

# Analysis of ENAM gene single-nucleotide polymorphism rs3796704 with caries susceptibility in young adult Tamil population

Niveditha Thampan<sup>1</sup>, R Ramya<sup>2</sup>, R Swarnalakshmi<sup>3</sup>, K Rajkumar<sup>1</sup>, S Savithri<sup>4</sup>, G Divyalakshmi<sup>1</sup>

Departments of <sup>1</sup>Oral and Maxillofacial Pathology and <sup>4</sup>Microbiology, SRM Dental College, <sup>2</sup>Department of Oral and Maxillofacial Pathology, Saveetha Dental College, Chennai, Tamil Nadu, India, <sup>3</sup>Department of Stomatognathic Function and Occlusal Reconstruction, Tokushima University, Tokushima, Japan

## Abstract

**Background:** Dental caries is as primeval as humanity, but still, investigations are undergoing regarding the etiopathogenesis behind this multifactorial disease. Genetics is known to play a vital role in the etiology behind dental caries in addition to environmental and socioeconomic factors. Genetic variations like single-nucleotide polymorphisms (SNPs) were extensively studied in the past decade to portray the etiopathogenesis contributing to dental caries.

**Aim:** This investigation was undertaken to analyze the ENAM gene SNP rs3796704 with caries susceptibility in ethnic young adult Tamil population of India.

**Materials and Methods:** Out of 370 patients included, 215 patients belonged to the high caries group (Decayed, Missing and Filled Tooth [DMFT]  $\geq 2$ ) and 155 patients belonged to the low caries group (DMFT  $\leq 1$ ). DNA was extracted from the blood of all the individuals. SNP genotyping was performed utilizing tetra-primer amplification refractory mutation system–polymerase chain reaction with specific primers.

**Results:** The genotyping results showed that there were no differences in allelic ( $P = 0.114$ ) and genotypic frequencies ( $P = 0.159$ ) between the high caries and low caries groups.

**Conclusion:** Future studies can be conducted in larger samples and different ethnicities around the globe to analyze the role played by SNPs of enamel formation genes in cariogenesis.

**Keywords:** Decayed, dental caries, ENAM, Missing and Filled Tooth, single-nucleotide polymorphism, tetra-primer amplification refractory mutation system polymerase chain reaction

**Address for correspondence:** Dr. Niveditha Thampan, Department of Oral and Maxillofacial Pathology, SRM Dental College, Bharathi Salai, Ramapuram, Chennai - 600 089, Tamil Nadu, India.

E-mail: thampan.niveditha@gmail.com

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## INTRODUCTION

Dental caries is an oral pathology of multifactorial etiology.<sup>[1]</sup> Environmental factors such as increased number of cariogenic bacteria in the mouth, poor oral hygiene,

dental plaque, cariogenic diet, poor salivary characteristics and insufficient fluoride exposure can collectively lead to formation of dental caries.<sup>[2]</sup> However, researches in the past decade had revealed that despite the individuals being

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exposed to the same levels of environmental risk factors, some of the individuals were more prone or more resistant to caries. Furthermore, the investigators could not explain the process of polarization of caries in a specific group of people.<sup>[3]</sup> All these problems prompted the researchers to analyze if genetics could be a causative factor for dental caries.

Genetic variations like single-nucleotide polymorphisms (SNPs) were extensively studied in the last decade, and it was concluded that genetics does play an important role in the etiology of dental caries. The major candidate genes responsible for dental caries belong to the following groups: enamel formation genes, genes related to saliva, genes related to immunological responses and genes related to carbohydrate metabolism.<sup>[4]</sup>

The largest enamel matrix protein is enamelin, which is expressed by ameloblasts in the secretory and early maturation stages.<sup>[5]</sup> It is the largest (~200 kDa) but the least abundant (3%–5%) of the three major secretory-stage enamel matrix proteins.<sup>[6]</sup> It is concentrated near the mineralization front and facilitates crystal elongation and organization through self-assembling nanostructures.<sup>[5]</sup> In the absence of enamelin protein, there is a lack of normal enamel crystal formation and only a disorganized mineral layer is developed within the intercellular spaces between the ameloblasts in the secretory stage.<sup>[7]</sup>

