ORIGINAL ARTICLE

CONTENTS

Molecular study of FAM20A gene and biochemical analysis for amelogenesis imperfecta patients

Mushtaq Ibraheem, Saif Abdulrazaq
DEPARTMENT OF ORAL DIAGNOSIS, COLLEGE OF DENTISTRY, UNIVERSITY OF BAGHDAD, BAGHDAD, IRAQ

ABSTRACT

Aim: This study aimed to diagnose Amelogenesis Imperfecta patients if have an isolated type or are related to a syndrome such as enamel renal syndrome.

Materials and Methods: This case-control study included (60 patients and 20 controls). DNA extraction from the blood sample then used the Conventional PCR reaction and Agarose Gel Electrophoresis. The generated PCR fragments were subjected to Sanger sequencing. Geneious software showed the genotypes after aligning with a reference sequence in the Gene Bank. In addition, biochemistry analyses are performed by using a spectrophotometer.

Results: The FAM20A gene was presented with three genotypes (TT, TC, CC) and two alleles (T and C), no significant variations were found. There was a positive correlation between the TT genotype with Amelogenesis Imperfecta; this means that patients whom caring TT genotype have a risk for Amelogenesis Imperfecta than other genotypes and non-significant relation regarding serum creatinine, potassium, and calcium while the serum urea and alkaline phosphatase have significant results.

Conclusions: patients who carry for TT genotype have a higher risk for Amelogenesis Imperfecta than other genotypes and no significant relation between creatinine, potassium, and calcium, while the serum urea and alkaline phosphatase show a significant relation.

KEY WORDS: Amelogenesis Imperfecta, nephrocalcinosis, enamel renal syndrome, FAM20A

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INTRODUCTION

Amelogenesis imperfecta (AI) is a term used to describe a group of genetically based developmental conditions that impact the composition and clinical look of enamel in all or nearly all of the teeth in a roughly equal way. These conditions may also be linked to morphologic or biochemical alterations in other parts of the body [1]. Amelogenesis imperfecta and nephrocalcinosis (NC) together may indicate pleiotropism or a contiguous gene syndrome. According to one theory, there may be an underlying anomaly in the interstitial architecture that causes the kidney's dystrophic calcification and the teeth's aberrant enamel formation [2]. Further studies are needed to determine the involvement of dental proteins in phosphate and calcium metabolism and renal function since another idea proposes that many of the proteins previously believed to be specific to tissue may also be found in non-dental tissues [3]. The autosomal recessive disorder known as enamel renal syndrome (ERS) is rare and poorly characterized. Numerous terms with comparable features have been suggested, including AI and NC syndrome [4, 5]. These conditions are believed to be signs of a single sickness caused by underlying abnormalities in the FAM20A gene [6]. Clinically, typical oral characteristics include hypoplastic (AI), postponed tooth eruption, calcification in the pulp, hyperplastic dental follicles, various degrees of gingival hyperplasia, and the presence of calcified nodules [7]. Other renal diseases, such as NC, have also been described as frequent findings, especially in early adulthood [8]. Additionally, a condition called NC and other renal disorders have been reported often, especially in adolescents and young adults. It follows that even those with oral symptoms but no-renal issues who have balletic FAM20A mutations are thought to eventually develop NC [9]. Because FAM20A encodes proteins that are produced in suprabasal cells in the gingiva, odontoblasts and dental pulp, as well as in ameloblast throughout the secretory and maturation phases of enamel formation, these proteins are essential for gingival homeostasis and enamel development. It has been demonstrated that many FAM20A variants, including it end gain, missense, frame shift, and splice-site mutations, are present in individuals with the ERS phenotype [10-14].

