Original article

Association between ENAM polymorphisms and dental caries in children

Darshan Devang Divakar a,b,⇑, Sultan Ali S. Alanazi c, Mohammed Yahya A. Assiri c, Shahad Mohammed Halawani c, Saleh Zaid Alshehri d, Saeed Ahmed Saeed Al-Amri e, Mohammed Mustafa f

a Dental Biomaterials Research Chair, Dental Health Department, College of Applied Medical Sciences, King Saud University, Riyadh 11433, Saudi Arabia
b Department of Oral Medicine and Radiology, KVG Dental College and Hospital, Sullia, Karnataka, India
c Alfarabi Dental College, Riyadh 11691, Saudi Arabia
d Department of Oral and Maxillofacial Surgery, College of Dentistry, King Saud University, Riyadh 11433, Saudi Arabia
e Dental Public Health and Advanced Education in General Dentistry Resident, Department of Preventive Dentistry, Riyadh College for Dentistry and Pharmacy, Riyadh and Prince Sultan Dental Center (Namas General Hospital) MOH, Asser, Saudi Arabia
f Division of Endodontics, Department of Conservative Dental Sciences, College of Dentistry, Prince Sattam bin AbdulAziz University, P.O. Box:153, AlKhurj 11942 Riyadh, Saudi Arabia

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Abstract

Aim: Dental enamel, the most rigid biological tissue of the tooth known to mankind, is the most integral and fundamental part of the tooth. Enamel matrices compile 5% of Enamelin peptides and at the time of tooth development, they are considered to effect the formation and elongation of enamel crystallites. ENAM plays critical role in enamel formation. Any changes in ENAM may affect the thickness of enamel and may lead to dental caries. The present study is aimed to evaluate the association of ENAM gene polymorphisms and susceptibility of dental caries development risk.

Material and methods: The present study was carried out on 168 South Indian children, children’s with dental caries were included in study. Written consent was taken from their parents/guardians. Additionally 193 healthy individuals were enrolled as controls. Sampling was done after dental examination of the individuals. Three ENAM gene single nucleotide polymorphisms (SNPs) were rs7671281, rs3796704 and rs12640848 was genotyped to check their role in susceptibility of dental caries development risk.

Results: Out of three SNPs rs7671281 showed statistically significant risk association with dental caries susceptibility in this ethnic population at heterozygous allele CT (OR: 1.939, p = .01865) and with minor allele T (OR: 1.451, p = .001292). SNP rs3796704 showed significant protective association with dental caries in Indian population at heterozygous allele GA (OR: 0.409, p = .0192) and with minor allele A (OR: 0.645, p = .00875). SNP rs12640848 showed significant protective association with dental caries in Indian population at heterozygous allele AG (OR: 3.041, p = .00642) and with minor allele G (OR: 1.478, p = .02184). Preliminary insilico analysis also showed that rs7671281 (Ile648Thr) amino acid change will cause the structural and functional changes in ENAM protein.

Conclusions: In the present study significant association was observed between ENAM gene SNP rs7671281 and dental caries susceptibility in South Indian children. These results suggested that ENAM gene variants may contribute to dental caries in children.

1. Introduction

Dental enamel, the most rigid biological tissue of the tooth known to mankind, is the most integral and fundamental part of the tooth, that is principally accountable for surmounting the mechanical resistance of foodstuffs, combating masticatory pressures and diminishing wear and tear. The density, consistency and allocation of the enamel are thus presumable to emulate the conditions of dental adaptations to the dietary regimen. Enamel thickness in posterior teeth has become a pivotal and indispensable feature in taxonomical and practical researches pertaining to...
the teeth in both living and already extinct primates (Shellis et al., 1998). There exists a large amount of conspicuous hypothesis in the anthropological writings with reference to the paramountcy of diet in human evolution. Diet and foraging motif are speculated to have had anesteric predominance on the human phenotype, and dietary dissimilarities have been contemplated to accord to the sensational morphological alterations seen in avant-garde human beings (Babbitt et al., 2011). Pragmatically, molar enamel consistency and density does turn up to correspond with diet (Hlusko et al., 2004).

