

Analysis of the *COL17A1* in non-Herlitz junctional epidermolysis bullosa and amelogenesis imperfecta

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Abstract. Non-Herlitz junctional epidermolysis bullosa (nH-JEB) disease manifests with skin blistering, atrophy and tooth enamel hypoplasia. The majority of patients with nH-JEB harbor mutations in *COL17A1*, the gene encoding type XVII collagen. Heterozygotes with a single *COL17A1* mutation, nH-JEB defect carriers, may exhibit only enamel hypoplasia. In this study, to further elucidate *COL17A1* mutation phenotype/genotype correlations, we examined two unrelated families with nH-JEB. Furthermore, we hypothesized that *COL17A1* mutations might underlie or worsen the enamel hypoplasia seen in amelogenesis imperfecta (AI) patients that are characterized by defects in tooth enamel formation without other systemic manifestations. We therefore conducted *COL17A1* mutational analysis in three patients from two AI families. One nH-JEB patient showed no *COL17A1* expression and was a compound heterozygote for the novel premature termination codon (PTC) mutations 1285delA and Q1387X. In addition, reduced *COL17A1* expression was found in a second nH-JEB patient who was homozygous for the novel PTC mutation 4335delC, the most carboxyl terminal PTC mutation thus far identified. Due to nonsense mediated mRNA decay, the position of these PTC mutations is thought not to influence the effect of *COL17A1* transcript loss and hence the severity of the nH-JEB phenotype. This study is the first to suggest that type XVII collagen carboxyl PTC mutations lead to restoration of

truncated polypeptide expression and to a milder clinical disease severity in nH-JEB. Conversely, we failed to detect any pathogenic *COL17A1* defects in AI patients, in either exon or within the intron-exon borders of AI patients. This study furthers the understanding of mutations in *COL17A1* causing nH-JEB, and clearly demonstrates that the mechanism of enamel hypoplasia differs between nH-JEB and AI diseases.

Introduction

Type XVII collagen, 180-kDa bullous pemphigoid antigen is a transmembrane glycoprotein expressed in stratified and other complex epithelia, such as skin, mucous membranes, and the eye (1,2). Type XVII collagen was initially identified as a major autoantibody target in bullous pemphigoid whereas mutations in the gene encoding this protein have been shown to cause junctional epidermolysis bullosa (JEB) (3). These facts indicate that type XVII collagen plays a crucial role in dermo-epidermal attachment and hemidesmosome stability within the basement membrane zone.

JEB comprises a clinically and genetically heterogeneous group of disorders with tissue separation occurring within the lamina lucida of the epidermal basement membrane. To date, two major clinical variants of this subtype have been described: the moderately severe non-Herlitz JEB subtype (nH-JEB) characterized by life-long blistering and a non-lethal course; and the Herlitz type of JEB, associated with more severe and extensive blistering and a high perinatal mortality (4-6).

nH-JEB is characterized by several integument manifestations, including: blistering with skin atrophy, diffuse alopecia with loss of some eyelashes, eyebrows and pubic hair, as well as nail dystrophy and dental abnormalities. Recent studies showed that the gene encoding type XVII collagen (*COL17A1*) contains mutations in the majority of the patients with nH-JEB (6-14). However, the precise pathogenic mechanisms of all these various skin symptoms have yet to be elucidated.

Amelogenesis imperfecta (AI) is a rare heterogeneous group of genetic disorders characterized by defects in tooth enamel formation in the absence of any generalized or systemic

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Abbreviations: AI, amelogenesis imperfecta; nH-JEB, non-Herlitz junctional epidermolysis bullosa; PCR, polymerase chain reaction; PTC, premature termination codon

