Biochem Genet (2011) 49:104–121
DOI 10.1007/s10528-010-9392-6

Defining a New Candidate Gene for Amelogenesis Imperfecta: From Molecular Genetics to Biochemistry

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Received: 22 February 2010 / Accepted: 23 July 2010 / Published online: 3 December 2010

Abstract Amelogenesis imperfecta is a group of genetic conditions that affect the structure and clinical appearance of tooth enamel. The types (hypoplastic, hypocalcified, and hypomature) are correlated with defects in different stages of the process of enamel synthesis. Autosomal dominant, recessive, and X-linked types have been previously described. These disorders are considered clinically and genetically heterogeneous in etiology, involving a variety of genes, such as *AMELX*, *ENAM*, *DLX3*, *FAM83H*, *MMP-20*, *KLK4*, and *WDR72*. The mutations identified within these causal genes explain less than half of all cases of amelogenesis imperfecta. Most of the candidate and causal genes currently identified encode proteins involved in enamel synthesis. We think it is necessary to refocus the search for candidate genes using biochemical processes. This review provides theoretical evidence that the human *SLC4A4* gene (sodium bicarbonate cotransporter) may be a new candidate gene.

Keywords Molecular genetics · Biochemistry · Amelogenesis · New candidate gene

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Introduction

Definition

Historically, amelogenesis imperfecta (AI) has been defined as a diverse group of hereditary disorders characterized by developmental abnormalities in the quantity and quality of enamel with an absence of generalized or localized metabolic or morphological defects in other systems of the body (Sauk et al. 1972; Witkop 1989). In its mild forms, AI causes discoloration and abnormal morphology of the tooth crowns; however, in its more severe forms, the enamel is very scarce, clinically almost imperceptible, or it is hypoplastic, hypomineralized, or hypomature and easily lost after dental eruption. These disorders are limited to the enamel and may affect the primary, permanent, or both dentitions (Neville et al. 1995; Sapp et al. 1998). Recently, AI has been redefined as a group of conditions of genetic origin that affect the structure and clinical appearance of enamel of all or almost all the dentition and that may be associated with morphological or biochemical changes in other parts of the body. This definition is no longer restricted to enamel abnormalities, and as a result, syndrome-type AI must also be considered, which contributes to an increase in the current knowledge regarding the pathogenesis of the disorder (Aldred et al. 2003).

Classification

The development of normal enamel is divided into two main stages, and the abnormalities that cause AI may occur in either stage: (1) development of the matrix, or secretory stage (functional ameloblasts), and its simultaneous primary mineralization; (2) maturing of the enamel, in which proteins are removed and secondary mineralization occurs (Neville et al. 1995; Sapp et al. 1998; Witkop 1989). The three main types of AI are correlated with defects in the stages of the enamel synthesis process. In the hypoplastic type, there is a defect in the enamel matrix caused by interference in the function of the ameloblasts. The enamel does not have the normal thickness; it is thin or presents cavities due to apposition defects in local or generalized areas. The radio opacity of the enamel is greater than that of the dentin. In the hypocalcified type, the enamel is of normal thickness, but there is defective mineralization of the matrix, with abnormal enucleation and mineralization of the crystals of the prism or rod, which results in soft enamel that is easily eliminated with an instrument. In these cases, the enamel has lower radio opacity than the dentin. In the hypomature type, although the enamel is of normal thickness, its hardness and transparency are abnormal. There is a defect in the growth of the crystals during the maturation phase because proteins are not completely removed. In these cases, the radio opacity of the enamel is approximately the same as that of the dentin (Neville et al. 1995; Sapp et al. 1998; Witkop 1989). This anomaly has been classified into three types with at least 14 subtypes based on the process of normal amelogenesis and its clinical manifestations (Witkop 1989).

Prevalence

The estimated frequency of AI in the general population varies between 1 in 700 (Bäckman and Holmgren 1988) and 1 in 14,000 (Witkop 1989), depending on the diagnostic criteria used and the demographics of the population under study. Some studies have reported that hypoplastic AI is the most frequent in some populations (Neville et al. 1995; Sapp et al. 1998; Witkop 1989).

Genetics of Amelogenesis Imperfecta

Autosomal dominant and recessive as well as dominant and recessive X-linked types of AI have been described. As a result, AI disorders are considered to be genetically heterogeneous and are thought to involve different mutations in the same or different genes (Crawford et al. 2007; Wright et al. 2003; Wright 2006).

X Chromosome-Linked Types

Genetic linkage analyses carried out in some families with X chromosome-linked AI have located mutations in the region Xp22.1-p22.3, which corresponds to the amelogenin locus (*AMELX*). Therefore, mutations in this locus would be responsible for the abnormal phenotype in those families. To date, 15 mutations in the gene coding for the enamel protein, amelogenin, are associated with hypoplastic and hypomineralized enamel defects (Table 1) (Lagerström et al. 1991; Lench et al. 1994; Lench and Winter 1995; Lagerström-Fermér et al. 1995; Collier et al. 1997; Hart et al. 2000, 2002; Kindelan et al. 2000; Sekiguchi et al. 2001; Greene et al. 2002; Kim et al. 2004; Kida et al. 2007).

