced by epithelial signals and expressed in dental mesenchyme. Accordingly, their abnormal function may affect tooth development (8). Located on chromosome 14, PAX9 is directly involved in the craniofacial development, particularly in the formation of the palate and teeth. It also establishes the time of organ initiation and morphogenesis. Furthermore, it has been reported that PAX9 would act marking mesenchymal specific sites where future teeth will form (9, 10). Defects in this gene are suggested to cause selective tooth agenesis affecting mostly permanent molars (11).

Despite the high prevalence of tooth agenesis, mutational screening for PAX9 gene has returned a restricted number of causative mutations so far (Table 1). The first one was identified by Stockton *et al.* (12) in a family segregating autosomal dominant oligodontia. To date, the majority of mutations identified are located at the paired domain coding region, which correspond to the DNA binding site of PAX9 factor. Those mutations have usually been described on congenital non– syndromic oligodontia and/or hypodontia studies (Table 1). In a general manner, these mutations affect all major signaling pathways mediated by PAX9 and other transcription factors during odontogenesis. A loss of function of PAX9 would be expected to cause an haploinsufficiency, particularly when nonsense or frameshift mutations are involved (3, 8, 12–16, 21). Accordingly, abnormalities in the odontogenesis could occur including the arrest of tooth bud.

In the present report, we studied six Brazilian nuclear families affected with non–syndromic oligodontia/hypodontia involving particularly premolars and third

molars. Mutational screening for PAX9 gene was performed aiming to associate genotype and phenotype.

Materials and methods

Selection of nuclear families and pedigree construction

Six unrelated orthodontic patients affected with non–syndromic oligodontia (OMIM #604625) or hypodontia (OMIM #167416) were selected from a private office to participate in this study. Their families have more than one individual presenting with tooth agenesis. All subjects from the six families are Caucasian and Italian descent from an immigrant community in the region of Veneto, Italy. Diagnosis of the anomaly was verified by clinical examination and panoramic radiograph. The same investigation was carried out in the six probands respective first–degree relatives allowing to determine the pedigrees (Fig. 1). Associated dental anomalies were also registered and custom questionnaires were applied in order to evaluate the medical and families history. The affected members of each family were reported to have had

Table 1. Identified mutations in PAX9 that have been associated with tooth agenesis.

Gene	Exon	Intron	Mutation		Reference	Phenotype	Type / Observations
			Nucleotide	Residue*			
PAX9			–	–	Das *et al.*(17)	Oligodontia	Gene deletion
	1		1A>G	Met1Val	Klein *et al.*(10)	Oligodontia	Missense
		•	109G>C 54A>G 41A>G	–	Pawlowska *et al.*(18) This study	Oligodontia / Hypodontia	Authors found a higher frequency of these mutations in affected patients. They are listed in dbSNP database as polymorphisms # rs12883298, #rs12882923 and #rs12883049
	2		16G>A	Gly6Arg	Wang *et al.*(19)	Hypodontia	Missense
	2		62T>C	Leu21Pro	Das *et al.*(13)	Oligodontia	Missense
	2		76C>T	Arg26Trp	Lammi *et al.*(11)	Oligodontia	Missense
	2		83G>C	Arg28Pro	Jumlongras *et al.*(20)	Oligodontia	Missense
	2		109–110insG	Ile37SerfsX41	Zhao *et al.*(21)	Oligodontia	Insertion
	2		128G>A 129C>A	Ser43Lys	Wang *et al.*(19)	Hypodontia	Missense
	2		139C>T	Arg47Trp	Zhao *et al.*(21, 22)	Oligodontia	Missense
	2		151G>A	Gly51Ser	Mostowska *et al.*(23)	Oligodontia	Missense
	2		175C>T	Arg59X	Tallón–Walton *et al.*(16)	Oligodontia / Dental anomalies	Nonsense
	2		175–176ins288	Arg59GlnfsX177	Das *et al.*(13)	Oligodontia	Insertion
	2		218–219insG	Ser74GlnfsX317	Stockton *et al.*(12)	Oligodontia	Insertion
	2		259A>T	Ile87Phe	Kapadia *et al.*(24)	Oligodontia	Missense
	2		271A>G	Lys91Glu	Das *et al.*(13)	Oligodontia	Missense
	2		340A>T	Lys114X	Nieminen *et al.*(8)	Oligodontia	Nonsense
	2		433C>T	Gln145X	Hansen *et al.*(15)	Oligodontia	Nonsense
	2		503C>G	Ala168Gly	This study	Oligodontia / Hypodontia	Missense
	2		619_621 delATCins24bp	Ile207Tyr_X211	Mostowska *et al.*(14)	Oligodontia	Deletion / Insertion
	4		792–793insC	Val265ArgfsX315	Frazier–Bowers *et al.*(3)	Oligodontia	Insertion

*change of residues referred by the authors or obtained from sequence alignment.

normal deciduous dentition. All subjects from the six families, including minors, signed consent form. The protocol for this research was approved by the Research Ethics Committee of the University of Caxias do Sul, Rio Grande do Sul, Brazil, according to the Resolution 196/96 of the National Health Council.