Key words: type XVII collagen, blistering, enamel hypoplasia, mutation

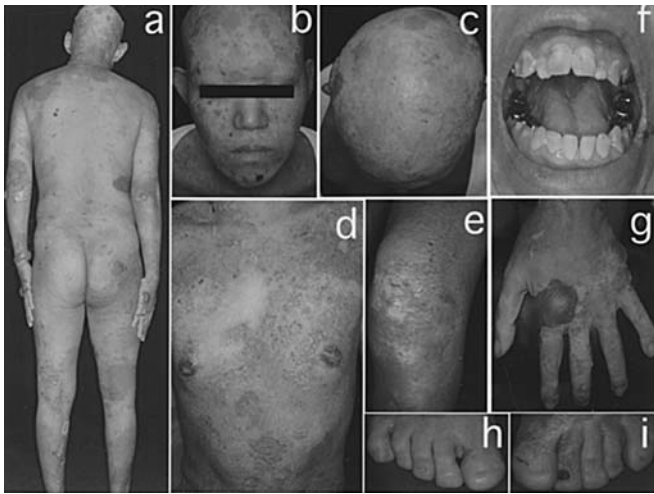


Figure 1. Clinical features of patient 1. There were many erosions and hyperpigmented or depigmented areas over his whole body (a), face (b), scalp (c) and trunk (d). Marked atrophic skin was found on his right knee (e). All his teeth showed abnormal enamel, with pitting and widespread dental caries (f). Large hemorrhagic blisters were seen on the left hand (g). Onychia is seen on all the toenails and some fingernails (h and i).

diseases (15,16). Significant progress has been made in determining the genetic background underlying AI. Mutations in the enamel gene cause autosomal-inherited forms of AI, while X-linked AI results from mutations in the amelogenin. Also, kallikrein-4, MMP-20 and DLX-gene have been shown to be causative genes in a limited number of cases. Congenital enamel hypoplasia and the resulting tooth pitting associated with severe dental caries are also characteristic features of nH-JEB. Particular *COL17A1* glycine substitution mutations can lead to enamel hypoplasia without skin blistering only when combined with a normal allele (9).

This study examined *COL17A1* mutations in two unrelated families with nH-JEB. Furthermore, to examine any possible correlation between enamel hypoplasia and nucleotide changes of *COL17A1*, we also searched for *COL17A1* mutations in three patients from two distinct families with amelogenesis imperfecta (AI).

Materials and methods

Families with nH-JEB

Patient 1. A proband was a 21-year-old male from family 1. He was the offspring of healthy nonconsanguineous parents and his elder brother was clinically unaffected. The patient had suffered recurrent blistering over his entire body since birth and had then suffered hair loss, dental problems, and dystrophic nails since infancy. Upon clinical examination (Fig. 1), bullae were seen on his extremities, abdomen, face, neck, and head, and there were depigmented or hyperpigmented skin lesions at recurrent sites of blistering without the presence of scars. The patient was bald, with an absence of eyelashes, eyebrows, pubic and axillary hair. His fingernails and toenails were dystrophic or missing. Marked hypoplasia of his permanent dentition was seen.

Patient 2. The second proband was a 41-year-old male from family 2. He was the offspring of healthy parents and there

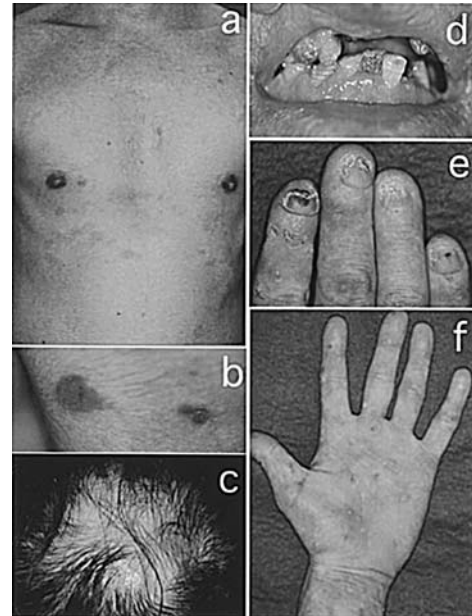


Figure 2. Clinical appearance of patient 2. There are several erosions and light hyperpigmentation on the trunk (a and b). On the parietal vertex of the scalp, there was focal non-scarring alopecia (c). Marked hypoplasia of the permanent dentition and dental caries were present (d), together with dystrophy of his fingernails (e), and small bullae (f) on his left palm were also seen.

was no history of consanguinity. No other family member had relevant skin manifestations. The patient had suffered from skin fragility over his whole body since birth. His hair growth was normal, but blisters and erosions had occurred on the scalp that led to patches of atrophic alopecia. The blisters healed without scars but some atrophy and hyperpigmentation had taken place. Upon clinical examination (Fig. 2), several bullae or erosions were seen on his extremities. Depigmentation or hyperpigmentation were present at recurrent sites of blistering without the presence of scars. Some of his fingernails were dystrophic or missing. There were irregular-shaped areas of alopecia on the parietal scalp, but his eyebrows, eyelashes, pubic and axillary hair were present. His teeth showed fragile enamel and had a tendency to exhibit dental caries. He also demonstrated marked focal hyperkeratosis on his palms and soles. We have previously published this patient highlighting his unusual hyperkeratotic lesions (17).