The phenotypes associated with defects in the amelogenin gene are variable. In general, the phenotype in affected men is the development of hypomature, yellowish, rough enamel that varies from a normal thickness to extremely thin or with local hypoplasia with neither prism structure nor retention of amelogenin-type proteins. Heterozygous women have vertical bands of hypoplastic and normal enamel arranged linearly, with changes in color as a result of X chromosome inactivation in the ameloblasts, which is known as Lyonization (Sauk et al. 1972). In addition, there is genetic evidence for a second AI locus on the X chromosome in the region Xq24-q27.1 (Aldred et al. 1992a, b; Chapman et al. 1991; Lagerström et al. 1990).

Genotype–Phenotype Correlations in Inherited, X-Linked Cases

The four mutations in the *AMELX* gene that affect the signal peptide and cause total loss of protein secretion result in the formation of thin enamel (Table 1), which indicates that the absence of amelogenin is not compatible with the formation of enamel of normal thickness (Lagerström-Fermér et al. 1995; Wright et al. 2003). This conclusion is supported by an amelogenin knockout mouse that displays a phenotype characterized by its very thin layer of enamel (Gibson et al. 2001).

Table 1 Mutations in genes that cause amelogenesis imperfecta

Causative gene	Pattern of inheritance	Gene location/ affected region	Mutation (cDNA; protein)	Clinical phenotype	Reference
Amelogenin (*AMELX*) Xp22.1-p22.3	X-linked	Exon 2/signal peptide	c.2T→C; p.M1T	Hypoplastic	Kim et al. (2004)
		Exon 2/signal peptide	c.11G→C; p.W4S	Hypoplastic	Kim et al. (2004)
		Exon 2/signal peptide	c.11G→A; p.W4X	Smooth hypoplastic	Sekiguchi et al. (2001)
		Exon 2/signal peptide	c.14_22del; p.I5_A8delinsT	Smooth hypoplastic	Lagerström-Fermér et al. (1995)
		Exon 3–7/all protein	c.55_54del; p.18del	Hypomineralized/ hypomaturation	Lagerström et al. (1991)
		Exon 5/terminal	c.152C→T; p.T51I	Hypomaturation	Lench and Winter (1995)
		Exon 5/terminal	c.152C→G; p.P52R	Smooth hypoplastic	Kida et al. (2007)
		Exon 5/terminal	c.155delC; p.P52fsX53	Hypomaturation	Aldred et al. (1992a); Lench et al. (1994)
		Exon 6/terminal	c.208C→A; p.P70T	Hypomaturation	Collier et al. (1997); Hart et al. (2000)
		Exon 6/terminal	c.230A→T; p.H77L	Hypoplastic/ hypomineralization	Hart et al. (2002)
		Exon 6/terminal	c.385delC; p.H129fsX187	Smooth hypoplastic	Sekiguchi et al. (2001)
		Exon 6/terminal	c.420delC; p.Y141fsx187	Smooth hypoplastic	Greene et al. (2002
		Exon 6/terminal	c.473delC; p.P158fsX187	Smooth hypoplastic	Lench and Winter (1995)
		Exon 6/terminal	c.541delC; p.L181fsX187	Smooth hypoplastic	Kindelan et al. (2000); Hart et al. (2002)
		Exon 6/terminal	c.571G→T; p.E191X	Smooth hypoplastic	Lench and Winter (1995)
Enamelin (*ENAM*) 4q11-q21	Autosomal dominant	Exon 5	c.157A→T; p.K53X	Local hypoplastic	Mardh et al. (2002)
		Intron 6	c.211-2A→C; p.M71_Q157del	Generalized hypoplastic	Kim et al. (2005a)
		Intron 8	c.534+1G→A; p.A158_Q178del	Smooth hypoplastic	Rajpar et al. (2001)

