Genetic variants in let-7/Lin28 modulate the risk of oral cavity cancer in a Chinese Han Population

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Let-7 and Lin28 establish a double-negative feedback loop to affect several biological processes, such as differentiation of stem cell, invasion and metastasis, and tumorigenesis. In this study, we systematically investigated the associations between 6 potentially functional SNPs of let7 and Lin28 genes and the risk of oral cavity cancer with a case-control study including 384 oral cavity cancer cases and 731 controls. We found that the variant allele (T) of rs221636 of Lin28B was significantly associated with a reduced risk of oral cavity cancer with a case-control study including 384 oral cavity cancer cases and 731 controls. We investigated the associations between 6 potentially functional SNPs of let7 and Lin28 genes and the risk of oral cavity cancer with a case-control study including 384 oral cavity cancer cases and 731 controls. We found that the variant allele (T) of rs221636 of Lin28B was significantly associated with a reduced risk of oral cavity cancer [odds ratio (OR) = 0.73, 95% confidence interval (CI) = 0.58–0.92, P = 7.55 × 10−3 in additive model]. Bioinformatics prediction indicated that rs221636 was located at the binding site of hsa-miR-548p in the 3’ UTR of Lin28B. Luciferase activity assay also showed a lower expression level for rs221636 T allele compared with A allele. These findings indicated that rs221636 located at Lin28B may contribute to the risk of oral cavity cancer through the interruption of miRNA binding.

Oral cavity cancer is a serious worldwide public health problem, with high incidence and mortality rates. Approximately 263,900 new cases and 128,000 deaths from oral cavity cancer (including lip cancer) occurred in 2008 worldwide1. Smoking and alcohol consumption have been established as the most common environmental risk factors; however, the fact that only a small portion of exposed individuals develop oral cavity cancer suggests that genetic susceptibility plays an important role in modulating the risk of oral cavity cancer2. Therefore, the identification of susceptibility biomarkers for screening the high-risk individuals is important for the prevention of oral cavity cancer in general population.

MiRNAs are an abundant class of ~22 nucleotide noncoding RNAs that post-transcriptionally regulate the expression of protein-coding genes by targeting the 3’ untranslated region of specific messenger RNAs for degradation or translational repression3. Accumulative evidence has demonstrated the critical role of miRNAs in a variety of physiological processes, such as cell growth, cell differentiation, epithelial morphogenesis and cell survival. Furthermore, the deregulation of miRNAs has been involved in the pathogenesis of human diseases including multiple cancers4. MiRNA let-7 is the first miRNA identified in humans, originally discovered in the nematode Caenorhabditis elegans5. Let-7 has been widely proposed as a tumor suppressor by regulating several oncogenes, such as K-Ras, STAT3, c-Myc, and HMGA26–9. It has been revealed that decreased let-7 expression can increase the tumorigenicity of cancer cells10. The RNA-binding protein Lin28 is a stem cell pluripotency factor that contributes to the maintenance of stem cell characteristics and the promotion of cell malignant transformation. Recently, Lin28A and its homolog, Lin28B have been found to regulate let-7 family members through maturation process and cellular differentiation11,12. Specially, Lin28 can bind to the terminal loops of pre-let-7 elements and induce terminal uridylation of let-7 precursor miRNA, thus blocking the biogenesis of let-7 miRNAs13. Lin28A and Lin28B share similar structures; however, different functions were explored in mammalian cells14,15. For example, Lin28A suppresses let-7 biogenesis at the Dicer step in cytoplasm16, but Lin28B accumulates in the nucleus and binds pre-let-7 miRNAs to block their processing by the Microprocessor14. Because let-7 directly targets 3’ UTR of Lin28A and Lin28B, this let-7/Lin28 axis establishes a double-negative feedback loop. The double negative feedback loop comprising let-7 and Lin28 has been involved in several biological processes, including differentiation of stem cell, tumorigenesis, invasion, metastasis and drug resistance and relapse17–19. Thus, it can be speculated that slight changes in let-7/Lin28 axis, such as sequence variants, may affect the interaction of let-7 and Lin28 and result in more significant alterations by the loop.
Many studies have shown that single nucleotide polymorphisms (SNPs) related to miRNAs may either create or disturb miRNA target interactions, and induce diverse functional consequences. Thus, SNPs related to miRNAs may either create or disturb miRNA target interactions. We found among nondrinkers (adjusted OR = 0.68; 95% CI = 0.44–0.81; P = 2.30 × 10⁻² for heterogeneity test) and subjects with squamous cancer (adjusted OR = 0.68, 95% CI = 0.53–0.87; P = 4.30 × 10⁻² for heterogeneity test), whereas no significant differences were found between other subgroups. We then did an interaction analysis and detected a significant multiplicative interaction between rs221636 and drinking on oral cavity cancer risk (P = 1.97 × 10⁻³). As shown in Table 5, compared with drinkers with AA genotype, significantly decreased risks of oral cavity cancer were observed for non-drinkers with AT or TT genotypes (AT: adjusted OR = 0.24, 95% CI = 0.16–0.38; TT: adjusted OR = 0.20, 95% CI = 0.08–0.49).

