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ORIGINAL ARTICLE

Association of COL1A2 (PvuII) gene polymorphism with risk and severity of dental fluorosis – A case control study

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KEYWORDS

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Abstract *Introduction:* Dental fluorosis is a foremost public health problem in many countries, including India. Very few studies investigated gene polymorphism and risk of dental fluorosis. Genetic polymorphisms in Collagen Type I, alpha 2 (COL1A2) gene, found to be linked with bone pathogenesis, may affect the tooth formation resulting in the vulnerability to dental fluorosis.

Aim: To assess the association between COL1A2 (PvuII) gene polymorphism and risk as well as severity of dental fluorosis.

Methods: The present case control study was conducted among participants with (n = 60) and without (n = 60) dental fluorosis. Dental fluorosis was assessed using Modified Dean's fluorosis index (1942). The PvuII polymorphisms (in exon 25) inside the COL1A2 gene were genotyped by Polymerase Chain Reaction-Restriction Fragment Length Polymorphism (PCR-RFLP) procedure. Statistical analysis were carried out with Chi-square test and Odds Ratio (OR) was determined with multivariate logistic regression analysis.

Results: The genetic polymorphism in COL1A2 PvuII was found to be associated with the risk of dental fluorosis which was highly significant ($p < 0.001$). The odds ratio was 31.4 times [OR = 31.9, 95% CI: 3.9–48.7] higher for the homozygous PP genotype group and 4.0 times [OR = 4.0, 95% CI: 1.0–10.7] higher for the heterozygous Pp genotype.

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Conclusion: Genetic polymorphism of COL1A2 was found to be associated with dental fluorosis. The present study provides an insight for identification of the population who may subsist at risk of developing dental fluorosis in their later life.

1. Introduction

G.V. Black's statement "An endemic imperfection of the enamel of the teeth here to fore unknown in the literature of dentistry" has become well known today in dentistry. The term 'mottled enamel' and 'denti di chiaie' were scientifically coined as dental fluorosis by Dean in 1934 (McKay and Zinder, 1916). Dental fluorosis is a fluoride-induced condition, which leads to specific interruption in tooth formation, mineralisation and an aesthetic condition, in which enamel formation is altered and hypo mineralized (Fejerskov and Manji, 1990). Since the time of inception, enormous scientific literature on dental fluorosis has been reported. However, the studies on dental fluorosis had a paradigm shift when an inverse association between dental fluorosis and dental caries was discovered. In 1942, an important milestone discovery was made by Dean. He reported that 60% diminution in dental caries at one ppm of fluoride in drinking water (Szpunar and Burt, 1988). Fluoridation of drinking water is considered as important public health measure for prevention of dental caries. Worldwide, dental caries prevalence has been declining due to fluoridation of drinking water. However, dental fluorosis is a major public health problem in several countries, including India, mostly of geological origin. Of the 85 million tons of fluoride stores on the earth's surface, 12 million are solely established in India (Teotia and Teotia, 1984).

World Health Organization committee on fluoride use stated that 1.0 mg/L should be an absolute upper limit, in cold climates, and that 0.5 mg/L may be an appropriate lower limit as a guideline value in drinking water (World Health Organization, 2004, 2011). According to a recent report, the disease is endemic in about 275 districts, an estimated 62 million population in India are affected with dental, skeletal and/or non-skeletal fluorosis (Vaish and Vaish, 2000). According to the National Oral Health survey and Fluoride Mapping (2002–2003), the overall prevalence of dental fluorosis is 10% which was mostly in the milder form. In fluoride rich districts, however the prevalence was as high as 18% (Bali et al., 2004). Several studies have established a disparity in the prevalence of fluorosis. Until recently, very few studies have explored the underlying genetic origin for fluoride susceptibility. Association between genetic polymorphism and dental fluorosis was studied among various genes like Pvu II and RsaI of COL1A2 gene (Huang et al., 2008), Hind III of Osteocalcin gene (Ba et al., 2009), RsaI of Estrogen Receptor gene (Ba et al., 2011), Bst BI of Parathyroid hormone gene (Wen et al., 2012).