Table 1 continued

Causative gene	Pattern of inheritance	Gene location/ affected region	Mutation (cDNA; protein)	Clinical phenotype	Reference
		Exon 9	c.817G→T; p.R179M	Generalized hypoplastic	Gutiérrez et al. (2007)
		Intron 9	c.588+1delG; p.N197fsX277	Smooth hypoplastic	Kida et al. (2002)
		Exon 10	c.737C→A; p.S246X	Local hypoplastic	Ozdemir et al. (2005a)
		Exon 10	c.2991delT; p.L998fsX1062	Local and generalized hypoplastic	Kang et al. (2009)
	Autosomal dominant and recessive	Exon 10	c.1258_1259insAG; p.P422fsX448	Heterozygote: phenotype LHED[a] Homozygote: generalized hypoplastic with malocclusion	Hart et al. (2003b)
	Autosomal dominant and recessive	Exon 10	c.1020_1021insAGTCA GTACCAGTA CTGTGTC; p.V340_M341insSSQYQYCV	Heterozygote: phenotype LHED[a] Compound heterozygote: generalized hypoplastic with malocclusion	Ozdemir et al. (2005a)
Enamelysin (*MMP-20*) 11q22.3-q23	Autosomal recessive	Exon 1	c.102G→A; p.W34X	Hypoplastic-hypomaturation	Papagerakis et al. (2008)
		Exon 5	c.678T→A; p.H226Q	Hypomaturation	Ozdemir et al. (2005b)
		Exon 6	c.910G→A; p.A304T	Hypomaturation	Lee et al. (2010)
		Intron 6	c.954-2A→T; p.I319X ó p.I319fs338X	Hypomaturation	Kim et al. (2005b)
Kallikrein 4 (*KLK4*) 19q13.3-q13.4	Autosomal recessive	Exon 4	c.458G→A; p.W153X	Hypomaturation	Hart et al. (2004)
Distal less (*DLX3*) 17q21.3-q22	Autosomal dominant	Exon 3	c.560_562delTC; p.Y188Q	Hypoplastic-hypomaturation with taurodontism	Dong et al. (2005)

Table 1 continued

Causative gene	Pattern of inheritance	Gene location/ affected region	Mutation (cDNA; protein)	Clinical phenotype	Reference
Family with sequence similarity 83, member H (*FAM83H*) 8q24.3	Autosomal dominant	Exon 5	c.860C→A; p.S287X	Hypocalcified generalized	Wright et al. (2009)
		Exon 5	c.891T→A; p.Y297X	Hypocalcified generalized	Lee et al. (2008a)
		Exon 5	c.923_924delTC; p.L308fsX323	Hypocalcified generalized	Wright et al. (2009)
		Exon 5	c.973C→T; R325X	Hypocalcified generalized	Kim et al. (2008)
		Exon 5	c.1192C→T; Q398X	Hypocalcified generalized	Kim et al. (2008), Hart et al. (2009)
		Exon 5	c.1243G→T; p.E415X	Hypocalcified generalized	Lee et al. (2008a)
		Exon 5	c.1330C→T; p.Q444X	Hypocalcified generalized	Hart et al. (2009)
		Exon 5	c.1354C→T; p.Q452X	Hypocalcified generalized	Hyun et al. (2009)
		Exon 5	c.1366C→T; p.Q456X	Hypocalcified generalized	Hart et al. (2009)
		Exon 5	c.1374C→A; p.Y458X	Hypocalcified generalized	El-Sayed et al. (2010)
		Exon 5	c.1379G→A; p.W/Q460X	Hypocalcified generalized	Wright et al. (2009)
		Exon 5	c.1380G→A; p.W460X	Hypocalcified generalized	Lee et al. (2008a)
		Exon 5	c.1408C→T; p.Q470X	Hypocalcified generalized	Wright et al. (2009)
		Exon 5	c.1872_1873delCC; p.L625fsX703	Hypocalcified localized	Wright et al. (2009)
		Exon 5	c.2029C→T; p.Q677X	Hypocalcified generalized	Lee et al. (2008a)
		Exon 5	c.2080G→T; p.E694X	Hypocalcified localized	Wright et al. (2009)
β-Propeller *WDR72* 15q21.3	Autosomal recessive	Exon 15	c.2348C→G; p.S783X	Hypomaturation	El-Sayed et al. (2009)
		Exon 16	c.2857delA; p.S953VfsX20	Hypomaturation	El-Sayed et al. (2009)
		Exon 17	c.2934G→A; p.W978X	Hypomaturation	El-Sayed et al. (2009)

[a] LHED, localized hypoplastic enamel defects

However, the deletion mutation of 5 kb described in humans, which essentially should also destroy the function of the gene, is expressed as a hypomineralized/hypomature phenotype with variable degrees of hypoplasia (Lagerström et al. 1991). This finding supports the function of amelogenin in determining the thickness of the enamel, but it does not explain the variation in the phenotypes.

The mutations in *AMELX* that cause alterations in the amino-terminal portion of the amelogenin protein are all associated with hypomineralization/hypomaturation phenotypes of AI, with variable degrees of hypoplasia, yellowish brown color, and porous enamel caused by the retention of amelogenin-type protein (Table 1) (Ravassipour et al. 2000; Wright et al. 2003). The amino-terminus of the amelogenin protein contains a tyrosine-rich amelogenin peptide (TRAP) region and a highly conserved potential binding site for carbohydrates and cytokeratins in addition to some important proteolytic-processing sites. It is believed that alterations in the binding capacities of the mutated amelogenin proteins could affect protein–protein interactions and/or its proteolytic processing, thereby contributing to the expression of the described phenotype (Wright et al. 2003). On the other hand, the loss of the amelogenin carboxy-terminus results in a phenotype of AI with smooth, hypoplastic enamel (Table 1). The carboxy-terminal domain of the protein is important in the self-assembly process to form the nanospheres and their interacting minerals. Thus, it is possible that alterations in any of these processes affect the normal development of the enamel and abruptly ceases its formation, which explains the hypoplastic phenotype found in all the mutations of this region of the gene (Wright et al. 2003).