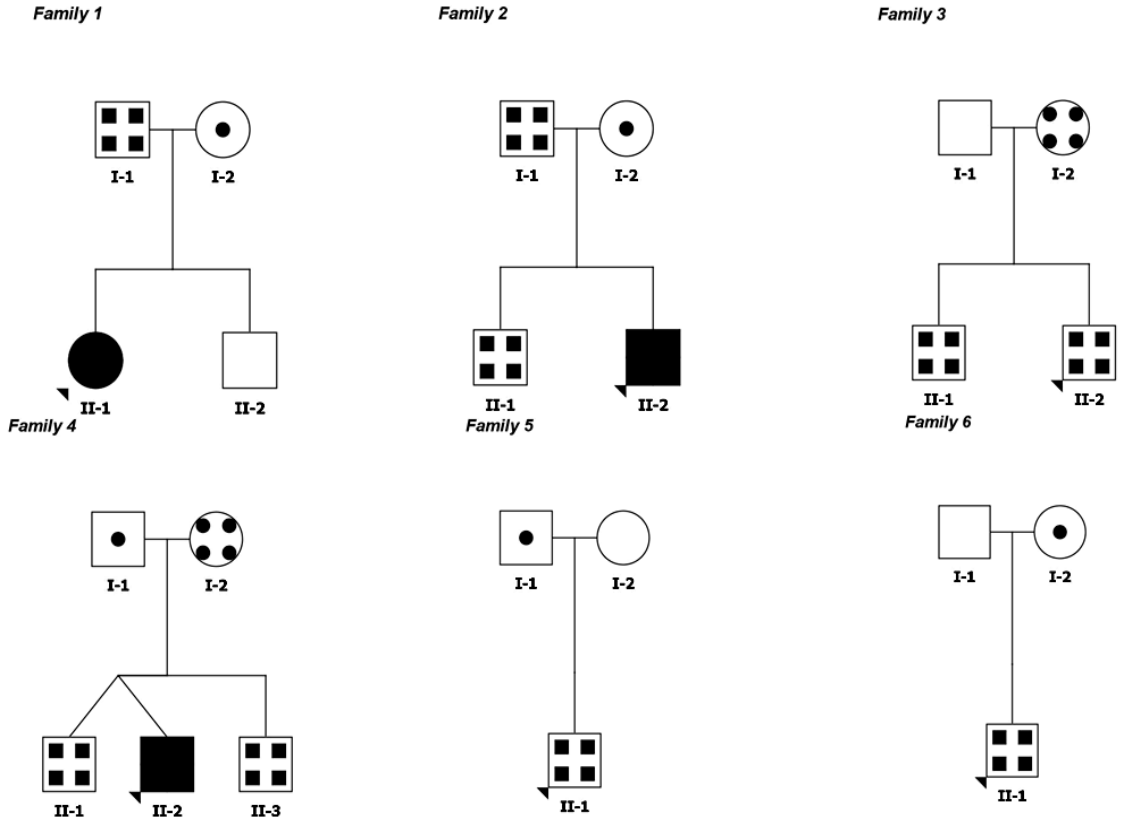

Figure 1. Pedigrees showing phenotypes and autosomal dominant mode of inheritance: squares, males; circles, females; full black, oligodontia; four black dots, hypodontia; one black dot, agenesis of one or more third molars; full white, unaffected. Arrows indicate probands.

DNA collection, screening and mutational analysis

All subjects had a sample of buccal epithelial cells collected with cytology brushes. DNA was isolated by using Wizard Genomic DNA Purification Kit (Promega, Madison, WI, USA) according to the manufacturer's instructions. The amplification process by polymerase chain reaction (PCR) was performed with five sets of primers

covering the four exons and boundaries of the PAX9 gene, as previously described (11). PCR was carried out in an Eppendorf Mastercycler® Gradient Thermal Cycler (Eppendorf, Hamburg, Germany). PCR reactions contained: 1.5 U Taq DNA Polymerase (Invitrogen, Karlsruhe, Germany); 1X PCR Buffer (Invitrogen, Karlsruhe, Germany); 2 mM $MgCl_2$; 0.2–0.8 mM dNTPs; 300 nM each primer; 5% DMSO and 20 ng of genomic DNA. Conditions for PCR were as follows: 94° C for 3 min; 94° C for 30 s; 56°–65° C for 30 s; 72° C for 30–45 s after 30 cycles. Afterwards, PCR products were submitted to an ExoI–SAP enzymatic purification process in order to remove non– incorporated dNTPs and primers. Sequencing of both strands of each purified PCR product was performed on a model ABI Prism®3130xl sequencer (Applied Biosystems, Foster City, CA, USA). All sequences were compared with GenBank accession numbers AJ238381, AJ238382 and AJ238383 by means of the BioEdit 7.0 software. Homology of the mutant sequences was also compared using BLAST (http://blast.ncbi.nlm.nih.gov/Blast.cgi). Screening for mutations was first performed in the six probands. Once detected a mutation, the respective relatives were screened for that genetic variant by the same method. PAX9 genes from ten unrelated control subjects were also sequenced.

Results

Phenotype analysis and pedigree

Three of the six probands were diagnosed with oligodontia (F1:II–1; F2:II–2; F4:II–2), whereas the other three presented with hypodontia (F3:II–2; F5:II–1; F6:II–1). Among those with oligodontia, subject F1:II–1 still had three deciduous teeth and the

lack of ten permanent teeth, representing the most severe phenotype in the sample. Interestingly, proband F2:II–2 was missing all eight premolars which is a rather rare oligodontia phenotype (Fig. 2). Clinical and radiographic examinations of proband F4:II–2 revealed absence of nine permanent teeth. Proband F5:II– 1 showed the most severe hypodontia phenotype in the sample, since all second premolars and third molars were missing, except the lower right third molar. Proband F3:II–2 was lacking upper lateral incisors and upper third molars, whereas F6:II–1 lacked one lower left second molar and four more permanent teeth. Moreover, microdontia of all first premolars and upper lateral incisors was found in subject F5:II–1, whereas only the upper left second premolar presented with severe microdontia in individual F6:II–1. No other associated dental anomaly was identified.

