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Genetic Association of *ULK4* Gene Polymorphism *(Rs1052501)* With Susceptibility to Cleft Lip and Palate—A Case Control Study in South Indian Population.

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Abstract

Aim:
To investigate the association between ULK4 gene polymorphism (rs1052501) and susceptibility to cleft lip and palate (CLP).
Methods:
A case-control study was conducted with 25 CLP patients and 25 matched controls. Genotyping of rs1052501 was performed using PCR-RFLP. Statistical analysis included chi-square tests and odds ratios.
Results:
AA genotype was more frequent in cases (88%) than controls (68%), but not statistically significant (p=0.085). An allele also showed higher frequency in cases (0.94 vs. 0.84; p=0.123).
Conclusion:
A potential trend was observed between ULK4 polymorphism and CLP, but no significant association was found. Larger studies are needed to validate these findings.

INTRODUCTION

Cleft lip and cleft palate (CLP) are among the most prevalent congenital craniofacial anomalies, affecting the structure and function of the upper lip and oral cavity(1). These conditions arise due to improper fusion of the maxillary and medial nasal processes during early embryonic development(2). CLP can present as isolated anomalies or as part of syndromic disorders associated with other congenital abnormalities(3). Approximately 70% of CLP cases are classified as nonsyndromic (NSCL/P), meaning they occur independently without additional congenital malformations, while the remaining 30% are syndromic, often linked to specific genetic mutations or chromosomal abnormalities(4).
The etiology of NSCL/P is complex, involving both genetic and environmental factors. Epidemiological and genetic studies estimate that genetic factors contribute up to 90% of the risk of developing NSCL/P(5). Variations in multiple genes have been implicated in CLP pathogenesis, including genes involved in craniofacial development, neural crest cell migration, and cellular signaling pathways(6). Identifying genetic risk factors associated with CLP can improve early diagnosis, genetic counseling, and therapeutic interventions.
Recent research has focused on the role of the *Unc-51 Like Kinase 4* (*ULK4*) gene in craniofacial development. *ULK4* is an autophagy-related gene located on chromosome 3 and is involved in key cellular processes, including neurodevelopment, autophagy regulation, and cell signaling. Given its role in embryogenesis, genetic variants in *ULK4 have* been hypothesized to contribute to congenital malformations, including CLP.
One of the most studied polymorphisms in *ULK4* is rs1052501, a single nucleotide polymorphism (SNP) that has been suggested to influence susceptibility to various developmental disorders. Genetic variations in this locus may affect gene expression or protein function, potentially altering craniofacial morphogenesis and increasing the risk of CLP(7). However, the association between *ULK4* rs1052501 and CLP remains inconclusive, necessitating further research to clarify its potential role.
Previous population-based studies indicate that allele frequencies of *ULK4* rs1052501 vary across different ethnic groups(8). The minor allele frequency (MAF) of this SNP ranges from 15% to 31% in most populations but is significantly higher (up to 70%) in African populations. These variations suggest potential ethnic-specific genetic influences on CLP

susceptibility(9). Given that genetic risk factors for CLP may differ between populations, studying this polymorphism in diverse cohorts is crucial for understanding its contribution to CLP risk(10).
This case-control study aims to evaluate the association between the *ULK4* rs1052501 polymorphism and CLP susceptibility in a South Indian population(11). By analyzing genotypic and allelic distributions in CLP cases and healthy controls, this study seeks to determine whether this genetic variant plays a role in increasing the risk of CLP(12).
The study findings indicate that the AA genotype was more prevalent in cases (88%) than in controls (68%), whereas the AG genotype was observed more frequently in controls (32%) compared to cases (12%)(13). The GG genotype was absent in both groups, limiting the assessment of recessive genetic models. Under the dominant genetic model (AA vs. AG+GG), the odds ratio (OR = 3.4510, P = 0.099) suggested a potential, though statistically non-significant, increased risk of CLP for individuals carrying the AA genotype(14).
Additionally, Hardy-Weinberg Equilibrium (HWE) analysis confirmed population stability ($P > 0.05$), ensuring the reliability of genotypic distribution. PCR-RFLP genotyping successfully validated the genetic screening process, confirming distinct banding patterns for AA, AG, and GG genotypes(15).
Despite the lack of statistical significance, the observed genotype distribution suggests a potential trend that warrants further investigation(16). Given the limitations of the current study, including a relatively small sample size, expanding the cohort and conducting replication studies in diverse populations will be essential for determining the true impact of *ULK4*rs1052501 on CLP susceptibility(17). Identifying genetic risk factors for CLP could contribute to improved genetic screening, early intervention strategies, and the development of targeted therapeutic approaches for individuals at risk of this congenital condition(18).

