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ORIGINAL ARTICLE

Unexpected identification of a recurrent mutation in the *DLX3* gene causing amelogenesis imperfecta

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OBJECTIVE: To identify the molecular genetic aetiology of a family with autosomal dominant amelogenesis imperfecta (AI).

SUBJECTS AND METHODS: DNA samples were collected from a six-generation family, and the candidate gene approach was used to screen for the enamelin (*ENAM*) gene. Whole-exome sequencing and linkage analysis with SNP array data identified linked regions, and candidate gene screening was performed.

RESULTS: Mutational analysis revealed a mutation (c.561_562delCT and p.Tyr188Glnfs*13) in the *DLX3* gene. After finding a recurrent *DLX3* mutation, the clinical phenotype of the family members was re-examined. The proband's mother had pulp elongation in the third molars. The proband had not hair phenotype, but her cousin had curly hair at birth.

CONCLUSIONS: In this study, we identified a recurrent 2-bp deletional *DLX3* mutation in a new family. The clinical phenotype was the mildest one associated with the *DLX3* mutations. These results will advance the understanding of the functional role of DLX3 in developmental processes.

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Introduction

Tricho-dento-osseous (TDO) syndrome is a highly penetrant, rare autosomal dominant genetic disorder which is characterized by inherited defects in the hair, tooth and bones (Hart *et al*, 1997). Curly/kinky hair at birth, hypoplastic enamel with less mineralization and thickening of the cortical bones are the main features of TDO syndrome (Shapiro *et al*, 1983). Other clinical phenotypes include brittle nails, mandibular prognathism and taurodontism (Wright *et al*, 1997). A common mutation responsible for TDO syndrome in the United States was identified (Price *et al*, 1998a,b), and the mutation was a 4-bp deletional mutation in the *DLX3* gene (c.571_574delGGGG and p.Gly191Argfs*66).

The hypomaturation and hypoplasia type of amelogenesis imperfecta with taurodontism (AIHHT) is a rare and unique type of genetic disorder with defective tooth enamel and elongated pulp chambers (Aldred *et al*, 2002). A 2-bp deletional mutation (c.561_562delCT and p.Tyr188Glnfs*13) in the *DLX3* gene was identified from an Australian family with AIHHT by linkage analysis and candidate gene sequencing (Dong *et al*, 2005). The affected individuals exhibited only the dental phenotype including taurodontism, without any hair and bone phenotypes. Subsequently, the same mutation was identified in a Korean and a Caucasian family (Lee *et al*, 2008; Wright *et al*, 2008). The affected individuals exhibited an enamel defect with pulp enlargement and hair phenotypes but without a bone phenotype. Pulp enlargement was moderate but variable among family members, and hair was curly and coarse.

The distal-less homeobox 3 (*DLX3*) gene is located on chromosome 17q21 and has 3 exons (all coding). It encodes a homeobox protein DLX3 that is 287 amino acids in length. DLX3 contains the homeodomain sequence in the middle of the protein (129–188 amino acids). The characteristic homeodomain sequence usually consists of a 60-amino acid-long helix-turn-helix structure with three alpha helices (Banerjee-Basu and Baxevanis, 2001).

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Homeobox proteins can bind to DNA and regulate the expression of targeted genes (Feledy *et al*, 1999). Therefore, these proteins have essential roles during early embryonic development and induce cellular differentiation by regulating gene expression through their DNA-recognition properties (Merlo *et al*, 2000).

Because *Dlx3* gene targeting in a mouse germ line resulted in early embryonic death due to a placental defect caused by altered development of the labyrinthine layer (Morasso *et al*, 1999), conditional and/or targeted deletions have been used to identify the functional roles of DLX3 (Hwang *et al*, 2008; Lezot *et al*, 2008; Duverger *et al*, 2012, 2013). DLX3 has an essential role during the development of hair follicles, bone, enamel and dentin.