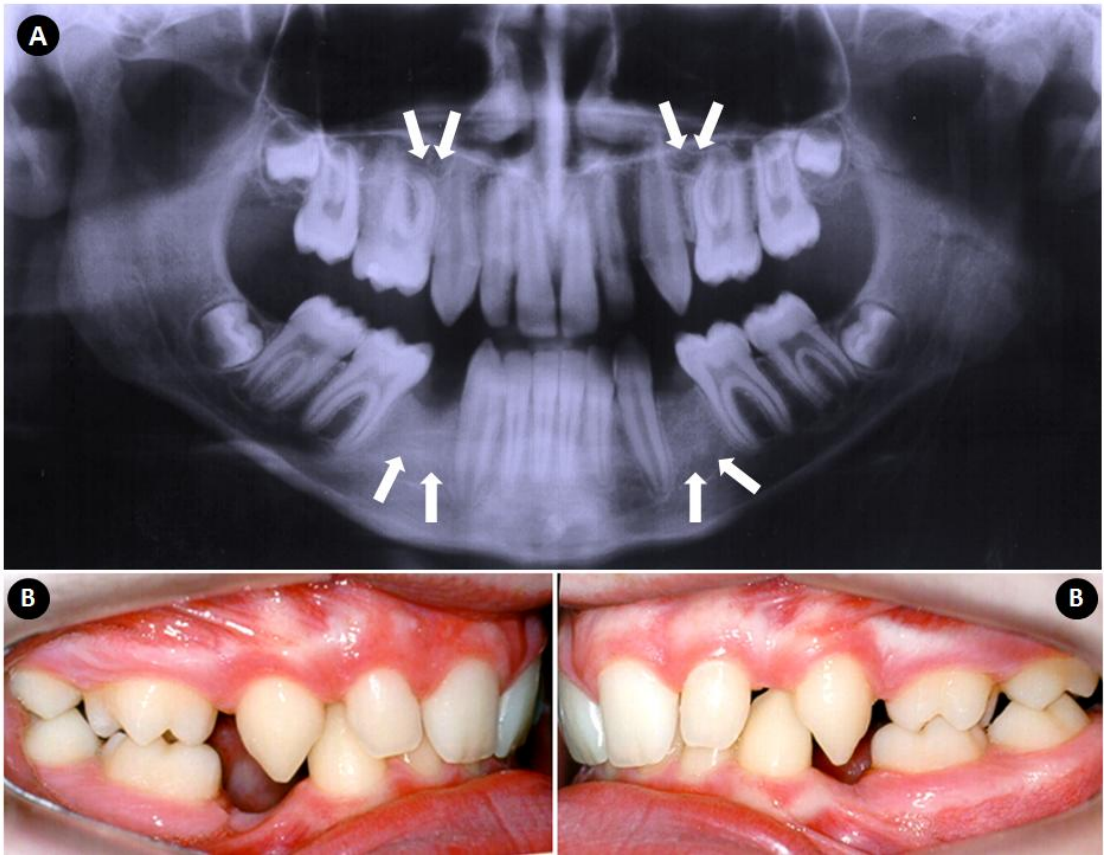

Figure 2. Images from proband 2. (A) Panoramic x–ray. Arrows indicate agenesis of all eight premolars (oligodontia). (B) Bilateral intraoral views.

Both parents in families 1, 2 and 4 were affected with tooth agenesis, whereas in families 3, 5 and 6 just one parent was affected. Among members of second generation in all families only subject F1:II–2 was unaffected. In family 3, proband F3:II–2 and his brother F3:II–1 and mother F3:I–2 were lacking upper lateral incisors. All affected members in family 4 were lacking at least upper second premolars, except for the father F4:I–1, whose agenesis involved only upper third molars. Regarding phenotypic status of affected members in families 5 and 6, third molars and second premolars were the most absent teeth. Although it is a small sample, a high number of missing teeth were observed: 96 out of 736 teeth were missing, which corresponds to 13%. Excluding third molar agenesis (43.5% of the lacking teeth), the most absent tooth was second premolar (31.2%), followed by first premolar (10.4%) and upper lateral incisor (9.4%).

As observed in Figure 1, phenotypes in the six nuclear families showed an autosomal dominant segregation pattern. Expressivity was found to be variable in families 1, 2 and 4. Medical records of all families did not reveal health problems or disorders related to hair follicles, nails or sweat glands. Probands F1:II–1 and F4:II–2, as well as their brothers F1:II–2 and F4:II–1, were prematurely born neonates.

Mutational analysis

Sequence analysis of the PAX9 gene of the six probands and relatives revealed one mutation in exon 2 and three in intron 2 (Table 2). A novel heterozygous mutation C503G (Fig. 3) in exon 2 was identified in probands of families 2, 4 and 6 resulting in an alanine– to– glycine amino acid change at residue 168 (Ala168Gly). All

affected members of these families revealed the same substitution, whereas sequencing of ten unrelated control subjects, as well as all members of the other three families, were negative for this alteration.

Intronic mutations IVS2–109G>C and IVS2–54A>G were observed in all six probands and their affected family members except for F2:II–1 and F4:I–2. However, these variations were also detected in three unaffected individuals (F1:II–2, F3:I–1 and F5:I–2) and in three out of the ten unrelated control subjects. Intronic mutation IVS2–41A>G was detected in probands of families 1, 3, 4 and 5, as well as in some affected and unaffected family members. This transition was absent from control subjects. All intronic variations were found in either homozygotic or heterozygotic state.

