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**RESEARCH NOTE**

# The association study of nonsyndromic cleft lip with or without cleft palate identified risk variants of the *GLI*3 gene in a Chinese population

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**Abstract.** Nonsyndromic cleft lip with or without cleft palate (NSCL/P) is a common birth defect due to abnormal orofacial development. Previous studies report abnormal sonic hedgehog (SHH) signalling activity during NSCL/P pathogenesis and propose several genes in the SHH pathway as candidate risk genes. As such, we focussed on *GLI3*, a downstream modulator of the SHH pathway. In the present study, we genotyped 34 tag SNPs covering *GLI3* and performed association analysis with NSCL/P in 504 cases and 455 healthy controls. Our preliminary results identified risk variants of *GLI3* that are associated with NSCL/P susceptibility in a Chinese population. In particular, rs3801161 and its haplotypes rs3801161–rs7785287 displayed significant association with NSCL/P and survived Bonferroni correction for multiple comparisons. The robustness of the association between *GLI3* and NSCL/P is worth further examination in the future across different populations.

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**Keywords.** nonsyndromic cleft lip with or without cleft palate; *GLI3* gene; association study; risk variants.

## Introduction

It is known that CL/P results from abnormal craniofacial development during embryogenesis (Ferguson 1988). Craniofacial developmental includes formation of the lip and palate, which involves a complex series of cellular and molecular events. As such, several signalling pathways and genes have been proposed as promising candidates to understand the aetiology of NSCL/P (Vieira 2006). SHH signalling is one of the most studied candidate pathways because it plays an important role in regulating palatal growth (Lan and Jiang 2009). Disruption of SHH signalling in animal models results in increased occurrence of CL/P (Lipinski *et al.* 2010). Therefore, it is believed that abnormal SHH signalling activity contributes to the aetiology and pathogenesis of NSCL/P. In humans, mutations in *PTCH*, a key gene in the SHH signalling pathway, have been identified in NSCL/P cases (Mansilla *et al.* 2006). Follow-up association and functional studies provide further evidence supporting the role of *PTCH* in NSCL/P (Letra *et al.* 2010). The identification of the SHH pathway and the *PTCH* gene provide new insight into the understanding of the aetiology of NSCL/P and imply that other genes in the SHH pathway might be candidate genes for NSCL/P as well.

The GLI family zinc finger 3 (*GLI3*) gene is located on chromosome 7p14.1 and encodes a zinc-finger transcription factor (Abbasi *et al.* 2009). As a key downstream modulator of the SHH pathway, appropriate regulation of *GLI3* expression is important for the development of embryonic craniofacial structures in mammals and other vertebrates (Rice *et al.* 2006). Animal studies indicate that *GLI3*-deficient mice have increased incidence of cleft palate due to abnormal tongue development (Huang *et al.* 2008). In humans, *GLI3* mutations have been identified in a group of phenotypes, including Greig cephalopolysyndactyly syndrome (GCPS), Pallister-Hall syndrome (PHS), and postaxial polydactyly, which are collective known as *GLI3* morphopathies (Radhakrishna *et al.* 1999). Recent molecular analysis of *GLI3* mutations further expand its phenotypic spectrum and propose robust genotype–phenotype correlations (Johnston *et al.* 2010; Démurger *et al.* 2015). Although *GLI3* morphopathies have distinct clinical characteristics, the craniofacial abnormalities such as CL/P are identified as primary features shared by different syndromes associated with *GLI3* mutations.

Thus, based on current genetic evidence from animal models and syndromic patients, it is believed that the *GLI3* gene plays an important role in the aetiology and pathogenesis of CL/P. Therefore, *GLI3* is another promising candidate gene in the SHH pathway for NSCL/P research. Although some *GLI3* mutations with deleterious and pathogenic effects have been identified, these mutations are rare and, therefore, seem unlikely to be prevalent among the more common form of CL/P (i.e., NSCL/P). As such, common genetic polymorphisms of *GLI3* might be risk factors contributing to NSCL/P susceptibility.