Among all the genes, COL1A2 (PvuII) gene polymorphism was observed to be significantly related with susceptibility to dental fluorosis. Type I collagen is the most abundantly distributed collagen family of proteins. It is a heterotrimer comprising of two pro- α1(I) chains and one pro-α2(I) chain which are encoded by the unlinked loci COL1A1 and COL1A2 respectively. *COL1A2* is ~38 kb and is located at 7q21.3–q22.1. and induce the pro-alpha2 (I) chain production (Dalgleish, 1997). Till date, variations in the COL1A2 gene were connected specifically to a extensive range of cartilage, bone and blood vessels pathologies. In sight of their vital role in bone formation, genetic variants in COL1A2 gene, may have impact on tooth formation among high-fluoride exposed population leads to dental fluorosis susceptibility. Detection of such genes can be novel biomarker for dental fluorosis. This information can be used in identifying person's susceptibility and there by alter an individual's risk of developing dental fluorosis. Scientific literature revealed very limited studies on genetic polymorphism of COL1A2 and dental fluorosis in India.

Thus, the current study was conducted to assess the association between COL1A2 (PvuII) gene polymorphism and risk of dental fluorosis among Indian inhabitants.

2. Materials and methods

2.1. Study design and sample calculation

This present case control study was approved by the Institutional Review Board of study institution. Before the commencement of the study all participants gave written Informed consent and parents consent was also obtained while involving the children. Sample size was calculated with 80% power and 5% alpha error using n-master software as 120 participants.

2.2. Eligibility criteria

A total of 120 subjects aged between 10 and 30 years and only those who were raised in the endemic fluorosis area, were selected from the Outpatient Department at Tertiary care Government Hospital, Chennai, Tamil Nadu. Endemic fluorosis area is defined as an area with fluoride in potable water beyond 2.0 mg/l. Individuals presenting with bone disorders, cases under medications such as bisphosphonates, hormone replacement therapy fluoride were excluded. Hence the data of fluoride level of water in power pump forces from districts of Tamil Nadu reported by Tamil Nadu Water Supply and Drainage Board (TWAD board) was obtained.

2.3. Examination of dental fluorosis

The entire study participants were assessed for dental fluorosis with the Modified Dean's fluorosis index (1942). According to that criteria, dental fluorosis was categorised based on 6 point ordinal scale ranged from 0 (normal) to 5 (severe). Participants those who were ascertained as grade 0 and 1 were control group without dental fluorosis (n = 60), whereas those who were ascertained from grade 2 to 5 were assigned as case group

with dental fluorosis (n = 60). The demographic data and details of their fluoride exposure were recorded through a pre-validated structured interview schedule.

2.4. Sample collection and DNA isolation method

Venous blood samples were obtained from the antecubital vein of all the participants in a 2 ml syringe and transferred to 3 ml vacutainers (BD Vacutainer® plus# 367899) and stored at 4 °C till DNA isolation (Rahila et al. (2017)) DNA sample was isolated from the stored blood using the buffers given in the DNA isolation kit (Favorprep Genomic DNA mini kit). The purified DNA was subsequently stored and the yield was recorded using UV spectrophotometer (NanoDrop 2000c).

2.5. DNA amplification

The isolated DNA samples were amplified through the PCR method (Bio Rad), (Thermo scientific Pfu DNA polymerase). Primers used in this study were obtained in accordance to previous study (Suuriniemi et al., 2003; Rahila et al. (2017)). The primers COL1A2 F (5′-GGG ATA TAA GGA TAC ACT AGA GG-3′) COL1A2 R (5′-GAA ATA TCG CCG CTG GAA- 3′) (Shrimpex) were utilised to amplify the 771-bp fragment of COL1A2 PvuII site supposed to show evidence of polymorphism. The obtained PCR product in 2% agarose gel was visulaised under UV transilluminator in gel documentation system.