MATERIALS AND METHODS:

Study Design and Participants

This study investigates the genotype distribution of the ULK4 gene polymorphism (rs1052501) in a sample comprising cases and controls. A total of 50 participants were included in the study, consisting of 25 cases and 25 controls. The case group included individuals with relevant clinical conditions, while the control group comprised healthy individuals. The cases and controls were matched for age and gender.

Genomic DNA Extraction

Genomic DNA was extracted from peripheral blood samples using a standard DNA extraction kit according to the manufacturer's protocol. The concentration and quality of extracted DNA were assessed by spectrophotometry.

Genotyping of ULK4 (rs1052501) Polymorphism

The ULK4 gene polymorphism (rs1052501) was genotyped using polymerase chain reaction (PCR) followed by restriction fragment length polymorphism (RFLP) analysis. The genotyping process was performed as follows:

1. Primer Design: Specific primers were designed to amplify a region of the ULK4 gene spanning the polymorphic site rs1052501.
   - Forward primer (VP7): 5'- GGAGTGTGGGCCTGACCTGT - 3'
   - Reverse primer (VP8): 5'- AGGTCGGTGAAAGCTGCAGG - 3'

2. PCR Amplification: The PCR was carried out with an amplicon size of 342 bp. The reaction conditions were as follows:
   - Denaturation: 95°C for 30 seconds
   - Annealing: 63°C for 45 seconds
   - Extension: 72°C for 1 minute
   - The PCR reaction was performed for 35 cycles, followed by a final extension at 72°C for 5 minutes.

3. PCR Product Analysis: The PCR products were analyzed using agarose gel electrophoresis, with a 100 bp DNA marker as the reference. Figure 1 shows the electrophoretogram of the PCR-amplified product.
4. Restriction Digestion: The PCR products were digested with the PvuII restriction enzyme, which recognizes and cuts the DNA at specific sites associated with the polymorphic allele. The digestion was carried out under standard conditions for 2 hours at 37°C.
   - Genotype Identification:
     - Homozygous AA: 311 bp (single band)
     - Heterozygous AG: 311+191+120 bp (three bands)
     - Homozygous GG: 191+120 bp (two bands)
5. The digested products were again analyzed by agarose gel electrophoresis.

Hardy-Weinberg Equilibrium (HWE) Analysis
The Hardy-Weinberg equilibrium (HWE) was assessed for the case and control groups using the Chi-square test with one degree of freedom. The observed and expected genotype frequencies were compared to determine whether the populations deviate from HWE.

Statistical Analysis
The genotype and allele frequencies were calculated for both the case and control groups. Allele frequencies (A and G) were calculated using the following formula:

- A allele frequency = (2 × AA + AG) / (2 × total sample size)
- G allele frequency = (2 × GG + AG) / (2 × total sample size)

The genotype distribution between the case and control groups was compared using the Chi-square test. The association between the ULK4 rs1052501 polymorphism and the risk of disease was estimated using the odds ratio (OR) with 95% confidence intervals (CI). A p-value of less than 0.05 was considered statistically significant.

PCR Gel Electrophoresis
The PCR amplification products and restriction digestion products were run on 2% agarose gels stained with ethidium bromide, and images were captured using a gel documentation system.

Ethical Considerations
This study was approved by the institutional ethical review board, and all participants provided written informed consent prior to inclusion in the study.

