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ASSOCIATION OF EDA GENE POLYMORPHISMS rs 372024, rs 3764746, rs 3795170 IN SKELETAL CLASS I CASES WITH DENTAL CROWDING

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ABSTRACT

The EDA gene, associated with X linked hydrotic form of Ectodermal Dysplasia, its mutations could potentially lead to differential gene expression that causes large tooth phenotype, which has been suggested to cause dental crowding. We analyzed the association of genetic polymorphisms in EDA gene variants rs 372024, rs 3764746, and rs 3795170 among Skeletal Class I crowding cases using blood samples of 30 cases and 30 controls, which were subjected to PCR amplification and DNA sequencing. Based on the statistical analysis using the Z test we found CG and GG genotype for rs3764746 and GT and TT genotype for rs3795170 showed a statistically significant result. These results suggest that EDA gene variants rs3764746 and rs3795170 could be genetic markers for dental crowding in our population while EDA gene variant rs372024 did not show any significant association in our population. These findings can provide in-depth knowledge, regarding the genetic influences on the incidence of crowding of teeth.

KEYWORDS : Dental Crowding, Class I, EDA Gene Variants (rs372024, rs 3964746 and rs 3795170), SNP, DNA sequencing

INTRODUCTION

Genetic polymorphisms have been successfully reported for several forms of dental anomalies including Amelogenesis Imperfecta and hyppodontia19. EDA gene and BMP2 gene are considered responsible for syndrome-like Ectodermal Dysplasia affecting teeth as well. Thus, this study attempts to determine possible genetic variations that are associated with dental crowding19. EDA gene is located on the long arm of the X chromosome between positions Xq12-q13.1 (region 1, band2 and region 1, band 3 sub-band 1). Mutations of this gene can lead to Ectodermal Dysplasia involving anomalies of development of ectodermal organs, which include sweat glands, hair follicle, and teeth. It could potentially lead to differential gene expression that causes large tooth phenotype, which has been suggested to cause dental crowding19. Therefore, by analyzing the association of the EDA gene with dental crowding, can give an in-depth knowledge, regarding the genetic influences on the incidence of crowding of teeth. Hence, we conducted a study to analyze the association of genetic polymorphisms in EDA gene variants rs 372024, rs 3764746, and rs 3795170 among Skeletal Class I crowding cases.

MATERIALS AND METHODS

Our study aimed at investigating the association of EDA gene variants rs 372024, rs 3764746, and rs 3795170 in skeletal Class I crowding cases using the Polymerase Chain Reaction (PCR) test followed by DNA sequencing. We collected blood samples from 60 subjects who visited the Department of Orthodontics and Dentofacial Orthopedics, D.A.P.M.R.V. dental college, Bangalore, after written informed consent. These were divided into two groups; Group A consisting of 30 subjects with Skeletal Class I crowding and Group B including 30 controls with Skeletal Class I profile without visible crowding or spacing (+/-2mm). Group A subjects were healthy and without orthodontic treatment before attending the orthodontic clinic, age less than 27 years and had full permanent dentition. These were diagnosed with a Skeletal Class I relation according to ANB angle (00 - 40)19, Witt's appraisal (0 to -3mm)9, and pleasant facial profile and had not less than 5mm of crowding in at least 1 arch.

Class II molar relation or Class II jaw relation, Class III molar relation or Class III jaw relation, Congenitally missing teeth or missing teeth due to caries, retained deciduous teeth, the spacing of more than 2mm and presence of supernumerary teeth were excluded from the study.

Genomic DNA was extracted from the blood of the subjects. Whole blood was taken with a graduated micropipette into a 1.5ml micro centrifuge tube. It was treated with 400 l of 10mmol/l TRIS HCl basic buffer (pH 7.5) solution containing 5mmol/l MgCl2, 0.32mmol/l sucrose and 1% Triton X-100. Later, Proteinase K (10mg/ml) and 10% SDS were added to the mixture and then incubated at 37°C for 30 min.

Then, the mixture was subjected to phenol treatment which dissolves all the proteins followed by chloroform treatment to remove the phenol. In this way, the genomic DNA was extracted by phenol and chloroform. Ethanol (100% alcohol) was added and ripped in the ultracentrifuge 12000rpm for five minutes at room temperature. The genomic DNA was precipitated by ethanol as a clear layer in the micro centrifuge tube.

The Polymerase chain reaction (PCR) is an in vitro technique which allows the amplification of a specific deoxyribonucleic acid (DNA).