To explore the functional implication of rs221636 in the development of oral cavity cancer, we used the in silico analysis tools (SNPinfo, http://snpinfo.niehs.nih.gov; PolymiRTS Database 3.0, http://compbio.uthsc.edu) to predict the potential function of this SNP and found that rs221636 was located at the target site of hsa-miR-548p, but not the let-7. Thus, we hypothesized that rs221636 might affect the expression of Lin28B by disturbing the binding of hsa-miR-548p and then the let-7/Lin28 double-negative feedback loop. To test this hypothesis, the luciferase reporter gene assay was performed. The results showed that two alleles had different effects on the expression levels of the luciferase gene when the rs221636 locus changed from the wide A allele to the variant T allele in three cell lines (Cal27, Tca8113 and 293T) (P = 2.13 × 10⁻⁴, 1.28 × 10⁻⁴, and 1.85 × 10⁻⁴, respectively) (Fig. 1). The results suggested that variant allele of rs221636 might affect the targeting of hsa-miR-548p to 3′ UTR of Lin28B in oral cancer cells.

### Discussion

To our knowledge, this is the first study to evaluate the effect of polymorphisms in let-7/Lin28 genes on oral cavity cancer risk in a Chinese Han population. We found that rs221636 of Lin28B, a SNP located at the binding site of some miRNA in Lin28, might affect oral cavity cancer risk through disturbing the interaction of miRNAs with Lin28.

Members of the let-7 family often promote the oncogenesis by depressing targets such as K-Ras, STAT3, c-Myc, and HMGA2 in numerous types of cancer. Specically, let-7a was down-expressed in the tissue of oral cavity cancer and might affect the metastasis and prognosis of oral cavity cancer. In contrast, Lin28 and its homolog, Lin28B, are often overexpressed in primary human tumors. Recent evidence has also reported that Lin28A/Lin28B block let-7 precursors from being processed to mature miRNAs, suggesting

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**Table 1 | Selected characteristics of oral cavity cancer cases and controls**

| Variables         | Cases          | Controls       | P     |
|-------------------|----------------|----------------|-------|
| N (%)             | N (%)          |                |       |
| **All subjects**  |                |                |       |
| Age, yr           |                |                |       |
| ≤60 (median)      | 198 (51.6)     | 366 (50.1)     | 0.163 |
| >60 (median)      | 186 (48.4)     | 365 (49.9)     |       |
| Sex               |                |                |       |
| Females           | 163 (42.4)     | 280 (38.3)     |       |
| Males             | 221 (57.6)     | 451 (61.7)     | 0.179 |
| **Smoking status**|                |                |       |
| No                | 213 (55.8)     | 437 (59.8)     | 0.196 |
| Yes               | 169 (44.2)     | 294 (40.2)     |       |
| **Drinking status**|                |                | <0.001|
| No                | 208 (54.5)     | 519 (71.0)     |       |
| Yes               | 174 (45.5)     | 212 (29.0)     |       |
| **Histology**     |                |                |       |
| Squamous          | 341 (88.8)     |                |       |
| Other             | 43 (11.2)      |                |       |