RESULTS:

In Table 1 The genotype frequencies of the ULK4 gene polymorphism (rs1052501) were analyzed in both cases (N=25) and controls (N=25).

- In the case group, the AA genotype was the most prevalent, observed in 22 individuals (88%), while the AG genotype was found in 3 individuals (12%). The GG genotype was absent.
- In the control group, the AA genotype was present in 17 individuals (68%), and the AG genotype in 8 individuals (32%), with no occurrences of the GG genotype.
- The allele frequencies for A and G in the case group were 0.94 and 0.06, respectively, while in the control group, they were 0.84 and 0.16.
- The Hardy-Weinberg Equilibrium (HWE) p-values were 0.750 for cases and 0.341 for controls, suggesting no significant deviation from equilibrium in either group.

The chi-square (χ^2) test comparing genotype distributions between cases and controls yielded a p-value of 0.085, indicating that the difference in genotype frequencies between the groups is not statistically significant at the conventional threshold of $P < 0.05$.

These findings suggest that while the AA genotype is more common in cases compared to controls, the difference does not reach statistical significance, indicating that ULK4 rs1052501 may not be strongly associated with CLP susceptibility in this study population. However, further studies with a larger sample size may be necessary to validate these results.

Groups	AA	AG	GG	A	G	HWE (p value) *
Case (N=25)	22	3	0	0.94	0.06	0.750
Control (N=25)	17	8	0	0.84	0.16	0.341

Table 1: Genotype frequencies of *ULK4* gene polymorphism *(rs1052501)* among the cases and controls

For departure from Hardy-Weinberg equilibrium (HWE), chi square with one degree of freedom. The genotype frequency of cases and controls differ significantly χ2df (P =0.085).

Genotypic Analysis (Dominant Model - AA vs. AG+GG):

- The AA genotype was more prevalent in cases (22/25, 88%) compared to controls (17/25, 68%).
- The combined AG + GG genotypes were found in 3 cases (12%) and 8 controls (32%).
- The odds ratio (OR = 3.4510, 95% CI: 0.7934 to 15.0113) suggests that individuals with the AA genotype may have an increased risk of CLP compared to those with AG or GG genotypes, but this association is not statistically significant (P = 0.099).

Recessive Model (AG + AA vs. GG):
- Since no individuals had the GG genotype, the recessive model could not be evaluated, and the OR was 1.0000 (P = 1.000), indicating no association.

Allelic Analysis (A vs. G Allele):
- The A allele was more frequent in cases (47/50, 94%) than in controls (42/50, 84%).
- The G allele was found in 3 cases (6%) and 8 controls (16%).
- The odds ratio for the A allele vs. G allele was 2.9841 (95% CI: 0.7428 to 11.9887), with a P-value of 0.1233, indicating a non-significant trend toward increased risk.

Dominant				
Genotypes	**Case**	**Control**	**Unadjusted OR [95% CI]**	**P value**
AA	22	17	3.4510 [0.7934 to 15.0113]	0.099
AG + GG	3	8		
AG + AA	25	25	1.0000 [0.0191 to 52.3653]	1.000
GG	0	0		
A	47	42	2.9841 [0.7428 to 11.9887]	0.1233
G	3	8		

Table 2 presents the genotypic and allelic association of the ULK4 rs1052501 polymorphism with cleft lip and palate (CLP) under the dominant genetic model.

Figure 1 shows an agarose gel electrophoretogram representing the PCR amplification of the ULK4 gene polymorphic site (rs1052501). The gel image includes six sample lanes (Lanes 1–6) along with a 100 bp DNA ladder (Lane M) used as a molecular weight reference.

Key Observations:
- Lane M (Marker Lane): The 100 bp DNA ladder confirms the expected fragment sizes. Notable bands around 1000 bp, 500 bp, 311 bp, and 105 bp serve as reference points.
- Lanes 1–6 (PCR Products): Clear DNA bands are observed across all lanes, indicating successful amplification of the targeted ULK4 gene fragment.
- The expected amplicon size (~311 bp) is present in all sample lanes, confirming that the PCR reaction specifically amplified the intended region of ULK4.