Discussion

Due to its key role during odontogenesis, PAX9 gene has been investigated regarding mutations associated with tooth agenesis, particularly with oligodontia involving permanent molars (3, 8). Although all six families of this study show different patterns of missing teeth, even when considering each family separately, the present results suggest that the novel missense mutation identified in exon 2 may be associated to the phenotypes of this sample. Located 119 base pairs downstream the PAX9 paired domain, the missense transversion C503G resulted in an alanine–to–glycine amino acid change (Ala168Gly) which has not been previously described neither in the literature nor in any known data base (Fig. 3). Analysis of sequence homology between man and other vertebrate species pointed out that the alanine–168 is an invariably conserved residue among several species, which is an indicative of

its structural or functional importance (Fig. 4). Even though alanine and glycine are small amino acids, an alanine–glycine change might lead to protein structural alteration due to the unique flexibility properties of glycine.

Table 2. Phenotype, missing teeth and PAX9 mutations in the studied families.

	Relatives	Phenotype	Missing Teeth[a]	PAX9[b]			
				109G>C	54A>G	41A>G	Ala168Gly
Family 1	I–1	Hypodontia	12,14,15,23,25,35	CC	GG	AG	
	I–2	Tooth Agenesis	18,28,38,48	GC	AG	AG	
	II–1*	Oligodontia	13,14,17,18,23,28,41, 44,45,48	CC	GG	GG	
	II–2	Non Affected		GC	AG	AG	
Family 2	I–1	Hypodontia	18,28,35,38,45,48	GC	AG		CG
	I–2	Tooth Agenesis	18,28,38,48	GC	AG		CG
	II–1	Hypodontia	18,28,35,38, 42,45,48				CG
	II–2*	Oligodontia	14,15,24,25, 34,35,44,45	CC	GG		CG
Family 3	I–1	Non Affected		GC	AG	AG	
	I–2	Hypodontia	12,22	GC	AG	AG	
	II–1	Hypodontia	12,22	GC	AG	AG	
	II–2*	Hypodontia	12,18,22,28	GC	AG	AG	
Family 4	I–1	Tooth Agenesis	18,28	GC	AG	AG	CG
	I–2	Hypodontia	15,18,25,28,35				CG
	II–1	Hypodontia	15,25,38	GC	AG		CG
	II–2*	Oligodontia	14,15,18,24,25,28, 35,44,45	GC	AG	AG	CG
	II–3	Hypodontia	15,18,25	GC	AG	AG	CG
Family 5	I–1	Tooth Agenesis	18,28,38,48	CC	GG	AG	
	I–2	Non Affected		GC	AG	AG	
	II–1*	Hypodontia	15,18,25,28, 35,38,45	CC	GG	AG	
Family 6	I–1	Non Affected					
	I–2	Tooth Agenesis	18,28,38,48	CC	GG	AG	CG
	II–1*	Hypodontia	15,35,37,38,45	GC	AG		CG

* indicates probands.
[a] missing teeth numeration follows FDI standards.
[b] same letters represent homozygotic mutations, whereas different letters represent heterozygotic mutations.

The C503G is the first missense mutation identified after the C– terminal subdomain of the paired domain within exon 2. The association between this missense mutation and agenesis reinforce the assumption that mutations outside the paired domain of PAX9 may also significantly affect odontogenesis. Three nonsense

mutations located beyond the PAX9 paired domain were also reported to be involved in the phenotype (3, 14, 15). These variants caused nonsense codons that result in premature termination of the translation leading to a truncated mutant protein. Thus, the PAX9 protein level of activity may be compromised (haploinsufficiency) contributing to the phenotype in all affected members of families 2, 4 and 6 in the present study. This assumption can be particularly applied to families 2 and 4, whose both parents present with tooth agenesis. Indeed, all their siblings were affected.

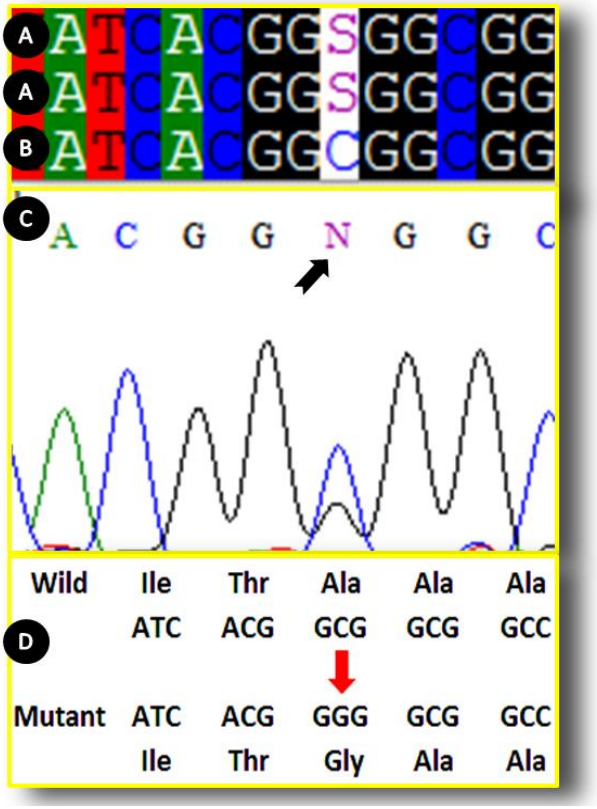

Figure 3. Sequencing and chromatogram of exon 2 of the proband 2. (A) Mutant sequences. (B) Wild–type sequence from GenBank. (C) Arrow indicates C503G heterozygous mutation in PAX9. (D) Comparison between gene and protein original sequences and nucleotide and amino acid mutant sequences: residue substitution Ala168Gly.