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**Table 2 | Primary information and genotyping results of selected SNPs**

| Gene | rs #      | Location | Base change | MAF in cases/controls | P for HWE test | Genotyping rate |
|------|-----------|----------|-------------|-----------------------|----------------|-----------------|
| Lin28A | rs4659441 | 1p36.11 | C > T       | 0.100/0.110           | 8.60 × 10⁻¹   | 96.9%           |
| Lin28B | rs3811463 | 1p36.11 | A > G       | 0.146/0.134           | 4.94 × 10⁻⁴   | 97.8%           |
|       | rs221636  | 6q21    | A > T       | 0.180/0.228           | 1.26 × 10⁻¹   | 98.4%           |
|       | rs221634  | 6q21    | T > A       | 0.430/0.413           | 9.50 × 10⁻¹   | 97.4%           |
| let-7p | rs10877887 | 12q14.1 | T > C       | 0.327/0.354           | 2.05 × 10⁻¹   | 98.1%           |
|       | rs13293512 | 9q22.32 | T > C       | 0.433/0.474           | 1.57 × 10⁻¹   | 98.6%           |

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*SNPs in the promoter region of let-7 family: rs10877887 (386 bp upstream of let-7) and rs13293512 (8496 bp upstream of let-7a/1/let-7f/1/let-7d cluster).
their overexpression might promote the malignancy through repression of let-7. Furthermore, studies show that let-7 represses the translation of Lin28 and the knockdown of Lin28 in cell culture restores levels of mature let-7 miRNAs. Thus, it is recognized that these two factors form a unique double-negative feedback, which may interact with other factors, such as RAS, MYC and NF-kB, to form a complex regulatory network and play a significant role in the tumorigenesis.

Up to date, the mechanisms by which let-7/Lin28 loop homeostasis is maintained remain largely unknown. However, some studies have investigated the associations between genetic changes of let-7/Lin28 and the risk of human cancer. For example, Chen et al. reported that a SNP rs3811463 located near the let-7 binding site in Lin28, could lead to differential regulation of Lin28 by let-7 and have a significant effect on the risk of breast cancer. This study provided the evidence that genetic variants could directly influence the interaction of Lin28 and let-7; however, other regulatory mechanisms may also contribute to the regulation of let-7/Lin28 loop and development of cancer. In our study, we investigated the associations between several functional SNPs of let-7/Lin28 and found that another SNP of Lin28B (rs221636) might affect the risk of oral cavity cancer through disturbing the interactions of other miRNAs with Lin28, such as hsa-miR-548p. Luciferase assay also indicated that the transcription activity of reporter gene with rs221636 A allele significantly increased than that with T allele. Such results can provide more clues supporting the speculation that some genetic changes in the let-7/Lin28 loop may induce the significantly biological alterations and even the development of cancer. But, the association between rs3811463 and the risk of oral cavity cancer was non-significant in this study, which was inconsistent with the results reported in breast cancer by Chen et al., possibly because of different mechanisms involved in the development of different types of cancer. Furthermore, in this study, we also found a significant multiplicative interaction between rs221636 and drinking on oral cavity cancer risk and the decreased risk of oral cavity cancer was observed for those non-drinkers with AT or TT genotypes compared with drinkers with AA genotype. While the sample is relative small, these findings suggested that the genetic variants of Lin28B and alcohol drinking may have synergistic effect in relation to the risk of oral cavity cancer.