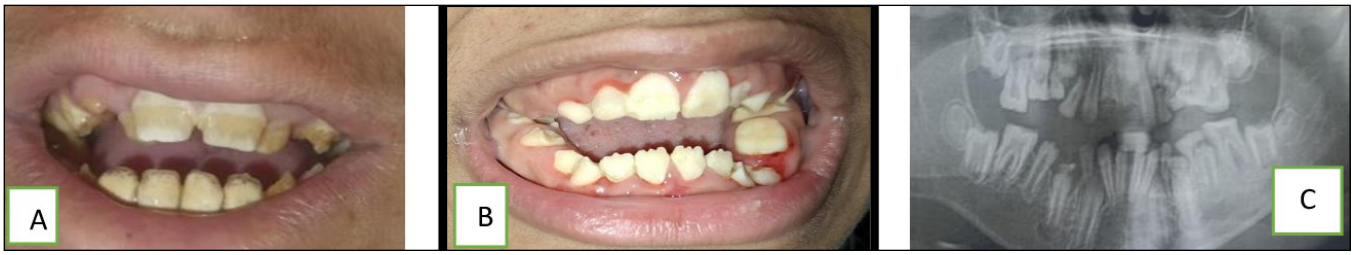

Fig. 1. A: clinical appearance of AI patient; B: AI patient with gengival enlargment; C: radiological appearance of AI patients in this study.

Table 1. Primers developed for the study

Primer	Sequence (5′→3′ direction)	primer size, bp	Product size, bp	TM (°C)
		FAM20A		
Forward	AAGGACCCCACAGGTGTTTT	20	711	60.64
Reverse	TGTGTCTCAGTTTCCCTGTCTG	20		60.33

AIM

This study aimed to diagnose patients with amelogenesis imperfecta if have an isolated type or are related to a syndrome such as enamel renal syndrome and this study represents the first study to take this number of patients with molecular and biochemical analyses.

MATERIALS AND METHODS

STUDY DESIGN AND PARTICIPANTS

The resident population of Al Salam Township in Diyala City was assessed for AI and enamel renal condition in a cross-sectional study conducted between April 25, 2022, and October 16, 2023. A study sample of sixty individuals with AI, diagnosed clinically as shown in (Fig.1-A and 1-B) and radiologically by take extraoral radioghraph as shown in (Fig. 1-C) and enamel kidney condition and 20 patients as a control group were then included in the study, the age of the sample was arranged between 21-45 years for both genders.

BLOOD COLLECTION

Five milliliters of blood were split into two tubes for our study: two milliliters were placed in an EDTA tube and frozen at -20°C, while a gel tube containing three milliliters was centrifuged for five minutes. After that, the serum was transferred to a biochemical lab in a tube that had been prepared for testing.

BIOCHEMICAL LABORATORY METHOD

Human creatinine, urea, calcium potassium and alkaline phosphatase levels in the serum samples were determined using the spectrophotometer.

STUDY PRIMERS

The University Code of Student Conduct (UCSC) programs double-checked the primers, which were created with Primer 3plus, V4, and had their reference sequences verified by the National Center for Biotechnology Information (NCBI) database. Alpha DNA Ltd. (Canada) produced and lyophilized them. All primer sequences used in the tests for this investigation are included in (Table 1).

PREPARING OF PRIMERS

For each assay in this study, the required primers, as shown in table 1, were prepared as follows: after dissolving the lyophilized sample in nuclease-free water according to the manufacturer's instructions, a stock solution with a concentration of 100µM was prepared and stored at -20°C. Diluting 10µL of each primer stock solution in 90µL of nuclease-free water yielded a working solution with a concentration of 10µM, which was maintained at -20°C until use.

DNA SEQUENCING AND GENOTYPING

DNA EXTRACTION FROM THE BLOOD SAMPLE

Using the *Easy Pure*® Genomic DNA Kit (Trans Gen, biotech EE101-01), one milliliter of whole blood was centrifuged for 1 minute/12000 rpm or 5 minutes/5000 rpm. After removal of the supernatant, blood was resuspended in 500 µl TE buffer and stored at -20 °C until ready for evaluation.

DNA CONCENTRATION AND PURITY ASSESSMENT

TE buffer was used as a blank solution before a Nano-drop 2000c spectrophotometer (Thermo

