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Transcriptional Dysregulation Of Enamel Formation Genes In Amelogenesis Imperfecta

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Abstract
Amelogenesis imperfecta (AI) is a hereditary enamel disease that is typified by aberrant enamel development, manifesting in several clinical phenotypes, including hypoplastic, hypomaturation, and hypocalcified variations. The mutations impacting enamel matrix proteins and related structural elements constitute the genetic etiology of AI. Despite lots of research, the expression profiles of the main genes linked to enamel development AMELX, AMBN, ENAM and LAMB3 in patients with AI are still unclear. This study evaluates the differential expression of AMELX, AMBN, ENAM, and LAMB3 genes in dental tissue from subjects with AI compared to controls without AI. The experiment was performed using qPCR with the SYBR Green protocol and run in triplicate, including negative controls. Fold changes derived from ΔΔCt were reported as median values. Analysis was done on enamel-matrix expression of genes levels in amelogenesis imperfecta patients and non-AI controls in this study. AMELX expression was also significantly impaired in AI (median = 0.658) versus controls (median = 1.17; p = 0.0002). AMBN levels were also significantly lower in these patients (median = 0.287 vs. control median = 1.16; p = 0.001), and there was a significant decrease in ENAM expression (median = 0.223 vs. control median = 1.1; p = 0.007). Conversely, no significant change in LAMB3 mRNA was observed (patient median = 0.944 vs. control median = 1.17; p = 0.24). These findings conclude that the crucial enamel maturation genes for enamel formation, AMELX, AMBN, and ENAM, are specifically diminished in AI, whereas LAMB3 seems to not be disturbed in these defects. The results revealed qPCR-induced dysregulation in AI's symptomatic enamel defects, highlighting the molecular mediators of biomineralization-related diseases.
Key Words: *Amelogenesis imperfecta, gene expression, AMELX, AMBN, ENAM, LAMB3*

INTRODUCTION

Amelogenesis imperfecta (AI) is a set of anomalies in enamel formation abnormalities that vary in both clinical and genetic characteristics with phenotypically variable structural and cosmetic consequences in the form of tooth hypersensitivity, caries susceptibility, and premature dentin exposure. These defects result from abnormalities in the pathways of enamel biomineralization, and the molecular details are only partially understood despite the recent advances in genetic diagnostics (1–3). AI may occur as isolated or syndromic forms that are inherited in X-linked, autosomal dominant, or autosomal recessive, and is phenomenologically divided into hypoplastic (decreased enamel thickness), hypomaturation (defective mineralization), hypocalcified (soft, disorganized matrix), and mixed phenotypes (mixed features with taurodontism) (4-6). More than 20 genes are involved in the pathogenesis of AI, including those that encode enamel matrix proteins (SECRET levels are in my genes: screening for amelogenesis imperfecta pathogenic mutations in a new kindred and proteases (KLK4, MMP20), and structural regulators (LAMB3) (7), and unique clinical phenotypes are often linked to mutations in each of these genes.

The formation of enamel depends on the matrix proteins enamelin (ENAM), amelogenin (AMELX), and ameloblastin (AMBN). AMELX (Xp22.2) makes the most common protein in predentin, and its early release helps shape the structure of hydroxyapatite. Mutations in the N-/C-terminal domains are related to X-linked hypoplastic AI, and mutations in the intermediate region have been associated with hypomaturation phenotypes (8). AMBN, produced from early to late enamel formation, mediates ameloblast differentiation, cell-matrix adhesions, and mineralization. Non-syndromic hypoplastic AI with thin enamel is primarily due to AMBN biallelic loss-of-function mutations (exon