Families with amelogenesis imperfecta (AI). We investigated 3 patients diagnosed with an autosomal-dominant form of AI. Two patients were related, a father (patient 3) and son (patient 4). Their clinical findings were published elsewhere (18). Mutation analysis of candidate genes revealed they harboured heterozygous single G deletion within a series of 7 residues at the exon 9-intron 9 boundary of the enamel gene (18). The other AI patient (patient 5) was a 22-year-old male and his family members were clinically unaffected. He was normally delivered and, at 2 years of age, abnormal teeth were first recognized (Fig. 3). All affected patients exhibited enamel hypoplasia in both the deciduous and permanent dentition without any other general complications, including blistering of the skin or oral mucosa.

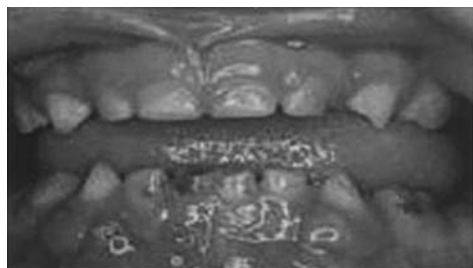


Figure 3. Clinical appearance of patient 5 with amelogenesis imperfecta. The AI patient exhibited enamel hypoplasia in both the deciduous and permanent dentition without any additional general complications, including skin blistering.

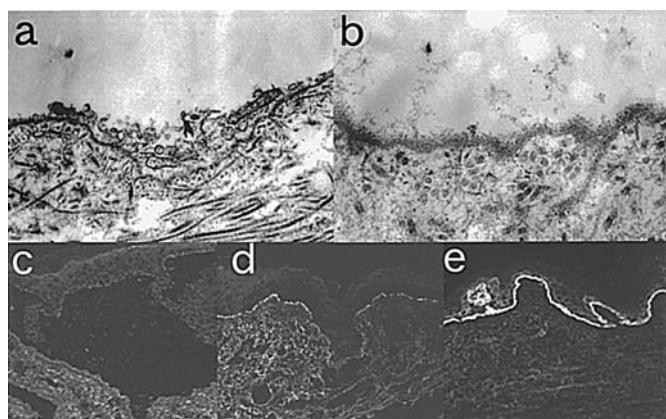


Figure 4. Ultrastructural and immunohistochemical analyses. Blistered skin from patients 1 (a) and 2 (b) showed tissue ultrastructural separation within the lamina lucida. Immunoreactivity of type XVII collagen was totally absent in patient 1 (c) while patient 2 showed decreased intermittent immunoreactivity at the base of the blister. Normal skin displayed bright, linear and continuous staining along the dermal-epidermal junction (e).

Immunohistochemical and ultrastructural analyses. Direct immunofluorescence analysis was performed using cryostat skin sections from patients as described previously (19). The monoclonal HDD 20 against human type XVII collagen was a kind gift from Dr Sonnenberg, The Netherlands Cancer Institute and used for these experiments. For electron microscopic examination, skin specimens from patients 1 and 2 were fixed in 5% glutaraldehyde and postfixed in 1% osmium tetroxide, stained en-block in uranyl acetate. They were dehydrated in a graded series of ethanol solutions, and then embedded in Araldite 6005. Ultrathin sections were cut, stained with uranyl acetate and lead citrate. The sections were examined with a transmission electron microscope (H-7100; Hitachi, Tokyo, Japan) at 75 kv.

Mutation analysis of COL17A1. Genomic DNA was isolated from peripheral lymphocytes from the patients and their families using standard procedures. The mutation detection strategy was performed after polymerase chain reaction (PCR) amplification of all exons including the intron-exon borders, followed by direct automated nucleotide sequencing using the BigDye terminator system (Applied Biosystems, Foster City, CA). The genomic DNA nucleotides were numbered based on the previous sequence information (GenBank

