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ORIGINAL ARTICLE

Exclusion of *p63* as a candidate gene for autosomal-dominant amelogenesis imperfecta

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Abstract

Objective. Mutations within the *p63* gene have been shown to cause ectodermal dysplasia syndromes affecting a spectrum of developmental abnormalities, including ectodermal appendages, e.g. enamel. The affected teeth have a similar phenotype as another dental disorder, amelogenesis imperfecta (AI), a disease of genetically determined abnormal enamel formation in the absence of systemic symptoms. The genetic basis of particular forms of AI has been found, although the gene(s) responsible for the most prevalent AI types has not been identified. ***Material and Methods***. DNA samples of 41 individuals (25 affected and 16 unaffected) from 6 Swedish families with autosomal-dominant AI were screened for mutations (by partially denaturing HPLC) and sequenced. ***Results***. No mutation in *p63* was found in these families. ***Conclusions***. *p63* is not responsible for different forms of autosomal-dominant AI in the Swedish families studied. The roles of *p63* in tooth development and in the genetic etiology of AI remain to be identified.

Key Words: *Amelogenesis imperfecta, enamel, p63*

Introduction

The *p63* gene acts as a key regulator in limb, epithelial, and craniofacial development. *p63* null mice have severe developmental abnormalities, such as deficiencies in the development of squamous epithelia and their derivatives, including teeth [1]. Distinct heterozygous mutations in the *p63* gene have been shown to cause five ectodermal dysplasia syndromes, including EEC (ectrodactyly ectodermal dysplasia-clefting), AEC (ankyloblepharon-ectrodactyly ectodermal dysplasia), ADULT (acro-dermato-ungual-lacrimal-tooth), LMS (limb-mammary syndrome), and SHFM (non-syndromic split hand/foot malformation) [2–4]. These conditions encompass a spectrum of developmental abnormalities including ectodermal appendages. Dental anomalies are frequently observed in the AEC and ADULT syndromes, with the main manifestations being absent or reduced numbers of teeth, some patients showing underdeveloped teeth and in addition various defects of the enamel, phenotypes similar to amelogenesis imperfecta (AI), another inherited tooth disorder.

The diagnosis AI implies a genetically determined abnormal enamel formation in the absence of systemic symptoms. It has a high degree of clinical and genetic diversity and is broadly divided into hypoplastic, hypocalcified, and hypomaturation types according to the thickness and hardness of the enamel layer. The prevalence of AI is reported at 1/700 in northern Sweden [5] and 1/14,000 in the USA [6]. AI can be inherited as an X-linked – an autosomal-dominant – or an autosomal-recessive trait. Development of the dental enamel is controlled by the action of enamel proteins and proteinases secreted by ameloblasts such as amelogenin, ameloblastin, enamelin, tuftelin, enamelysin, and kallikrein-4. Fourteen different mutations within the amelogenin gene (*AMELX*) on the X chromosome have been identified in individuals with X-linked AI [7]. Mutations responsible for autosomal-recessive AI have also been found in the enamelin gene

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(*ENAM*) [8], the kallikrein-4 gene (*KLK4*) [9], and in the enamelysin gene (*MMP20*) [10].

The most common AI types in Europe and the USA are autosomal-dominant AI (ADAI). Several mutations of *ENAM* have been detected in some ADAI types and the same mutation resulted in variable severity of the enamel defects [11–14]. So far, the gene(s) responsible for most ADAI cases has not been identified, though several candidate genes encoding enamel matrix proteins are proposed. In addition, a hitherto unknown gene or a gene not considered to be a major contributor to enamel formation has been suggested [15]. Recently, one type of ADAI, hypoplastic-hypomaturation with taurodontism (AIHHT), was found to be associated with a mutation in *DLX3* [16], a gene known to be responsible for the Tricho-dento-osseous (TDO) syndrome [17], indicating that particular syndromes and AI may have similar pathogenesis.

It has been shown that different ectodermal dysplasia syndromes have mutations within different locations of the *p63* gene [2], so the aim of the present study was to perform mutation analysis of the *p63* gene in six families with ADAI to evaluate whether a disease affecting one of these ectodermally derived structures, such as AI, had any genetic defect within this gene.

Material and methods

Patients

The study was performed on 6 ADAI families with a total of 41 individuals (25 affected and 16 unaffected), all of whom, except family R, have been described previously [18]. Patients with AI were clinically characterized according to the criteria of Witkop & Sauk (1976) and none had ectodermal dysplasia syndromes. All the individuals included had agreed to provide their DNA for the purpose of finding genes and mutations causing AI and this was approved by the ethics committee of the medical faculty. The pedigrees of the ADAI families in this study and their clinical features are shown in Figure 1.