Autosomally Inherited Types

Theoretically, the potential AI candidate genes with autosomal inheritance patterns include tuftelin (*TUFT*, 1q21-q23), ameloblastin (*AMBN*, 4q13.3), enamelin (*ENAM*, 4q11-q21), enamelysin (*MMP-20*, 11q22.3-q23), kallikrein 4 (*KLK4*, 19q13.3-q13.4), amelotin (*AMTN*, 4q13.3), distal less 3 (*DLX3*, 17q21.3-q22), genes from the family with sequence similarity 83, member H (*FAM83H*; 8q24.3), and the gene encoding beta propeller *WDR72* (Crawford et al. 2007; Stephanopoulos et al. 2005; Wright 2006; El-Sayed et al. 2009). Of these genes, *ENAM*, *MMP20*, *KLK4*, *DLX3*, *FAM83H*, and *WDR72* are considered causal genes because mutations in their coding regions are responsible for diverse clinical phenotypes of AI. No mutations have been reported in the *TUFT*, *AMBN*, and *AMTN* genes. As a result, these genes continue to be as candidate genes (Crawford et al. 2007; Stephanopoulos et al. 2005; Wright 2006).

Autosomal dominant AI (ADAI) presents great variability in its clinical expression, making it difficult to define the relationship between genotype, pathogenesis, and phenotype (Hart et al. 2003a). To date, 9 mutations have been reported in the enamelin gene (Table 1) (Rajpar et al. 2001; Mardh et al. 2002; Kida et al. 2002; Hart et al. 2003b; Kim et al. 2005a; Ozdemir et al. 2005a; Gutiérrez et al. 2007; Kang et al. 2009); 1 mutation has been reported in *DLX3*, which is responsible for the ADAI cases associated with taurodontism (Dong et al. 2005); and 16 mutations have been reported in the recently nominated *FAM83H* gene. This

series of mutations within these three genes are all associated with ADAI of the hypocalcified type (Table 1) (Kim et al. 2008; Lee et al. 2008a; Hart et al. 2009, Wright et al. 2009; Hyun et al. 2009; El-Sayed et al. 2010).

The autosomal recessive AI (ARAI) phenotypes have been explained mainly by mutations in the genes coding for enamel proteases, *MMP-20* and *KLK4*, which are responsible for the hypomature and pigmented AI phenotypes (Table 1) (Hart et al. 2004; Kim et al. 2005b; Ozdemir et al. 2005b; Papagerakis et al. 2008; Lee et al. 2010). Nevertheless, a study of three Turkish families has shown that an insertion of 2 base pairs (bp) (g.13185_13186insAG) in the *ENAM* gene has a dose-dependent effect such that ARAI associated with an anterior, open bite cosegregates as a recessive trait and localized, hypoplastic defects of the enamel segregate as a dominant trait (Hart et al. 2003b; Ozdemir et al. 2005a). In another study, the authors reported that two subjects, also from a Turkish family, affected with severe and generalized hypoplastic-type ARAI, contained the same 2 bp insertion mutation (g.13185_13186insAG) and a new insertion mutation of 21 bp (g.12946_12947ins AGTCAGTACCAGTACTGTGTC) in the *ENAM* gene (Ozdemir et al. 2005a). This last observation emphasizes the need to carry out molecular analyses in all of the causal genes to confirm the presence and/or absence of the previously described mutations. Recently, three mutations (Table 1) in the gene encoding the beta propeller *WDR72* have been reported, all of which cause hypomaturation-type AI (El-Sayed et al. 2009).

Phenotype–Genotype Correlations in Autosomally Inherited Cases

Autosomal dominant AI is the most frequently reported form of AI in some epidemiological studies (Bäckman and Holmgren 1988; Wright et al. 1997). The main gene involved is *ENAM*. Of all the mutations described in *ENAM*, five correspond to single base changes (four transversions and one transition), two are insertion mutations (2 and 21 bp), and two are deletions of one base (Table 1). The mutations located in introns 6, 8, and 9 affect the processing of ENAM mRNAs, and they become degraded by nonsense-mediated mRNA decay, which results in a reduction of the amount of protein or causes alterations in one of its proteolytic-processing products. The two mutations classified as nonsense, within exons 5 and 10, and the deletion mutation of a single base that affects exon 10 would result in a truncated protein, thereby causing haploinsufficiency of ENAM or its processed products (Table 1). The missense mutation that affects exon 9 would result in an ENAM protein that is unable to interact with amelogenin. The two base insertion mutations would alter the reading frame and are therefore considered frameshift mutations, which cause dose-dependent phenotypic effects. Both mutations would affect the structure of the ENAM protein and subsequently alter its function (Table 1). In brief, independent of the area of the *ENAM* gene that is affected by a mutation, the global result is a significant reduction in the amount of wild-type ENAM available during the amelogenesis process, which translates into enamel hypoplasia.