## Materials and methods

### *Subject*

In the present study, we used *GLI3* as a candidate gene and performed case–control-based association analysis in a large, unrelated Chinese NSCL/P cohort. In total, 504 NSCL/P cases and 455 healthy controls were recruited from the Western China Hospital of Stomatology, Sichuan University. This study was approved by the Ethical Committee of Sichuan University. Written consent and peripheral blood samples were obtained from each participant. All participants were genetically unrelated Han Chinese. They were interviewed and clinically assessed by an experienced oral surgeon based on detailed diagnostic information from medical records and physical examinations to exclude individuals with syndromic orofacial clefts or other major congenital defects. Subjects in the control group were healthy individuals without a family history of orofacial clefts or other major congenital defects. The age and sex of control samples were matched with case samples.

### *Marker selection and genotyping*

In total, 34 tag SNPs covering the *GLI3* gene were selected using the Tagger program (de Bakker *et al.* 2005) with parameters of minor allele frequency >5% and pairwise $r^2$ threshold of 0.8. Linkage disequilibrium analysis and haplotype selection were performed using Haploview software (Barrett *et al.* 2005). Genotyping of all tag SNPs was performed at CapitalBio Corporation (Beijing, China) with the MassARRAY platform (Sequenom, San Diego, USA) according to the manufacturer's protocol. Briefly, genomic DNA was extracted from whole blood from each individual using a Wizard® Genomic DNA Purification kit (Promega, Madison, USA). The DNA concentration was determined using a NanoDrop 1000 (Thermo Fisher Scientific, Waltham, USA). Multiplex reaction primers were designed using the MassARRAY Assay Design software package (v3.1). Mass determination was performed with a MALDI-TOF mass spectrometer, and

MassARRAY® TYPER 4.0 genotyping software was used for data acquisition.

***Statistical analysis***

All of the selected SNPs were individually evaluated by Hardy–Weinberg equilibrium (HWE) in controls. Their associations with NSCL/P were analysed under different models (additive, dominant, recessive and genotype) and in haplotypes using PLINK software (Purcell *et al.* 2007). The odds ratio (OR) and its corresponding 95% confidence intervals (CI) were used to indicate the effective size of each SNP and haplotype. The association results were also adjusted by sex through logistic regression during single marker and haplotype analysis. Bonferroni correction was applied for multiple comparisons.

## Results

***Single marker analysis***

In the present study, we performed genotyping of 34 tag SNPs of *GLI3* followed by association analysis with NSCL/P. The complete *GLI3* genotyping results are listed in table 1 in electronic supplementary material at http://www.ias.ac.in/jgenet/. The raw genotyping data were adjusted for age and sex, and Bonferroni correction was then performed for multiple comparisons.

Here, table 1 shows selected SNPs exceeding the significance threshold ($P < 0.05$) after data adjustment. The association analysis under the additive model identified the G allele of rs3801161 showing strong association ($P = 0.00097$) and the G allele of rs846401 showing marginal association ($P = 0.05$). In addition, several SNPs emerged as significant ones under the genotype, dominant, and recessive models. As such, rs3801161 in the AG genotype ($P = 0.004$) and under the dominant model ($P = 0.001$), rs2237421 in the TT genotype ($P = 0.05$), rs846290 under the recessive model ($P = 0.04$), rs10951667 under the recessive model ($P = 0.05$), rs1003876 in the AT genotype ($P = 0.05$) and under the dominant model ($P = 0.05$), rs846401 in the GG genotype ($P = 0.02$) and under the recessive model ($P = 0.01$), rs3801212 in the GG genotype ($P = 0.03$) and under the recessive model ($P = 0.02$), and rs3801228 in the GG genotype ($P = 0.03$) and under the recessive model ($P = 0.03$) displayed nominal significant association with NSCL/P. However, after Bonferroni correction for multiple comparisons, only rs3801161 was significant.

***Haplotype analysis***

We built eight blocks within the *GLI3* gene using Haploview software (figure 1). Table 2 shows the eight haplotypes of *GLI3* and their association with NSCL/P. The rs3801161–rs7785287 haplotype (block 3) is the only *GLI3* haplotype that showed overall significant association (OMINIBUS, $P = 0.007$) and survived Bonferroni correction for multiple comparisons in the GC genotype ($P = 0.0009$).