In this study, we identified a family with amelogenesis imperfecta with hypomaturation and hypoplastic type but seemingly lacking any other clinical phenotypes. Mutational analysis identified the same 2-bp deletional *DLX3* mutation through a long roundabout way. This family could represent the mildest clinical phenotype associated with a recurrent *DLX3* mutation.

Materials and methods

Recruitment of the family

The protocol of this study was independently reviewed and approved by the institutional review board of the Seoul National University Dental Hospital and the University of Istanbul. The participating individuals were informed of the study and agreed to participate. Clinical examinations and blood collections were performed with written consent according to the Declaration of Helsinki.

DNA isolation and candidate gene sequencing

DNA was isolated from peripheral whole blood with the QuickGene DNA whole blood kit S (Fujifilm, Tokyo, Japan). The quantity and quality of the DNA were measured with spectrophotometry by the OD_{260}/OD_{280} ratio. Candidate gene sequencing of the exons and adjacent intron sequences of the *ENAM* gene were performed using the DNA of the proband. PCR amplifications were performed according to a previous report (Kim *et al*, 2005) with the HiPi DNA PCR premix (ElpisBio, Taejeon, Korea). The amplification products were purified with a PCR purification kit (ElpisBio) and submitted to a DNA sequencing centre for sequencing (Macrogen, Seoul, Korea). Mutation Surveyor® (SoftGenetics, State College, PA, USA) was used for sequence analyses.

Whole-exome sequencing

The DNA samples of two affected (V:8 and VI:7) and one unaffected (V:9) individuals were captured with SureSelect Target Enrichment System (Agilent Technologies, Santa Clara, CA, USA). Ninety-bp paired-end sequencing reads were generated with the Illumina HiSeq 2000. The Burrow–Wheeler Aligner was used to align the sequencing reads to the human reference genome (NCBI build 37.2, hg19). After series of bioinformatic analyses with SAMtools and GATK (Li *et al*, 2009; McKenna *et al*, 2010), single-nucleotide variants and short insertions/deletions were obtained. The single-nucleotide polymorphism database (dbSNP build 138) was used to annotate the sequence variations.

SNP array and linkage analysis

SNP array analysis was performed for all participating members (four affected and seven unaffected). Data obtained from the Affymetrix Genome-Wide Human SNP array 6.0 (DNA LINK Inc., Seoul, Korea) were used to detect any pathologic copy number variations (deletion or duplication). SNPs were annotated for all individuals, and linkage analysis was performed with Superlink online SNP ver. 1.1 (http://cbl-hap.cs.technion.ac.il/superlink-snp/) (Silberstein *et al*, 2013).

Polymerase Chain Reaction (PCR) and sequencing

The identified variation in the *DLX3* gene was confirmed with Sanger sequencing, and segregation within family was confirmed with primers previously described (*DLX3* exon 3; sense: 5′-ATT GGGTTCTGGCCTTTCTT, antisense: 5′-GCCTTCTGCCTGGTCCTG) (Kim *et al*, 2006). PCR amplification and sequencing were performed as described above.

Results

The proband was a 9-year-old female (Figure 1a). According to the six-generation family pedigree, it was obvious that the disease was transmitted as an autosomal dominant inheritance pattern, based on the male-to-male transmission. The proband had localized enamel hypoplasia with hypomaturation but was otherwise healthy without any