Another gene, *DLX3*, has recently been reported to be involved in cases of hypoplastic/hypomature ADAI associated with taurodontism (HHADAIT). This gene codes for the protein homeodomain of the same name that acts as transcription

factor during development. A study involving an Australian family with HHADAIT found a deletion of 2 bp in exon 3 of the *DLX3* gene (Table 1; Dong et al. 2005). In a recent study of a Korean family with superimposed phenotypes of the syndrome tricho-dento-osseous (TDO) and HHADAIT, the same mutation was observed, and the authors postulated that the phenotypic variation was due to environmental effects, modifier genes, or the residual effect of genotype on the mutated gene (Lee et al. 2008b). On the other hand, in a detailed study of the phenotypic traits found in a family of unknown race (possibly of Swedish origin) with the same mutation in *DLX3*, Wright et al. (2008) proposed that this mutation causes an attenuated phenotype of TDO syndrome and not HHADAIT. In contrast, the work of Pavlic et al. (2007), who studied a case with similar characteristics of superimposed traits of AI and TDO, reported the absence of mutations in *DLX3*. Despite the controversy raised by the diagnosis of the two clinical types, both present affected enamel, and it is proposed that the mutation c.560_562delCT would affect the carboxy-terminus of the homeodomain DLX3 protein, which would cause a frameshift that inserts a premature stop codon and shortens the protein to 88 amino acids. Previously, the exact role of this gene in amelogenesis was unknown; however, work by Lézot et al. (2008) using mice with *dlx* knockouts shows that these genes participate in differentiation and spatial organization of the ameloblast and in the formation of enamel, thereby regulating the expression of amelogenin.

Hypocalcified AI is another type of ADAI. These types can be inherited as dominant and/or recessive autosomal traits, with constant expression of the clinical phenotype and complete penetration. Hypocalcified AI is present in Caucasian and Negroid races, generally in both dentitions, with thermal sensitivity and with or without anterior open bite (Chabora et al. 1972; Giansanti 1973). Clinically, the teeth are characterized by a yellowish brown color and enamel of normal thickness. The cervical areas are typically less affected than the crown areas, and the phenotype may appear as a rotten tooth with a claylike appearance (Wright et al. 1993a, b; 1997). The most prevalent form of AI in North America is the hypocalcified type, and three years ago (2007), the genetic basis of this type of AI was completely unknown. A study carried out in 2003 with two large families in the United States who had the hypocalcified ADAI phenotype excluded linkage of genetic markers in the genes *ENAM*, *AMBN*, *MMP20*, *TUFT1*, and *KLK4* (Hart et al. 2003a). Subsequently, a large Brazilian family with hypomineralized AI was studied using broad genomic screening and showed linkage with chromosomal region 8q24.3 (Mendoza et al. 2007). Another report analyzed the known candidate genes *ENAM*, *AMBN*, *AMELX*, *MMP20*, *KLK4*, and amelotin (*AMNT*) in two Brazilian families by PCR and sequencing and excluded the participation of these genes in the hypomineralized AI phenotype (Santos et al. 2007). Surprisingly, at the beginning of 2008, two studies carried out by the same group of researchers reported 6 mutations in the *FAM83H* gene, found in region 8q24.3, which is responsible for hypocalcified ADAI (Lee et al. 2008a). With the recent work of Hart et al. (2009), Wright et al. (2009), Hyun et al. (2009), and El-Sayed et al. (2010), the number of mutations in the *FAM83H* gene has increased to 16 (Table 1). All of these mutations are located in exon 5 of the gene, and they have been described in families of different ethnic origins. Nine of the mutations cause transitions, 5 cause

transversions, and 2 are deletions of two base pairs that each result in the premature termination of protein. The genotype–phenotype correlation analysis of these mutations has revealed that the more likely explanation for the molecular etiology of hypocalcified ADAI is that FAM83H protein is truncated to lengths less than 700 amino acids exerts a dominant negative effect. Truncated proteins that are greater than 700 amino acids would be responsible for a less severe clinical phenotype (Wright et al. 2009).