6–7 deletions) (9, 10). Similarly, mutations in ENAM, such as c.1258_1259insAG, cause severe recessive hypoplastic AI with normal thickness but incomplete assembly of the enamel matrix (11). In contrast, LAMB3 (laminin-332; β3 subunit) is under-studied in AI; however, a frameshift mutation (p.E1133Gfs*27) was found in a single familial autosomal dominant case of AI, raising consideration of a likely mechanism involving basement membrane integrity in mediating enamel pathology (12). Although most of the previous studies are centered on genetic mutations, the transcription part of enamel-associated genes in AI is poorly understood. So mRNA expression is a quantitative trait that provides biologically meaningful information about diseases beyond genomic variations, which can help bridge the gap between genotype and phenotype. This study's objective is to investigate the profiles of AMELX, AMBN, ENAM, and LAMB3 expression in AI patients compared to healthy individuals using qPCR. We hypothesize that the differential downregulation of AMELX, AMBN, and ENAM, rather than LAMB3, in AI is associated with enamel biomineralization disorder. Our study of ten unrelated enamel dysplasia individuals provides insights into transcriptional contributors to AI pathogenesis, which may be applied to molecular diagnosis and targeted interventions in the future, despite difficulties recruiting large cohorts because of the rarity of AI.

METHODOLOGY

Sampling

The study involved a total of 10 patients diagnosed with amelogenesis imperfecta (AI) and 10 age-matched healthy controls (aged 17–50 years). Based on a clinical assessment of enamel abnormalities, which involved a thorough clinical examination and x-ray study to assess the phenotypic characteristics of amelogenesis imperfecta (AI), the diagnosis of AI was made. The Iraqi Ministry of Health and the Council of the University of Al-Qadisiyah's College of Biotechnology for Postgraduate Studies gave their approval to this research. Every patient gave their formal consent after being briefed about the study. Dental tissues were collected from AI patients during routine therapeutic procedures (extractions). Control samples were obtained from healthy individuals undergoing unrelated dental treatments. All specimens were immediately preserved in RNA stabilization solution and stored at −80°C until molecular analysis.

RNA Isolation and cDNA Synthesis

Total RNA was extracted from dental tissue samples using a Total RNA MiniKit Tissue (Geneaid, Taiwan), followed by cDNA synthesis with reverse transcriptase (UnionScript First-strand cDNA Synthesis Mix; Genesand, China). RNA quantification was verified via Denaturing MOPS agarose gel electrophoresis (13) and microvolume spectrophotometer (Colibri, Germany), and the cDNA was kept at -80°C until it was analyzed.

Quantitative Real-Time PCR (qPCR)

Gene-specific primers for AMELX, AMBN, ENAM, and LAMB3, as well as the housekeeping gene, were designed using Primer-BLAST (NCBI) (Table 1). qPCR was conducted on a RealTime PCR System (qTOWER3 G; Analytik Jena, Germany) using SYBR Green chemistry. The reaction (20 µL total volume) consisted of 1 µL cDNA template, 10 µL of 2× PerfectStart Green SuperMix (TransGen Biotech, China), 0.4 µL of each of the forward and reverse primers (10 µM), and a final volume completed of nuclease-free water. All samples were measured in triplicate, and negative and no-template controls, as well as inter-run standards, were included in every run to reduce technical variation. Thermal cycling parameters consisted of an initial denaturation at 94°C for 30 seconds, followed by 40 cycles of denaturation (94°C, 5 seconds), annealing/extension (60°C, 30 seconds), and a melt curve analysis (60–95°C, 0.5°C increments, 10 seconds/step), allowing confirmation of amplification specificity.

Table (1): The gene expression primer sequences utilized in this investigation.

Gene	Sequence (5'-3')	
AMELX	F	5'-GATCCCCCAGCAACCAATGA-3'
	R	5'-GATCAGGAAGCATGGGAGGC-3'
AMBN	F	5'-CCAAAGGCCCTGAGAACGAA-3'
	R	5'-CCACGGATGTGGTCATGTCA-3'
ENAM	F	5'-GCCAGAATTTGCCCAAAGGG-3'
	R	5'-ACCATGGGAAGGATGGGGTA-3'
LAMB3	F	5'-TGCGCAGCGATCAGCAT-3'
	R	5'-GGTGGGGGAGATCACAAACT-3'
GAPDH	F	5'-CCACCCATGGCAAATTCCATGGCA-3'
	R	5'-TCTAGACGGCAGGTCAGGTCCACC-3'

Data Analysis

To quantify gene expression, the comparative ΔΔCt technique was employed (Livak & Schmittgen, 2001), and the relative fold changes between AI patients and controls were calculated. To evaluate the data distribution, the Shapiro-Wilk test was applied. The findings are shown using median values with ranges. The Mann–Whitney U test was used to determine whether group differences were significant. To explore the relationship between expression and etiological info, Fisher's exact test was employed. A difference or correlation was considered significant if the p-value was less than 0.05. The research project was carried out using the an internet program called Bricks (AI spreadsheet).