Regarding association between all subjects having the C503G mutation in the studied sample and their most absent teeth, it was possible to report agenesis of 25 third molars and 22 second premolars. Since there is a high amount of premolars lacking, it can be speculated that the substitution Ala168Gly might have generated similar pathogenic mechanisms to arrest development of these second premolars and third molars.

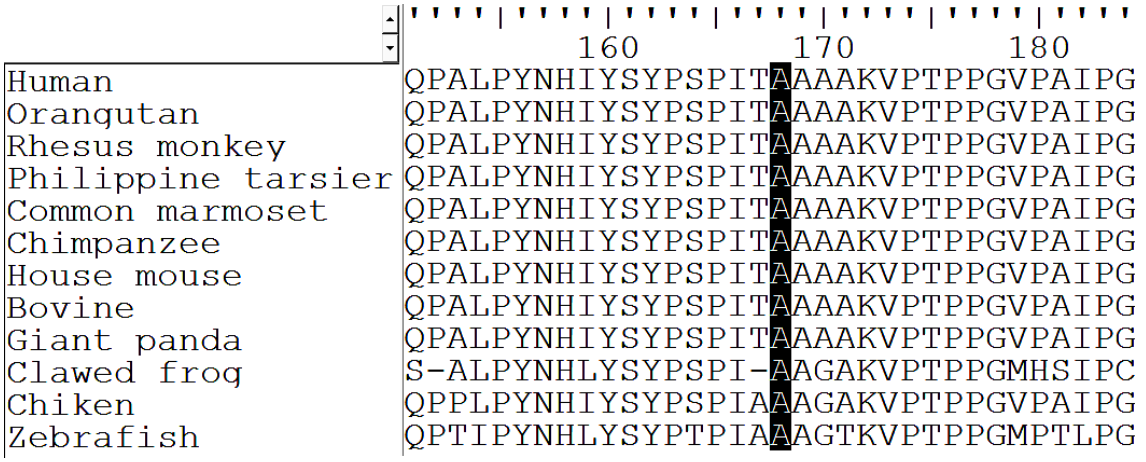

Figure 4. Comparison of amino acid sequences of PAX9 across different species. Black row indicates evolutionary conservation of the alanine 168 which was replaced by a glycine in the affected subjects of families 2, 4 and 6.

Other three mutations occurring at the end of the intron 2 were also detected. Genetic variants IVS2–109G>C, IVS2–54A>G and IVS2–41A>G are located 109, 54 and 41 base pairs upstream exon 2, respectively. All three are polymorphisms previously identified and deposited under the accession numbers #rs12883298, #rs12882923 and #rs12883049 in the dbSNP database (http://www.ncbi.nlm.nih.gov/snp). Association between phenotype and these polymorphisms in the present study was rather variable. All individuals with at least one homozygous polymorphism were affected, whereas 21.5% of unaffected subjects had all three variants in heterozygous state. Recently, these three intronic polymorphisms were reported to be somehow related to agenesis since they were present in higher frequency among affected subjects (18). The same study suggested that intronic sequences may play a significant role in the regulation of alternative and normal splicing thus altering pre–mRNA splicing. It is widely known that cis– acting sequences and hnRNP proteins are also involved in recognition of the translation initiation sites, but how they interact with splicing sites negatively affecting them is still a matter of further investigation.

The severe phenotype of proband F1:II–1 can be associated to the presence of all three intronic mutations in homozygosis (this was the only individual showing this genotype) or defects in other genes, as no mutation events within PAX9 exons were detected. As odontogenesis is a highly complex process, knowledge of gene interactions in several levels might better clarify such cases.

It is remarkable the high frequency of all four mutations within the evaluated sample. The C503G mutation was detected in 47.8% of analyzed individuals, whereas 65.2% of them showed all three intronic variants. These data may be related to the ethnic origin of the sample as all subjects descend from a same location, north Italy. Isolation of small populations and inbreeding may have contributed to reduce the genetic variation thus increasing the risk of genetic defects being inherited.

Conclusions

This report describes six families segregating non–syndromic oligodontia/hypodontia as an autosomal dominant trait. A novel missense mutation C503G was detected in PAX9 gene in all affected members of families 2, 4 and 6 leading to amino acid substitution in residue 168 (Ala168Gly). Although it has occurred in an evolutionarily conserved amino acid close to the end of the paired domain, further experimental research is required to quantify transcript levels as well as to evaluate functional capabilities of mutant protein. Other three intronic mutations (IVS2–109G>C, IVS2–54A>G and IVS2–41A>G) were identified in both affected and unaffected members of families, however, these polymorphic variants may be somehow involved in the phenotype as one proband showing all three intronic

mutations in homozygosis was affected with the most severe oligodontia within the sample. The high frequency of mutations and missing teeth in these six families may indicate inbreeding. Further studies evaluating gene expression in larger samples may be valuable in order to clarify the pathogenesis of tooth agenesis.