Autosomal recessive is the other type of autosomally inherited AI. Linkage studies have reported the association of syndromic ARAI with a locus on chromosome 2 in one family (Downey et al. 2002), but results in another four families that segregate with nonsyndrome ARAIs do not show linkage with this region (Michaelides et al. 2004). To date, only four genes have been demonstrated to possess causal roles in the etiology of this type of AI: the *ENAM* gene, the genes that code for the enamel proteases *MMP-20* and *KLK4*, and the gene encoding *WDR72* (El-Sayed et al. 2009). The four mutations described in the *MMP-20* gene (Table 1) are substitutions of a single base that affect exon 1, exon 5, exon 6, and intron 6. The final consequence of all the mutations is the significant reduction or the complete loss of MMP-20 catalytic activity, which explains the recessive phenotype observed in all four cases (Kim et al. 2005b; Ozdemir et al. 2005b; Papagerakis et al. 2008; Lee et al. 2010). On the other hand, the only mutation reported in the *KLK4* gene causes the hypomature ARAI clinical phenotype (Hart et al. 2004). This mutation (Table 1) is a base substitution in exon 4 of the gene that results in a nonsense mutation, replacing a conserved tryptophan residue by a stop codon at amino acid position 153. The effect of this change is the synthesis of a truncated protein that lacks 101 amino acids, including functionally important domains for the catalytic activity of the enzyme, which makes it nonfunctional. The loss of KLK4 function affects the maturation of the enamel by causing retention of proteins and resulting in the formation of crystals that do not complete their final growth and do not reach their normal degree of mineralization (Hart et al. 2004). The KLK4-induced changes in enamel maturation are consistent with the recessive phenotype.

New Candidate Genes

In many cases, the mutational analyses of families affected with AI fail to identify causative or associated mutations (Kim et al. 2008). Furthermore, it has been known for some time that the defects in causal genes explain less than half of all cases of AI (Hart et al. 2003a; Kim et al. 2006; Santos et al. 2007). The recent discovery of the 8q24.3 chromosomal locus, and the participation of genes such as *DLX3*, *FAM83H*, and *WDR72* in the etiology of AI, reveals that the selection of candidate genes is biased (Kim et al. 2006, 2008; Mendoza et al. 2007). Therefore, it is worth asking, "What could be the other relevant candidate genes in AI?" In an attempt to refocus the selection of candidate genes, we have identified an interesting aspect of the biomineralization phenomenon in the literature: the process of pH regulation during the formation of crystals in the secretory and enamel maturation stages.

Regulation of pH During Amelogenesis

During the secretory stage of amelogenesis, the earliest event is the formation and stabilization of thin, apatite crystals within an extracellular, organic matrix rich in amelogenins. The lengthening of the crystals occurs as the ameloblast retracts toward the crown from the dentin–enamel union and builds the complete thickness of the tissue layer by appositional growth. Once the secretory stage is complete, the ameloblast finishes depositing the matrix, shortens its cellular body, and, after a brief transitional stage, enters the maturity stage. During the secretory stage, the mineral deposited is mainly octacalcium phosphate, which results in a low rate of mineralization accompanied by large quantities of proteic material (mainly composed of amelogenins and, to a lesser degree, enamelin, ameloblastin, tuftelin, and enamelysin) with high buffering capacities in the enamel extracellular space that neutralize the production of protons from the formation of octacalcium phosphate. The net pH during the secretory stage is close to neutral (Smith et al. 2005; Lyaruu et al. 2008).

In the maturation stage, the situation is different because the ameloblast enters a process of cyclic modulation of its activity in which it fluctuates between two functionally relevant morphologies: the ameloblast with a smooth apical end and the ameloblast with a ruffled apical end (Simmer and Fincham 1995; Ten Cate 1998). The enamel matrix under the smooth-ended ameloblasts has been associated with a pH of 7.0–7.2, and the enamel layer under the ruffled-ended ameloblasts has an acidic pH of 5–6. During maturation, there is a high mineralization rate, and therefore the rate of proton generation is also high. At the same time, an accelerated phase of protein degradation is triggered by the serine proteases found in the matrix (KLK4), which allows a faster volumetric growth of the apatite crystals, especially with regard to their thickness. Eventually, the crystals expand to occupy 80% of the enamel layer volume and comprise 95% of the mature enamel weight. The degradation of proteins that maintain the pH of the extracellular space leaves the ameloblast without buffering capacity, and it has been hypothesized that the maturing ameloblast should be actively involved in the buffering of protons by secreting bicarbonate to the interior of the enamel during formation. The molecular mechanism that regulates the pH maintenance during enamel synthesis is unknown (Lyaruu et al. 2008; Smith et al. 2005).