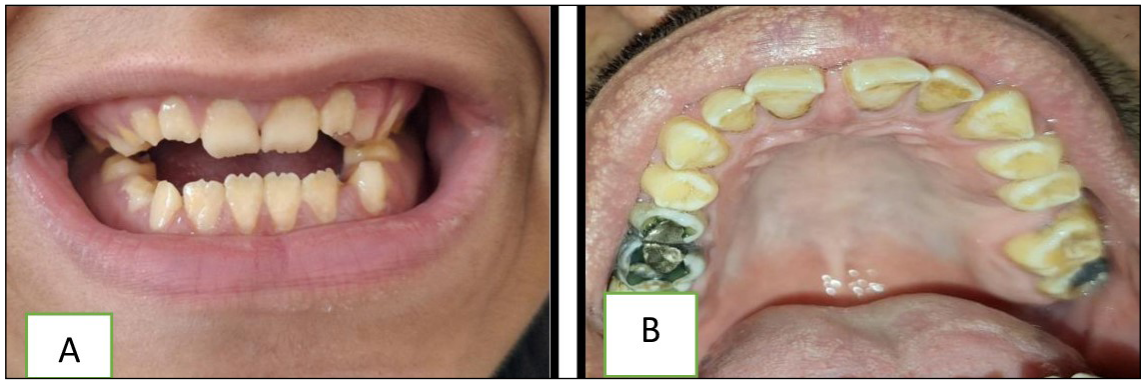

Fig. 2. A: female amelogenesis imperfcta patient; B: male amelogenesis imperfcta patient.

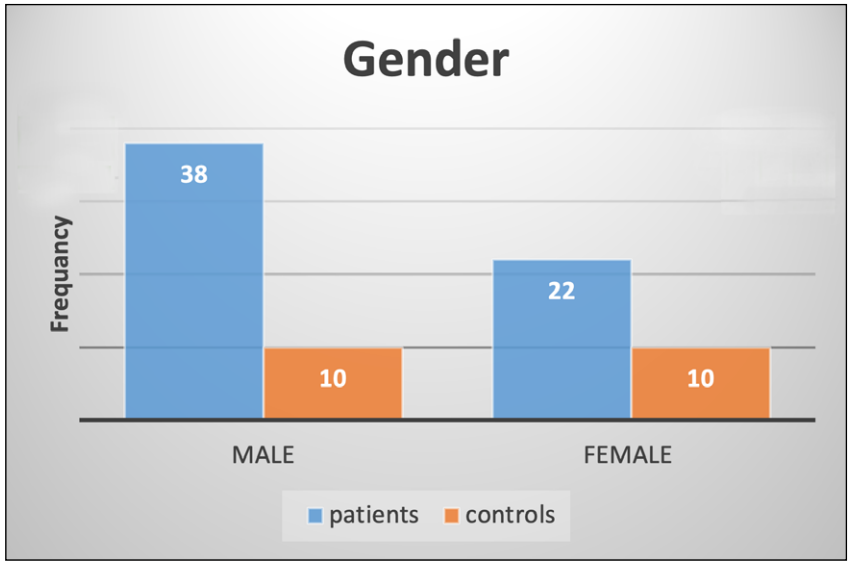

Fig. 3. Distribution of patients into groups according to gender.

Fisher Scientific) with basic computer control and data logging software was used to determine the concentration of DNA samples that demonstrated acceptable integrity. To find the concentration in ng/µl, two microliters of the isolated DNA were put into the Nano-drop.

CONVENTIONAL PCR REACTION

When running the PCR, the reaction was tuned by testing four annealing temperatures: 56, 58, 60 and 62°C. An annealing temperature of 58 °C was found to be optimal for producing clear and sharp bands in agarose gel, hence it was used in the current study. This protocol employs 2xEasyTaq® PCR Super Mix. All PCR reactions were carried out in a 25 µl final volume and according to the manufacturer's instructions.

AGAROSE GEL ELECTROPHORESIS

Extracted DNA and amplified PCR fragments were separated on an agarose gel and then examined under UV light after ethidium bromide staining.

DNA LOADING AND ELECTROPHORESIS

A mixture of 3 µl loading dye and 7 µl isolated genomic DNA (or PCR product) was loaded into the wells of the gel. Following the loading of all wells, the electrical power was turned on at 100 volts (5V/cm^2) for 60 minutes. This caused DNA with a negative charge to migrate from the cathode (-) to the anode (+) poles.

AGAROSE GEL STAINING AND UV VISUALIZATION

After staining the electrophoretic gels with ethidium bromide, which was made by adding 70µl of the 10 mg/ml ethidium bromide to 300 ml of D.W., the gel was stained by soaking in the solution for 20-30 minutes, and then the gel was placed into the gel documentation system to view the DNA bands at a 365 nm wavelength. Special software was used to save the photos captured by the device on the computer [15].