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5. CONCLUSÕES

A partir dos métodos utilizados para a investigação da amostra estudada, foi possível concluir que:

- Os dentes mais ausentes foram: 43,5% – terceiros molares; 16,2% – segundos pré–molares; 7,7% – incisivos laterais superiores permanentes, sendo a agenesia mais frequente nos familiares de probandos com oligodontia em comparação aos que apresentam hipodontia.
- Observou–se que o fator prematuridade ao nascimento não apresentou influência na manifestação da agenesia dentária na presente amostra.
- Foram diagnosticadas duas anomalias dentárias associadas: caninos impactados e microdontia.
- A agenesia dentária segregou de modo autossômico dominante em todas as famílias da amostra.
- A mutação missense em heterozigose C503G foi identificada no gene PAX9 de três famílias resultando em troca de aminoácido no resíduo 168 (Ala168Gly). Essa substituição pode determinar uma alteração estrutural na proteína devido às propriedades únicas de flexibilidade da glicina, levando à agenesia dentária. Tal mutação não encontra–se registrada em nenhum banco de dados conhecido sendo, portanto, inédita.
- A mutação C503G no PAX9 esteve associada a um alto índice de agenesias de segundos pré–molares, muito próximo do número de ausências de terceiros molares (em toda a amostra foram 22 para 25 agenesias, respectivamente).

- Foram identificados quatro polimorfismos: *6C>T no gene MSX1 e 109G>C, 54A>G, 41A>G no gene PAX9. Todos apresentam registro no GenBank, estando associados a maior incidência de agenesia dentária. O polimorfismo no MSX1 esteve relacionado particularmente aos incisivos laterais superiores permanentes, enquanto que os polimorfismos no PAX9 mostraram grande variabilidade em relação aos dentes afetados.
- As proteínas codificadas por MSX1 e por PAX9 apresentaram alto índice de correlação e interação.
- A alta frequência de mutações e de dentes ausentes nas seis famílias (entre 23 indivíduos, apenas quatro não são afetados) pode sugerir a ocorrência de casamento consanguíneo nos ascendentes dos familiares estudados, uma vez que as famílias não apresentam grau de parentesco conhecido atualmente.

6. ANEXOS

ANEXO 1 – TERMO DE CONSENTIMENTO LIVRE E ESCLARECIDO

UCS UNIVERSIDADE DE CAXIAS DO SUL	**Termo de Consentimento Livre e Esclarecido**

IDENTIFICAÇÃO DO PROJETO DE PESQUISA	
Título da Pesquisa: Associação de Polimorfismos nos Genes MSX1 e PAX9 com Agenesia Dentária	
Curso: Doutorado em Biotecnologia	Unidade: Instituto de Biotecnologia
Instituição Onde Será Realizado: Universidade de Caxias do Sul	
Pesquisadores: Prof. MSc. Breno Ramos Boeira Júnior (Doutorando) Prof. Dr. Sergio Echeverrigaray Laguna (Orientador)	

Você está sendo convidado(a) a participar do projeto de pesquisa científica acima identificado. O documento abaixo contém todas as informações necessárias sobre a pesquisa que estamos realizando e sua colaboração será de fundamental importância.

Eu, indivíduo participante da pesquisa, abaixo assinado, após receber informações e esclarecimento sobre o projeto de pesquisa, concordo de livre e espontânea vontade em participar como voluntário(a) e estou ciente:

1. Da justificativa e dos objetivos para a realização desta pesquisa

Os(as) senhores(as) estão sendo convidados a participar de um estudo científico, realizado pela Universidade de Caxias do Sul. Esta pesquisa científica é uma importante contribuição para a identificação de genes envolvidos na alteração da face mais freqüente no ser humano: a agenesia dentária, que significa dentes que não se formaram e que não se formarão, implicando na ausência definitiva desses dentes.

A principal causa está relacionada a modificações na função de determinados genes responsáveis pela formação dos dentes. Assim, o objetivo deste estudo é procurar saber qual gene seria responsável pela falta de dentes. Além disto, este estudo quer comparar pessoas que possuem seus dentes com aquelas em que vários dentes não foram formados. Estas informações poderão contribuir para o surgimento de um exame de DNA que pode mostrar, mesmo durante a infância, se a pessoa tem possibilidade de não formar alguns dentes. Se esse resultado for positivo, ou seja, se a pessoa tiver possibilidade de não formar alguns dentes, um tratamento mais específico pode ser elaborado para essas pessoas. A grande diferença é que, atualmente, um exame assim não existe e só é possível comprovar que dentes não se formaram depois que isso já ocorreu.

Antes de decidir se deseja participar, queremos que os(as) senhores(as) saibam mais sobre o estudo.

Perguntas poderão ser feitas a qualquer momento.

Se participar do estudo, e após a sua assinatura nesse formulário de consentimento, receberá(ão) uma cópia.

2. Do objetivo de minha participação

O objetivo da minha participação neste estudo é tão somente responder o questionário descrito abaixo e fornecer saliva para posterior análise dos genes envolvidos na formação dos dentes.

É fundamental ressaltar que a coleta de saliva será realizada através de escovação com uma escova macia esterilizada na parte interna da boca, o que não lhe causará dor ou sangramento, somente um pequeno desconforto. O material obtido – incluindo as informações obtidas pelos questionários – estará sob a responsabilidade do pesquisador que o obteve, e

será encaminhado, no mesmo dia, para o Laboratório de Biotecnologia do Instituto de Biotecnologia da Universidade de Caxias do Sul. A saliva será congelada e armazenada imediatamente no laboratório citado. O questionário e a amostra de saliva permanecerão sob sigilo total e absoluto e, após a conclusão do estudo, ambos serão destruídos.