Primers used in the study for the EDA gene (rs372024):
F: 5'GTGCCACCCAGCAAGGAGAC'3
R: 5'CCAAGGTCTCAGCTCCAGTAT'3
Primers for EDA gene (rs 3764746):
F: 5'CTTTCATTACCCTACAGACAGC'3
R: 5'GCTACCTATTGCTTCCCTCATC'3
Primers for EDA gene (rs 3795170):
F: 5'ATGGCATGGCAGGAGTCTCT'3
R: 5'GGTGGAAGAAAGAAGATGGC'3

Genomic DNA was amplified by 0.2 Units of Taq polymerase which carries out the polymerization reaction in a final volume of 20 l TRIS HCl buffer containing 25mmol/l KCl, 0.25 mmol/l dNTP, 2.5mmol/l MgCl2 and 250mmol/l PCR primers. The balance volume is added up by the distilled water until it reaches 20 l volume in the PCR tube.

This reaction mixture in the PCR tubes was placed in the PCR machine which was set programmed to repeat the following three staged cycle 35 times.

Stage1: Separation/Denaturation: The double-stranded DNA is denatured by heat, by disrupting the hydrogen bonds between complementary bases, yielding single-stranded DNA templates at 95 °C for 3 min, followed by actual denaturation at 95 °C for 1 min

Stage 2: Priming/Annealing: The primers anneal to the single-stranded DNA template at 58 °C for 1 min.

Stage 3: Polymerization/Elongation: The DNA polymerase recognizes the primer and makes a complimentary copy of the template at 72 °C for 1 min.

The DNA sequencing was performed using Frederick Sanger's dideoxy sequencing method in an automated ABI sequencer machine based on the selective incorporation of chain-terminating dideoxynucleotides by DNA polymerase during in vitro DNA replication.

DNA to be sequenced, complimentary primer, DNA polymerase, 4 different dNTPs, 4 different fluorochrome-labeled ddNTPs (chain terminators) are taken and this reaction mixture is heated (96oC) so that the double-stranded DNA separates into single-stranded DNA. Then the temperature is reduced to 50oC so that the primers bind to the complementary sequence on the now single-stranded DNA. The temperature is again raised and DNA polymerase binds to the DNA and Chain synthesis reaction take place until ddNTP (chain terminators) binds.

Thus Strands of DNA of varying lengths are formed which are again denatured at (96oC) to form single strands of DNA.

This is subjected to Capillary electrophoresis and Laser is used to excite the fluorochrome dye which forms images on computer termed as "Electropherogram"

The results obtained were tabulated and subjected to Z- test for statistical analysis.

It can be applied for qualitative as well as quantitative data. Here it was applied to test the difference between two proportions (cases and controls).

Z-test for proportions formula:

$$z = \frac{\hat{P}1 - \hat{P}2}{SEDp}$$

$$SEDp = \sqrt{\hat{P}(1-\hat{p})(1/n1 + 1/n2)} \quad \text{and} \quad P = \frac{X1+X2}{n1+n2}$$

p1,proportion1 = x1/n1
p2,proportion2 = x2/n2
x1 = number of cases with the 3 genotypes of each gene.
x2 = number of controls with the 3 genotypes of each gene.
n1 = total number of cases
n2 = total number of controls

Statistical interpretation:
+ Suggestive significance $0.05 < p < 0.10$ * Moderately significant $0.01 < p < 0.05$
** Strongly significant $p < 0.001$
Statistical software: The Statistical software namely SPSS 11.0 and Systat 8.0 were used for the analysis of the data and Microsoft Word and Excel have been used to generate graphs, tables, etc.

RESULTS

Results for EDA gene rs 372024:

The results of these sixty subjects were tabulated based on the presence or absence of CC, GG and CG genotype of EDA gene variant rs 372024 (Table no.1). The number of cases and controls with the difference in their genotype frequencies has been tabulated (Table no.2, Graph1). Z-test was applied for evaluating the statistical significance between groups A, B, and genotypes. (Table no.3)

Also, the results of the sixty subjects were tabulated based on the presence or absence of CC, GG and CG genotype of EDA gene variant rs 3764746 (Table no.4). The number of cases and controls with the difference in their genotype frequencies has been tabulated (Table no.5, Graph2). Z-test was applied for evaluating the statistical significance between groups A, B, and genotypes. (Table no.6)