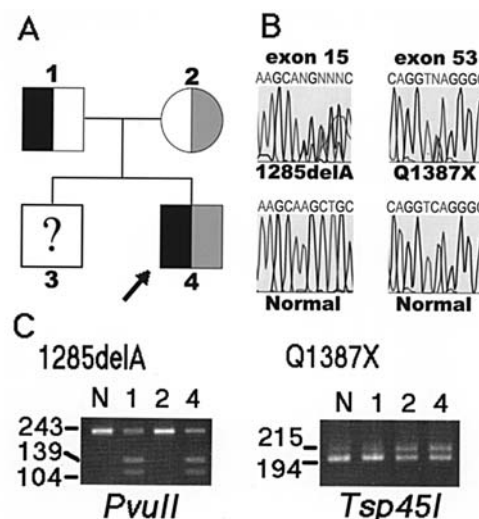


Figure 5. Identification of *COL17A1* mutations in patient 1 with non-Herlitz junctional epidermolysis bullosa. A: family pedigree; black areas, the deletion mutation 1285delA; gray areas, the nonsense mutation Q1387X. We could not obtain samples from the brother (3). B: Direct nucleotide sequencing of exon 15 in the father (1) and the proband patient (4) revealed a heterozygous 1-bp deletion of nucleotide A at position c.1285, whereas exon 53 of the mother (2) and the patient (4) showed a heterozygous C-to-T transition at position c.4264, which changes a glutamine 1387 to a stop codon (Q1387X). C: 1285delA generated the PvuII site within the 243-bp PCR product (g.37 to g.279; GenBank accession no.: U76578), which was digested to 139-bp and 104-bp fragments. Q1387 abolished the Tsp45I site of the 215-bp PCR product (g.-77 to g.128; U76603), which failed to be digested to 194-bp and 21-bp fragments in control samples. N: normal individual.

accession no.: U76564-U76604). PCR amplification of all 56 exons of the *COL17A1* gene was carried out as described previously (11).

Results

Immunohistochemical and ultrastructural results. Electron microscopic examination of the skin samples from patients 1 and 2 revealed that the tissue separation was localized within the lamina lucida (Fig. 4a and b). The lamina densa was well formed at the base of the split whereas the basal cell membrane was present at the roof of the separation opposing the lamina densa. The ultrastructural findings were consistent with these cases being classified as JEB. Immunoreactivity using the monoclonal antibody HDD20 against type XVII collagen was totally absent in patient 1 while patient 2 showed reduced intermittent or granular immunoreactivity along the dermal-epidermal junction (Fig. 3c and d). Normal skin demonstrated bright, linear and continuous staining of the dermal-epidermal junction (Fig. 3e).

Detection of COL17A1 mutations. Mutation screening included direct PCR amplification of *COL17A1* exons from genomic DNA. Patient 1 was compound heterozygous for a deletion of the base A at position c.1285 in exon 15 and for a C-to-T transition at position c.4264 in exon 53 (Fig. 5). The 1285delA deletion led to a premature termination codon (PTC) 26-bp downstream from the site of deletion, and the C→T transition caused the conversion of codon CAG (Gln) to a PTC at amino acid 1387 and was designated Q1387X.

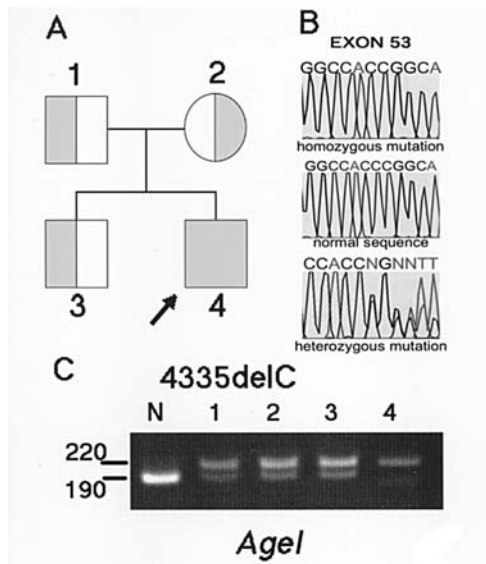


Figure 6. Identification of *COL17A1* mutations in patient 2 with non-Herlitz junctional epidermolysis bullosa. A: family pedigree: the gray area: the deletion mutation 4335delC. B: direct nucleotide sequencing of exon 53 revealed homozygous 1-base deletions of a C nucleotide at c.4335 in the patient (4), and a heterozygous mutation in the other family members (1, 2, 3). C: 4335delC abolished the *AgeI* site of the 210 PCR product (g.-10 to g.200; GenBank accession no.: U76603), which was not digested to 190 bp and 20 bp fragments. N: normal individual.