### Table 3 | Associations between the selected SNPs and risk of oral cavity cancer

| SNP            | Cases (N = 384) | Controls (N = 731) | Adjusted OR (95%CI) | P       |
|----------------|----------------|-------------------|---------------------|---------|
| rs4659441      |                |                   |                     |         |
| CC             | 306            | 555               | 1.00                |         |
| CT             | 70             | 138               | 0.92 (0.66–1.28)    | 6.28 × 10⁻¹ |
| TT             | 3              | 8                 | 0.90 (0.23–3.49)    | 8.73 × 10⁻¹ |
| Additive model | -              | -                 | 0.91 (0.68–1.25)    | 6.05 × 10⁻¹ |
| Dominant model | -              | -                 | 0.90 (0.23–3.49)    | 8.73 × 10⁻¹ |
| Recessive model| -              | -                 | 0.90 (0.23–3.49)    | 8.73 × 10⁻¹ |
| rs3811463      |                |                   |                     |         |
| AA             | 268            | 524               | 1.00                |         |
| AG             | 110            | 187               | 1.16 (0.87–1.54)    | 3.14 × 10⁻¹ |
| GG             | 0              | 2                 | 1.14 (0.86–1.51)    | 3.66 × 10⁻¹ |
| Additive model | -              | -                 | 1.15 (0.87–1.53)    | 3.34 × 10⁻¹ |
| Dominant model | -              | -                 | -                   | -       |
| Recessive model| -              | -                 | -                   | -       |
| rs221636       |                |                   |                     |         |
| AA             | 256            | 418               | 1.00                |         |
| AT             | 116            | 266               | 0.72 (0.54–0.95)    | 1.86 × 10⁻² |
| TT             | 11             | 30                | 0.58 (0.29–1.20)    | 1.14 × 10⁻¹ |
| Additive model | -              | -                 | 0.73 (0.58–0.92)    | 7.55 × 10⁻³ |
| Dominant model | -              | -                 | 0.70 (0.53–0.91)    | 8.26 × 10⁻³ |
| Recessive model| -              | -                 | 0.64 (0.31–1.31)    | 2.18 × 10⁻¹ |
| rs221634       |                |                   |                     |         |
| TT             | 121            | 244               | 1.00                |         |
| TA             | 190            | 342               | 1.12 (0.84–1.50)    | 4.30 × 10⁻¹ |
| AA             | 68             | 121               | 1.24 (0.85–1.82)    | 2.65 × 10⁻¹ |
| Additive model | -              | -                 | 1.11 (0.92–1.34)    | 2.66 × 10⁻¹ |
| Dominant model | -              | -                 | 1.15 (0.87–1.51)    | 3.23 × 10⁻¹ |
| Recessive model| -              | -                 | 1.15 (0.82–1.61)    | 4.20 × 10⁻¹ |
| rs10877887     |                |                   |                     |         |
| TT             | 172            | 291               | 1.00                |         |
| TC             | 165            | 343               | 0.80 (0.61–1.05)    | 1.09 × 10⁻¹ |
| CC             | 41             | 82                | 0.78 (0.51–1.20)    | 2.56 × 10⁻¹ |
| Additive model | -              | -                 | 0.86 (0.70–1.04)    | 1.18 × 10⁻¹ |
| Dominant model | -              | -                 | 0.80 (0.62–1.03)    | 8.65 × 10⁻² |
| Recessive model| -              | -                 | 0.88 (0.59–1.33)    | 5.49 × 10⁻¹ |
| rs13293512     |                |                   |                     |         |
| TT             | 114            | 191               | 1.00                |         |
| TC             | 197            | 380               | 0.87 (0.64–1.17)    | 3.49 × 10⁻¹ |
| CC             | 64             | 153               | 0.68 (0.46–1.00)    | 4.78 × 10⁻² |
| Additive model | -              | -                 | 0.84 (0.69–1.01)    | 6.08 × 10⁻² |
| Dominant model | -              | -                 | 0.82 (0.62–1.09)    | 1.63 × 10⁻¹ |
| Recessive model| -              | -                 | 0.76 (0.54–1.05)    | 9.82 × 10⁻² |

*Adjusted by age, sex, smoking status and alcohol status. Significant values (p < 0.05) are in bold.
Some limitations are inherent in our study design. Firstly, it is a hospital-based, case-control study, and inherent selection bias cannot be completely excluded. However, we applied a rigorous epidemiological design in selecting study subjects and used further statistical adjustment for known risk factors to minimize potential biases. Second, the sample size in this study (384 cases and 731 controls) is relatively small, which may have limited statistical power to detect the weak genetic effect of some SNPs. Thirdly, although we have demonstrated that rs221636 was associated with the risk of oral cavity cancer through disturbing the binding of Lin28 with other miRNAs, we are still unclear about the precise function of this SNP. Furthermore, though we have detected a significant multiplicative interaction between rs221636 variant and alcohol consumption on oral cavity cancer risk, this result is in statistical scale and future studies are required to validate this finding.

In summary, this case-control study from a Chinese population reported that the functional SNP rs221636 of the Lin28B may modify the risk of oral cavity cancer. More rigorous studies with larger sample sizes and SNP functional relevance are warranted to replicate our findings and identify the underlying mechanism of the SNPs in the etiology of oral cavity cancer.

Methods

Ethics statement. This case-control study was approved by the institutional review board of Nanjing Medical University. Informed written consent was obtained from all the subjects. The experimental protocol was carried out in accordance with the approved guidelines.