2.6. Restriction fragment length polymorphism analysis

Following PCR amplification, the DNA products were subjected to gel purification, digestion by the restriction enzyme *PvuII* which is derived from Proteus vulgaris (Pvu) gene coding for the PvuII restriction enzyme. (Imperial Life Sciences Private Limited, Gurgaon, Haryana, India) and incubation at 37 °C for 16 h. The resulting fragments obtained were subjected to electrophoresis; the observable bands were visualized and recorded. The PCR products enclosing p alleles were cleaved by PvuII, into two separate bands 541 bp and 239 bp, while uncleaved P alleles contains only one band of 771 bp was observed (Fig. 1). After the post electrophoresis gel treatment, the purified DNA of three samples of different genotype frequencies were sent for direct sequencing analysis to confirm the RFLP findings.

2.7. Statistical analysis

Data obtained was analyzed using Statistical package for Social Sciences (SPSS, ver. 18.0; SPSS Inc., Chicago, IL, USA). The association between polymorphism in the COL1A2 gene with severity of dental fluorosis was assessed by chi-square test. The control only chi square test was employed to examine for departures from the Hardy-Weinberg equilibrium (HWE) among controls. The majority of the genotype in control subjects were predictable with the HWE (p = 0.506). This shows that allele and genotype frequencies at the COL1A2 PvuII (in exon 25) in control participants were in Hardy-Weinberg equilibrium. Odds ratios (ORs) was calculated with multivariate logistic regression analysis. In the present study, the level of significance (α) was set at 5%.

3. Results

Among the study participants, the age appropriation was comparable among the cases and control group. The sex distribution was observed to be similar among case and controls

Table 1 Distribution of demographic characteristics in study group and control group.

	Case group [n = 60]	Control group [n = 60]
Age* [years]	25.2 ± 6.8 years	24.3 ± 4.6 years
Gender [n]		
Male	36	38
Female	24	24

* Values are means ± SD.

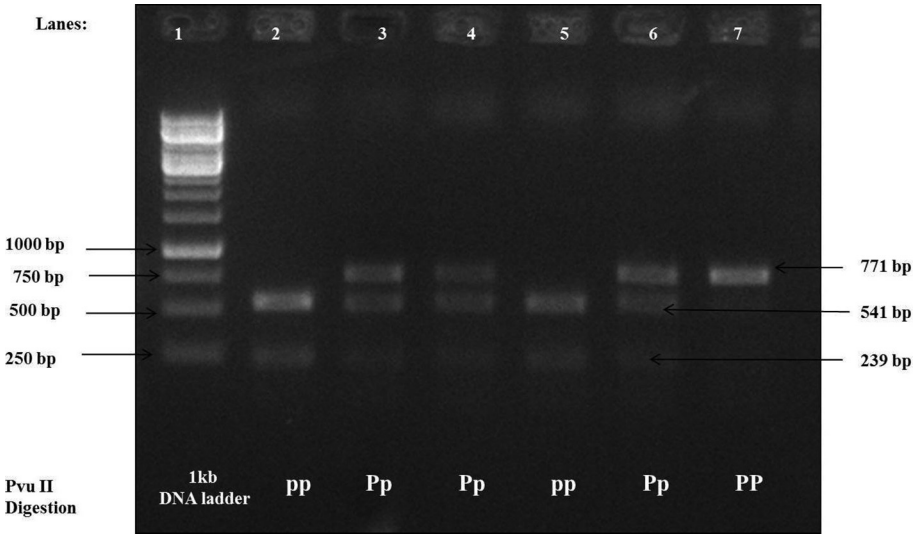

Fig. 1 Agarose gel picture showing PVU II digested amplicons showing genotypes of COL1A2.

Table 2 Association between dental fluorosis and COL1A2 polymorphisms.