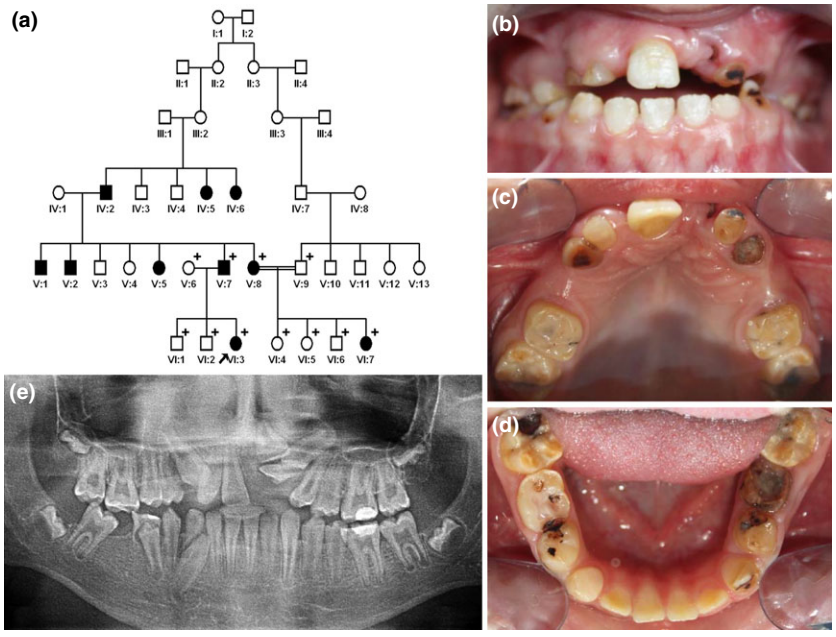

Figure 1 Pedigree, clinical photographs and panoramic radiograph of the family. (**a**) The disease was transmitted to the proband (VI:3) from her father (V:7). The + symbols indicate family members who participated in this study. (**b–d**) Clinical photographs of the proband at age 9. Localized enamel hypoplasia was noted on the labial sides of the anterior teeth and hypomaturation in the molar teeth caused excessive attrition. (**e**) Panoramic radiograph of the proband (VI:7) at age 13. There was no taurodontic feature in any tooth.

other medical conditions (Figure 1b–e). There were no other clinical symptoms in the hair, nails and bones.

Therefore, we screened for the *ENAM* gene as a candidate gene approach, based on the autosomal dominant inheritance and hypoplastic enamel defect. Candidate sequencing of the *ENAM* gene did not reveal any pathologic variations. Then, we did whole-exome sequencing and analysed data with conventional filtering conditions for SNP and indel calling. Candidate variants, which were common in the affected individuals but not present in the unaffected individual, were selected and tested for all participating family members. However, no candidate variant was found to cosegregate with the disease phenotype in the family.

Multipoint linkage analysis was performed to locate the chromosomal region linked to the disease, even though the maximum LOD score would be <3 with a limited number of family members. Among the five linked regions obtained with a maximum LOD score of 2.1 (Figure S1), chromosome 17 had the longest linked region spanning 17.5 cM (chr17:29,784,258–49,844,513) (Figure 2). Whole-exome data were re-analysed without any filtering options. Variants within the linked regions were searched, and a recurrent 2-bp deletional variant (c.561_562delCT and p.Tyr188Glnfs*13) in the *DLX3* gene was identified. Sanger sequencing of all family members confirmed this variant as a disease-causing mutation in this family.

After finding the mutation, the clinical phenotype of the affected individuals was re-examined (Figure 3). The proband's father (V:7), who was 35 years old, had lost many teeth and wore partial dentures. Panoramic radiograph and clinical examination showed thin enamel with less mineralization. The proband's aunt (V:8), who was 33 years old, also had generalized enamel hypoplasia with hypomaturation. Her third molars showed elongated pulp chambers. Panoramic radiograph of the proband's cousin (VI:7) revealed slight elongation of the pulp chamber in the left second permanent molars. A cephalometric radiograph, taken at age 16, showed no bone-related abnormality, including increased bone density or sclerotic changes. The proband's hair was not curly at birth, but her cousin had curly hair at birth but is now less curly.