Enamelin protein is coded by the ENAM gene.<sup>[5]</sup> Functional and evolutionary studies have confirmed that ENAM gene plays a very important role in amelogenesis. Till date, 10 mutations and 406 SNPs have been reported for ENAM gene. Mutation in ENAM gene leads to a genetic disorder with generalized and severe enamel defects called type 2 autosomal dominant amelogenesis imperfect (ADAI).<sup>[6]</sup>

Out of 406 SNPs identified so far in ENAM gene, SNP rs3796704 was analyzed in the present study. This SNP is encoded by exon 10 region of the ENAM gene (chromosome 4q 13.3), and it is a missense variant present in the coding region. In this SNP, G allele is a wild-type allele and A allele is a minor allele.<sup>[6]</sup> This SNP was selected for the present study, because at position 763 of this SNP, there is a substitution of amino acid arginine with glutamine (Arg > Gln). This amino acid substitution can lead to alteration in size and charge of the protein. The hydrophobicity of the protein is also modified. These changes may influence the enamel structure and hardness, thereby rendering the enamel vulnerable to caries.<sup>[8]</sup>

Previous studies had confirmed that SNPs in ENAM gene can alter the phosphorus levels in saliva,<sup>[9]</sup> increase the levels of mineral loss under acidic conditions and also can facilitate bacterial attachment and biofilm deposition.<sup>[10]</sup> Researches conducted in Turkey<sup>[11]</sup> and France<sup>[6]</sup> had identified that rs3796704 polymorphism in ENAM gene has a strong association with dental caries. Although the researches concerning the management of dental caries are steadily improving, the studies associated with the preventive measures are yet to gain momentum. Thus, polymorphisms related to ENAM gene are of utmost importance to identify the caries-susceptible group in a population which will aid as a preventive measure in the management of dental caries. Till date, no study has been reported linking the polymorphisms in ENAM gene and dental caries in ethnic young adult Tamil population. Therefore, this exploration has been undertaken to check whether there is any association between rs3796704 SNP of ENAM gene and dental caries in young adult ethnic Tamil population in India.

## MATERIALS AND METHODS

### Sample size calculation

The power analysis was done with the help of Openepi software (Dean AG, Sullivan KM, Soe MM, Atlanta, GA, USA, OpenEpi: Open Source Epidemiologic Statistics for Public Health, Version.3.01 www.OpenEpi.com). A minimum sample size of 150 was required for both high caries and low caries groups with a confidence interval of 95% and with a power of 90% in an unmatched case–control study. It was decided by the researchers to include 370 patients in the present study with 155 patients belonging to the low caries group (Decayed, Missing and Filled Tooth [DMFT] score ≤1) and 215 patients belonging to the high caries group (DMFT score ≥2).

### Ethical considerations

This study was approved by the Institutional Review Board and Ethical Committee (Ref No: SRMDC/IRB/2017/MDS/No. 604).

### Study population and patient selection

The patients were selected from the Outpatient Department of SRM Dental College, Ramapuram, Tamil Nadu, from December 2017 to May 2019. The patients were given oral and written information notes regarding the objectives, methodology and scope of the study and the required consent forms were signed. The patients within the age group of 18–30 years were only included in the present study. A survey questionnaire was prepared and validated to analyze the ethnicity of the individuals. Patients with

Tamil as mother tongue and those with three generations of family residing in Tamil Nadu were included in the present study. Patients with any genetic diseases, such as amelogenesis imperfecta, dentinogenesis imperfecta, osteogenesis imperfecta, dysplasia of enamel from medications or chemical origins or with any chronic systemic illness or eating disorders were excluded from the study. Patients undergoing orthodontic treatment were also excluded from the present study.

### Patient examination and grouping

Oral examination was performed in dental chair using a sterile mouth mirror (Dentsply Sirona Mouth Mirror #5), explorer (Dentsply Sirona Ash Lustra combination probe/explorer AMP No. 5) and an overhead dental lamp. DMFT index was used to categorize patients into low caries group (DMFT score  $\leq 1$ ) and high caries group (DMFT score  $\geq 2$ ). Panoramic and bitewing radiographs were also taken for each patient to confirm the diagnosis of decayed and filled teeth.