COL1A2 Pvu II polymorphism	Severity of dental fluorosis		Chi square value	p value
	Normal and questionable [control group] [%]	Very mild, mild, moderate, severe [case group] [%]		
PP genotype	1.9	28.4	24.541	0.000*
Pp genotype	16.9	32.8		
pp genotype	81.2	38.8		

* $p \leq 0.05$.

Table 3 Sub group comparison of grades of dental fluorosis in cases with controls with Bonferroni correction.

COL1A2 *Pvu* II polymorphism	Severity of dental fluorosis			
	Verymild and control [%]	Mild and control [%]	Moderate and control [%]	Severe and control [%]
PP	23.6	31.2	37.5	22.2
Pp	35.2	31.2	37.5	27.8
pp	41.2	37.5	25	50
Chi square value	13.332	16.714	23.430	10.463
p value	0.001†	0.000†	0.000†	0.005†

† Bonferroni-adjusted p value.

Table 4 Association between risk of dental fluorosis and COL1A2 polymorphisms.

COL1A2 Pvu II polymorphism	Based on severity of dental fluorosis		OR [95%CI]	p value
	Control group [%]	Case group [%]		
pp	81.2	38.8	1.0	
PP	1.9	28.4	31.4 [3.9–48.7]	0.000*
Pp	16.9	32.8	4.0 [1.6–10.1]	0.003*

* $p \leq 0.05$.

(Table 1). The genetic polymorphism in COL1A2 PvuII was found to be associated with dental fluorosis (Table 2).

The most common way to control the error rate is with the Bonferroni correction. There are four possible pair wise case group comparisons with control group, the Bonferroni-adjusted *p* value essential for significance is 0.05/4, or 0.00125. The association within grades of severity of dental fluorosis and COL1A2 polymorphisms were established to be significant ($p < 0.00125$†), but the risk was not increased with severity of fluorosis (Table 3). Compared to participants who conceded pp genotype of COL1A2 (PvuII), participants who conceded PP genotype and heterozygous Pp genotype, had a considerably increased possibility of developing dental fluorosis (OR = 31.4, 95% CI: 3.9–48.7) and (OR = 4.0, 95% CI: 1.6–10.1) respectively (Table 4).

4. Discussion

To date, investigations of fluorosis primarily focused on assessing disease prevalence, dental, skeletal and non skeletal manifestations of fluorosis, and aesthetic treatment modalities of dental fluorosis. Only very few studies on the genetic predisposition to dental fluorosis were published. This study brings some crucial attention to the prospective consequence of genetics in determining an individual's vulnerability to dental fluorosis.

Any individual affected by dental fluorosis above ten years of age were included because dental fluorosis is uncommon in deciduous dentition. This disparity may exist because mineralization of primary teeth occurs in foetal stage and the placenta serves as a barrier for the transfer of plasma fluoride to foetus. Erstwhile that the enamel formation stage for primary teeth is shorter than the permanent teeth (Murray et al., 2003). The genetic variants of COL1A2 can end result in the connective tissue disorder (Dalgleish, 1997). So patients with known syndromes, and connective tissue disorders were excluded in the present study. Type I collagen is a omnipresent fibrous protein that gives tensile strength to bone and other tissues. Hence, patients with drug history known to affect bone metabolism were excluded from the study.

Though salivary collection is a non invasive procedure for DNA isolation. Conversely, potential disadvantages include lower mean DNA yield and contamination with DNA of oral bacteria, proteins and food was reported (Philibert et al., 2008). Hence in the current study, whole blood sample was chosen. Cushwa and Medrano et al. reported that the best possible storage temperature was found as 4 °C for up to 4 weeks (Cushwa and Medrano, 1993). Richardson et al showed that storage of blood samples at 4 °C for even up to 2 years will yield a doable DNA. (Richardson et al., 2006). Hence the collected samples were stored at 4 °C till DNA isolation. According to Suuriniemi M et al. the COL1A2 gene polymorphism in pvuII site was detected. In this study 771 bp were amplified using the primers as specified method in above mentioned study (Suuriniemi et al., 2003).