Discussion

Among the clinical phenotypes, the enamel defect is the most constant feature of TDO syndrome. The thin and less mineralized enamel results in discoloration and excessive

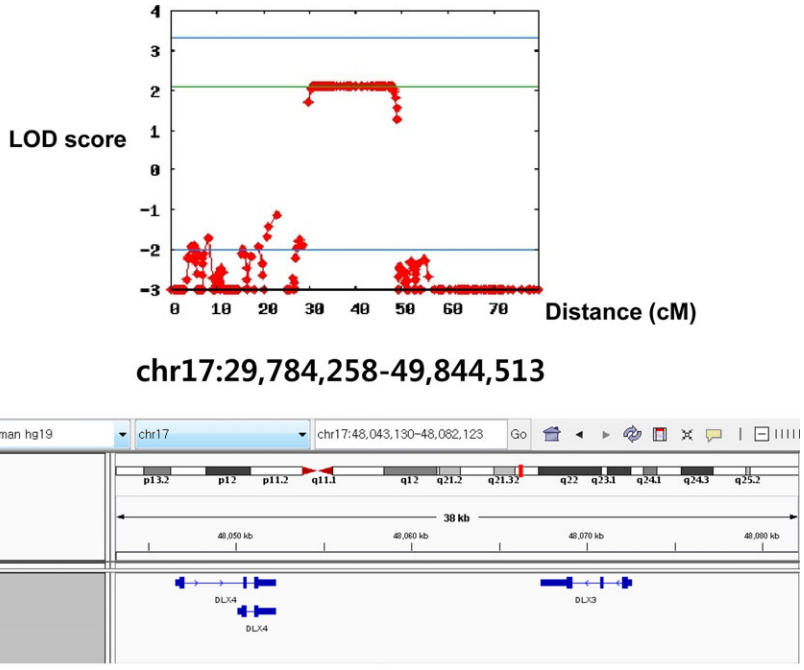

Figure 2 Mutational analysis. Superlink online analysis revealed linkage to the long region of the chromosome 17 with a LOD score of 2.1. The linked region is shown below the LOD plot. The *DLX3* gene is located within the linked region, shown with the Integrated Genome Viewer 2.3. Sanger sequencing chromatogram of a wild-type individual (VI:6) is shown above, and the chromatogram of the proband is shown below. The position of the deletion is indicated by a red arrow.