DNA SEQUENCING

The generated PCR fragments were subjected to Sanger sequencing using an ABI3730XL automated DNA sequencer (Macrogen Corporation, Korea). After

Table 2. Comparison between patients and control groups by age

Groups	Mean	Std. Deviation	mean P-value	p-value
Patients	31.63	8.42	1.09	0.4 NS
Control	30.00	7.90	1.78	

NS - non-significant

Table 3. Comparison of data between patient and control groups for urea, creatinine, calcium, potassium and alkaline phosphatase

Groups		Urea	Creatinine	Calcium	potassium	ALP
Patients	Mean	37.61	0.66	9.75	4.46	58.11
	Std. Deviation	8.88	0.11	0.87	0.33	8.88
	Std. Error of Mean	1.15	0.01	0.11	0.055	1.15
Control	Mean	30.67	0.61	9.48	4.37	67.85
	Std. Deviation	6.33	0.15	0.62	0.49	11.45
	Std. Error of Mean	1.41	0.03	0.14	0.11	2.56
p-value		0.002**	0.07 NS	0.1 NS	0.4 NS	0.001**

** - significant; NS - non-significant.

Table 4. Pearson correlation between patients and controls according to age for urea, creatinine, calcium, potassium and alkaline phosphatase

		Age	Urea	Creatinine	Calcium	Potassium	ALP
Age	Pearson Correlation	1	0.385	0.244	0.181	-0.158	0.128
	Sig. (2-tailed)		0.000	0.029	0.108	0.160	0.260
Urea	Pearson Correlation		1	0.541	0.019	0.075	0.051
	Sig. (2-tailed)			0.000	0.866	0.506	0.651
Creatinine	Pearson Correlation			1	-0.119	0.393	0.081
	Sig. (2-tailed)				0.293	0.000	0.475
Calcium	Pearson Correlation				1	-0.014	-0.022
	Sig. (2-tailed)					0.899	0.845
Potassium	Pearson Correlation					1	-0.162
	Sig. (2-tailed)						0.151
ALP	Pearson Correlation						1
	Sig. (2-tailed)						-

matching the genotypes with the reference sequence in the GenBank, clever software displayed the genotypes.

STATISTICAL ANALYSIS

The IBM SPSS Statistics 26 application was utilized to determine how various factors affected the study's parameters. The T-test and one-way ANOVA were used to statistically compare means. A meaningful comparison between percentages (0.05 and 0.01 probability) was made using the chi-square test. Graph Pad Prism 9 was used to create the figures in this investigation.

RESULTS

DISTRIBUTION OF STUDIED SAMPLES ACCORDING TO GENDER

The study sample consisted of 60 AI patients of both sexes: there were 22 (36.7%) females and 38 (63.3%) males (Fig.2A-B).

Control group with comparable age and gender makes up the second group. These twenty-odd healthy-looking people 10 males and 10 females showed no signs of illness or noticeable anomalies.

There are no significant results when comparing patients and control individuals with a p-value equal to 0.2 (Fig.3).

DISTRIBUTION OF SAMPLES ACCORDING TO AGE GROUP

Table 2 shows the association status between the two groups of AI patients and control individuals according to age. The outcome showed that the observed frequency distributions in the two samples among various age groups showed non-significant differences at P>0.05. The mean age of AI patients is 31.63 ± 8.42 and the mean for control individuals was 30.84 ± 7.90 (Table 2).