Various studies have been reported the role of genetic variants and its vulnerability to dental fluorosis which includes following genes, Collagen type I alpha 2 [COL1A2 gene] PvuII,

RsaI (Huang et al., 2008), Osteocalcin [OC gene] Hind III (Ba et al., 2009), Estrogen receptor [ESR gene] RsaI (Ba et al., 2011), Parathyroid hormone [PTH gene] Bst BI (Wen et al., 2012). Among all the genes, Collagen 1 alpha 2 (COL1A2) [PvuII] gene polymorphism was observed to be significantly linked with susceptibility of dental fluorosis. This gene plays a important role in bone and hard tissues formation sof the tooth which in turn influences an individual's susceptibility to dental fluorosis.

The plaucibility of causal relationship between COL1A2 PvuII polymorphisms and the risk dental fluorosis are not well recognized. Hence in the current study COL1A2 gene was chosen to assess the genetic susceptibility to dental fluorosis. The foremost evidence of an association between COL1A2 gene polymorphisms with dental fluorosis among high fluoride exposed populations was shown by Huang et al. (2008). The association between PP genotype and dental fluorosis was significantly higher [OR = 31.9, 95% CI: 3.9–48.7] in the current study. The association between Pp genotype and dental fluorosis was also substantially higher [OR = 4.0, 95% CI: 1.0–10.7] which is not in agreement with previous study on Chinese population (Huang et al., 2008).

In the present study, the genetic susceptibility had no influence on the severity of dental fluorosis, which was relatively similar to a previous study (Huang et al., 2008). Suuriniemi M et al. stated that COL1A2 polymorphism is concurrent with non osteoporotic fractures among prepubertal finnish females (Suuriniemi et al., 2003). They reported that subjects with the homozygous P allele had higher chances of fracture than those with the homozygous p allele. COL1A2 polymorphism shown strongest and most consistent association with decreased bone mineral density and hypomineralisation (Willing et al., 2003). The present study also demonstrates similar association with COL1A2 polymorphism.

The fluoride exposure of every selected individual could not be accurately ascertained in the present study. Rather the fluoride exposure measurement was based on the data from fluoride registry averaged for place they lived. The odds ratio obtained in the present study had less precision due to a wide range in 95% confidence interval, owing to the relatively smaller sample size.

5. Conclusion

The current study demonstrated a considerably significant association of COL1A2 gene polymorphism and the susceptibility for dental fluorosis among population who are exposed to high fluoride. Apart from environmental factor, genetic variant also increases the vulnerability to dental fluorosis. This may help in identification of a subset of the population who are at possibility of developing dental fluorosis.

CRediT authorship contribution statement

C. Rahila: Conceptualization, Data curation, Formal analysis, Funding acquisition, Investigation, Methodology, Project administration, Resources, Software, Supervision, Validation, Visualization, Writing - original draft, Writing - review & editing. **M.B. Aswath Narayanan:** Conceptualization, Formal analysis, Investigation, Methodology, Project administration, Resources, Supervision, Validation, Visualization, Writing - review & editing. **S.G. Ramesh Kumar:** Conceptualization, Formal analysis, Methodology, Project administration, Supervision, Validation, Visualization, Writing - review & editing. **A. Leena Selvamary:** Conceptualization, Formal analysis, Methodology, Project administration, Supervision, Validation, Visualization, Writing - review & editing. **A. Sujatha:** Conceptualization, Formal analysis, Methodology, Project administration, Supervision, Validation, Visualization, Writing - review & editing. **J. John Kirubaharan:** Methodology, Project administration, Resources, Supervision, Validation, Visualization, Writing - review & editing.

Ethical Statement

This study was approved by the Institutional Review Board of Tamil Nadu Government dental college and Hospital (Ref no. 0430/DE). This article was reported based on STROBE guidelines. All author approved the manuscript writing and submission of this article.

Declaration of Competing Interest

None.

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