### Blood collection and single-nucleotide polymorphism genotyping

Under strict aseptic conditions, 2-mL venous blood was drawn from all the patients in both the groups and collected in K3 ethylenediaminetetraacetic acid-coated nonvacuum blood collection tubes, labeled and stored at 4°C till further use. Four primers (two inner primers – forward and reverse, two outer primers – forward and reverse) were designed after analyzing the ENAM gene sequence from SNP database in NCBI (<http://www.ncbi.nlm.nih.gov/snp/>), for performing tetra-primer amplification refractory mutation system–polymerase chain reaction (TP ARMS-PCR). The characteristics of the primers used in the study are summarized in Table 1. The DNA isolation was performed by salting out method. Isolated DNA from venous blood was subjected to TP ARMS-PCR technique. The product size of PCR amplicons for G allele was 210 bp, for A allele was 312 bp and for the outer primers was 501 bp. The PCR product was electrophoresed on 2% standard agarose gel at 100 volts for 20–30 min. The DNA fragments were visualized by ethidium bromide on an ultraviolet transilluminator (Biobase BK02S) to identify the alleles.

### Statistical analysis

Statistical analysis was done using the Statistical Package

for the Social Sciences (SPSS Inc. Released 2008. SPSS Statistics for Windows, Version 17.0. Chicago: SPSS Inc). Mean and standard deviation (SD) values were utilized to compare the age and DMFT scores between the high caries and low caries groups. The Hardy–Weinberg equilibrium, gender, allele and genotypic differences between the high caries and low caries groups were analyzed using Chi-square test.  $P < 0.05$  was considered statistically significant.

## RESULTS

The results evaluating the demographic characteristics are summarized in Table 2. The mean DMFT score  $\pm$  SD for the high caries group was  $4.79 \pm 2.568$  and for the low caries group was  $0.43 \pm 0.507$ . The mean age of patients in the high caries group was  $26.85 \pm 3.585$  years and that in the low caries group was  $26 \pm 3.847$  years.

Out of the 215 patients included in the high caries group, 92 (43.21%) patients were male and 123 (56.79%) patients were female. There were 67 (42.86%) males and 88 (57.14%) females in the low caries group. A total of 159 (43.14%) males and 211 (56.86%) females were included in the present study. Females were slightly more than males in the studied population, but it was not statistically significant.

The comparison of alleles and genotypes between high caries and low caries groups is summarized in Table 3. All the genotypes were in Hardy–Weinberg equilibrium. 95.06% of individuals in the high caries group and 99.02% of individuals in the low caries group carried G allele whereas 20.99% individuals in the high caries group and 0.98% of individuals in the low caries group displayed A allele. 79.01% in the high caries group and 95.24% in the low caries group displayed homozygous GG genotype whereas 4.94% in the high caries group and none of the individuals in the low caries group showed homozygous AA genotype. 16.05% in the high caries group and 4.76% in the low caries group showed heterozygous GA genotype [Figure 1]. The results were not statistically significant.

## DISCUSSION

Enamelin is an 1142 amino acid secretory protein with a 39 amino acid signal peptide.<sup>[12]</sup> Hydrophobic, acidic and basic domains are present in different regions of the

**Table 1: The characteristics of the primers used in the study**

Primer	Nucleotide sequence	Length	Annealing temperature (°C)	Expected products (bp)
Forward inner	GAAGAAAGCACTCTATTCCTTCCCG	30	65	G allele - 210
Reverse inner	GTATCCTGTGGTCCAGGAATGCT	27	66	A allele - 312
Forward outer	TCTTCCTGGACAAAATAGATGGGGT	27	65	Common - 501
Reverse outer	AGTTCAAATTCTCACCTTCATGCCA	28	65	

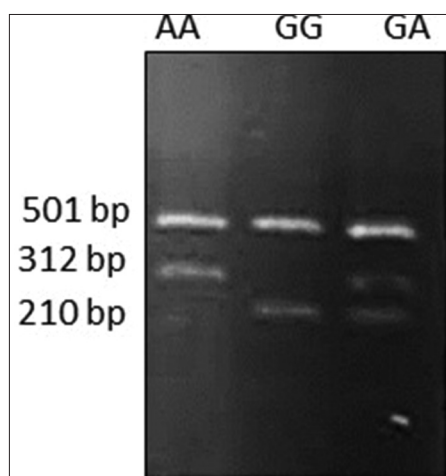
**Table 2: The demographic characteristics of the studied population**