Recently, two models have been described to explain the regulation of pH during the secretory and maturation stages of amelogenesis. Both of these mechanisms are based on the specific expression profile of two transport proteins that could be involved in the regulation mechanism of the acid charge generated after the formation of apatite. These are SLC4A4 (NBCe1) and SLC4A2 (AE2), which belong to the solute-linked carrier (SLC4) family of transport proteins (Lyaruu et al. 2008; Paine et al. 2008). In model 1 (Fig. 1), the polarized expression of the transporters *NBCe1* and *AE2* suggests that they play a role in the vectorial transcellular transport of bicarbonate from the basolateral side to the apical side of the secretory ameloblast. The incorporation of basolateral bicarbonate is mediated by *NBCe1*, and the secretion of apical bicarbonate, by *AE2*, is coupled to the transport of chlorine, which is mediated by the cystic fibrosis transmembrane

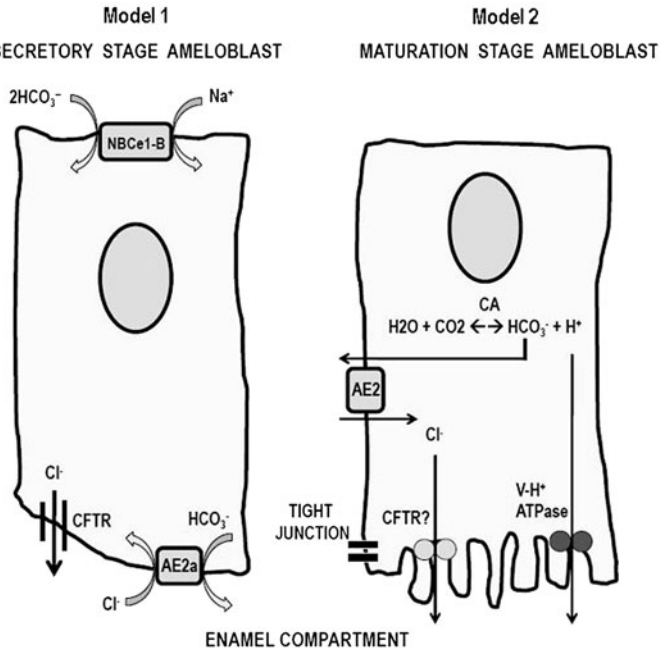

Fig. 1 Model 1: pH regulation in secretory ameloblasts in incisor teeth of mice with bicarbonate transport performed by the SLC4A4 (NBCe1) and SLC4A2 (AE2) transporter proteins (adapted from Paine et al. 2008). Model 2: pH regulation in the maturation-stage ameloblast conducted by the transport protein SLC4A2 (AE2) (from Lyaruu et al. 2008). *CA* carbonic anhydrase II; *V–H*$^+$ *ATPase* vacuolar hydrogen ATPase; CFTR cystic fibrosis transmembrane conductance regulator

regulator (*CFTR*) gene. Model 2 (Fig. 1) assumes that the ruffled ameloblasts in the maturation stage maintain a low pH environment in the enamel layer to catalyze the hydrolysis of proteins and/or direct the process of mineralization. To maintain this low pH, ameloblasts pump protons generated by carbonic anhydrase II toward the enamel space using the vacuolar H^+-ATPase. Then, the transporter, AE2, which is associated with the basolateral membrane, exchanges extracellular Cl^- for intracellular HCO_3^-. The accumulated Cl^- is transported toward the enamel by CFTR (Lyaruu et al. 2008). Obviously, there are differences with respect to the results of the expression of the AE2 transporter in both models, but they are not explained by the authors of the most recent model (Paine et al. 2008).

These findings allow us to propose the existence of new candidate genes for AI represented by their products: the transporter proteins AE2 (SLC4A2), NBCe1 (SLC4A4), vacuolar H^+-ATPase, CFTR, and the enzyme carbonic anhydrase II. Based on a bibliographic study evaluating some parameters, such as expression profiles of the mRNAs and/or proteins in the secretory-maturation stage of amelogenesis, known genic structure, existence of knockout mice for the gene, and the condition of the enamel in humans carrying mutations for these genes, we propose that *SLC4A4* (*NBCe1*), the transporter family of solute 4, member A4, is the most likely gene.

A New Candidate Gene, *SLC4A4*

In eukaryotes, intracellular and extracellular pH is regulated by membrane proteins that mediate the transport of bases (HCO_3^-, CO_3^{-2}). In mammals, there are two unrelated multigenic families, the transporters *SLC4* and *SLC26*. Recently, roles in the ion transport process during enamel formation have been attributed to some members of the *SLC4* family of transporters (Lyaruu et al. 2008; Paine et al. 2008). In humans, the genic *SLC4* family comprises nine members, including *SLC4A1–SLC4A5* and *SLC4A7–SLC4A10* (Pushkin and Kurtz 2006). Based on the nature of the specific transport process (e.g., exchange and/or cotransport) and the specific ions that are transported, the members of this family may be separated into three functional groups with potential transport modes for HCO_3^- and/or CO_3^{-2}: (1) Na^+-independent Cl^-/HCO_3^- exchangers that mediate the exchange of Cl for bases (HCO_3^-, CO_3^{-2}), (2) Na^+–HCO_3^- cotransporters that mediate the cotransport of Na^+ and bases, and (3) Na^+ driven Cl^-/HCO_3^- exchangers that mediate the exchange of Cl^- for Na^+ and bases. The SLC4 proteins all transport bases (HCO_3^-, CO_3^{-2}), but they differ in their ability to mediate the concomitant transport of Na^+ and Cl^-. The transporter SLC4A4 (also called NBC1, NBCe1) belongs to the group 2 transporter proteins that simultaneously transport Na^+–HCO_3^-, which mediates an electrogenic-type exchange with a stoichiometry of 2:1 or 3:1 (Pushkin and Kurtz 2006).