## Discussion

The complex aetiology underlying NSCL/P remains elusive. Given the proposed role of the SHH signalling pathway during craniofacial development, genes connected to the SHH pathway such as *PTCH* have emerged as promising candidates to identify genetic aetiologies underlying NSCL/P (Mansilla *et al.* 2006). In the present study, we identified risk variants of *GLI3*, namely rs3801161 and its haplotype rs3801161–rs7785287, that exceeded the significance threshold ($P < 0.05$) and survived Bonferroni correction for multiple comparisons. The OR values implicated that the G allele of rs3801161 and G–C haplotype of rs3801161–rs7785287 block may raise the risk of NSCL/P. In the significant level of $P < 0.05$, the power of the study of the association of rs3801161 is 71% which is calculated using CaTS software (Skol *et al.* 2006). Therefore, *GLI3* is likely another candidate gene identified in the SHH pathway that contributes to NSCL/P susceptibility.

These results are in accordance with the described role of *GLI3* in previous reports (Abbasi *et al.* 2013). It is known that CL/P results from abnormal development of the secondary palate during embryonic development (Ferguson 1988). The SHH signalling pathway is believed to play an essential role for palatal outgrowth by triggering appropriate signals to repress or activate its downstream targets (Lan and Jiang 2009). The *GLI3* gene belongs to a family of GLI transcriptional factors that plays a secondary role in mediating SHH signals (Abbasi *et al.* 2009). In mice, the *GLI3* gene is widely expressed in the palatal mesenchyme (Rice *et al.* 2006). The *GLI3* deficiency in mice results in abnormal development of the tongue and increased incidence of cleft secondary palate (Huang *et al.* 2008). In humans, the *GLI3* mutations have been identified in a group of developmental disorders in which CL/P is a shared primary phenotype (Radhakrishna *et al.* 1999; Johnston *et al.* 2010; Démurger *et al.* 2015). Syndromic CL/P is usually caused by rare and deleterious genetic mutations that are unlikely to be prevalent among large populations. In contrast, for the most common form of CL/P (i.e. NSCL/P), common SNPs may contribute to disease susceptibility with modest effects. Therefore, we selected tag SNPs of *GLI3* to perform association analyses. It should be noted that tag SNPs are usually used as genetic markers representing a cluster of SNPs in linkage disequilibrium, and therefore, the actual causal variant might be tagged. As such, SNP imputation could be an option to estimate the association of