Similarly, the results of the sixty subjects were tabulated based on the presence or absence of GG, GT and TT genotype of EDA gene variant rs 3795170 (Table no.7). The number of cases and controls with the difference in their genotype frequencies has been tabulated (Table no.8, Graph3). Z-test was applied for evaluating the statistical significance between groups A, B, and genotypes. (Table no.9)

Tables and graphs:

TABLE 1: TABULATED RESULTS FOR SIXTY SUBJECTS SHOWING VARIATION IN PRESENCE OF GENOTYPES OF EDA2R rs372024 GENE VARIANT AMONG PATIENTS AND CONTROLS

Group A	CC GENOTYPE	CG GENOTYPE	GG GENOTYPE	Group B	CC GENOTYPE	CG GENOTYPE	GG GENOTYPE
PATIENT 1	PRESENT	ABSENT	ABSENT	Control 1	PRESENT	ABSENT	ABSENT
PATIENT 2	PRESENT	ABSENT	ABSENT	Control 2	PRESENT	ABSENT	ABSENT
PATIENT 3	PRESENT	ABSENT	ABSENT	Control 3	PRESENT	ABSENT	ABSENT
PATIENT 4	PRESENT	ABSENT	ABSENT	Control 4	PRESENT	ABSENT	ABSENT
PATIENT 5	PRESENT	ABSENT	ABSENT	Control 5	PRESENT	ABSENT	ABSENT
PATIENT 6	PRESENT	ABSENT	ABSENT	Control 6	PRESENT	ABSENT	ABSENT
PATIENT 7	PRESENT	ABSENT	ABSENT	Control 7	PRESENT	ABSENT	ABSENT
PATIENT 8	PRESENT	ABSENT	ABSENT	Control 8	PRESENT	ABSENT	ABSENT
PATIENT 9	PRESENT	ABSENT	ABSENT	Control 9	PRESENT	ABSENT	ABSENT
PATIENT 10	PRESENT	ABSENT	ABSENT	Control 10	PRESENT	ABSENT	ABSENT
PATIENT 11	ABSENT	ABSENT	PRESENT	Control 11	PRESENT	ABSENT	ABSENT
PATIENT 12	PRESENT	ABSENT	ABSENT	Control 12	PRESENT	ABSENT	ABSENT
PATIENT	PRESEN	ABSENT	ABSENT	Control	PRESEN	ABSENT	ABSENT

TABLE 2: THE PRESENCE OF CC, CG AND GG GENOTYPE OF EDA2R rs372024 GENE VARIANT AMONG CASES AND CONTROLS

Genotype of EDA GENE (rs372024) Gene Variant	GROUP A (PATIENTS)	GROUP B (CONTROLS)
CC	25	29
CG	0	0
GG	5	1

TABLE 3:-THE TABLE DENOTES THE STATISTICAL SIGNIFICANCE OF THE GENOTYPE WHEN CASES AND CONTROLS ARE COMPARED USING Z- TEST.

Genotype of EDA GENE (rs372024) Gene Variant	Cases		Controls		Difference in Proportion	Z	P value
	N	%	N	%			
CC	25	83.3	29	96.7	-0.134	-1.72	0.085 (Not
CG	0	00	0	00	NA	NA	NA
GG	5	16.7	1	3.3	0.134	1.72	0.085 (Not Significant)

Note: p value <0.05 indicates statistically significant

The difference in the proportion of positive results between cases and controls for genotype CC was not statistically significant (P>0.05).

The difference in the proportion of positive results between cases and controls for genotype GG was found to be statistically not significant (P>0.05).

4: TABULATED RESULTS FOR SIXTY SUBJECTS SHOWING VARIATION IN PRESENCE OF GENOTYPES OF EDA rs3764746 GENE VARIANT AMONG PATIENTS AND CONTROLS