In humans, the *SLC4A4* gene located on 4q21 gives rise to two main protein variants. One is 1,035 amino acids in length and is expressed in the kidney (kNBCe1 and NBCe1-A). The other, 1,079 amino acids, is expressed in the pancreas (pNBCe1, NBCe1-B). The isoforms differ in their extreme amino termini and are derived from the gene by the use of alternative promoters. A smaller variant, rb2NBCe1 (NBCe1c), has also been cloned from rat brains. This variant is almost identical to the pancreatic form, and a very similar form has recently been identified in the human and porcine vas deferens (Pushkin and Kurtz 2006). *SLC4A4* covers a region of 23.2 kb in human genomic DNA and contains 23 exons and 22 introns. It has recently been cloned.

A great deal of evidence supports the nomination of *SLC4A4* as a new and likely candidate gene for AI in humans. *SLC4A4* is found on chromosome 4, where a group of genes associated with tooth development has been described (Abuladze et al. 2000; Burnham et al. 1997). Immunohistochemical analyses of rat incisors and molars have revealed that the SLC4A4 protein is expressed in the basolateral membrane of ameloblasts in the secretory stage in both types of teeth (Paine et al. 2008). RT-PCR analysis in cultured cells of the ameloblast-like LS8 cells identified the expression of the mRNA corresponding to the NBCe1-B isoform of the cotransporter. Furthermore, using the same cellular model system, this study showed that the NBCe1-B variant was expressed in a pH-dependent form and could be detected only at pH 6.0 (Paine et al. 2008).

On the other hand, there are reports of patients with confirmed mutations in *SLC4A4* (*NBC1)* that cause the disease known as proximal renal tubular acidosis (pRTA), which typically causes ocular abnormalities but also caused dental abnormalities in these patients (Gawenis et al. 2007; Paine et al. 2008). The patient with the homozygous mutation c.2311delA in *NBC1* showed multiple areas of

defective enamel, where distinct white stains similar to chalk appeared (Inatomi et al. 2004). The patient with the homozygous mutation c.1429C→T (the only one affected in that family) presents teeth with hypoplastic enamel with large areas of fractured enamel and white stains distributed homogeneously in all the teeth (Dinour et al. 2004). Furthermore, there is an earlier report of two patients with pRTA of unknown cause who had similar dental abnormalities (Gawenis et al. 2007; Winsnes et al. 1979). Similarly, it has been shown that mice lacking *SLC4A4* present severe metabolic acidosis, retardation of growth, low levels of plasmatic Na^+, hyperaldosteronism, splenomegalia, intestinal obstructions, abnormal dentition, and death before weaning. The dentition abnormality in these mice consists of white-clay teeth and easily detached enamel with a tendency to fracture (Gawenis et al. 2007).

In addition, the presence of the enzyme cytoplasmic carbonic anhydrase type 2 (which generates HCO_3^- and protons) in ameloblasts during the maturation stage sustains the existence of a system that generates bases and protons within the cell, which suggests the need for transport of protons or secretion of bicarbonate to the extracellular space of the enamel (Lyaruu et al. 2008). In two previously presented models, mice that were deficient in another gene appear to be linked to the mechanism of pH regulation during amelogenesis also present dental phenotypes that affect the enamel. CFTR knockout mice show an altered mineral content (hypomineralization) and the retention of matrix proteins in their teeth. On the other hand, it has been demonstrated that the expression of CFTR mRNA occurs in the bud of the apical tooth and in other mineralized tissues. Likewise, the extracellular pH of enamel in the incisors of mice showed that CFTR-deficient mice had more acidic enamel (Gawenis et al. 2007; Arquitt et al. 2002; Sui et al. 2003).

Finally, the clinical appearance described in patients with pRTA, who were homozygous for the two mutations reported in the *SLC4A4* gene (Inatomi et al. 2004; Dinour et al. 2004), the dental phenotype found in knockout mice (Gawenis et al. 2007), and the earlier report of two patients with pRTA who had dental abnormalities (Winsnes et al. 1979) allow us to speculate that mutations in other regions of this gene could alter the mechanism of pH regulation and interfere with the process of mineralization during amelogenesis. This interference could manifest itself most obviously in clinical phenotypes of hypoplastic or hypocalcified AI.

Acknowledgments This research has been supported by grants PRI-ODO 07/03 and FIOUCh 09-1 from the Faculty of Dentistry of the University of Chile.

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