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Association Between MMP9 Gene Polymorphisms and Nonsyndromic Cleft Lip/Palate in an Indian Population

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Abstract

Cleft lip/palate (CL/P) is among the most common congenital anomalies worldwide. This study investigates the association between matrix metalloproteinase 9 (MMP9) gene polymorphism (rs3918242) and nonsyndromic CL/P in an Indian population. The study involved 120 individuals with nonsyndromic CL/P and 140 healthy controls. DNA was extracted from blood samples, and genotyping for MMP9 polymorphisms was performed using polymerase chain reaction (PCR) and restriction fragment length polymorphism (RFLP) techniques with SphI enzymes. Statistical analysis included univariate and multivariate logistic regression models to calculate odds ratios and 95% confidence intervals, with significance set at P<0.05. Results indicated a significant association between MMP9 polymorphism and nonsyndromic CL/P, showing a higher prevalence of the T allele and TT genotype in cases compared to controls. This study suggests a potential link between MMP9 polymorphism and nonsyndromic CL/P in the Indian population, emphasizing the need for further research with larger and more diverse samples to provide more robust evidence. Expanded studies across different ethnic groups are crucial for a deeper understanding of the genetic factors contributing to CL/P.

Keywords:

Introduction

One of the most frequently occurring birth defects, which is believed to have a multifactorial etiology is the Cleft lip and palate (CL/P). It is known to be an amalgamation of various environmental as well as genetic factors [1]. This malformation has an incidence of 1.3 per 1000 live births in India [2] while the state of Andhra Pradesh had the maximum number of infants affected with this malformation with an incidence of 1.09 per every 1000 live births [3]. The analysis of genomic sequences has identified 18 risk loci which are associated with CL/P [4]. It was observed that early detection of the candidate genes for patients with nonsyndromic CL/P would enable the prediction and prevention of this disorder [5].

The significance of matrix metalloproteinases (MMPs) in the formation of CL/P is noteworthy because of their contribution to craniofacial structure development [6]. Research indicates that MMPs 2, 3, 7, 9, and 13 are present during the fusion of the palate in mice, both in terms of timing and location [7]. Furthermore, MMPs are involved in palate formation by breaking down the extracellular matrix (ECM), a process essential for lip and palate fusion. Alterations in MMP levels could potentially hinder fusion, thereby contributing to the development of clefts in the lip and/or palate.[8] These findings suggest a potential link between MMPs and the origins of CL/P. The MMP9 gene, situated on chromosome 20q12.2, is known for its ability to break down type IV collagen, a key component of the ECM, and promote cell movement.[9] Thus far, scientists have discovered a minimum of 12 potential single nucleotide polymorphisms (SNPs) within the promoter

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and coding sections of the MMP9 gene, which could hold clinical importance in how it is expressed and functions. One specific polymorphism found in the MMP9 gene's promoter region at position 1562 (C/T) has been shown to affect transcription functionally. This change from C to T interrupts the binding of a nuclear protein in this area of the MMP9 gene, leading to heightened transcriptional activity [9].

The MMP2 gene, found on chromosome 16q13.21, is another matrix metalloproteinase involved in conditions impacting the dentin-pulp complex. It might also play a role in dental irregularities seen in individuals with cleft lip and/or palate (CL/P) [10]. A prevalent single nucleotide polymorphism (SNP) in the CCACC box of the MMP2 gene promoter, 1306 C/T, has been linked to cleft lip and/or palate (CL/P) in various populations. This SNP alters the amino acid sequence from alanine to valine at position 429 of the protein [11–13]. In some studies, the valine allele has been linked to reduced MMP2 activity and a heightened risk of cleft lip and/or palate (CL/P) [14, 22]. Given the role of MMP2 and MMP9 in craniofacial development and dental abnormalities, it is crucial to examine if genetic variations in these genes could influence the occurrence of cleft lip and/or palate (CL/P). Therefore, this study analyzed the presence of MMP9 gene polymorphisms in individuals with CL/P compared to healthy controls. Identifying any associations between specific genetic variations and CL/P may offer insights into the genetic factors contributing to this common congenital condition.

Methodology

Selection of the Area The study was conducted within an Indian population, focusing on individuals diagnosed with nonsyndromic cleft lip and/or palate (CL/P) and healthy controls.

Sample Size Determination The study included 260 participants: 120 patients with nonsyndromic CL/P and 140 healthy controls. The minimum required sample size for the CL/P group was determined to be 120 patients.