During the secretory phase of amelogenesis, thickness of the enamel is resolute. A multitude of evidences point out the significance of enamelin (ENAM), enamelysin (MMP20), ameloblastin (AMBN), and amelogenin (AMELX) in determining the space for the appropriate growth in length of the enamel crystallite ribbons and enamel rods preceding to maturation (Horvath et al., 2014). Thickness of the Enamel differs appreciably between existing hominoids and is an essential characteristic with importance for explicating dietary adaptation, phylogeographic affiliations and life history trajectory (Horvath et al., 2014). Several studies put forward an intricate mechanism for enamel thickness, with a probable dietary constituent of intertaxon disparity overlapped on allometric trends. Attenuated enamel correlates with smoother foodstuffs, and bulkier enamel associated with harder food stuffs (Hlusko et al., 2004).

ENAM gene plays a crucial role in enamel formation. Enamel matrixes compile 5% of Enamelin peptides and at the time of tooth development, they are considered to effect the formation and elongation of enamel crystallites (Kelley and Swanson, 2008). ENAM is a member of P/Q-rich secretory calcium-binding phosphoprotein cluster genes, (Al-Hashimi et al., 2009). In spite of the salience of enamel thickness in modern primate and hominoid evolutionary studies and genotypic studies, only one study elucidated the phenotypic effects of polymorphism.

Recent genome wide association studies in dental caries patients from European Americans revealed the role of ENAM gene variants in dental caries formation which created more interest to study the SNPs in ENAM gene. Exon10 of ENAM gene plays key role in causing dental caries. Previous studies reported that the ENAM gene variants rs3796704 and rs7671281 may affect the microstructure of enamel which may leads to development of the caries (Lucas et al., 2008) and they have role in causing dental caries. Additional research of the ENAM gene led to the detection of a non-synonymous SNPs (rs7671281) and (rs3796704) which might be associated with dental caries formation (Lucas et al., 2008).

The current study is deliberated to explore the phenotypic effect of SNPs rs12640848, C14625T (rs7671281), and G14971A (rs3796704) on the dental enamel thickness of humans.

2. Materials and methods

In this case control study 168 children with caries attending outpatient Department of Oral Medicine and Radiology, KVG, Dental College and Hospital, Karnataka, India were included after obtaining the necessary approval from the institutional review board. Written consent was taken from the children's parents/guardians. Additionally 193 caries-free patients attending the Departments were enrolled as controls. Saliva samples were collected from both the groups (with caries and caries free). The criteria for inclusion were children between 2 and 15 years with minimum 3 active caries lesions with cavitation, for permanent dentition, decayed, missing, filled tooth (DMFT/DMFS) ≥ 4, and for primary dentition decayed, filled tooth (dft/dfs ≥ 4), and for mixed dentition both were done. Patients with genetic diseases, chronic illness and other disorders were not included. Control individuals were included based on following that their age must be in between 18 to 25 years old with no caries lesions, without DMFT.

2.1. Sample collection

Saliva samples were collected in PBS solution, then immediately utilized for DNA extraction using the PureLink® Genomic DNA Mini Kit (Invitrogen™, USA). The DNA concentration was quantified using a Nano Drop 8000 (Thermo Fisher Scientific, USA).

2.2. Genotyping

Three SNPs in ENAMEL gene rs7671281 C>T (Ile648Thr), rs3796704 G>A (Arg763Gln) and rs12640848 (C>T) were selected based on literature. Genotyping analysis was performed using TaqMan genotyping assays. Each genotyping reactions were performed as described by Alani et al. (2017) using a QuantStudio™ 7 Flex RT PCR System (Applied Biosystems).