Parameters	Percentage and mean		Mean	SD		$\chi^2$	P
	High caries	Low caries		High caries	Low caries		
n	215	155					
Age	26.85	26.00	26.676	3.585	3.847		
DMFT score	4.79	0.43	3.892	2.568	0.507		
Gender (%)							
Males	92 (42.86)	67 (43.21)	159 (43.14)			0.001	0.977
Females	123 (57.14)	88 (56.79)	211 (56.86)				

SD: Standard deviation, DMFT: Decayed, Missing and Filled Tooth

**Table 3: The comparison of the alleles and the genotypes present in high caries and low caries groups**

Parameter	Group		$\chi^2$	Degrees of freedom	P
	High caries (%)	Low caries (%)			
Allele					
G	95.06	99.02	2.49	2	0.114
A	20.99	0.98			
Genotype					
GG	79.01	95.24	1.98	2	0.159
GA	16.05	4.76			
AA	4.94	0			



**Figure 1:** Tetra-primer amplification refractory mutation system– polymerase chain reaction for the detection of ENAM rs3796704 genotypes. The product sizes were 312 bp for A allele, 210 bp for G allele and 501 bp for internal control

enamelin molecule. It is a glycosylated, phosphorylated protein that is promptly sliced into smaller particles following its secretion. Immunohistochemical studies had revealed that enamel is most commonly seen at the developing tip of the enamel crystallites whereas its cleavage products are mostly seen in the rod and interrod enamel. The 186-kDa glycoprotein is the primary parental enamel protein secreted by ameloblasts which is typically appreciated near the secretory face of the Tomes's process of the ameloblasts. This protein is then cleaved to produce many small-molecular-weight proteins, out of which the stable one is 32 kDa.<sup>[13]</sup> The 32-kDa enamel, with its two phosphorylated serines and three glycosylated asparagines,

is a hydrophilic and acidic glycoprotein which has increased attraction for calcium and hydroxyapatite crystals, thereby helping in the progression of enamel mineralization. The 32-kDa enamel along with amelogenin amplified the dimensions of octacalcium phosphate crystals and improved the stability of the transient amorphous calcium phosphate phase. The enamel also stimulated the kinetics of nucleation of hydroxyapatite crystals in a pattern which is dose dependent. *In vitro* studies had confirmed that enamel and amelogenin interact with each other for amelogenesis.<sup>[14]</sup>

Enamelin protein is encoded by the ENAM gene, positioned on chromosome 4q 13.3, the same region as that of AMBN gene, signifying that this region could comprise a cluster of genes that encrypt for enamel matrix proteins.<sup>[15]</sup> This gene belongs to P/Q-rich secretory calcium-binding phosphoprotein cluster gene family.<sup>[11]</sup> The gene spans over 20 kb, and it is found to have 10 exons and 8 introns. Out of the 10 exons, 8 of them are in the coding region. Till date, mutations were described in 7 exonic and 3 intronic regions of this gene, with a maximum number of them related to hypoplastic ADAI. A few of these mutations amend the areas related to the sequence of the 32-kDa cleavage product of the ENAM gene, and *in vitro* studies have exposed that this particular area is vital in the collaboration of enamel with amelogenin. Thus, the amelogenin–enamel interactions were affected because of a weak 32-kDa polypeptide, which consecutively have a direct effect on the phenotype of enamel.<sup>[16]</sup>

Females were more affected by dental caries than males in the present study, however, results were not statistically significant. Increased prevalence among women was in accordance with studies conducted by Antunes *et al.*<sup>[17]</sup>, Lukacs and Largaespada,<sup>[18]</sup> and Demirci *et al.*<sup>[19]</sup> Increased occurrence of dental caries in females may be attributed to early eruption of teeth, hormonal fluctuations in women leading to disparities in salivary flow rate and composition and hormonal changes during pregnancy.

The SNP genotyping was done utilizing TP ARMS-PCR technique which reinforces the fact that this technique

is more simple, specific and cost-effective in detecting SNPs when compared to other currently employed techniques.

There are various mechanisms by which variations in ENAM gene can make an individual susceptible to dental caries. Gerreth *et al.*<sup>[10]</sup> had suggested that disparities in ENAM gene can result in increased loss of minerals from the enamel during acidic conditions. Furthermore, deposition of biofilm and attachment of bacteria is enabled in such a situation. Kuchler *et al.*<sup>[9]</sup> had confirmed that genetic alterations in the ENAM gene can alter the phosphorous levels in saliva, thereby disturbing the remineralization process. Patir *et al.*<sup>[11]</sup> hypothesized that discrepancy in ENAM gene may intermingle with the presence of *Streptococcus mutans* infection. Babbitt *et al.*<sup>[20]</sup> had confirmed that any alterations in the enamel matrix protein-encoding genes can show hypoplastic or thin enamel, thus rendering the tooth susceptible for dental caries.