RESULTS

Demographic and Clinical Characteristics

The study sample consisted of 10 amelogenesis imperfecta (AI) patients and 10 controls with matched age, as shown in Table 2 with their demographics and clinical features. The distribution of sexes in the groups was the same (Fisher's exact test, p = 0.41). The median age of AI patients was 28.1 (IQR:12–51) years versus 31.1 (IQR: 16–55) for controls (p = 0.50, Mann-Whitney U test).

The smoking rate of AI patients (40%) was higher compared to controls (20%), but there was no statistically significant difference (Fisher's exact test, p = 0.60). On the other hand, a positive family history of AI was very dissimilar between groups: 100% of patients vs. none of the controls (Fisher's exact test, p ≈ 0.001). These data highlight familial aggregation as the predominant risk for AI, and that did not differ according to age, sex, or smoking status within this group.

Table 2: The demographic group attributes of both the controls and patients.

Item	Characteristic	Patients (N=10)	Controls (N=10)	p-value
Sex, n (%)	Male	7 (70%)	5 (50%)	0.41*
	Female	3 (30%)	5 (50%)	
Age (years)	Mean ± SD	28.1 ± 14.9	31.1 ± 12.6	0.62**
	Median (Range)	20.5 (12–51)	27.5 (16–55)	0.50***
	Min–Max	12, 51	16, 55	
Smoking Status, n (%)	Smoker	4	2	0.60 *
	- Non-smoker	6	8	
Family History, n (%)	-	10	10	p ≈ 0.001

*** Fisher's exact test (sex comparison).**

**** Independent samples t-test (assuming normal distribution).**

***** Mann-Whitney U test (non-parametric, if age is skewed.**

Expression of Genes in Dental Tissue

Participants were categorized into two groups: the patient in the AI group. and without AI control group, Analysis of the expression of the genes LAMB3 (laminin), AMELX (amelogenin), AMBN (ameloblastin), and ENAM (enamelin) was carried out. In light of the findings, AMELX was stated.

The study demonstrated that the median relative AMELX gene expression in the patients with the illness group was 0.658 (0.41–0.87) in comparison with 1.17 (1.01–1.2) in the control group. The control group's AMELX levels of expression were significantly higher than those of AI patients (p-value = 0.0002), as shown by Figure 1, The AMELX messenger RNA (mRNA) expression data sets fold change and log_2 fold change are demonstrated.