Group A	CC GENOTYPE	CG GENOTYPE	GG GENOTYPE	Group B	CC GENOTYPE	CG GENOTYPE	GG GENOTYPE
PATIENT 1	ABSENT	PRESENT	ABSENT	Control 1	ABSENT	PRESENT	ABSENT
PATIENT 2	ABSENT	ABSENT	PRESENT	Control 2	ABSENT	PRESENT	ABSENT
PATIENT 3	ABSENT	ABSENT	PRESENT	Control 3	ABSENT	PRESENT	ABSENT
PATIENT 4	ABSENT	ABSENT	PRESENT	Control 4	ABSENT	ABSENT	PRESENT
PATIENT 5	ABSENT	PRESENT	ABSENT	Control 5	ABSENT	ABSENT	PRESENT
PATIENT 6	ABSENT	ABSENT	PRESENT	Control 6	PRESENT	ABSENT	ABSENT
PATIENT 7	ABSENT	PRESENT	ABSENT	Control 7	PRESENT	ABSENT	ABSENT
PATIENT 8	ABSENT	ABSENT	PRESENT	Control 8	PRESENT	ABSENT	ABSENT
PATIENT 9	ABSENT	ABSENT	PRESENT	Control 9	PRESENT	ABSENT	ABSENT
PATIENT 10	PRESENT	ABSENT	ABSENT	Control 10	PRESENT	ABSENT	ABSENT
PATIENT 11	PRESENT	ABSENT	ABSENT	Control 11	PRESENT	ABSENT	ABSENT
PATIENT 12	ABSENT	PRESENT	ABSENT	Control 12	PRESENT	ABSENT	ABSENT
PATIENT 13	ABSENT	ABSENT	PRESENT	Control 13	PRESENT	ABSENT	ABSENT

TABLE 5: THE PRESENCE OF CG, CC AND GG GENOTYPE OF EDA GENE (rs3764746) GENE VARIANT AMONG CASES AND CONTROLS.

Genotype of EDA GENE (rs3764746) Gene Variant	GROUP A (PATIENTS)	GROUP B (CONTROLS)
CC	2	25
CG	12	3
GG	16	2

TABLE 6:- Comparison of EDA (rs3764746) Gene Variant Genotypes among Cases & Controls (Z test)

Genotype of EDA GENE (rs3764746) Gene Variant	Cases		Controls		Difference in Proportion	Z	P value
	N	%	N	%			
CC	2	6.7	25	83.3	-0.76	-5.97	0.001 (highly Significant)
CG	12	40	3	10	0.30	2.68	0.007 (Significant)
GG	16	53.3	2	6.7	0.466	3.94	0.001 (highly Significant)

Note: p value <0.05 indicates statistically significant

The difference in the proportion of positive results between cases and controls for genotype CC was highly statistically significant (P<0.001).
The difference in the proportion of positive results between cases and controls for genotype CG was found to be statistically significant (P = 0.007).
The difference in the proportion of positive results between cases and controls for genotype GG was found to be highly statistically significant (P<0.001).

7: TABULATED RESULTS FOR SIXTY SUBJECTS SHOWING VARIATION IN PRESENCE OF GENOTYPES OF EDA rs3795170 GENE VARIANT AMONG PATIENTS AND CONTROLS

Group A	GG GENOTYPE	GT GENOTYPE	TT GENOTYPE	Group B	GG GENOTYPE	GT GENOTYPE	TT GENOTYPE
PATIENT 1	PRESENT	ABSENT	ABSENT	Control 1	PRESENT	ABSENT	ABSENT
PATIENT 2	PRESENT	ABSENT	ABSENT	Control 2	PRESENT	ABSENT	ABSENT
PATIENT 3	ABSENT	ABSENT	PRESENT	Control 3	PRESENT	ABSENT	ABSENT
PATIENT 4	ABSENT	ABSENT	PRESENT	Control 4	ABSENT	ABSENT	PRESENT
PATIENT 5	ABSENT	PRESENT	ABSENT	Control 5	ABSENT	ABSENT	PRESENT
PATIENT 6	PRESENT	ABSENT	ABSENT	Control 6	PRESENT	ABSENT	ABSENT
PATIENT 7	PRESENT	ABSENT	ABSENT	Control 7	PRESENT	ABSENT	ABSENT
PATIENT 8	ABSENT	PRESENT	ABSENT	Control 8	PRESENT	ABSENT	ABSENT
PATIENT 9	ABSENT	PRESENT	ABSENT	Control 9	PRESENT	ABSENT	ABSENT
PATIENT 10	PRESENT	ABSENT	ABSENT	Control 10	PRESENT	ABSENT	ABSENT
PATIENT 11	PRESENT	ABSENT	ABSENT	Control 11	PRESENT	ABSENT	ABSENT
PATIENT 12	ABSENT	PRESENT	ABSENT	Control 12	PRESENT	ABSENT	ABSENT
PATIENT 13	ABSENT	PRESENT	ABSENT	Control 13	PRESENT	ABSENT	ABSENT

TABLE 8: THE PRESENCE OF CG, CC AND GG GENOTYPE OF EDA GENE (rs3795170) GENE VARIANT AMONG CASES AND CONTROLS.