2.3. Statistical analysis

The case-control comparisons were performed by calculating Odds ratios (ORs), and 95% confidence intervals (CIs) and chi-square test. SPSS version 22.0 (Statistical Package for the Social Sciences) was used to carry out all the statistical analysis. I-Mutant2.0 (Capiotti et al., 2005) was used for Protein stability change estimation and HOPE software (Venselaar et al., 2010) was used to check the functional and structural effects of amino acids.

3. Results

A total of 361 individuals were included in the present study. ENAM gene polymorphisms were analyzed in children with dental caries (n = 168) and caries-free young adult controls (n = 193). Table 1 gives the patients attributes. Males were largely represented in caries free patients (53.33%), whereas 47.67% of female patients were caries free. But in children with caries, females exhibited less caries (47.62%) than males (52.38%). Around 32.74% of children brushed their teeth twice with fluoridated toothpaste as compared with caries free young adults (33.16%). Also children's with caries showed extrinsic factors for caries and had 5.7 ± 2.9 active caries lesions (mean ± SD). Plaque index showed 59.52% in children's with caries whereas caries free young adults showed 16.06%.

3.1. Genotyping

To investigate the risk of predisposition to dental caries among Indian population with the ENAM gene polymorphisms, three different SNPs rs7671281 C>T (Ile648Thr), rs3796704 G>A (Arg763Gln) located in exon 10 and rs12640848 located in intron 8 were selected and were examined on 168 dental caries children cases and 193 healthy adult controls. The clinicopathological characteristics of case controls are presented in Table 1.

The distribution of genotype and allele frequencies between dental caries cases and healthy controls of the ENAM polymorphisms along with odds ratio, 95% CI and p-values are shown in Tables 2, 3 and 4. Ancestral homozygous allele has been used as reference for comparison. SNP rs7671281 of the three SNPs in ENAM gene showed statistically significant risk association with dental caries susceptibility in this ethnic population at heterozygous allele CT (OR: 1.939, p = .01865) and with minor allele T (OR: 1.451, p = .001292). Table 2. SNP rs3796704 showed significant protective association with dental caries in Indian population.
at heterozygous allele GA (OR: 0.409, p = .0192) and with minor allele A (OR: 0.645, p = .00875) Table 3. SNP rs12640848 showed significant risk association with dental caries in Indian population at heterozygous allele AG (OR: 3.041, p = .00642) and with minor allele G (OR: 1.478, p = .02184) Table 4. Even after Bonferroni correction most of the p-values showed significant association (Tables 2–4).

3.2. Effect of SNP rs7671281 mutation on ENAM protein structure

The Fig. 1 illustrates the structures of the original (left) and the mutant (right) amino acids and its backbone (red colored) (Fig. 1). The side chain which is unique for each amino acid, is colored black. The wild and mutant amino acids vary in size, charge and hydrophobicity value. Threonine is smaller than the Isoleucine amino acid which is wild type position. Isoleucine is more hydrophobic than the Threonine. Due to these changes protein may lose central functional part or on the surface interactions.

3.3. Effect of SNP rs3796704 mutation on ENAM protein structure

The Fig. 2 illustrates the structures of the wild (left) and the mutant (right) amino acids and its backbone (red colored) (Fig. 2). The side chain of each amino acid is in black color and it’s unique for every amino acid. There is a difference in charge between the Arginine and Glutamine (Fig. 2). If there is any change in amino acid charge it may lose interactions with other molecules. The Arginine and Glutamine differ in size. Glutamine residue is smaller, which may lead to loss of interactions.

4. Discussion

ENAM gene plays a crucial role in teeth enamel formation. Any modifications in genes that encode proteins of the enamel may lead to malformation of the enamel (Babbitt et al., 2011). Any aberrations during the primary stage of enamel formation may result in hypoplastic or thin enamel (Babbitt et al., 2011). As Patir et al. reported, the enamel in individuals with mutations in genes associated in enamel formation may have excessive levels of mineral loss under acidic condition as well as biofilm deposition and microbes attachment is much aided under these circumstances (Al-Hashimi et al., 2009).