Study subjects. All newly and histologically confirmed oral cavity cancer patients were consecutively recruited from Jiangsu Stomatological Hospital and the First Affiliated Hospital of Nanjing Medical University, Nanjing, China, since January 2009 to April 2012. There were no age, sex, histology or stage restrictions, but patients with second oral cavity cancer primary tumors, primary tumors of the nasopharynx or sinonasal tract, metastasized cancer from other organs, or any histopathologic diagnosis other than oral cavity cancer were excluded. Cancer-free controls that were frequency matched to the cases on age (±5 years) and sex were randomly selected from a cohort of more than 30,000 participants in a community-based screening program for non-infectious diseases in the Jiangsu Province, China. All participants were genetically unrelated, ethnic Chinese Han population. When written informed consent was obtained, a structured questionnaire was used by trained interviewers to collect information on demographic data and environmental exposure history, such as age, sex, smoking, and drinking consumption. Individuals who smoked one cigarette per day for over 1 year were considered as smokers and those who had three or more alcohol drinks a week for over 6 months were defined as alcohol drinkers. After the interview, approximately 5 ml of venous blood sample was collected from each study participant. Finally, 384 incident oral cavity cancer cases and 731 frequency-matched controls were included in this study.

SNPs selection. The dbSNP database and International HapMap Project database were first used to search all common SNPs [MAF (minor allele frequency) >0.05 in China populations] located in 3′UTR region of Lin28 genes (Lin28A and Lin28B), which is the primary binding site of miRNAs. Furthermore, the members of let-7 miRNAs (let-7a, 7b, 7c, 7d, 7e, 7f, 7g, 7i, 7k, 7l) were determined through miBase and Gene database of NCBI. In this study, we mainly selected common SNPs located in the sequence encoding the precursors of let-7 plus 10-kb upstream region. However, no common SNP was found in the coding sequence of let-7g gene, indicating it is very conservative. Then, we used the web-based analysis tools (SNPinfo, http://snpinfo.niehs.nih.gov; PolymiRTS Database 3.0, http://compbio.uthsc.edu; TFSEARCH 1.3, http://www.cbrc.jp/research/db/TFSEARCH.html) to predict the functional implications of these SNPs. Additionally, the linkage disequilibrium analysis was conducted to optimize the selection of SNPs (r² > 0.8). As a result, six SNPs (Table 2) of let-7 and Lin28 were selected for genotyping.

Genotyping. Genomic DNA was extracted from leukocyte pellet by protease K digestion and followed by phenol-chloroform extraction and ethanol precipitation. SNPs were genotyped by using the TaqMan allelic discrimination assay on the platform of 7900HT Real-time PCR System (Applied Biosystems, Foster City, CA). Genotyping was performed without knowing the subjects’ case or control status, and two negative controls (no DNA) included in each 384-well plate was used for quality assurance.

Table 4 | Stratified analysis for rs221636 and oral cavity cancer risks in additive model

| Variables     | Case |           | Control |           | Adjusted OR [95% CI]a | P*  | Phetogeneity |
|---------------|------|-----------|---------|-----------|-----------------------|-----|--------------|
| Age, yr       |      |           |         |           |                       |     |              |
| ≤60           | 130  | 61        | 7       | 200       | 142                   | 17  | 0.72 (0.53–0.99) | 4.40 × 10⁻² | 9.07 × 10⁻¹ |
| >60           | 126  | 55        | 4       | 218       | 124                   | 13  | 0.74 (0.53–1.04) | 8.65 × 10⁻² |              |
| Sex           |      |           |         |           |                       |     |              |
| Females       | 116  | 41        | 6       | 177       | 77                    | 23  | 0.72 (0.51–1.00) | 5.16 × 10⁻² | 7.80 × 10⁻¹ |
| Males         | 140  | 75        | 5       | 241       | 189                   | 7   | 0.77 (0.55–1.06) | 1.05 × 10⁻¹ |              |
| Smoking status|      |           |         |           |                       |     |              |
| Never         | 148  | 58        | 6       | 267       | 135                   | 26  | 0.72 (0.53–0.96) | 2.76 × 10⁻² | 7.09 × 10⁻¹ |
| Ever          | 107  | 57        | 5       | 151       | 131                   | 4   | 0.79 (0.54–1.17) | 2.40 × 10⁻¹ |              |
| Drinking status|     |           |         |           |                       |     |              |
| Never         | 152  | 48        | 7       | 293       | 189                   | 26  | 0.60 (0.44–0.81) | 9.28 × 10⁻⁴ | 2.30 × 10⁻² |
| Ever          | 103  | 67        | 4       | 125       | 77                    | 4   | 1.06 (0.72–1.55) | 7.77 × 10⁻¹ |              |
| Histology     |      |           |         |           |                       |     |              |
| Squamous cell carcinoma | 233 | 98        | 9       | 238       | 96                    | 9   | 0.68 (0.53–0.87) | 2.17 × 10⁻³ | 4.30 × 10⁻³ |
| Othersb       | 23   | 18        | 2       |           |                       |     |              |

aAdjusted by age, sex, smoking status and alcohol status. Significant values (p < 0.05) are in bold.
bIncluding adenocarcinoma, undifferentiated carcinoma and undetermined cancer.