Sampling Technique Participants were selected based on specific inclusion criteria. For the CL/P group, inclusion criteria were adult patients with nonsyndromic CL/P of Indian ethnicity, and consent for blood collection. For the control group, inclusion criteria were no family history of CL/P, matching the CL/P patients in terms of age, gender, and geographical region, absence of any syndrome, and consent for blood collection.

Data Collection Written consent was obtained from all participants after thoroughly informing them about the study (Ethics code: IHEC/SDC/ORTHO-2107/24/051). Blood samples were collected from each participant for DNA extraction using the salting-out method. The quality and quantity of the isolated DNA were assessed using gel electrophoresis and spectrophotometry. Polymerase chain reaction (PCR) and restriction fragment length polymorphism (RFLP) techniques were employed for genotyping. Specific primers for the MMP9 gene were used, with PCR settings optimized for gene amplification. The PCR products were confirmed via agarose gel electrophoresis, and genotyping was performed using the SphI enzyme to digest the PCR products, which were then analyzed on a 2% agarose gel to determine the various genotypes associated with CL/P.

Data Analysis Statistical analysis was conducted using SPSS 11.5. Univariate and multivariate logistic regression models were used to calculate odds ratios and 95% confidence intervals (CIs). The level of statistical significance was defined at $P<0.05$.

Genotyping

The salting-out technique was used to extract DNA from blood samples, followed by quality and quantity assessment through gel electrophoresis and spectrophotometry. PCR was performed using Taq DNA polymerase, 10-mM dNTP mix, 50-mM $MgCl_2$ solution,

and primers specific to MMP9 genes. The PCR settings included an initial denaturation at 94°C for 5 minutes, followed by 35 cycles of denaturation at 94°C for 45 seconds, annealing at 62°C for 15 seconds, extension at 72°C for 45 seconds, and a final extension at 72°C for 10 minutes. PCR products were confirmed using agarose gel electrophoresis. RFLP was employed to genotype the MMP9 genes, with SphI enzyme digestion of PCR products, and analysis on 2% agarose gel to identify different genotypes linked to CL/P.

Table 1. The Diameters of the RFLP Fragments, PCR Product Sizes, and Primer Sequences

SNPs	Primer Sequence (5'----> 3')	Product Size (bp)	RFLP Fragments (bp)
MMP9 (rs3918242 C/T)	F: TGGTCAACGTAGTG AAACCCCATCT R: CCAGCCCCAATTATC ACACTTAT	385	CC:385 bp TT:317 bp+68 bp CT:385bp+317bp+68bp

Data Analysis Statistical analysis was performed using SPSS 11.5. Univariate and multivariate logistic regression models calculated odds ratios and 95% confidence intervals (CIs), with statistical significance set at $P<0.05$.

Results

To examine the MMP9 (rs3918242C/T) gene polymorphisms in the case and control groups, PCR amplification and RFLP-PCR were used. Using certain enzymes (SphI for MMP9), the PCR products were separated on a 2% agarose gel and the RFLP patterns were observed. It was discovered that the genotype frequencies in both groups fell within the Hardy-Weinberg equilibrium ($P<0.001$ in both cases). An investigation of MMP9 gene polymorphisms identified three genotypes: Forty patients (33.3%) in the case group and ninety-two individuals (65.7%) in the control group had the CC (wild-type) genotype; twenty-four patients (20%) in the case group and eighteen individuals (12.9%) in the control group had the CT (heterovariant) genotype; and thirty patients (21.4%) in the case group and forty patients (36.7%) in the control group had the TT (variant) genotype. In the case group (56.7%), the prevalence of the T allele, which corresponds to the variant genotype, was substantially greater than in the control group (27.9%) ($P<0.001$).

The genotype and allele distribution of MMP9 gene polymorphisms in the case and control groups is displayed in Table 2.

Figure 1 shows the RFLP products generated by the enzymatic digestion for MMP9 gene polymorphisms in a few selected samples of the case group.