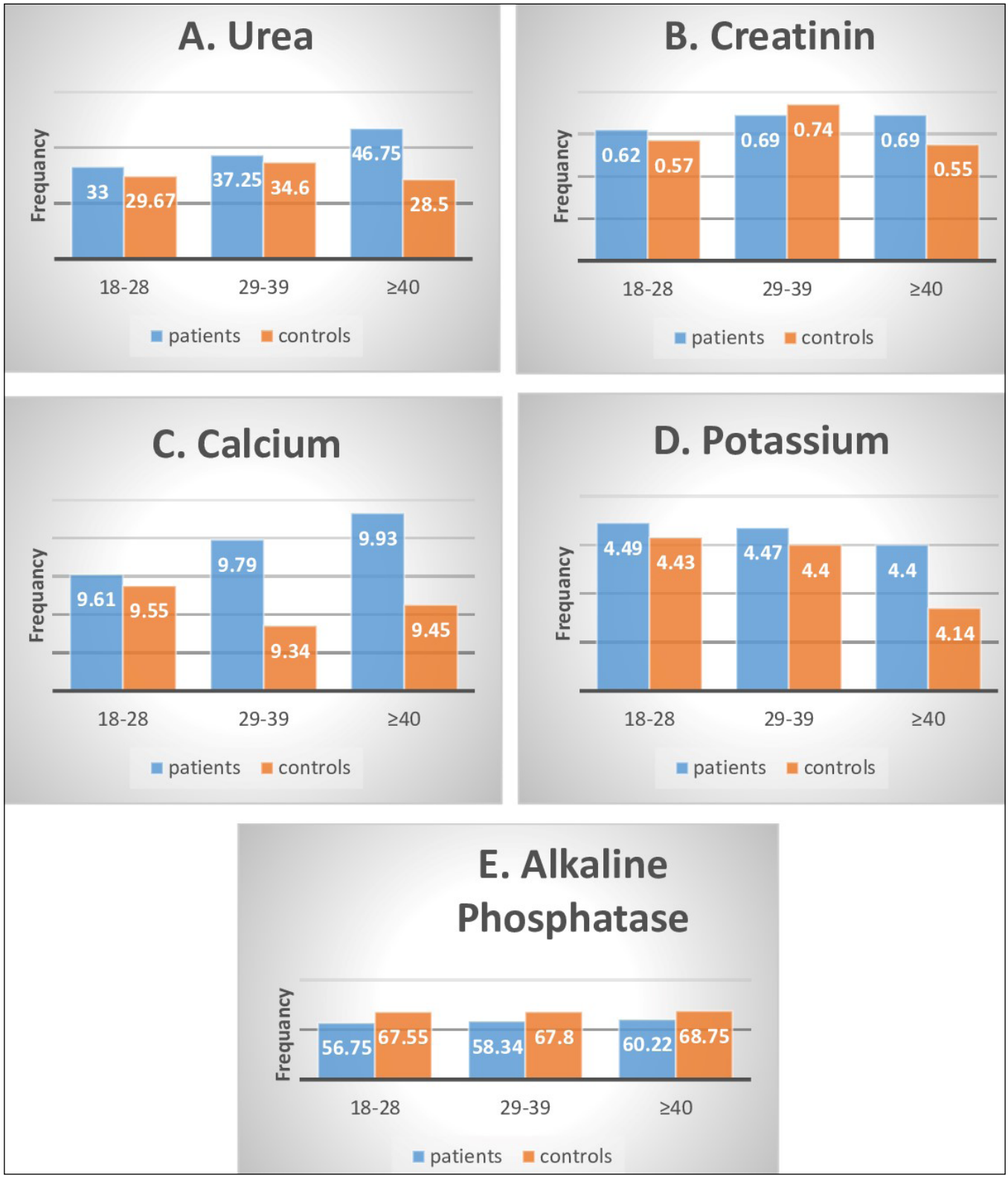

Fig. 4. Comparison between patients and controls for (A) urea, (B) creatinine, (C) calcium, (D) potassium, (E) alkaline phosphatase.

Figure 4 shows the comparison between age groups in amelogenesis imperfecta patients and healthy individuals, so when observed non-significant between two different age groups for biochemical markers (creatinine= 0.4, calcium=0.6, potassium = 0.005, and alkaline phosphatase=0.5) while the P-value for urea= 0.001 represent significant result (Fig.4 A-E).

Table 3 investigates the serum urea in the patient and control group with the mean and SD. The value was evident, with a mean value of 37.61, while the mean

Fig. 5. FAM20A gene sequencing was received from the Gene Bank for both cases and controls. Samples are represented by the query, while the subject is represented by the data from the NCBI database.

value of serum urea for the control group was 30.67 and significant difference was found between them. On the other side, the serum creatinine for AI patients was equal to 0.66 and that was non-significant when compared with control individuals who have a mean of serum creatinine equal to 0.61. Another test taken in this study was serum calcium and this test recorded a mean for the patient of 9.75 and had non-significant relation with the control individual who has a mean equal to 9.48. On the other hand, we investigate the serum potassium and found that its means for patient and control groups equal 4.46 and 4.37 respectively, and the relation between them was non-significant. The last analysis performed, the alkaline phosphatase biochemical analysis, showed a significant correlation between the mean value for patients 58.11 and the mean value for controls 67.85 (Table 3).