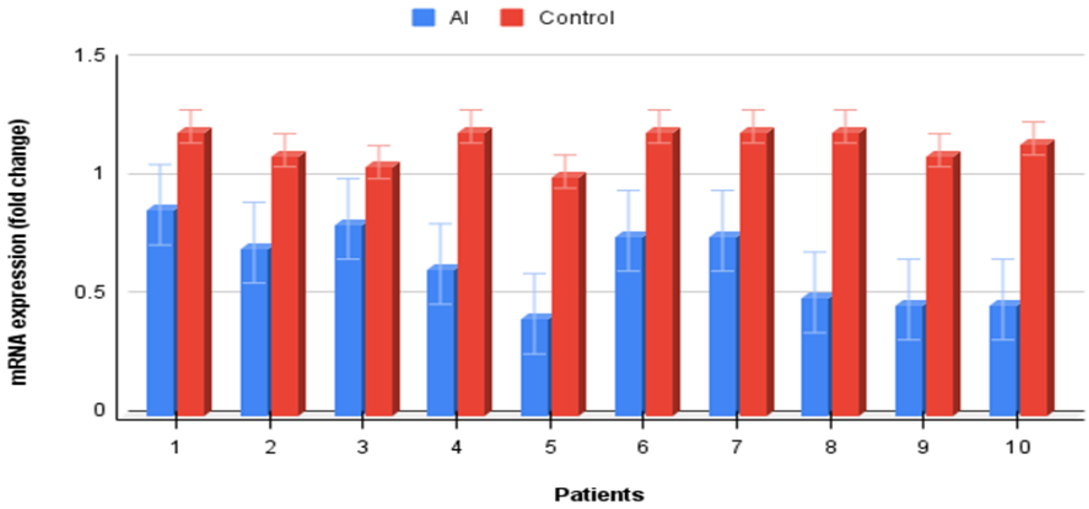

Figure 1:Compared to the control group without AI, the tooth tissue samples from patients 1–10 with AI showed a relative AMELX gene expression fold change.

In comparison to the control group, In the ill group, the average relative AMBN gene expression was 0.29 (range: 0.13–0.71). Moreover, compared to the control group 1.16 (1.02-1.19), Al patients had significantly lower levels of AMBN expression. Figure 2 illustrates both the fold change and the log2 fold change in the AMBN mRNA expression investigation.

. Based on this study, patients' mRNA expression of the AMBN gene is significantly lower (p-value = 0.001), which reveals that it might be employed as an AI diagnostic indicator.

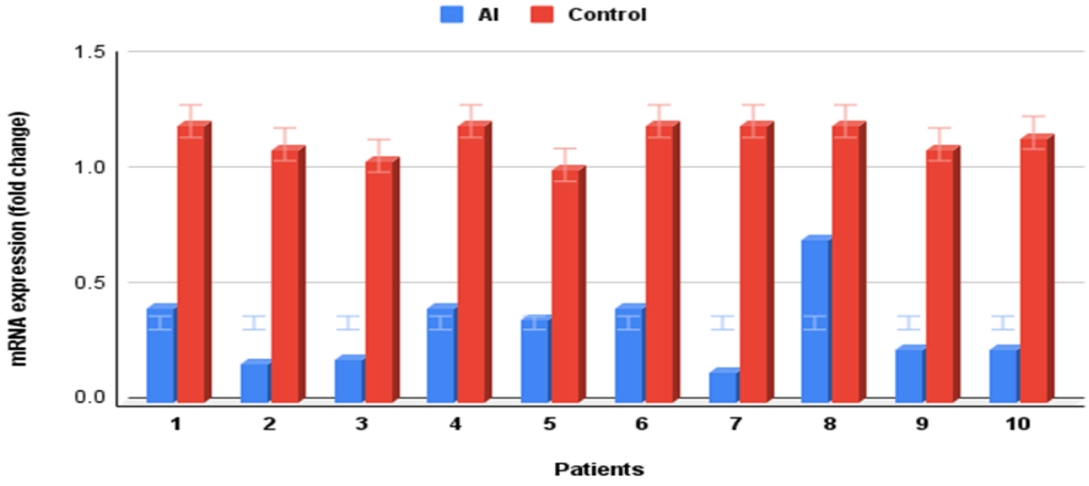

Figure 2: The dental tissue samples from patients 1–10 with AI showed a a relative fold change in AMBN gene expression folds as compared to the control group without AI.

According to measurements of ENAM expression, the study's patients' median relative ENAM gene expression was (0.223), with a range of (0.017 - 0.66). The ENAM expression The Al study group's levels differed significantly from the control group's 1.1 (1.07-1.28). Figure 3 displays these findings, the AMBN mRNA expression analysis's fold change and Log2 fold change. According to the study findings, ENAM mRNA levels are significantly lower in Ai patients (p-value = 0.007), which might suggest that the pathogenesis of Al is related to these expression changes.