Table 2. The Genotype And Allele Distribution of MMP9 Gene Polymorphisms

Gene	**Genotype**	**Case (n=120)**	**Control (n=140)**
MMP9	CC	40 (33.3%)	92 (65.7%)
	CT	24 (20.0%)	18 (12.9%)
	TT	56 (46.7%)	30 (21.4%)

	Allele	C: 104 (43.3%) T: 136 (56.7%)	C: 202 (72.1%) T: 78 (27.9%)

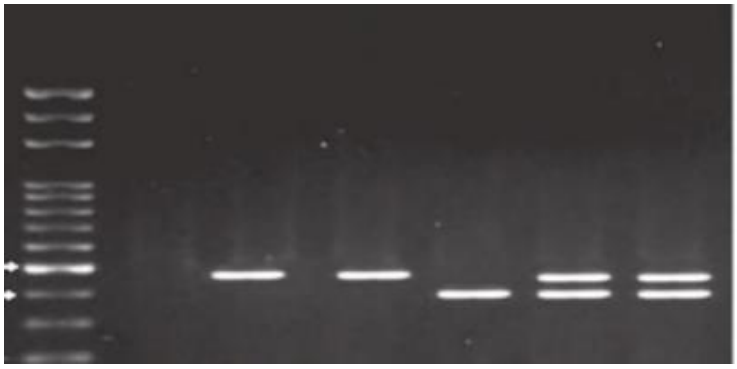

Figure 1. RFLP Product Analysis Using The SphI Enzyme for Mmp9 Gene Polymorphism (CC: 385 bp, CT: 317, 385 bp, and TT: 317 bp)

Discussion

Determining the genetic and environmental factors involved in the development of CL/P requires an understanding of the intricate mechanisms underlying the condition. Research on humans and biology both suggest the relevance of MMPs and their inhibitors as relevant genes for nonsyndromic CL/P[17]. In the past, most studies on nonsyndromic orofacial clefts focused on looking at SNPs separately. In light of this, examining the numerous polymorphisms in these genes and their correlation with CL/P has emerged as a critical strategy in both basic and clinical research. The current investigation assessed the MMP9 gene's rs3918242 polymorphism in nonsyndromic CL/P patients and healthy controls. The two proteases MMP2 and MMP9 play a major part in the breakdown of extracellular matrix (ECM), which includes type IV collagen and laminin, the two main building blocks of the basal membrane [18]. Degeneration of the palatal shelves' inner epithelium during palate fusion has been directly linked to the upregulation of MMP2, MMP3, MMP9, and MMP13 in the midface [19].

The MMP9 gene's promoter region has the functional rs3918242 polymorphism (C/T), which has an immediate effect on transcriptional activity and nuclear protein binding. The T allele increases promoter activity by around 1.5 times, which leads to an overexpression of MMP9 [9]. Prior research on palatal fusion has demonstrated that MMP9 is upregulated during the latter phases of secondary palate development and then downregulated following fusion [8]. Additionally, the current study looked at the relationship between nonsyndromic CL/P in the Indian population and the MMP9 gene's rs3918242 polymorphism. The current findings showed a substantial correlation, with patients having a higher frequency of faulty T allele than healthy controls. Furthermore, the case group had a higher prevalence of the TT genotype. These results are in line with the 1.5-fold increase in promoter activity, possible overexpression of MMP9, and maybe elevated risk of nonsyndromic CL/P. The results of a study conducted in Brazil conflict with the conclusions of this investigation. Their findings indicated that in nonsyndromic CL/P patients, the CC genotype was substantially more prevalent than the CT genotype, and no group exhibited the TT genotype [20]. Moreover, nonsyndromic CL/P and MMP9 gene polymorphism were not significantly associated in India [21]. However, it is important to remember that the reported

discrepancies in the findings of various research could be the consequence of various factors, including sample size, environmental and geographic changes, and the multifactorial nature of these genes in the development of CL/P. All things considered, a thorough grasp of the variables that contribute to the development of CL/P is essential for creating more accurate preventative and treatment plans. The current study made clear how important it is to continue researching more genes and how, to draw more firm conclusions, a larger sample size and thorough assessments of a variety of groups are required. Potential treatment plans and preventative measures for CL/P may be created with a better understanding of the role that MMPs and their suppressors play in the development of this congenital condition.

Conclusion

In the same cohort, this study discovered a strong correlation between the MMP9 (rs3918242) gene polymorphism and nonsyndromic CL/P. Because these genes are multifactorial in the development of CL/P, variations in the findings of various research may be due to variations in sample size as well as geographic and environmental factors. It is necessary to do more extensive and varied population-based studies to confirm and clarify the genetic contributions to nonsyndromic CL/P in different ethnic groups.

Conflict of Interest

The authors declare no conflict of interest.

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Nil.

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