Table 4 presents the results of Pearson correlation between patients and control groups depending on age for urea, creatinine, calcium, potassium and alkaline phosphatase. Regarding t the testing of the relationships between age factors and biochemical markers, the results showed a non-significant difference among them at $P>0.05$, while highly significant differences were obtained between the levels of each factor's tests independently at $P<0.001$, (i.e., among age factors, and biochemical markers).

FAM20A GENE

Two alleles (T and C) and three genotypes (TT, TC, and CC) were found for the FAM20A gene. The genotypes in the sick and healthy groups were found to be compatible with the Hardy-Weinberg equilibrium (HWE),

and there were no discernible variations between the predicted and observed genotype frequencies in blood samples (Table 5).

Inspecting of FAM20A gene genotypes and allele frequencies in patient groups and control group revealed that the homozygous genotype frequency of T-allele showed non-significant variation in patients compared to control (35 vs. 58.4%), and there was a positive correlation (etiological factor) between TT genotype with AI, which means that patients whom caring TT genotype have a risk for AI than other genotypes, although patients had a decreased frequencies of the T allele (93 vs. 77%) and an increased frequency of the C allele (27 vs. 23%) compared with the control group (Table 6).

FAM20A GENE SEQUENCING RESULTS

In the current investigation, the targeted area of the FAM20A gene of AI and control was amplified using a PCR reaction on 80 blood samples. Amplification of this region was confirmed by agarose gel electrophoresis. Using ethidium bromide dye, the FAM20A gene PCR amplified products were electrophoresed on an agarose gel and the resulting figure shows that all samples had a successful double band amplified fragment size of 220 bp. DNA samples (PCR product) were obtained by a certain company (Macrogen). Analysis has been done on the sample DNA sequencing data. Alignment was performed using the NCBI nucleotide alignment tool, Nucleotide BLAST (Basic Local Alignment Search Tool), whereby the software Geneious Provision: 7.0.0 comparing the DNA sequence acquired in this investigation with the sequence of the human FAM20A reference gene NG_029809 (Table 7, Fig.5).

DISCUSSION

There are a few limitations to this study because AI is an uncommon condition. The purpose of this study was to investigate the relationships between biochemical analysis and AI. The patients' ages varied from 21 to 45 years, and there was no discernible difference in age across the groups (p=0.4) , so the comparison between the two groups is more accurate because the biochemical analyses changed as a result of age [16]. Regarding gender, the findings can be the consequence of disparities in the sample size and ethnic variation. The gender distribution in the patient as well as control groups was not identical. Of the 60 patients with AI of both sexes in the research sample, 38 (63.3%) were men and 22 (36.7%) were women, while Vani et al. [17] found 33 (47.82%) were women and 36 (52.17%) were men.

SERUM UREA

When comparing patients with renal illness with the control group, there were extremely significant increases in the levels of urea and creatinine [18], so when comparing the AI patients' thoughts about having enamel renal syndrome to the healthy control group, the current study demonstrated a significant prevalence of blood urea within the normal range. This finding is consistent with the data published by Ergun and Ataol [19], which demonstrates that the patient's clinical examination revealed inadequate enamel thickness and that the serum urea level in the laboratory was normal. This outcome is consistent with Nitayavardhana et al. [7], which investigated the diagnostic oral profile of recessive FAM20A mutation-induced enamel renal disease and came to the same conclusion.

SERUM CREATININE

The current study found no differences between the levels of serum creatinine in AI patients suspected of having enamel renal syndrome when compared with healthy control group and this was represented by the no significance of the result and this matches with another research [7, 19, 20], while Roomaney et al. [8] found that 7.2% of patients have elevated serum creatinine.

SERUM CALCIUM

Since calcium makes up a majority of bones and teeth, it is not unexpected that abnormalities in the metabolism of calcium are linked to the majority of chronic illnesses, such as osteoporosis, renal disease, and periodontal tissues [21]. In this study, the result of serum calcium was within the normal range for AI patients suspected to have renal enamel syndrome and non-significant difference when compared with healthy individuals, and this result same as the result of Vogel et al. [22]. Also, this result matches to some extent the results of other studies [7, 19, 20].