Table 5 | Interaction analysis between rs221636 genotypes and alcohol drinking on oral cavity cancer

| rs221636 | Drinking status | Cases | Controls | Adjust OR [95% CI] | P*    |
|----------|----------------|-------|----------|-------------------|-------|
| AA       | Ever           | 103   | 125      | 1.00              |       |
| AT       | Ever           | 67    | 47       | 1.03 (0.68–1.57)  | 8.94 × 10⁻¹ |
| TT       | Ever           | 4     | 4        | 1.21 (0.30–5.05)  | 7.90 × 10⁻¹ |
| AA       | Never          | 152   | 293      | 0.45 (0.31–0.66)  | 4.97 × 10⁻⁵ |
| AT       | Never          | 48    | 189      | 0.24 (0.16–0.38)  | 3.39 × 10⁻¹ |
| TT       | Never          | 7     | 26       | 0.20 (0.08–0.49)  | 5.01 × 10⁻⁴ |

*Derived from logistic regression with an adjustment for age, sex and smoking status.
control. The genotyping results were determined by using SDS 2.3 Allelic Discrimination Software (Applied Biosystems). Moreover, 10% of samples (40 cases and 70 controls) were randomly selected to repeat and the accordance rate reached 100%.

**Lin28 3′-UTR promoter luciferase reporter plasmid.** The Lin28 3′-UTR containing the putative recognition site rs221636 was amplified from the sample DNA, then cloned into the pMIR-REPORTTM vector with Mlu I and Hind III digestions. The primers were GACGCGTCACTTTGCAGGGATTA (sense) and CCAAGCTTGAGATTTCCCATGTCCTGT (antisense), which were then ligated by T4 DNA ligase (New England BioLabs) to generate the recombinant constructs. Plasmids containing the different alleles of rs221636 were generated using site-specific mutagenesis. The restriction map and sequencing were used to confirm the authenticity of all constructs in this study.

**Transient transfections and luciferase assays.** The Cal27, Tca8113 and 293T cells were maintained in DMEM medium supplemented with 10% heat-inactivated fetal, 10% heat-inactivated fetal bovine serum (Gibco) and 50 μg/ml streptomycin (Gibco) and incubated at 37°C in an incubator with 5% CO₂. Cells were seeded at 1 × 10⁴ cells per well in 24-well plates (BD Biosciences, Bedford, MA). Transfections were performed with cells using Lipofectamine2000 according to manufacturer’s introduction (Invitrogen) after 24 h. The luciferase plasmids (empty vector for control and vectors with different rs221636 alleles) were co-transfected, respectively, into different cells with synthesized mature hsa-miR-548p mimic. The pRL-SV40 plasmid (Promega) was also co-transfected as an internal control. Six replicates for each group and the experiment repeated at least three times. After 24 hours of incubation, cells were collected and analyzed for luciferase activity with the Dual-Luciferase Reporter Assay System (Promega).

**Statistical analysis.** Differences in the distributions of demographic characteristics, selected variables, and frequencies of the genotypes between the cases and controls were analyzed by using the χ² test (categorical variables) and student T test (continuous variables). The associations of variant genotypes with oral cavity cancer risk were estimated by computing odds ratios (ORs) and 95% confidence intervals (CIs) from both univariate and multivariate logistic regression analyses in different genetic models. The adjustment factors for the associations included age, sex and smoking and drinking status. The Hardy-Weinberg equilibrium was tested by a χ² test (categorical variables) and student T test (continuous variables). Two-sided tests were generally used for statistical analysis and p < 0.05 was considered as the level of statistical significance.

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**Author contributions**

H.Y., H.M. and N.C. conceived and designed the experiments. Y.Z., L.Z. and R.W. performed the experiment. H.Y., R.W. and H.J. analyzed the data. R.W., L.Z. and L.M. prepared the samples. Y.Z., R.W. and L.Z. wrote the manuscript.

**Additional information**

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