The present study suggests that ENAM is a candidate gene in susceptibility for primary dental caries in South Indian children. We have evaluated three ENAM gene SNPs namely rs7671281, rs12640848 and rs3796704. Out of three SNPs studied, two were located in exon 10. SNPs rs7671281 was significantly associated

### Table 1
The clinicopathological characteristics of case controls.

|                      | Children with caries | Young adults caries-free |
|----------------------|----------------------|--------------------------|
| Patients             | 168                  | 193                      |
| Age mean ± 1 SD      | 6.9 ± 1.9            | 23.2 ± 2.5               |
| Gender               |                       |                          |
| Male (%)             | 88 (52.38)           | 101 (52.33)              |
| Female               | 80 (47.62)           | 92 (47.67)               |
| Family history of decay (%) | at least 1 member of the family with decay | 61 (36.30) | 41 (21.24) |
| Tooth brushing (%)   |                       |                          |
| Brushing without brushing aids | 10 (5.95) | 22 (11.39) |
| Once a day (Fluoridated paste) | 98 (58.33) | 110 (56.99) |
| Twice a day (Fluoridated paste) | 55 (32.74) | 64 (33.16) |
| Consumption of soft drinks (%) | Once a week | 52 (30.95) | 60 (30.09) |
|                        |                      |                          |
|                        | (Citrated drinks)    |                          |
|                        | Once to three times a day | 38 (22.61) | 49 (25.39) |
|                        | (Citrated drinks)    |                          |
|                        | More than three times a day | 22 (13.09) | 18 (9.32) |
|                        | (Non-Citrated drinks) |                          |
|                        | Once to three times a day | 30 (17.85) | 39 (20.20) |
| Consumption of sweets |                        |                          |
|                        | Once a week (unusual time) | 61 (36.30) | 58 (30.5) |
|                        | Consumed before food | 22 (13.10) | 34 (17.61) |
|                        | Consumed after food | 80 (47.62) | 98 (50.78) |
| Dental examination     |                        |                          |
| DMT (mean ± 1 SD)     | 3.67 ± 3.1           | 0                        |
| dsf (mean ± 1 SD)     | 8.1 ± 2.8            | 0                        |
| DFS (mean ± 1 SD)     | 5.9 ± 6.2            | 0                        |
| dfs (mean ± 1 SD)     | 17.8 ± 10.6          | 0                        |
| Number of active caries lesions (D + d) | 5.7 ± 2.9 | 0 |
| Plaque index (%)      |                        |                          |
| Low                  | 21 (12.5)            | 106 (54.92)              |
| Moderate             | 42 (25)              | 56 (29)                  |
| High                 | 100 (59.52)          | 31 (16.06)               |

### Table 2
Genotype frequencies of ENAM gene SNP rs7671281 polymorphism in children with caries and healthy controls.

| SNP                  | Variant | Caries | Healthy | OR   | CI   | χ² Value | p-Value |
|----------------------|---------|--------|---------|------|------|----------|---------|
| rs7671281            | CC      | 44(0.26) | 64(0.33) | Ref |      |          |         |
|                      | CT      | 68(0.40) | 87(0.45) | 1.137 | 0.691–1.871 | 0.26 | 0.61352  | 1       |
|                      | TT      | 56(0.34) | 42(0.22) | 1.939 | 1.114–3.376 | 5.53 | 0.01865  | 0.0373  |
|                      | C       | 156(0.46) | 215(0.56) | Ref |      |          |         |
|                      | T       | 180(0.54) | 171(0.44) | 1.451 | 1.081–1.946 | 6.18 | 0.01292  | 0.02584 |