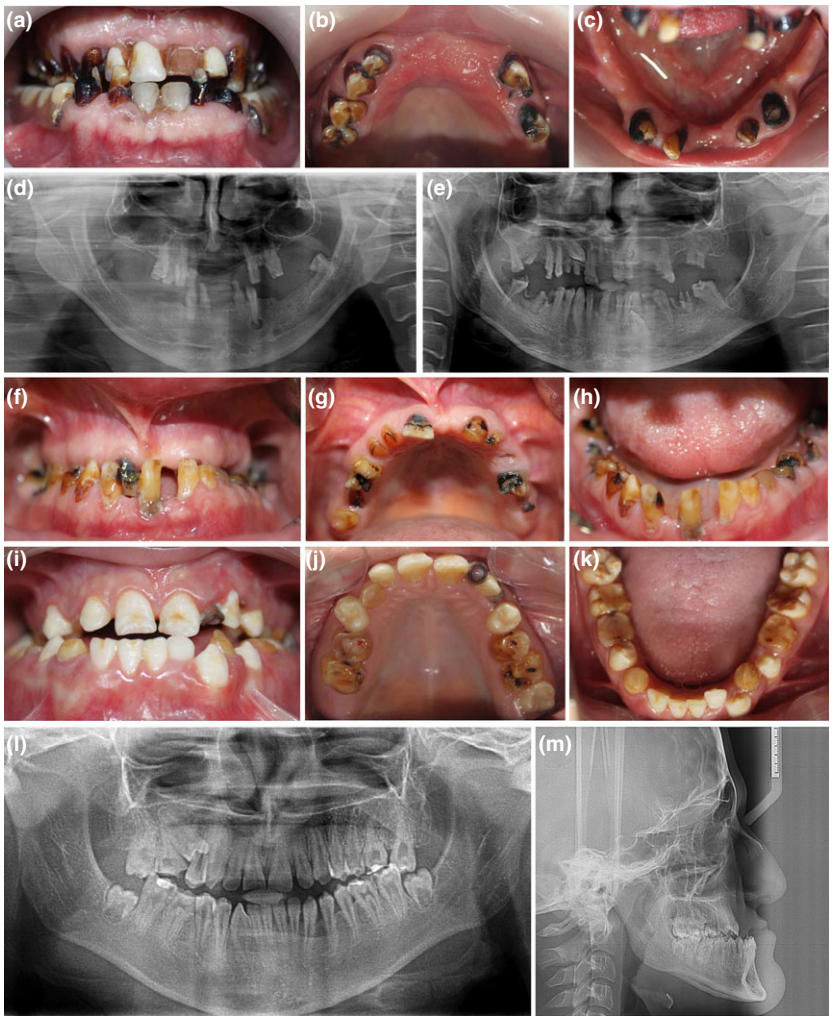

Figure 3 Clinical photographs and radiograph of the other affected individuals. (**a–d**) Clinical photographs and panoramic radiograph of the proband's father (V:7) at age 35. He retained only several teeth and wore partial dentures. Remaining teeth also showed general destruction with attritions due to hypomineralization and hypoplasia. (**e–h**) Clinical photographs and panoramic radiograph of the proband's aunt (V:8) at age 33. Her dentition also showed excessive attrition and discoloration due to hypomaturation and hypoplasia of the affected enamel. Remaining third molars showed elongated pulp chambers. (**i–l**) Clinical photographs of the proband's cousin (VI:7) at age 11 and panoramic radiograph at age 15. Hypoplastic enamel with hypomaturation resulted in excessive attrition and discolorations, especially in the posterior teeth. Left second permanent molars had slight pulp chamber elongation. (**m**) Cephalometric radiograph of the proband's cousin (VI:7) at age 16. There was no bone-related abnormality, including increased bone density or sclerotic changes.

attrition, causing an early destruction of the tooth materials and pathologic pulp involvement (Hyun and Kim, 2009). The expression of *DLX3* has been shown in both tooth epithelium and mesenchyme (Zhang *et al*, 2015). Especially, *DLX3* is constantly expressed in the ameloblast throughout all amelogenesis processes (presecretory, secretory and maturation stages) (Ghoul-Mazgar *et al*, 2005). Therefore, a defective DLX3 can affect both the thickness and degree of maturation of the enamel.

DLX3 has three main domains: an N-terminus and C-terminus transactivation domain and a central homeodomain. The homeodomain can directly interact with DNA in a sequence-specific manner and regulates the expression of target genes during many development processes (Feledy *et al*, 1999). The deletion of the transactivation domain has been shown to cause the loss of Dlx3 function as a transcription factor, indicating that these subdomains have important roles in homeodomain-DNA binding (Feledy *et al*, 1999).

To date, six *DLX3* mutations are known to exist (Table 1). In general, missense mutations, which change conserved amino acids in the homeodomain (129–188 amino acids), result in the most severe clinical phenotypes (Mayer *et al*, 2010; Nieminen *et al*, 2011; Li *et al*, 2015). In addition to the tooth phenotypes including enamel hypoplasia and hypomaturation, defective dentin formation and taurodontic features, the hair and bone phenotypes are usually clearly manifested in these cases. In some cases, the hair is sparse, resembling lanugo or uncombable (Mayer *et al*, 2010; Nieminen *et al*, 2011). It is believed that these clear phenotypes are caused by the loss of the DNA-binding ability due to missense mutations.

The first identified 4-bp deletional mutation (c.571_574delGGGG and p.Gly191Argfs*66) right after the homeobox region is the most common aetiology in Virginia and North Carolina TDO families and transmitted from a common ancestor (Price *et al*, 1998a). The clinical variability is believed to be from environmental factors and/or other genetic modifiers (Price *et al*, 1998b). This mutation does not change the homeodomain but results in frameshift changing a glycine at the 191 amino acid position to arginine and encodes a novel 65 amino acid sequence instead of a normal 97 amino acid sequence.