Genotype of EDA GENE (rs3795170) Gene Variant	GROUP A (PATIENTS)	GROUP B (CONTROLS)
GG	6	28
GT	13	0
TT	11	2

TABLE 9:-Comparison of EDA (rs3795170) Gene Variant Genotypes among Cases & Controls (Z test)

Genotype of EDA GENE (rs3795170) Gene Variant	Cases		Controls		Difference in Proportion	Z	P value
	N	%	N	%			
GG	6	20	28	93.3	-0.733	-5.7	0.001 (highly Significant)
GT	13	43.3	0	00	0.433	4.1	0.001 (highly Significant)

TT	11	36.7	2	6.7	0.30	2.82	0.0048 (highly Significant)

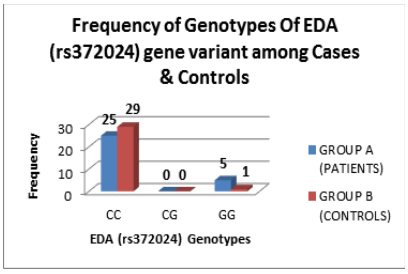
Note: p value <0.05 indicates statistically significant

The difference in the proportion of positive results between cases and controls for genotype TT was highly statistically significant (P = 0.0048).

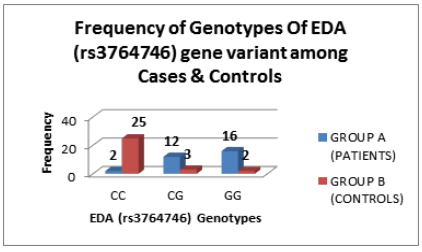
The difference in the proportion of positive results between cases and controls for genotype GT was found to be highly statistically significant (P<0.001).

The difference in the proportion of positive results between cases and controls for genotype GG was found to be highly statistically significant (P<0.001).

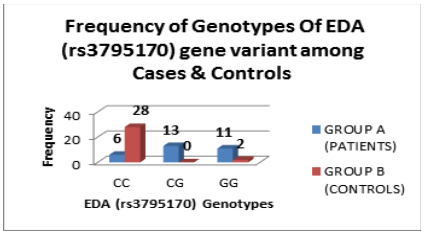
GRAPH1: GRAPH SHOWING THE VARYING FREQUENCIES OF GENOTYPE OF EDA2R rs372024 GENE VARIANT AMONG CASES AND CONTROLS.



GRAPH2: GRAPH SHOWING THE VARYING FREQUENCIES OF GENOTYPE OF EDA rs3764746 GENE VARIANT AMONG CASES AND CONTROLS.



GRAPH3: GRAPH SHOWING THE VARYING FREQUENCIES OF GENOTYPE OF EDA rs3795170 GENE VARIANT AMONG CASES AND CONTROLS.



DISCUSSION

EDA gene belongs to the tumor necrosis factors family and is traditionally known to participate in cell death, inflammation, and several other functions. By contrast, the EDA signaling pathway was shown to act in a morphogenetic role in teeth and other ectodermal organs. Mutations in the EDA gene cause Hypohidrotic Ectodermal Dysplasia in mice and in affected humans, the condition displayed defects in several ectodermal organs, example, teeth, hair, and sweat glands. EDA gene and BMP2 gene are considered responsible for syndrome-like Ectodermal Dysplasia affecting teeth as well.[18]

The EDA gene mutations could also result in X linked hydrotic form of ectodermal dysplasia and it causes a selective type of tooth agenesis which is syndromic1. However, only a few studies have been conducted to identify genetic associations with crowding. Thus this study attempts to determine possible genetic variations that are associated with dental crowding in our population.

In the present study, the role of EDA gene variants rs 372024, rs 3764746 and rs 3795170 with dental crowding were assessed in 30 cases (Group A) and 30 controls (Group B) in the local population. For this purpose, we collected blood samples from 30 cases and 30 controls as per the inclusion and exclusion criteria as mentioned previously.

Samples were subjected to PCR amplification of the desired segment according to rs 372024, rs 3764746 and rs 3795170 and were then analyzed by DNA sequencing and capillary electrophoresis.