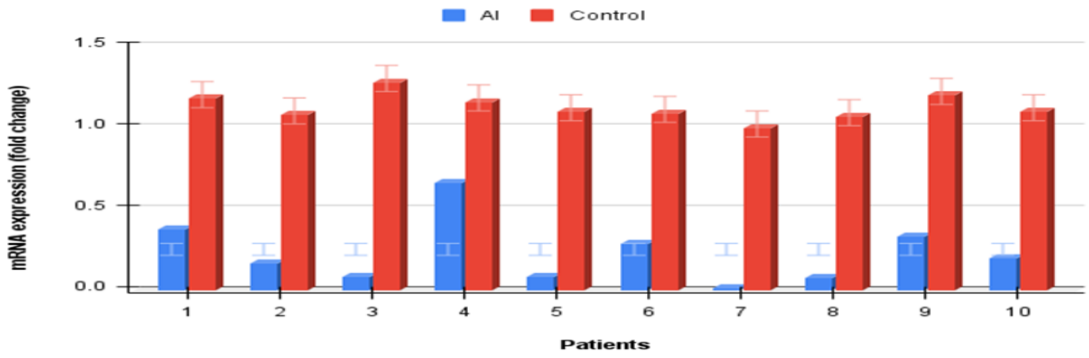

Figure 3: The dental tissue samples from patients 1–10 with AI showed a relative change in ENAM gene expression folds as compared to the control group without AI.

Analysis was done on (LAMB.3) expression. The ill group's average relative LAMB3 gene expression ranged from (0.71 - 1.07), with a value of 0.93 compared to the control group. There was no discernible change in LAMB.3 expression levels between the study group's AI patients and the control group 1.17 (1.01-1.2). Figure 4 displays these findings, showing the (LAMB.3) mRNA expression analysis's fold change and Log2 fold change (p-value = 0.24). According to the results, LAMB.3 might not be significantly involved in the pathophysiology of the patients under study.

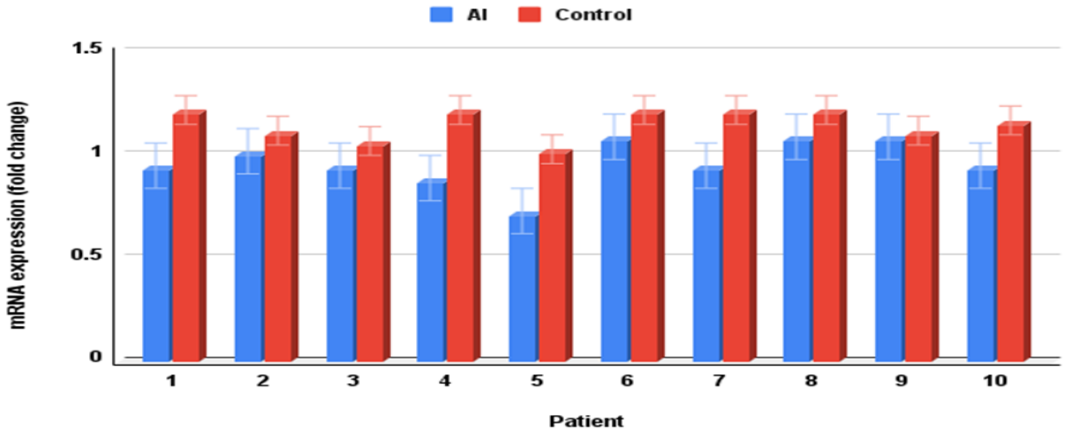

Figure 4: The dental tissue samples from patients 1–10 with AI revealed When compared to the control group, AI showed a relative fold change in LAMB3 gene expression.

Determining the differential expression of the genes: (AMELX, AMBN, ENAM, and LAMB.3) in individuals with AI—a genetic enamel deficit that prevents the formation of enamel—was the goal of our study. The results showed that AI patients had lower levels of (AMELX, AMBN, and ENAM) than healthy controls. However, there was no discernible difference in LAMB.3 levels between the two groups. These discoveries help understand the molecular mechanisms behind AI as well as potential genetic targets for diagnosis and treatment.

