Association of *Amelogenin* with High Caries Experience in Indian Children

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***Objective**: The purpose of this study was to identify alterations in amelogenin gene that are associated with dental caries susceptibility and to develop a non-invasive early screening test for caries risk. **Study design**: 60 individuals were selected for the study based on the inclusion and exclusion criteria and were divided into two groups based on DMFT score. DMFT was scored according to World Health Organization guidelines. Saliva obtained from all participants was stored in Indogenix DNA Self-Collection kits at 4°C. DNA was extracted according to the manufacturer's instructions. Once the entire DNA was isolated from each sample it was put forward for PCR amplification. The amplified amelogenin gene was then run on 2% agarose gel stained with ethidium bromide. The amplified gene was processed by SSCP technique to find out the altered bands and then subjected to DNA sequencing for identification of alterations in the amino acid sequence of amelogenin gene. **Results**: The sequencing data showed the presence of mutation. Samples showing mutation (43.3%) showed high correlation with caries (80.7%) experience which was statistically significant. **Conclusion**: Understanding the genetics of dental caries susceptibility will provide new insights into the caries process in individuals and will facilitate the development of targeted preventive strategies.*

***Key words**: Amelogenin, Dental caries, Enamel, Genetics, Mutation*

INTRODUCTION

Dental caries is an outcome of an interaction between the environmental factors and host factors. In spite of a number of advancements in diagnostic technologies the detection of individuals at risk prior to the occurrence of the disease has limited accomplishment. It has already been proved that a number of environmental factors contribute to the development of dental caries which is a multifactorial and infectious disease but at the same time there is emerging strong evidence; both by studies on humans and animals; on the genetic component in the etiology of this disease.[1, 2, 3, 4, 5]

The structure of enamel is dependent on the basic odontogenesis, more importantly amelogenesis, for formation of enamel. Genetics has shown major role in the formation of tooth structure. *Amelogenin* represents 95% of the total genetic constituents responsible for enamel formation.[6] The remaining constituents are *enamelin*, *ameloblastin*, *tuftelin, tuftelin interacting protein 11*, *MMP20* and *KLK4*, etc.[7] Due to the exclusivity and complexities of enamel formation and development, the recognition and functional description of enamel forming genes in humans is significantly limited.

Amelogenin is processed by proteinases shortly after its secretion, the intact full length parent molecule is found exclusively in the newly formed enamel. Specific mutations in the *amelogenin* gene have been shown to be associated with amelogenesis imperfecta.[8] Soluble and insoluble forms of *amelogenin* might play roles in the regulation of enamel mineralization.[9]

The purpose of this study was to identify an association between alterations in *amelogenin* gene and dental caries. We hypothesize that variation in *amelogenin* gene involved in enamel formation contributes to increased caries susceptibility in children.

MATERIALS AND METHOD

The study was conducted in the Department of Pedodontics & Preventive Dentistry in collaboration with department of Biotechnology, ITS College of Dental Sciences & Research, Muradnagar, Ghaziabad, U.P, India.

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The objective of the study was to identify the role of genetic factors in the etiology of dental caries by isolating alterations in the *amelogenin* gene.

To give 80% power and at 5% significance level the minimum number of samples required per group were calculated. A minimum sample of 7 per group was found to be sufficient to demonstrate a significant difference. To generate minimum 7 samples per group a convenient sample size of 30 subjects per group was selected according to the inclusion criteria.

Institutional ethical committee approval was obtained from the Ethical Committee of ITS College of Dental Sciences & Research, Muradnagar. A written informed consent based on the ethical committee guideline was obtained from the parents of the children included in the study.

The children included in the study were in an age range of 5-12 years, reporting as outpatients to the department of Pedodontics and Preventive Dentistry. It was also taken care to maintain an almost 1:1 sex ratio in both the experimental group and control groups to eliminate sexual variations. Children were selected based on the inclusion and exclusion criteria that are as follows:

Inclusion criteria

- individuals in the range of 5-12 years
- individuals with no systemic illness
- individuals not on a long term drug therapy

Exclusion criteria

- individuals presenting an oral cleft were not considered for these analyses
- individuals with any systemic disease or taking any medication
- individuals with enamel hyperplasia / fluorosis

Children were examined by a single examiner in pediatric dental clinic on a dental chair with attached shadow less light with low and high beam intensity. The teeth were examined in a wet and dry condition using air syringe. Experimental group was defined as children with 4 or more decayed or filled tooth surfaces (DMFT/DMFS + dmft/dmfs), while controls were defined as caries free children. Caries experience was scored by the DMFT/ dmft and DMFS/dmfs indexes according to World Health Organization guidelines, 2004.