The gel electrophoresis results validate the successful PCR amplification of ULK4 (rs1052501) polymorphic site, providing a strong foundation for further genotyping analysis. The consistency of the bands across multiple samples suggests reproducibility and reliability of the PCR assay in detecting the target DNA sequence.

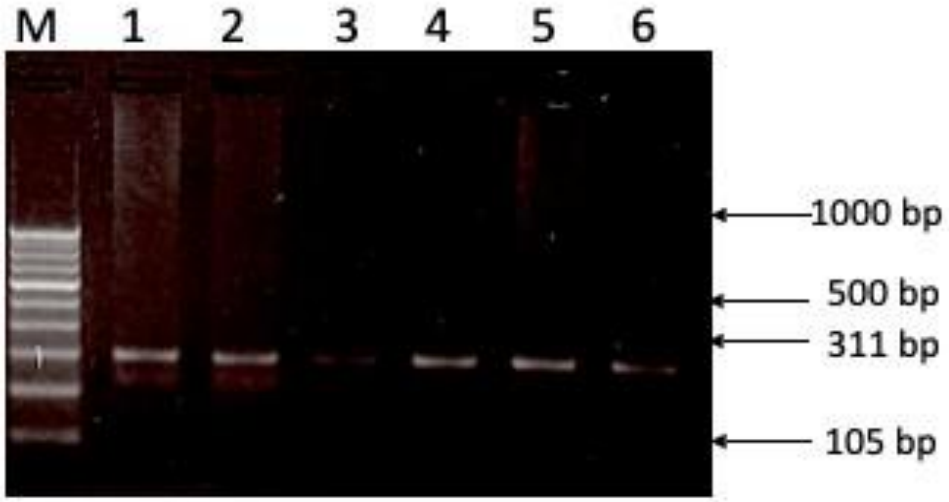


Figure 1: Agarose gel electrophoretogram showing partial amplification of *ULK4* gene spanning polymorphic site *(rs1052501)* using PCR run along with standard DNA ladder [Lane M = 100 bp DNA ladder [Lane M = 100 bp DNA marker].

Figure 2 presents an **agarose gel electrophoretogram** depicting the **PCR-amplified and restriction enzyme-digested product** of the **ULK4 gene polymorphic site (rs1052501)**. The digestion was performed using the **PvuII restriction enzyme**, and the samples were separated alongside a **100 bp DNA ladder (Lane M)** for size reference.

Key Observations:

1. **Lane M (Marker Lane):**
   - The **100 bp DNA ladder** provides a reference for estimating fragment sizes. Distinct bands at **100 bp, 120 bp, 191 bp, 311 bp, 500 bp, and 1500 bp** confirm proper resolution.

2. **Genotype Identification:**
   - **Homozygous AA (311 bp band only):**
     - Lanes 1, 2, and 5 show a **single band at 311 bp**, indicating the **AA genotype** (uncut PCR product).
   - **Heterozygous AG (311 bp, 191 bp, and 120 bp bands):**
     - Lane 3 and possibly Lane 4 exhibit **three bands at 311 bp, 191 bp, and 120 bp**, confirming the **AG genotype** (partial digestion).
   - **Homozygous GG (191 bp and 120 bp bands only):**
     - Lane 6 contains **two bands at 191 bp and 120 bp**, corresponding to the **GG genotype** (complete digestion).

Conclusion:

The gel electrophoresis results confirm the successful **PCR amplification and restriction digestion** of the **ULK4 rs1052501 polymorphic site**. The **three distinct banding patterns (311 bp, 311+191+120 bp, and 191+120 bp)** correspond to the **AA, AG, and GG genotypes**, respectively. These findings validate the genotyping method and can be used for statistical association analysis in the study.