DISCUSSION

The significantly reduced AMELX expression in AI patients (0.658 - fold, p= 0.0002) may point to an issue with amelogenin synthesis that is preventing appropriate assembly. as well as enamel matrix mineralization.This result is consistent with earlier studies showing that AI is impacted by mutations or changed AMELX expression. Hypomineralized or hypoplastic enamel is a characteristic of these phenotypes (14). Furthermore, ameloblastin, or AMBN, is another protein present in the matrix of enamel that is essential for ameloblast formation as well as enamel mineralization. (15).The significant decrease in AMBN expression (0.287-fold, p=0.001) supports the role of AMBN in preserving the integrity of the enamel matrix. Moreover, as seen in AI instances, reduced AMBN levels may have a deleterious effect on ameloblast efficiency and enamel matrix secretion, resulting

in impaired enamel development. This result is consistent with other studies that discovered that AI traits are associated with variations in AMBN expression (16). ENAM is necessary for the mineralization and structural arrangement of enamel. The crucial role ENAM contributes in enamel matrix development is highlighted by the notable drop in ENAM expression (0.223-fold, p=0.007). (A.I.) is known to result from mutations in ENAM, especially in hypoplastic variants (17). The observed downregulation can be the consequence of secondary consequences connected to disturbed ameloblast activity, or it might be a sign of genetic changes that affect transcriptional regulation. On the other hand, there was no significant difference in LAMB3 (laminin beta-3) expression levels between AI subjects and control subjects (p=0.24, 0.944-fold).

Pollio et al. (2010) reported that laminin-332, which is encoded by LAMB3, is mainly involved in promoting epidermal-dermal adhesion and preserving the integrity of the epithelial basement membrane. Although junctional epidermolysis bullosa is associated with mutations in LAMB3, Its exact role in enamel development is still unclear. (10).The pathophysiology of AI may not be directly influenced by LAMB3, or its regulatory processes during amelogenesis may be less affected, as evidenced by the lack of a discernible change in expression (18). The coordination of AMELX, AMBN, and ENAM downregulation in AI suggests a possible shared pathway influencing the formation of enamel matrix and ameloblast function. Important proteins that are encoded by these genes come together to create a strong enamel matrix that can mineralize (19). Alterations in their expression are expected to result in inadequate mineral deposition, impaired enamel matrix secretion, and ultimately the phenotypic signs of AI. The results support the theory that regulatory deficits or genetic mutations in important enamel matrix genes are the cause of AI. The present gene expression findings arebacked by past genetic studies that found AI patients to have mutations in AMELX, ENAM, and AMBN (20). A highly significant p-value of almost 0.001 was obtained since all patients in the current study had a positive family history of AI, but none of the controls did. In the study, 40% of AI patients smoked compared to 20% of controls, although the difference was not significant (p=0.60) (21; 22). Furthermore, the control group's absence of a family history highlights AI's genetic foundation. These results highlight the value of genetic counseling and family screening in AI management (23). The signs of AI, a genetic condition that disrupts enamel synthesis, include poor structure, color changes, thin enamel, and low mineral content (24). The different looks of AI are depicted in Figure 2, including a yellowish-brown color, small holes, rough surfaces, and thin enamel. These traits put the teeth's look and functionality in danger, which could lead to increased sensitivity, increased susceptibility to dental cavities and other cosmetic problems. The result could be a significant decline in the individual's quality of life (25).

CONCLUSION

This research highlights the important function that AMELX, AMBN, and ENAM genes play in enamel development by showing that these genes are significantly downregulated in individuals with Amelogenesis Imperfecta (AI) as compared to healthy controls. However, the expression of LAMB3 did not differ statistically significantly, indicating a little role in the pathophysiology of AI. The hereditary aspect of AI was highlighted by the substantial correlation between disease occurrence and familial history, although sex also seemed to have an impact on disease manifestation. However, in this group, there were no significant associations between AI and smoking or age. By highlighting the significance of genetic screening and family history evaluation in clinical diagnoses, these findings advance our knowledge of the genetic and epidemiological aspects underlying artificial intelligence. Larger sample numbers are necessary for future research to confirm these findings and investigate other molecular pathways.

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