Polymerase chain reaction

Exons of the *p63* gene from affected and unaffected individuals were amplified by gradient PCR on genomic DNA. Intron-specific primers previously used for amplification of exons 1–15, exon 3′, and flanking introns of the *p63* gene were modified in some cases [14]. PCR was performed in a 50-μl volume using AmpliTaq Gold DNA polymerase (Applied Biosystems). Amplification was performed in a GeneAmp 9600 thermal cycler (Perkin-Elmer). After the initial incubation, for 7.5 min at 95°C, samples were subjected to 35 cycles of 1 min at 94°C, 2 min at different annealing temperatures, and

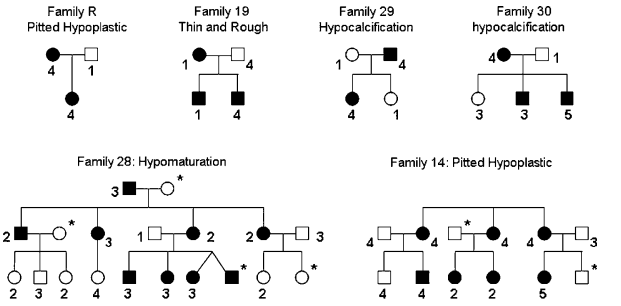

Figure 1. Pedigrees of the six families with different clinically characterized ADAI. Females are represented by circles and males by squares. Affected individuals are indicated by filled symbols and non-examined individuals by asterisks. Profiles of elution peaks of exon10 PCR products are also shown: 1 represents single peak, 2 represents the same single peak with changed elution time, 3 represents 2 peaks, 4 represents 3 peaks, 5 represents 3 peaks with different elution times from the parent.

1 min at 72°C. The final extension step was performed at 72°C for 10 min. Resulting fragments were analyzed by gel electrophoresis.

Heteroduplex analysis and sequence analysis

PCR products from the *p63* gene were analyzed by partially denaturing HPLC (dHPLC) using the WAVE DNA fragment analysis system (Transgenomic). The presence of heteroduplex and hence of a possible mutation in the sample is visualized as a change in the pattern of elution peaks. The pattern of peaks of the unaffected individuals of the family were set as wild-type controls and compared with those of the affected members.

PCR products containing heteroduplex detected by dHPLC and being considered as possible mutant alleles were purified using High Pure PCR Product Purification Kit (Roche) and sequenced by the use of BigDye Terminator v3.1 Cycle Sequencing Kit (Applied Biosystems). After purification on AutoSeq G-50 columns (Amersham Biosciences) the reactions were analyzed using an ABI PRISM 377 sequencer (Applied Biosystems).

Results

Six ADAI families with a total of 41 individuals (25 affected and 16 unaffected) were analyzed in this study. Results of electrophoresis analysis of PCR products showed the successful amplification of the different *p63* exon/introns.

Screening for *p63* gene mutations was performed using dHPLC analysis, which exploits the differential melting properties of homo- and heteroduplex DNA in the detection of potential mutations. The presence of genomic alterations in some exon/introns of *p63* was detected in this study. The profiles of elution peaks of exon10 amplicons are shown in Figure 1. Altered peak patterns were also found on

other exon amplicons in some family members. A mutation with the potential to cause disease should have a different peak pattern compared to unaffected individuals from the same family. In the present study, most changes were seen not only in affected but also in unaffected members, indicating presence of a polymorphism that does not associate with disease. However, results from exon 10 indicated a possible mutation, as in families R, 29 and 28, the heterozygote was only seen in AI patients and not detected in other unaffected family members. However, no mutation was identified in exon 10 at sequencing; instead, the heterozygote detected in dHPLC was due to intronic variants in intron 10, previously reported as single nucleotide polymorphisms (refSNP ID: rs9840359 (C/G) and rs9840360 (A/G)). Figure 2 shows the dHPLC elution profiles (left) and the corresponding sequencing results (right) of *p63* exon 10/intron 10 PCR products from three family R members.

Discussion

Ectodermal dysplasia syndromes such as AEC and ADULT show manifestations, e.g. absent or reduced numbers of teeth, underdeveloped teeth, and various defects of the enamel – phenotypes similar to those seen in AI, another inherited tooth disorder. In patients with these syndromes, mutations within different locations of the *p63* gene have been found. We therefore performed mutation analysis of the *p63* gene in six families with ADAI in order evaluate whether a disease affecting only one of these ectodermally derived structures, e.g. AI, also had genetic defects within the *p63* gene.