1 Bonferroni corrected p-value.
2 p-value < 0.05

### Table 3
Genotype frequencies of ENAM gene SNP rs3796704 polymorphism in children with caries and healthy controls.

| SNP                  | Variant | Caries | Healthy | OR   | CI   | χ² Value | p-Value |
|----------------------|---------|--------|---------|------|------|----------|---------|
| rs3796704            | GG      | 99(0.59) | 92(0.48) | Ref |      |          |         |
|                      | GA      | 58(0.34) | 76(0.39) | 0.709 | 0.455–1.106 | 2.30 | 0.129  | 0.258  |
|                      | AA      | 11(0.07) | 25(0.13) | 0.469 | 0.190–0.878 | 5.49 | 0.019  | 0.038  |
|                      | G       | 256(0.76) | 260(0.67) | Ref |      |          |         |
|                      | A       | 80(0.24) | 126(0.33) | 0.645 | 0.464–0.896 | 6.87 | 0.00875  | 0.0175 |

1 Bonferroni corrected p-value.
with the caries susceptibility. SNP rs3796704 showed protective association. Due to amino acid replacement key properties like size, charge and hydrophobicity were changed, which may lead
to effect the ENAM gene function, increasing enamel susceptibility to caries. The intronic SNP rs12640848 showed risk significant association with dental caries in young South Indian population.

Our results in contrast with Wang et al. (2012), study which reported that there is no correlation between ENAM gene polymorphisms and primary tooth caries. But Chaussain et al. (2014) and Gerreth et al. (2016) reported than six ENAM gene SNPs are significantly associated with caries susceptibility, particularly the C allele in SNP rs7671281 and the A allele in SNP rs3796704. These results are strongly supporting our present study.

Chaussain et al. (2014) reported significant association between caries susceptibility and ENAM gene SNP rs12640848 with an OR = 3.89; but the OR was not non-significant after multiple correction. They also reported that, there is significant association between dental caries susceptibility and ENAM coding region variant rs3796704.

We reported that SNP rs7671281 is leading to threonine (polar hydrophilic) substitution to isoleucine (non-polar hydrophobic). These both amino acids differed in their properties which is leading to change the structure and function of ENAM protein. This SNP showed significant difference in allele and genotype frequencies among dental caries samples and controls.

Furthermore, we generated linkage disequilibrium (LD) plots for the three ENAM SNPs rs7671281 and rs3796704 using SNP Annotation and Proxy Search (SNAP). The $r^2$ values for rs7671281 and rs3796704 were (0.958) and (0.965), respectively. The LD plot indicated that SNP rs3796704 showed high association with SNP ($r^2$ value = 0.965).

### Table 4

| SNP        | Variant | Caries | Healthy | OR   | CI    | $\chi^2$ Value | p-Value | 1-p-Value |
|------------|---------|--------|---------|------|-------|----------------|---------|-----------|
| rs12640848 | AA      | 89(0.53) | 116(0.60) | Ref  |       |                |         |           |
|            | AG      | 58(0.35) | 68(0.35)   | 1.112 | 0.712–1.737 | 0.22  | 0.64172 | 1         |
|            | GG      | 21(0.12) | 9(0.05)    | 3.041 | 1.328–6.962 | 7.43  | 0.00642 | 0.128     |
|            | A       | 236(0.70) | 300(0.78)   | Ref  |       |                |         |           |
|            | G       | 100(0.30) | 86(0.22)    | 1.478 | 1.057–2.066 | 5.26  | 0.02184 | 0.042     |

Bonferroni corrected p-value.
5. Conclusion

The present study reports that the evaluated ENAM gene SNPs are significantly associated with caries susceptibility. ENAM gene polymorphisms can be predictors of caries susceptibility in South Indian children. We report that ENAM gene SNP rs7671281 is a strong candidate for caries susceptibility in primary teeth of South Indian children. This SNP can be used a potential predictor factor for dental caries diagnosis in deciduous dentition. Further studies are required to confirm these results in other cohorts with large number of samples.

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Fig. 3b. Regional LD plot for the ENAM gene rs3796704 SNP.

Fig. 3c. Regional LD plot for the ENAM gene rs12640848 SNP.
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