While comparing the association of ENAM gene SNP rs3796704 among the high and low caries groups, the present study infers lack of association between the allelic or genotypic frequencies and caries phenotype, which is in agreement with the researches done by Gerreth *et al.*,<sup>[10]</sup> Koohpeima *et al.*,<sup>[21]</sup> and Chaussain *et al.*<sup>[6]</sup> However, Divakar *et al.*<sup>[8]</sup> concluded that the heterozygous GA and minor allele A of SNP rs3796704 act as a defending factor against dental caries, which is not in accordance with the present study.

Various researchers analyzed environmental factors such as cariogenic bacterial count, tooth brushing frequency and consumption of cariogenic diet along with the genetic polymorphisms. Patir *et al.*<sup>[11]</sup> inferred that rs3796704 polymorphism does not contribute directly toward dental caries in Turkish children, which is in accordance with the present study, but the researchers also concluded that there is a possible interaction of this polymorphism with *S. mutans*. Abbasoğlu *et al.*<sup>[22]</sup> also concluded that this SNP of ENAM gene along with exposure to environmental factors is not associated with early childhood caries in Turkish children, which is in accordance with the present study.

The occurrence of multiple SNPs together can also contribute to increased caries susceptibility. Daubert *et al.*<sup>[23]</sup> conducted studies among African American adults and observed that when SNP rs7671281 was examined along with rs3796704, the caries predisposition amplified 2.66 times independent of other risk factors.

The SNP rs3796704 is not only associated with dental caries but also plays a major role in the occurrence

of molar-incisor hypomineralization (MIH). Studies conducted by Jeremias *et al.*<sup>[24]</sup> concluded that individuals who carry the G allele in this SNP were 17 times more likely to be affected by MIH than those who carry the A allele.

The SNPs occurring in the exon 10 region of chromosome 4 are known to play a very crucial role in increasing the susceptibility of an individual to dental caries. This is confirmed following the observations by Wang *et al.*<sup>[5]</sup> in Chinese children where the researchers examined rs3796703 SNP of ENAM gene and they concluded that this neighboring SNP may also be involved in caries susceptibility.

## CONCLUSION

After comparing the allelic and genotypic frequencies in both the high caries and low caries groups, the current study concluded that rs3796704 ENAM gene SNP lacks a relationship with caries phenotype in young adult ethnic Tamil population in India. In addition to genetic factors, environmental factors were also known to play a significant role in the pathogenesis of caries. Since the participants included in the present study were from different socioeconomic backgrounds and followed varied dietary and oral hygiene practices, these factors may also contribute to caries. This may be one of the major reasons for the lack of association with the caries phenotype. But still, the study was able to rule out the role of an important SNP which was found to play a significant role in cariogenesis in other populations across the globe.

Future studies can aim to analyze other SNPs of ENAM gene in exon 10 region such as rs3796703, rs2609428, rs7671281, rs36064169 and intronic variants such as rs12640848, rs144929717 and rs139228330 which were all known to contribute to cariogenesis. SNPs of other enamel formation genes such as AMELX, AMBN, TUFT and KLK4 can also be analyzed in further studies. Not only the enamel formation genes but gene families contributing to caries like those related to saliva, immune responses and carbohydrate metabolism can also be evaluated. The environmental factors contributing to this multifactorial disease such as cariogenic bacterial count, saliva flow rate and pH, diet, fluoride content and dental plaque must be inspected along with genetic variations so that the complete depiction of the etiopathogenesis behind this complex disease can be analyzed. Moreover, epigenetic factors can also be inspected, in future, to examine its role in the pathogenesis of dental caries. These researches, when established, will be able to unravel the complex etiopathogenesis of dental caries, thereby

rendering custom-made prevention programs for different individuals around the globe against dental caries. An extra care and a word of caution can be prearranged to the caries-susceptible patients. This helps in reducing the mortality, morbidity and economic burden associated with this multifaceted and the most prevailing disease among human beings – the dental caries.

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### Conflicts of interest

There are no conflicts of interest.

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