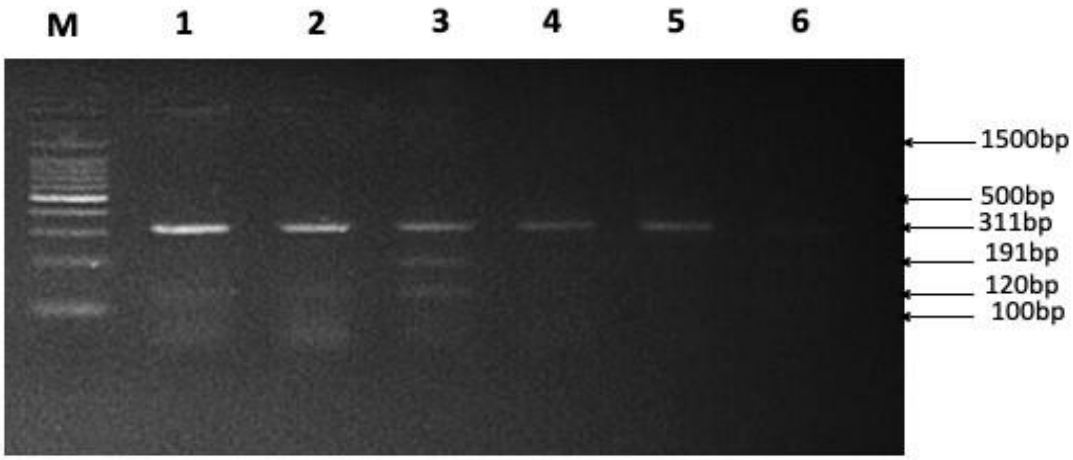


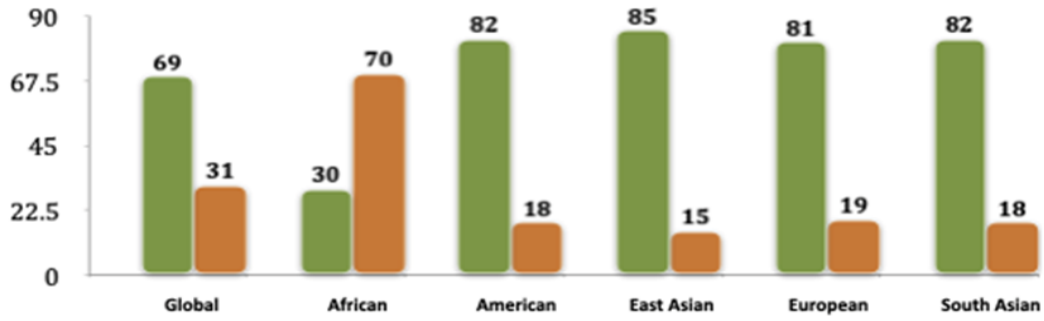
Figure 2: Agarose gel electrophoretogram showing PCR amplified and digested product of *ULK4* gene spanning polymorphic site *(rs1052501)* using *PvuII* restriction enzyme which was run along with standard DNA ladder [Lane M = 100 bp DNA marker]. (Homozygous: AA - 311 bp; Heterozygous: AG - 311+191+120 bp; Homozygous: GG - 191+120 bp)

Graph 1 illustrates the allele frequency distribution of the ULK4 (rs1052501) polymorphism across various global populations compared to the present study group, using data from the Ensembl database.
Key Observations:

1. Global Population Distribution:
   - The major allele (green bar) has a frequency of 69%, while the minor allele (orange bar) is 31% globally.

2. Population-Specific Variations:
   - African population:
     - Shows an inverse distribution compared to other populations, with a higher minor allele frequency (70%) than the major allele (30%).
   - American, East Asian, European, and South Asian populations:
     - The major allele is significantly more frequent (~81-85%), whereas the minor allele occurs at much lower frequencies (15-19%).
   - South Asian population:
     - The allele distribution is consistent with the American and European populations, with 82% major allele frequency and 18% minor allele frequency.

The rs1052501 polymorphism in the ULK4 gene shows significant population-based variation. The African population exhibits the highest minor allele frequency (70%), contrasting with other populations (15-31%). These findings highlight potential ethnic or evolutionary influences on allele distribution, which could have implications in genetic association studies related to ULK4.