SERUM POTASSIUM

Obtel et al. [23] found low serum potassium concentrations in patients who displayed severe enamel wear, which is not similar to our result that found non-significant relation between patients and healthy individuals.

ALKALINE PHOSPHATASE (ALP)

ALP is linked to mineralization, promoting and/or accelerating the mineralization process. Amelogenesis

Table 5. Numerical and percentage frequencies of FAM20A gene genotypes and their Hardy-Weinberg equilibrium (HWE) in the control group and patients group.

FAM20A	Patient group		Control group	
	Observed	Expected	Observed	Expected
Wild TT	35	36.038	12	12.800
Hetero TC	23	20.925	8	6.400
Mutant CC	2	3.038	0	0.800
Total	60	60	20	20
p-value	0.4		1.00	

Table 6. Genotype and allele frequencies of the FAM20A gene in control and patient groups in blood samples.

Genotype FAM20A	Patient group (n=60)	Control group (n=20)	P-value	OR	CI 95%
TT	35 (58.4%)	12 (60%)	--	1.00	(Reference)
TC	23 (38.3%)	8 (40%)	0.9	0.98	0.3492 to 2.7828
CC	2 (3.3%)	0 (0%)	0.7	1.6	0.0790 to 39.2411
Total	60	20	--	--	--
		Allele Frequency			
T	93 (77%)	32 (80%)	--	1.00	(Reference)
C	27 (23%)	8 (20%)	0.7	1.1	0.4791 to 2.8148

Table 7. Sequencing ID at gene bank, score, expects, and compatibility of DNA sequences obtained

Score	Expect	Identities	Gaps	Strand
1055 bits (571)	0.0	579/582(99%)	3/582(0%)	Plus/Plus

may be impacted by a potential pathway of phosphate movement through cells and matrix, similar to what has been proposed for the bone [24]. Enzymes like alkaline phosphatase are currently useful biochemical indicators for detecting of chronic periodontitis [25]. Ali et al. found that the ALP enzyme level was significantly higher than the non-periodontitis group [26]. In this study, there is a significant relationship between patients' alkaline phosphatase and control alkaline phosphatase, and this disagrees with Vogel et al. [22]. The increased activity of ALP indicates that the pathological destructive process has affected the alveolar bone, which means that the periodontal disease has significantly advanced [27]. Ozdas et al. discovered that blood ALP levels in AI subjects are statistically significantly aberrant, and the results are consistent with our investigation even in the occurrence of an additional condition [28].

FAM20A

Ding et al. identified autosomal recessive mutations in FAM20A as the cause of ERS [6], and also Kantaputra et al. found a relationship between a mutation in FAM20A and ESR [14]. Wang et al. found that FAM20A, a putative Golgi kinase with a kinase homology domain that locates in the Golgi, is important for regulating biomineralization processes, and those polymorphisms in FAM20A result in ERS [11].

CONCLUSIONS

Patients who carry for TT genotype have a higher risk for amelogenesis Imperfecta than other genotypes and have no significant relation between creatinine, potassium and calcium, and the biochemical analysis done for serum urea and alkaline phosphatase shows a significant relation between the patients and the control individuals.

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This investigation was carried out by local laws and the ethical guidelines provided in the Declaration of Helsinki. The local ethics committee provided ethical approval and provided the research protocol, informed permission forms, and subject information, University of Baghdad (Ref. 445, Jan 2022).
Contributions from MTI were gathering of data, statistical analysis, findings, discussion, draft, and final writing. The primary concept and critical revision were brought to by SSA.

CONFLICT OF INTEREST
The Authors declare no conflict of interest

CORRESPONDING AUTHOR
Mushtaq Ibraheem
College of Dentistry, University of Baghdad, Baghdad, Iraq
e-mail: mushtaq.taleb1106a@codental.uobaghdad.edu.iq

ORCID AND CONTRIBUTIONSHIP
Mushtaq Ibraheem: 0009-0003-0881-894X B C D E
Saif Abdulrazaq: 0000-0002-5553-3694 A E F

A – Work concept and design, B – Data collection and analysis, C – Responsibility for statistical analysis, D – Writing the article, E – Critical review, F – Final approval of the article

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