3. Do procedimento para coleta de dados

A coleta dos dados será processada em três fases: 1ª) Seleção e identificação dos grupos de estudo – grupo com agenesias dentárias (famílias) e grupo com presença de todos os elementos dentários (controle). Realizada através de análise de radiografia panorâmica previamente disponível na documentação do paciente que já se encontra em tratamento ortodôntico. 2ª) Questionário a ser respondido oralmente, pelo participante e/ou responsável, sobre informações relevantes relacionadas ao período de formação dos germes dentários do indivíduo participante do estudo. 3ª) Coleta de saliva. Efetuada por meio de suave raspagem, com escova esterilizada, sobre a superfície interna da bochecha de maneira rápida e indolor.

4. Da utilização, armazenamento e destruição das amostras

Os dados obtidos – informações do questionário e saliva coletada – estarão sob sigilo absoluto e serão utilizados exclusivamente para os objetivos dessa pesquisa. Após a coleta, a saliva será imediatamente congelada e armazenada no Laboratório de Biotecnologia do Instituto de Biotecnologia da Universidade de Caxias do Sul, em câmara fria a –80° C. A saliva e o questionário ficarão armazenados até 2011, quando serão destruídos.

5. Dos desconfortos

É fundamental ressaltar que a coleta de saliva será realizada através de escovação com uma escova macia esterilizada na parte interna da boca (bochecha), o que não lhe causará dor ou sangramento, somente um pequeno desconforto ou sensibilidade durante aproximadamente 5 minutos após a coleta. Caso seja necessário, o pesquisador oferecerá atendimento imediato no local da coleta.

6. Dos riscos

Não existem riscos importantes envolvidos na sua participação nesse estudo. Não haverá dor ou sangramento, mas poderá haver desconforto ou sensibilidade no local da coleta na parte interna da sua boca (bochecha). Poderá ocorrer algum tipo de inflamação, alguma reação de sensibilidade no local; entretanto, não é comum. Em caso de irritação ou desconforto maior, o pesquisador oferecerá atendimento clínico imediato pós–coleta. Qualquer um desses sinais deverá desaparecer em 1ou 2 dias.

7. Dos benefícios

Não existem benefícios diretos na participação neste estudo. As informações produzidas por este estudo poderão ajudar a conhecer melhores formas – exames – que possam identificar mais cedo as pessoas que, no futuro, não tenham formado um ou mais dentes facilitando seu tratamento. Por exemplo, se comprovada a hipótese deste estudo, no futuro o DNA poderá identificar precocemente a pessoa que não formará um ou mais dentes. E para esta pessoa, será possível manter os dentes de leite ou até mesmo recorrer a implantes dentários ou terapia de células–tronco para substituir os dentes que não se formaram. Assim, evitará problemas na mastigação, na fala, na musculatura relacionada à boca e pescoço, além de inconvenientes de origem estética.

Não receberá nenhum pagamento ou outro benefício direto, por participar deste estudo. Entretanto, não estará renunciando a nenhum direito legal ao assinar este formulário de consentimento.

8. Das indenizações

O ato da coleta de saliva pode gerar apenas desconforto passageiro. As informações do questionário e a amostra de saliva serão mantidas em sigilo. Assim, não havendo danos previstos aos indivíduos que participam da amostra, igualmente não há indenizações previstas.

9. Da isenção e ressarcimento de despesas

A minha participação é isenta de despesas e não receberei ressarcimento porque não terei despesas no fornecimento de material para coleta dados e durante todo o processamento dos resultados.

10. Da liberdade de recusar, desistir ou retirar meu consentimento

Tenho a liberdade de recusar, desistir ou de interromper a colaboração nesta pesquisa no momento em que desejar, sem necessidade de qualquer explicação. A minha desistência não causará nenhum prejuízo à minha saúde ou bem estar físico. Minha desistência também não gerará represálias, rancores ou consequências no meu atendimento e de meus familiares no futuro.

11. Da garantia de sigilo e privacidade

Os seus dados, bem como os resultados obtidos durante este estudo serão mantidos em sigilo absoluto, podendo ser revistos apenas pela equipe responsável pelo estudo. Concordo que o estudo seja divulgado em publicações científicas, desde que meus dados pessoais não sejam mencionados.

12. Da garantia de esclarecimento e informações a qualquer tempo

Tenho a garantia de tomar conhecimento e obter informações, a qualquer tempo, dos procedimentos e métodos utilizados neste estudo, bem como dos resultados, parciais e finais, desta pesquisa. Para tanto, poderei consultar o **pequisador responsável** (abaixo identificado) ou o **Comitê de Ética em Pesquisa da UCS – Caxias do Sul (RS)**, com endereço na Rua Francisco Getúlio Vargas, 1130, Bloco 46, sala 102, Bairro Petrópolis, CEP 95070–560, telefone (54) 3218–2118, e–mail: cpcoelho@ucs.br.