**Table 1.** The selected *GLI3* genotyping results.

| SNP | Patient | Control | Crude OR (95% CI) | Unadjusted *P* value | Adjusted OR (95% CI) | Adjusted *P* value |
|---|---|---|---|---|---|---|
| rs3801161 | | | | | | |
| A | 838 | 801 | 1 | | 1 | |
| G | 166 | 105 | **1.51 (1.16–1.97)** | **0.002** | **1.57 (1.20–2.04)** | **0.00097*** |
| AA | 349 | 354 | 1 | | 1 | |
| AG | 140 | 93 | **1.53 (1.13–2.06)** | **0.006** | **1.56 (1.15–2.11)** | **0.004** |
| GG | 13 | 6 | 2.2 (0.83–5.85) | 0.11 | 2.52 (0.94–6.78) | 0.07 |
| Dom | | | **1.57 (1.17–2.10)** | **0.003** | **1.61 (1.20–2.17)** | **0.001*** |
| Rec | | | 1.98 (0.75–5.26) | 0.16 | 2.25 (0.84–6.02) | 0.11 |
| rs2237421 | | | | | | |
| C | 556 | 462 | | | | |
| T | 452 | 444 | 0.85 (0.71–1.01) | 0.07 | 0.84 (0.70–1.01) | 0.06 |
| CC | 150 | 117 | 1 | | 1 | |
| CT | 256 | 228 | 0.88 (0.65–1.18) | 0.39 | 0.88 (0.48–1.19) | 0.41 |
| TT | 98 | 108 | 0.71 (0.49–1.02) | 0.06 | **0.7 (0.48–1.00)** | **0.05** |
| Dom | | | 0.82 (0.62–1.09) | 0.18 | 0.82 (0.62–1.10) | 0.18 |
| Rec | | | 0.77 (0.57–1.05) | 0.1 | 0.75 (0.55–1.03) | 0.08 |
| rs846290 | | | | | | |
| A | 616 | 526 | | | | |
| C | 392 | 380 | 0.88 (0.73–1.06) | 0.17 | 0.87 (0.73–1.05) | 0.15 |
| AA | 183 | 158 | | | | |
| AC | 250 | 210 | 1.03 (0.78–1.36) | 0.85 | 1.03 (0.78–1.37) | 0.84 |
| CC | 71 | 85 | 0.72 (0.49–1.06) | 0.09 | 0.71 (0.48–1.03) | 0.07 |
| Dom | | | 0.94 (0.72–1.22) | 0.64 | 0.94 (0.72–1.22) | 0.62 |
| Rec | | | **0.71 (0.50–1.00)** | **0.05** | **0.69 (0.49–0.98)** | **0.04** |
| rs10951667 | | | | | | |
| G | 727 | 649 | | | | |
| A | 279 | 259 | 0.96 (0.79–1.17) | 0.7 | 0.96 (0.78–1.18) | 0.72 |
| GG | 251 | 234 | | | | |
| GA | 225 | 181 | 1.16 (0.89–1.51) | 0.27 | 1.16 (0.89–1.52) | 0.26 |
| AA | 27 | 39 | 0.65 (0.38–1.09) | 0.1 | 0.65 (0.38–1.10) | 0.11 |
| Dom | | | 1.07 (0.83–1.38) | 0.61 | 1.07 (0.83–1.38) | 0.59 |
| Rec | | | **0.6 (0.36–1.00)** | **0.05** | **0.61 (0.36–1.01)** | **0.05** |
| rs1003876 | | | | | | |
| A | 770 | 664 | | | | |
| T | 236 | 240 | 0.85 (0.69–1.04) | 0.12 | 0.83 (0.68–1.03) | 0.08 |
| AA | 301 | 245 | | | | |
| AT | 168 | 174 | 0.79 (0.60–1.03) | 0.08 | **0.76 (0.58–1.00)** | **0.05** |
| TT | 34 | 33 | 0.84 (0.50–1.39) | 0.5 | 0.82 (0.49–1.37) | 0.45 |
| Dom | | | 0.79 (0.61–1.03) | 0.08 | **0.77 (0.59–1.00)** | **0.05** |
| Rec | | | 0.92 (0.56–1.51) | 0.74 | 0.91 (0.55–1.50) | 0.72 |
| rs846401 | | | | | | |
| A | 625 | 527 | | | | |
| G | 383 | 383 | 0.84 (0.70–1.01) | 0.07 | **0.83 (0.68–1.00)** | **0.05** |
| AA | 179 | 151 | | | | |
| AG | 267 | 225 | 1 (0.76–1.32) | 0.99 | 0.99 (0.75–1.32) | 0.96 |
| GG | 58 | 79 | **0.62 (0.41–0.93)** | **0.02** | **0.61 (0.41–0.92)** | **0.02** |
| Dom | | | 0.9 (0.69–1.18) | 0.45 | 0.89 (0.68–1.17) | 0.41 |
| Rec | | | **0.62 (0.43–0.89)** | **0.01** | **0.62 (0.43–0.89)** | **0.01** |
| rs3801212 | | | | | | |
| A | 801 | 714 | | | | |
| G | 207 | 196 | 0.94 (0.76–1.17) | 0.59 | 0.95 (0.76–1.19) | 0.66 |
| AA | 311 | 286 | | | | |
| AG | 179 | 142 | 1.16 (0.88–1.52) | 0.29 | 1.18 (0.89–1.55) | 0.24 |
| GG | 14 | 27 | **0.48 (0.25–0.93)** | **0.03** | **0.48 (0.25–0.94)** | **0.03** |
| Dom | | | 1.05 (0.81–1.36) | 0.71 | 1.07 (0.82–1.39) | 0.64 |
| Rec | | | **0.45 (0.23–0.87)** | **0.02** | **0.46 (0.24–0.88)** | **0.02** |
| rs3801228 | | | | | | |
| A | 890 | 779 | | | | |
| G | 118 | 131 | 0.79 (0.60–1.03) | 0.08 | 0.79 (0.60–1.03) | 0.08 |
| AA | 390 | 336 | | | | |

**Table 1** (*contd*)

| SNP | Patient | Control | Crude OR (95% CI) | Unadjusted $P$ value | Adjusted OR (95% CI) | Adjusted $P$ value |
|---|---|---|---|---|---|---|
| AG | 110 | 107 | 0.89 (0.65–1.20) | 0.43 | 0.89 (0.67–1.21) | 0.47 |
| GG | 4 | 12 | **0.29 (0.09–0.90)** | **0.03** | **0.28 (0.09–0.88)** | **0.03** |
| Dom | | | 0.83 (0.61–1.11) | NA | 0.83 (0.62–1.12) | 0.22 |
| Rec | | | 0.3 (0.09–0.92) | NA | **0.29 (0.09–0.90)** | **0.03** |

All SNPs were analysed under the additive, genotype; dom, dominant; rec, recessive models. The adjusted OR and adjusted $P$ value are the association results adjusted by sex. $P < 0.05$ are indicated in bold.
* Significant results after Bonferroni correction.