The 2-bp deletional mutation (c.561_562delCT and p.Tyr188Glnfs*13) has been identified in multiple families with different ethnicities, indicating this is a mutational hotspot in the *DLX3* gene (Dong *et al*, 2005; Lee *et al*, 2008; Wright *et al*, 2008). However, clinical phenotypes are different among the families with different ethnicity. An Australian family exhibited AIHHT without any hair

Table 1 Disease-causing mutations in *DLX3* gene

Location	*cDNA*	*Protein*	*Mode of inheritance*	*References*
Exon 2	c.398G>C	p.Arg133Pro	AD	Nieminen *et al* (2011)
Exon 3	c.524T>C	p.Ile175Thr	AD de novo	Mayer *et al* (2010)
Exon 3	c.533A>G	p.Gln178Arg	AD de novo	Li *et al* (2015)
Exon 3	c.545C>T	p.Ser182Phe	AD	Nieminen *et al* (2011)
Exon 3	c.561_562delCT	p.Tyr188Glnfs*13	AD	Dong *et al* (2005), Lee *et al* (2008), Wright *et al* (2008)
Exon 3	c.571_574delGGGG	p.Gly191Argfs*66	AD	Price *et al* (1998a,b)

*Sequences based on the reference sequence for mRNA (NM_005220.2) and protein (NP_005211.1), where the A of the ATG translation initiation codon is nucleotide 1.

and bone phenotypes (Aldred *et al*, 2002), but two other families (Korean and Caucasian) exhibited attenuated phenotype of TDO syndrome (Lee *et al*, 2008; Wright *et al*, 2008). In this report, we identified the same mutation in a Turkish family. The clinical phenotype was the mildest among the families with the same mutation. There was no bone-related phenotype, and pulp enlargement was absent or minimal except for the third molars. The proband did not have curly hair, but another affected individual had curly hair at birth. The clinical variability among families with the same 2-bp deletional mutation could be from the environmental and/or other genetic modifiers as in the cases of the 4-bp deletional mutation. However, it is obvious that the 2-bp deletional mutation is milder than the other mutations.

How can we explain the mild phenotype of the recurrent 2-bp deletional *DLX3* mutation? The CT deletion results in a frameshift changing a tyrosine at the 188 amino acid position, the last amino acid in the homeobox, to glutamine and encodes a novel 12 amino acid sequence instead of a normal 100 amino acid sequence. Both deletional mutations have a normal N-terminal domain and homeodomain. With an intact nuclear localization signal in the homeobox domain, these two truncated DLX3 would translocate into the nucleus (Nieminen *et al*, 2011). Without a normal C-terminus transactivation domain, both mutant DLX3s would lose their transcriptional activity by themselves. However, it is well known that certain transcription factors can interact to modulate developmental processes (Jolma *et al*, 2015). Therefore, the shorter C-terminal novel sequence from the 2-bp deletion would result in less harmful effects than that of the longer one from a 4-bp deletion for the interactions between transcription factors, resulting in a milder phenotype.

In conclusion, we identified a recurrent 2-bp deletion in the *DLX3* gene in a new family and described their mild clinical phenotype related to the *DLX3* mutation. Further molecular genetic and functional biochemical studies would be necessary to better understand the essential developmental role of normal and mutant DLX3 proteins.

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Author contributions

Y.-J. Kim designed the study and drafted the manuscript. F. Seymen designed the study and revised the article. F. Seymen, M. Koruyucu, Y. Kasimoglu and K. Gencay involved in sample collection and screened the patients. T.J. Shin, H.-K. Hyun and Z.H. Lee analysed the data and revised the article. J.-W. Kim designed study, and supervised and revised the article.

Conflicts of interest

The authors declared no conflict of interests.

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Supporting Information

Additional Supporting Information may be found in the online version of this article:

Figure S1. Multipoint linkage analysis result.