60 children were divided into two groups of 30 each. Group I had 30 children with no visible caries experience (DMFT/DMFS & dmft/dmfs: 0; n = 30) and Group II had 30 children with higher caries experience (DMFT/dmft= 4 & DMFS/dmfs ≥4; n = 30). Individuals who participated in this study were residents of villages around Muradnagar. It was seen that they had a similar income group. They also had no access to artificially fluoridated drinking water. It was also taken care to maintain an almost 1:1 sex ratio in both the experimental and control groups to eliminate sexual variations. Unstimulated saliva samples were obtained from all participants (subjects were asked to spit) and stored at -20°C; then DNAs were isolated through Genei DNA isolation Kit at room temperature until being processed. DNA was extracted according to the manufacturer's instructions and then it was checked on 1% agarose gel electrophoresis stained with ethidium bromide. Once the entire DNA was isolated from each sample it was put forward for Polymerase Chain Reaction to amplify *amelogenin* gene. The amplified *amelogenin* gene was then run on 2% agarose gel stained with ethidium bromide. The amplified gene was then processed by Single Strand Conformation Polymorphism technique (Chromous Biotech, Bangalore) to find out the altered bands and then subjected to DNA sequencing for identification of mutations in the amino acid sequence of *amelogenin* gene.

Candidate gene to be studied

Gene	Primer name	5'-3' Sequence
Amelogenin	AMEL F	GTTTCTTCCCTGGGCTCTGTAAAGAATAGTG
	AMEL R	ATCAGAGCTTAAACTGGGAAGCTG

RESULTS

The presence of decayed teeth was compared between the patients with and without mutation using the chi-square test. Mutation was found to be present significantly (p-value = 0.000) more frequently among the patients with caries (80.8%) in comparison to the patients without caries (26.5%) (Table 1)

The comparison of DMFT scores was done between the patients with and without mutation using the independent t-test. The DMFT score was found to be significantly (p-value = 0.000) more among patients with mutation (4.88±2.61) in comparison to the patients without mutation (1.47±2.53) (Table 2).

The mutation of *amelogenin* gene was noticed in 43.3% (26) children out of a total group of 60 in our study. Twenty one of these 26 children (80.7%) showed caries and had a high correlation between mutation and caries (Table 1, 2).

Yet another group of five children (19.2%) did not show caries in spite of presence of mutation (Table 1). The absence of decay can probably be due to protection through genetic means in males through Y chromosome. The products of the gene located on the X and Y chromosomes are both quantitatively and qualitatively different due to variations in the amino acid sequences of both X and Y *amelogenin* genes and the Y chromosome locus encodes a functional protein with an expression of only 10% of that of the locus on the X chromosome. [10, 11]

Altered band mobility shift was noted once the amplified gene was processed by Single Strand Conformation Polymorphism technique. On DNA sequencing mutations were identified in the amino acid sequence of *amelogenin* gene.

DISCUSSION

Tooth formation is a complex process involving sequential and reciprocal interactions of dental epithelium and mesenchyme. Ameloblasts at different stages of its life cycle; pre-secretory, secretory and mature ameloblasts; express several (i) secreted proteins, such as *ameloblastin*, *amelogenin*, *enamelin*, *tuftelin*, dentin sialoprotein, apin, amelotin (ii) enzymes such as kallikrein 4 and enamel proteinases, such as matrix metalloproteinase 20, (iii) signalling molecules like BMPs, TGFβ1, SHH and WNTs and (iv) transcription factors like Msx2, Sp3, Sp6 and Dlxs.[7]

Amelogenin is the most abundant extracellular matrix (ECM) protein in developing enamel. *Amelogenin*s are encoded by 2 single copy genes on chromosome Xp22.3–p22.1 (AMELX) and on chromosome Yp11 (AMELY).[11, 12] **Margolis** et al [9] have suggested that full length *amelogenin* plays a unique role in the control of crystal

Table 1: Table shows correlation between dental caries and mutations in *amelogenin* gene and gender distribution of the groups. The presence of decayed teeth was compared between the patients with and without mutation using the chi-square test. The decayed teeth were found to be present significantly (p-value = 0.000) more frequently among the patients with mutation (80.8%) in comparison to the patients without mutation (26.5%).