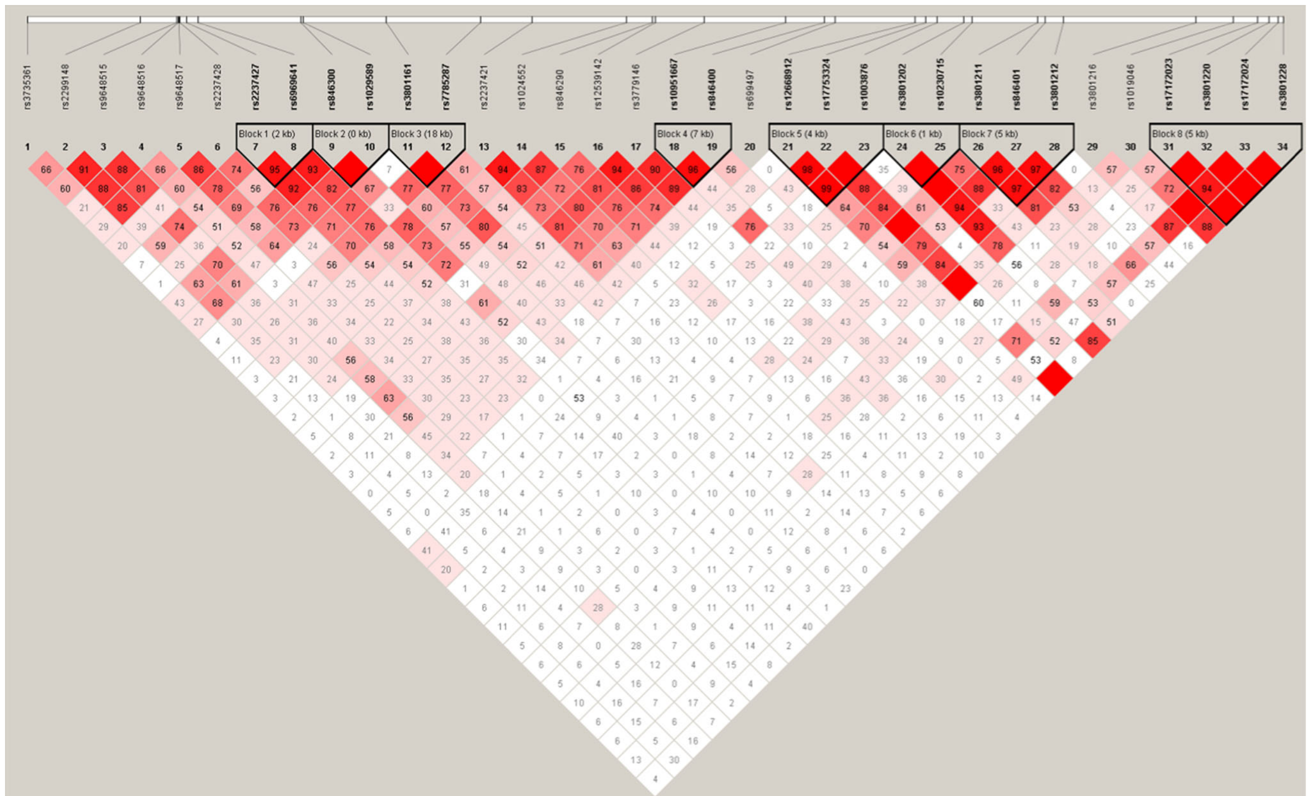


**Figure 1.** Calculated haplotype within the *GLI3* gene. Eight blocks were identified using Haploview software.

the rest of the SNPs tagged by rs3801161 (Halperin and Stephan 2009). The significant SNP rs3801161 can tag four SNPs in a 23 kb region and the significant haplotype block rs3801161–rs7785287 can tag seven SNPs in a 27 kb region. These SNPs are in high LD with rs3801161 or rs7785287 ($r^2 > 0.8$). This region is in the 8th intron of *GLI3* gene.