Sequencing of human and other genomes has been at the center of interest in the biomedical field over the past several decades and is now leading towards an era of personalized medicine. During this time, DNA sequencing methods have evolved from the labor-intensive slab gel electrophoresis to automated multi capillary electrophoresis systems using fluorophore labeling. Dye-terminator based DNA sequencing allowed the use of four dideoxynucleotide chain terminators, tagged with dyes of different fluorescent emission wavelengths, in a single sequencing reaction which is depicted by a graph called Electropherogram or Chromatogram. This graph contains a sequence of peaks in four colors which are universally color coded for each nucleotide (thymine-red, adenine-green, guanine-black and cytosine- blue). Any change in normal nucleotide sequencing will be shown by different color peak and if it is homozygous it will be shown as a single peak while if it is heterozygous it will be shown as a double peak.

EDA gene variant rs372024

This study indicates that the CG and GG genotype of EDA gene (rs372024) gene variant does not contribute to the occurrence of dental crowding in our population. This is contrary to the study done by T Y Ting, Ricky Wing Kit Wong, and A. Bakr M. Rabie in Hong Kong Chinese population.18 Contradictory results are probably due to genetic heterogeneity, incomplete penetrance, limited sample sizes, and different study designs. The discrepancy of the results between these two studies may be due to population diversity that may be responsible for the inconsistency. Genetic polymorphisms often show ethnic variation. Therefore, further studies of different ethnic populations are warranted to ascertain the association between genetic polymorphisms of EDA gene variant rs 372024 and dental crowding. The associated SNP (rs372024) occurring on the human EDA gene is a silent mutation that does not cause a change of the amino acids being translated. Therefore, it was suggested that those silent SNPs have no functional role but recent evidence has shown that silent SNPs can alter protein structure, function, and expression level through a series of mechanisms[18]

EDA gene variant rs 3764746

In this study done on our population, the number of samples with genotypes CG and GG were found to be more in cases compared to controls, but the genotypes CC are found to be more in controls. This is in confirmation to the study done by T Y Ting, Ricky Wing Kit Wong, and A. Bakr M. Rabie in Hong Kong Chinese population.[18]

EDA gene variant rs 3795170

In this study done on our population, the number of samples with genotypes GT and TT was found to be more in cases compared to controls, but the genotypes GG are found to be more in controls. This is in confirmation to the study done by T Y Ting, Ricky Wing Kit Wong, and A. Bakr M. Rabie in Hong Kong Chinese population.[18]

Thus, these results affirm that the novel heterozygous transition found in the EDA gene rs 3795170 and 3764746 might be responsible for dental crowding.

Penetrance in genetics is the proportion of individuals carrying a particular variant of a gene (allele or genotype) that also expresses an associated trait (phenotype). In medical genetics, the penetrance of a disease-causing mutation is the proportion of individuals with the mutation who exhibit clinical symptoms. Penetrance is said to be reduced or incomplete when some individuals fail to express the trait, even though they carry the allele. The phenomenon of incomplete penetrance of EDA gene variants rs 3795170 and rs 3764746 supports the view that dental crowding and its traits can be caused by mutations of several genes.

The above findings suggest that EDA rs3764746 and rs 3795170 may be implicated as genetic markers for dental crowding in our population. This can be confirmed by further studies with larger sample size. In contrast, EDA gene rs 372024 did not show any significant statistical association with dental crowding in our population. A Meta-analysis in a wider range of patient groups with more complete clinical descriptions of affected individuals may be required to more completely characterize the association between genetic markers and dental crowding. It is likely that advances in our understanding of both genetic and environmental etiology of dental crowding will continue. Ultimately all of these advances will allow more accurate methods of genetic screening, the identification of high-risk individuals or family groups and improved prenatal diagnosis.

In the near future, with rapid advances in the science of gene manipulation, the correction or alteration of genetic defects at the molecular level remains a possibility. Gene manipulation can be employed to control the expression of any gene in several orthodontically relevant issues. In turn, we may witness the introduction of both preventive and in vivo foetal therapy for such issues.

CONCLUSION

The conclusions drawn from this study are:-

1. This study suggests that the likelihood of dental crowding is higher in subjects having CG and GG genotypes of EDA gene variant rs3764746 and GT and TT genotypes of EDA gene variant rs3795170 in our population.

2. The findings of this study suggest that EDA gene variants rs3764746 and rs3795170 can be considered as genetic markers for dental crowding in our population.

3. This study indicates that there is no association between the presence of EDA gene variants rs 372024 with the incidence of dental crowding in our population.

4. A Meta analysis in a wider range of patient groups with more complete clinical descriptions of affected individuals may be required to more completely characterize the association between genetic markers and dental crowding.

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