		Mutation	Total	
Caries		**Mutation absent**	**Mutation present**	
	Absent	25	5	30
		73.5%	19.2%	50.0%
	Present	9	21	30
		26.5%	80.8%	50.0%
Total		34	26	60
		100.0%	100.0%	100.0%
		Value	df	P-value
Pearson Chi-Square		17.376(b)	1	0.000
	Experimental group		**Control group**	
Sex distribution	**Male**	**Female**	**Male**	**Female**
	14	16	15	15

Table 2: The table shows comparison of DMFT scores between the patients with and without mutation using the independent t-test. The DMFT score was found to be significantly (p-value = 0.000) more among patients with mutation (4.88±2.61) in comparison to the patients without mutation (1.47±2.53).

	DMFT			t-value	p-value
Mutation	N	Mean	S.D.		
Mutation absent	34	1.47	2.525	-5.112	0.000
Mutation present	26	4.88	2.613		

shape and organization. The proteolytic processing of *amelogenin* is essential for proper enamel formation. According to various authors, genetic susceptibility to caries is manifested majorly when the enamel undergoes post eruptive maturation, 2yrs following eruption in the oral cavity. [13, 14]

According to **Patir et al,** [15] it can be hypothesized that the variations in the X component of *amelogenin* gene contributes significantly in altering the enamel structure and thereby increase susceptibility to caries and the higher caries experience shown by females can be attributed to the absence of additional 10% *amelogenin* expression from AMELY. Other possible reasons for the absence of decay in spite of presence of mutation can be non-conducive environmental factors for dental caries and maintenance of good oral hygiene. However it is also possible that these children have non-cavitated difficult to diagnose caries lesions thereby representing a false clinical well being. These children may be genetically predisposed to caries and it is strongly felt that stronger preventive strategies shall be in order for these children. There is also likelihood for existence of any alteration in the genetic makeup of an individual that makes him/her caries resistant. [16]

Mutations of *amelogenin* might affect proper enamel formation. [17] One mutation in AMELX was identified in our study that resulted in a change to the amino acid sequence of a protein.

The abnormal protein function or decreased amounts of protein might cause structural alterations of the enamel like disorganization of the enamel prisms resulting in greater amount of loss of inorganic tooth structure under low pH conditions of oral cavity and/or aid in the bacterial attachment and biofilm deposition thereby increasing the individual's susceptibility to caries.[18] Therefore, it can be assumed that mutations in the *amelogenin* may cause discrete changes in enamel microstructure and hence increase an individual's susceptibility to dental caries.[17, 18]

Dental caries was observed in the absence of mutation in a group of nine children (26.5%; Table 1). The role of a number of other secreted proteins, enzymes and signalling molecules other than *amelogenin* that are involved at various stages of enamel formation needs to be evaluated. The presence of environmental factors that lead to development of caries is already well documented.[19]

Recent studies of relationship between genetics and dental caries have found one negative and two positive associations.[20, 21, 15] The present study has found a significantly positive correlation with genetic basis-*amelogenin* gene and dental caries. However, the role of other genes in enamel formation can be examined and there relations to health and disease can be evaluated.

Genetic basis of tooth formation has been well studied.[7] The absence of amino acids in one of the proteins can lead to the defects in mature enamel predisposing it to dental caries. An early detection of such defects may contribute in determining the at risk population group and ensuring positive preventive measures to counter the disease. Since determination of *amelogenin* is a non-invasive procedure it may be suggested as a basic tool in the risk assessment protocol for dental caries. In future studies, caries diagnosis can be more emphatic with the use of dry field area, magnification loopes, fiberoptics, etc to augment the findings of the present study indicating a relationship between mutation and caries.

ACKNOWLEDGEMENT

The authors would like to thank the individuals who participated in this study. The authors are grateful to I.T.S Centre for Dental Studies & Research, Muradnagar, Ghaziabad, U.P, India for providing the Institutional Research Grant facility to the team. The funds were provided by the Institutional Research Grant for the entire study. The funders had no role in study design, data collection and analysis, decision to publish, or preparation of the manuscript. Work has been done in I.T.S Centre for Dental Studies & Research, Muradnagar, Ghaziabad, U.P, India in collaboration with Department of Biotechnology, I.T.S Paramedical College, Muradnagar, Ghaziabad, U.P, India and associated labs.

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