Meanwhile, functional investigation of *GLI3* genetic variants could be direct and convincing. It was reported that conserved noncoding sequences within *GLI3* can act as intron enhancers with *cis*-regulatory effects on *GLI3* expression in embryonic craniofacial structures and internal organs (Abbasi *et al.* 2013). Therefore, the role of rs3801161 and its tagged region during *GLI3* expression is worthy of future characterization.

In conclusion, we used *GLI3* as candidate NSCL/P gene and performed an association study in a large unrelated Chinese cohort. Our preliminary results indicated that rs3801161 and its haplotype rs3801161–rs7785287 are risk variants contributing to NSCL/P susceptibility. To our knowledge, this is the first study reporting a significant association between *GLI3* and NSCL/P. Our results provide further evidence supporting the involvement of the SHH pathway, and in particular the *GLI3* gene, underlying the genetic aetiology of NSCL/P. As is a preliminary research, the observed association should

**Table 2.** The haplotype analysis results of *GLI3*.

| Haplotype | $OR_{unadjusted}$ | $P_{unadjusted}$ | $OR_{adjusted}$ | $P_{adjusted}$ |
|---|---|---|---|---|
| Block1 rs2237427–rs6969641 | | | | |
| OMNIBUS | NA | 0.32 | | |
| CG | 0.92 | 0.35 | 0.91 | 0.29 |
| TG | 0.85 | 0.41 | 0.86 | 0.44 |
| TC | 1.14 | 0.15 | 1.15 | 0.13 |
| Block2 rs846300–rs1029589 | | | | |
| OMNIBUS | NA | 0.13 | | |
| GA | 1.12 | 0.24 | 1.13 | 0.21 |
| CG | 1.19 | 0.23 | 1.2 | 0.21 |
| GG | 0.84 | 0.05 | 0.83 | 0.04 |
| Block3 rs3801161–rs7785287 | | | | |
| OMNIBUS | NA | **0.007** | | |
| AT | 0.95 | 0.61 | 0.95 | 0.596 |
| **GC** | **1.53** | **0.02** | **1.58** | **0.0009*** |
| AC | 0.85 | 0.09 | 0.84 | 0.07 |
| Block4 rs10951667–rs846400 | | | | |
| OMNIBUS | NA | 0.94 | | |
| AT | 1 | 0.96 | 1.01 | 0.96 |
| GT | 1 | 1 | 1 | 0.98 |
| GG | 1.03 | 0.75 | 1.03 | 0.76 |
| Block5 rs12668912–rs17753324–rs1003876 | | | | |
| OMNIBUS | NA | 0.47 | | |
| TGT | 0.85 | 0.12 | 0.83 | 0.08 |
| TTA | 0.97 | 0.86 | 0.98 | 0.92 |
| TGA | 0.95 | 0.85 | 0.92 | 0.74 |
| CGA | 1.15 | 0.13 | 1.17 | 0.09 |
| Block6 rs3801202–rs10230715 | | | | |
| OMNIBUS | NA | 0.86 | | |
| AA | 0.97 | 0.82 | 0.99 | 0.95 |
| GA | 0.95 | 0.65 | 0.93 | 0.54 |
| GG | 1.06 | 0.58 | 1.06 | 0.57 |
| Block7 rs3801211–rs846401–rs3801212 | | | | |
| OMNIBUS | NA | 0.31 | | |
| CGG | 0.92 | 0.48 | 0.93 | 0.54 |
| TGA | 0.81 | 0.1 | 0.8 | 0.08 |
| CGA | 0.98 | 0.92 | 0.95 | 0.83 |
| CAA | 1.17 | 0.1 | 1.18 | 0.09 |
| Block8 rs17172023–rs3801220–rs17172024–rs3801228 | | | | |
| OMNIBUS | NA | 0.55 | | |
| CATG | 0.79 | 0.08 | 0.79 | 0.08 |
| CGCA | 1.02 | 0.89 | 1.04 | 0.81 |
| CATA | 1 | 1 | 0.98 | 0.89 |
| TGTA | 1.02 | 0.84 | 1.02 | 0.83 |
| CGTA | 1.09 | 0.37 | 1.09 | 0.37 |

The $OR_{adjusted}$ and $P_{adjusted}$ are the association results adjusted by sex. $P < 0.05$ is indicated in bold.
* Significant results after Bonferroni correction.

be further validate in expanded cohorts across different populations. The functional characterization of the risk variant of *GLI3* is also important to uncover the explicit role of *GLI3* underlying NSCL/P aetiology and pathology.

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