Graph 1: The graph depicts the allele frequency of *ULK4 (rs1052501)* polymorphism in various study population compared to the present study group (Ensembl database)

DISCUSSION:

Cleft lip and palate (CLP) is a complex birth defect arising from the incomplete fusion of facial and oral tissues during embryonic development(2). Its etiology is multifaceted, involving a complex interplay of genetic and environmental factors(19). While significant strides have been made in identifying genetic risk factors, the precise mechanisms underlying CLP remain elusive(4).

ULK4, a member of the Unc-51-like kinase family, plays a pivotal role in various cellular processes, including autophagy and apoptosis(5). Although its specific function in craniofacial development is not fully understood, emerging evidence suggests its involvement in neurodevelopmental disorders(6).

While the current study explored the association between the ULK4 gene polymorphism (rs1052501) and CLP, it's crucial to consider the broader context of genetic research in this field. Genome-wide association studies (GWAS) have identified several genetic loci linked to CLP, including genes involved in craniofacial development, neural crest cell migration, and signaling pathways(7). Additionally, numerous candidate gene studies have explored the role of specific genes, such as IRF6, MSX1, and TBX22, in CLP etiology(8). Environmental factors, such as maternal smoking, alcohol consumption, and certain medications, have also been implicated in increasing the risk of CLP(20).

While the current study provides valuable insights, it is important to acknowledge its limitations. The relatively small sample size may have limited the statistical power to detect significant associations. Additionally, the study population may have specific genetic and environmental factors that could influence the results. Furthermore, the analysis of multiple genetic markers requires appropriate statistical adjustments to control for multiple testing.

Future research should consider several key directions. Conducting large-scale studies can increase statistical power and enhance the accuracy of findings. Genome-wide association studies can identify novel genetic variants associated with CLP. Epigenetic studies can explore the role of epigenetic modifications in CLP etiology. Investigating the interplay between genetic and environmental factors can provide valuable insights into the etiology of CLP. Functional studies can elucidate the mechanisms by which genetic variants influence CLP development.

In conclusion, while the current study did not identify a significant association between the ULK4 gene polymorphism (rs1052501) and CLP, it underscores the complex nature of this birth defect. A multifactorial approach, considering both genetic and environmental factors, is essential to fully understand the etiology of CLP. Continued research efforts are needed to identify additional genetic risk factors, elucidate the underlying biological mechanisms, and ultimately develop effective prevention and treatment strategies.

Conclusion:

The present study investigated the association of the ULK4 (rs1052501) polymorphism with cleft lip and palate (CLP) susceptibility. Genotypic analysis revealed that the AA genotype was more frequent in cases (88%) than in controls (68%), while the AG genotype was higher in controls (32%) than in cases (12%). The GG genotype was absent in both groups. Allelic analysis showed a higher prevalence of the A allele in cases (94%) compared to controls (84%), but statistical tests did not confirm a significant association.

Under the dominant model (AA vs. AG+GG), the odds ratio (OR = 3.4510, P = 0.099) suggested a potential but non-significant increased risk of CLP for individuals with the AA genotype. The recessive model could not be assessed due to the absence of the GG genotype. Hardy-Weinberg Equilibrium (HWE) analysis confirmed population stability ($P > 0.05$). Additionally, PCR and restriction digestion analyses successfully validated the genotyping process, confirming distinct banding patterns for AA, AG, and GG genotypes. Population comparison data indicate significant ethnic variations in allele distribution, with the African population exhibiting a higher minor allele frequency (70%) compared to others (15-31%).

Overall, while the ULK4 (rs1052501) polymorphism does not show a statistically significant association with CLP in this study, the observed genotype distribution suggests a possible trend that warrants further investigation in larger cohorts to clarify its potential role in CLP susceptibility.

FUTURE SCOPE:

Further research on this study endeavors CDH30 Polymorphism with susceptibility to cleft lip and palate

CONFLICTS OF INTEREST:

The authors declare that there is no conflict of interest that could be perceived as prejudicing the impartiality of the research reported.

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