No association was found between these intronic variants and AI. In some families, two variants in intron 10 were seen, but only in patients not in healthy members of the family, whereas in other families the prevalence of intronic variants was the same in patients and normal members, indicating that the variants do not account for the disease but are common single nucleotide polymorphisms as previously reported.

Identification of genes responsible for particular AI types has improved our understanding of this disease and indicates its genetic complexity. Multiple enamel matrix proteins have been found to participate in the highly controlled enamel formation and perform specific functions. Most studies have focused on these molecules and found that mutations in four genes (*AMELX*, *ENAM*, *KLK4*, and *MMP20*) cause different types of AI, whereas only mutations in *ENAM* have been shown in association with hypoplastic ADAI. A nonsense mutation in *ENAM* caused local hypoplastic ADAI, which accounts for 27% of AI cases in Sweden. The ADAI family members in this study have no mutation in this gene [13]. Hence, the genes responsible for AI in these ADAI families remain unknown.

The results of the present study show that *p63*, a gene responsible for some ectodermal dysplasia

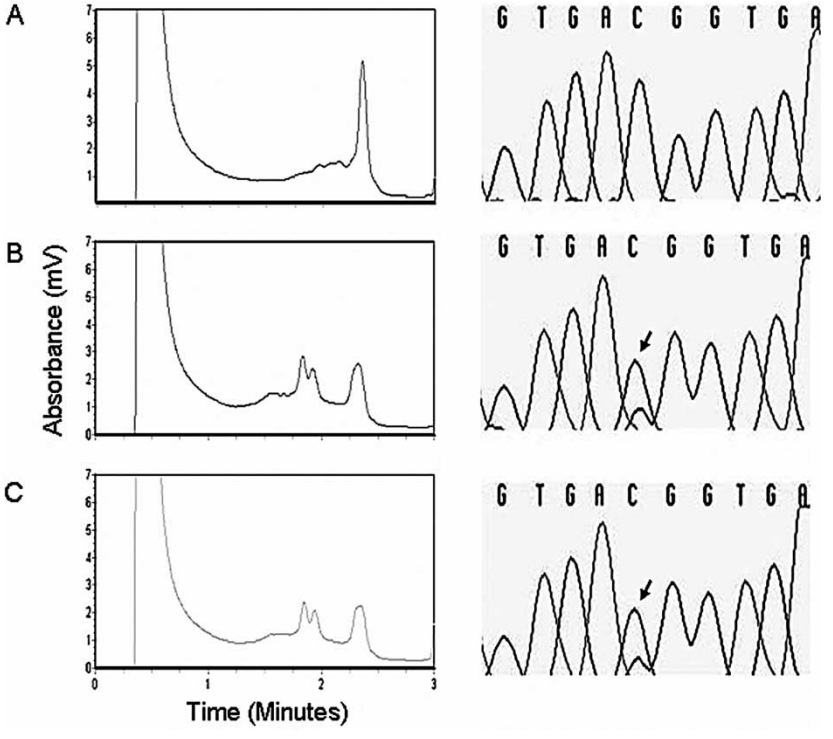

Figure 2. WAVE elution profiles (*left*) and the corresponding DNA sequencing chromatograms (*right*) of *p63* exon/intron10 from family R with pitted hypoplastic ADAI. A. Sample 1 of the unaffected father shows a single peak. B. Sample 2 of the affected mother shows three peaks and the sequence result showed an intronic variant (C/G) indicated by arrow. C. Sample 3 of the affected daughter also has this variety. The heterozygous site resulting in the formation of heteroduplexes was resolved as three peaks on the dHPLC chromatogram.

syndromes and for proper development of teeth, is not associated with AI. It seems that the dental features common to both AI and these ectodermal dysplasia syndromes are caused by different genes and that there may be other so far unidentified genes responsible for these diseases. Previously it has been shown that patients with mutation in the *COL1A1 (collagen type 1 A)* gene have both dentinogenesis imperfecta and osteogenesis imperfecta [19], whereas those with mutation in the *DSPP* gene have DI only [20,21]. Using today's criteria for definition of AI, we included patients with enamel disturbances only. However, based on the present data, it cannot be excluded that, if patients with AI and an additional ectodermal syndrome were analysed, disturbances within the *p63* gene could be found to play a role.

Acknowledgments

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