IDENTIFICAÇÃO DO INDIVÍDUO PARTICIPANTE DA PESQUISA

Nome:			Raça: B ( ) NB ( )
Naturalidade:	Estado:	Profissão:	
Gênero: M ( ) F ( )	Idade: anos	Data de Nascimento: / /	
RG/CPF Responsável:		E–mail:	
Telefones: ( )			
Endereço:			Cidade:

Anamnese (Referente ao período de gênese dentária)

- ➲ Estado de Saúde Geral:

Fator Nutricional: normal ( ) deficiente ( )

Fator Traumático (Facial):

Fator Infeccioso:

- ➲ Medicamentos:
- ➲ Fator Hereditário:

Mãe:

➲Gestação e Lactação: parto normal ( ) cesariana ( ) prematuro ( )	Tabagismo( ) Etilismo( )

Desordens orgânicas: endócrinas ( ) infecciosas ( )

Desordens nutricionais ( )

Medicamentos:	
Outras Complicações:	
Pai:	
Irmãos:	
Análise da Radiografia Panorâmica	
Grupo com Agenesias Dentárias ___,___,___,___,___,___,___,___,___,___,___	Total: elementos
Grupo com Presença de Todos os Elementos Dentários ( )	

IDENTIFICAÇÃO DO PESQUISADOR RESPONSÁVEL	
Nome: Prof. Dr. Sergio Echeverrigaray	Telefone: (54) 3218–2100 ramal 2075
Profissão: Biólogo	Registro no Conselho (CRB–RS): 17245
Endereço: Av. Francisco Getúlio Vargas – 1130 – Caxias do Sul – RS	
E–mail: selaguna@yahoo.com	

Declaro que obtive todas as informações necessárias e esclarecimento quanto às dúvidas por mim apresentadas e, por estar de acordo, assino o presente documento.

_____ (RS), _____ de _____ de 20___.

_____
Indivíduo participante da pesquisa

_____
Prof. Dr. Sergio Echeverrigaray
Pesquisador Responsável pelo Projeto

_____
Responsável Legal

Testemunhas:

_____
_____

Nome:
RG/CPF:
Telefone:

Nome:
RG/CPF:
Telefone:

ANEXO 2 – APROVAÇÃO DO COMITÊ DE ÉTICA EM PESQUISA/UCS

Comitê de Ética em Pesquisa
Universidade de Caxias do Sul

Caxias do Sul, 04 de Maio de 2011.

Parecer CEP/UCS Nº 001/2011
Ilmo Sr.
Breno Ramos Boeira Júnior

O Comitê de Ética em Pesquisa da Universidade de Caxias do Sul acompanhou o desenvolvimento ético da pesquisa com o título "Estudo da Associação de Polimorfismo nos Genes MSX1 e PAX9 com Agenesia Dentária", protocolo SISNEP CAAE0002.0.123.000-09, e não identificou falhas éticas.

Atenciosamente,

Prof. Dr. Wilson Paloschi Spiandorello
Coordenador do Comitê de Ética em Pesquisa

Universidade de Caxias do Sul. Cidade Universitária. Bloco A. Sala 321. Fone 054 32182100 Ramal 2829/2269. Email cep-ucs@ucs.br

ANEXO 3 – CERTIFICADO DE PREMIAÇÃO: 1° Lugar na 90th Annual Session of the Southwestern Society of Orthodontists at Austin, Texas, USA em 29 de Outubro de 2010

ANEXO 4 – CONFIRMAÇÃO DE ARTIGO SUBMETIDO AO *JOURNAL OF ORAL PATHOLOGY & MEDICINE* – Fator de Impacto 2.1

Journal of Oral Pathology and Medicine - Manuscript ID JOPM-11-11-RE-1914 Entrada x

ame@dadlnet.dk por manuscriptcentral.com 18:22 (35 minutos atrás)

para mim

12-Nov-2011

Dear Dr. Breno Boeira Junior,

Your manuscript entitled "Dentistry and molecular biology: a promising field for tooth agenesis management" has been successfully submitted online and is presently being given full consideration for publication in the Journal of Oral Pathology and Medicine. Should your manuscript not comply with the Journal's requirements, however, the Journal's administrator will notify you via email that you need to make specific changes to your manuscript before it can be considered for publication in the Journal of Oral Pathology and Medicine.

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Thank you for submitting your manuscript to the Journal of Oral Pathology and Medicine.

Sincerely,
Anne-Marie Engel
Administrator, Journal of Oral Pathology and Medicine

ANEXO 5 – CONFIRMAÇÃO DE ARTIGO SUBMETIDO AO *JOURNAL OF DENTAL RESEARCH* – Fator de Impacto 4.1

Manuscript JDR-11-0849 Submitted to the Journal of Dental Research

1 mensagem

kskinner@iadr.org <kskinner@iadr.org> **4 de novembro de 2011 16:24**
Para: ortoboeira@gmail.com

04-Nov-2011

Dear Dr. Boeira Junior:

Your manuscript, "Polymorphism in MSX1 gene in a family with autosomal dominant hypodontia," has been successfully submitted online to the Journal of Dental Research.

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Thank you for submitting your manuscript to Journal of Dental Research.

Sincerely,

Editorial Staff
Journal of Dental Research

ANEXO 6 – CONFIRMAÇÃO DE ARTIGO SUBMETIDO AO *JOURNAL OF DENTAL RESEARCH* – Fator de Impacto 4.1

Manuscript JDR-11-0848 Submitted to the Journal of Dental Research

kskinner@iadr.org <kskinner@iadr.org> **4 de novembro de 2011 15:38**
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04-Nov-2011

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Your manuscript, "Novel missense mutation in PAX9 gene associated with familial tooth agenesis," has been successfully submitted online to the Journal of Dental Research.

Your manuscript ID is JDR-11-0848.

Please mention the above manuscript ID in all future correspondence or when calling the office for questions. If there are any changes in your contact information, please log in to SAGETrack at http://mc.manuscriptcentral.com/jdr and edit your user information as needed. You can also view the status of your manuscript at any time by checking your Author Center after logging into system.

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Editorial Staff
Journal of Dental Research