Nucleotide sequencing showed that the 1285delA mutation was paternal and the Q1387X mutation was maternal. A DNA sample from the unaffected brother was not available. Patient 2 was homozygous for a deletion of C at position c.4335 in exon 53 (4335delC) that led to a PTC 35-bp down-stream from the deletion (Fig. 6). His father, mother, and older brother were all heterozygous for this mutation. The mutations were confirmed by restriction enzyme digestion (Figs. 5 and 6).

No significant nucleotide changes were noted in COL17A1 in AI patients. Analysis of all 56 exons of the *COL17A1* gene by direct sequencing failed to uncover any mutations in patients 3, 4 and 5 except for common gene polymorphisms (data not shown).

Discussion

Herein, we report two nH-JEB patients with tissue separation within the lamina lucida. The patients showed common phenotypic attributes including generalized blistering, skin atrophy, diffuse alopecia, nail dystrophy and dental abnormalities. Patient 1 was compound heterozygote for a deletion mutation (1285delA) in the intracellular domain and a nonsense mutation (Q1387X) in the extracellular domain, both of which are novel. Patient 2 also harboured a novel homozygous deletion mutation 4335delC.

As far as we know, >60 mutations in the *COL17A1* gene have been reported thus far (20). The majority of the reported mutations in the *COL17A1* gene cause premature termination codons (PTC) as a result of either nonsense or small insertion/deletion mutations. In all these cases, an absence of immunofluorescence staining was found in the patients' skin for type

XVII collagen. Thus, nonsense mediated decay of the abnormal transcripts are responsible for the lack of type XVII collagen at both the mRNA and protein level in these patients (3,10). In this respect, the position of the nonsense mutations might not be associated with the severity of nH-JEB phenotype, since any nonsense mutations will lead to a drastically reduced level of both collagen XVII mRNA and protein levels.

Although the clinical features of patients 1 and 2 were typical for nH-JEB, the second patient demonstrated fewer blisters and erosion, and milder alopecia with some normal hair growth compared to patient 1. Furthermore, patient 2 showed discontinuous immunoreactivity along dermal-epidermal junction, which indicated expression of type XVII collagen. The homozygous 4335delC mutation in patient 2 was the most carboxyl terminal homozygous mutation of all previous *COL17A1* mutations (20). The literature concerning nH-JEB cases with homozygous nonsense mutations suggests that the position of mutations on either the collagenous or the noncollagenous domains might affect the severity of the nH-JEB phenotype (6). In this study, the most carboxyl terminal homozygous mutation showed a milder phenotype. This suggests that more carboxyl PTC mutations in the type XVII collagen polypeptide may lead to a restoration of truncated polypeptide expression, resulting in the milder clinical severity.

Enamel hypoplasia and the resulting tooth pitting associated with severe caries, are also characteristic features of nH-JEB. As previously reported, both the ligand laminin 5 and its receptor in skin, the $\alpha 6\beta 4$ integrin are thought to be important in tooth morphogenesis (21-23). Mutations in the genes encoding type XVII collagen, laminin 5 and $\alpha 6\beta 4$ integrins result in JEB, and some interaction between those molecules has been suggested. Furthermore, the dental abnormality may also be caused by the hemizygosity of a dominant negative *COL17A1* missense mutation G627V (9). Therefore, we hypothesized that any mutations in the *COL17A1* gene might be associated with a genetic enamel hypoplasia and severely disrupted enamel formation. Thus, we have searched for nucleotide changes in the *COL17A1* gene of patients with amelogenesis imperfecta (AI).

AI comprises a clinical and genetically heterogeneous group of inherited defects with dental enamel formation but without the significant involvements of other organs and this disease group can be classified into a further 14 phenotypic subtypes. The process of dental enamel formation involves proteins secreted by ameloblast cells, which have an epithelial origin. To date, mutations have been identified in the enamelin gene that causes autosomal-inherited forms of AI, while X-linked AI results from mutations in the amelogenin. In addition, defects in kallikrein-4, MMP-20 and DLX-gene can also underlie AI genetic mutations in some cases (16,18,24). This study analyzed, by direct sequencing, all 56 exons of the *COL17A1* gene in 3 AI patients. As a result, we failed to identify any association between AI and collagen XVII and no significant *COL17A1* nucleotide changes were present in any AI cases.

In summary, we have examined *COL17A1* genes in 2 nH-JEB families and 2 AI families with enamel hypoplasia. We demonstrated three novel mutations in nH-JEB families, but no significant nucleotide changes in *COL17A1* in AI pedigrees.

This suggests that the mechanisms causing enamel hypoplasia are different between nH-JEB and AI.

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