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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 [odds ratio (OR) = 0.73, 95% confidence interval (CI) = 0.58–0.92, $P = 7.55 \times 10^{-3}$ 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 rs221236 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 worldwide[1]. 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 cancer[2]. 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 repression[3]. 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 cancers[3]. MiRNA *let-7* is the first miRNA identified in humans, originally discovered in the nematode Caenorhabditis elegans[4]. *Let-7 has been widely proposed as a tumor suppressor by regulating several oncogenes, such as K-Ras*, *STAT3*, *c-Myc*, and *HMGA2*[5–9]. It has been revealed that decreased *let-7* expression can increase the tumorigenicity of cancer cells[10]. 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 differentiation[11,12]. 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* miRNAs[13]. *Lin28A* and *Lin28B* share similar structures; however, different functions were explored in mammalian cells[14,15]. For example, *Lin28A* suppresses *let-7* biogenesis at the Dicer step in cytoplasm[16], but *Lin28B* accumulates in the nucleus and binds *pre-let-7* miRNAs to block their processing by the Microprocessor[14]. 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 *Lin28* and *let-7* has been involved in several biological processes, including differentiation of stem cell, tumorigenesis, invasion, metastasis and drug resistance and relapse[13,17,18]. 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.

Table 1 | Selected characteristics in oral cavity cancer cases and controls

Variables	Cases N (%)	Controls N (%)	P[a]
All subjects	384(100)	731(100)	
Age, yr			0.163
≤60(median)	198(51.6)	366(50.1)	
>60(median)	186(48.4)	365(49.9)	
Sex			0.179
Females	163(42.4)	280(38.3)	
Males	221(57.6)	451(61.7)	
Smoking status[b]			0.196
No	213(55.8)	437(59.8)	
Yes	169(44.2)	294(40.2)	
Drinking status[b]			<0.001
No	208(54.5)	519(71.0)	
Yes	174(45.5)	212(29.0)	
Histology			
Squamous	341(88.8)		
Other[c]	43(11.2)		

[a]Two-sided χ^2 test.
[b]2 subjects were absent of smoking and drinking information.
[c]Adenocarcinoma, undifferentiated carcinoma and undetermined cancer were included.

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[19–23]. Thus, in this study, we performed the genotyping of several potentially functional SNPs in *let-7* and *Lin28* and assessed their associations with risk of oral cavity cancer in an ongoing hospital-based case-control study of 384 cases and 731 cancer-free controls in a Chinese Han population.

Results

The selected characteristics of the cases and controls are shown in Table 1. There was no significant difference in the distributions of age, sex and smoking between the cases and controls ($P = 0.163$, 0.179 and 0.196, respectively). As expected, more drinkers were observed in the case group compared with that in the control group ($P < 0.001$). Among all oral cavity cancer cases, 341 (88.8%) were presented with squamous cell carcinoma.

Primary information and genotyping results of the 6 selected SNPs were showed in Table 2. The observed genotype frequencies of these variants were in agreement with the Hardy–Weinberg equilibrium among the controls, except rs3811463 ($P = 4.94 \times 10^{-4}$). The genotype frequencies of these 6 SNPs in the cases and controls are summarized in Table 3. After the adjustment for age, sex, smoking and alcohol status, multivariate logistic regression analysis revealed that rs221636 in the 3′UTR of *Lin28B* was significantly associated with a decreased risk of oral cavity cancer (additive model: adjusted OR = 0.73, 95% CI = 0.58–0.92, $P = 7.55 \times 10^{-3}$; dominant model: adjusted OR = 0.70, 95% CI = 0.53–0.91, $P = 8.26 \times 10^{-3}$). Even after the Bonferroni adjustment, the association remained significant (adjusted $P = 0.045$). Additionally, rs13293512 had a borderline association with a decreased risk of oral cavity cancer (CC *vs.* TT: adjusted OR = 0.68, 95% CI = 0.46–1.00, $P = 4.78 \times 10^{-2}$); however, the significance disappeared after the Bonferroni correction (adjusted $P > 0.05$). No significant association was observed between variant genotypes of the other SNPs and oral cavity cancer risk.

We further conducted the stratification analysis on the associations between rs221636 and oral cavity cancer by age, sex, smoking and drinking. As shown in Table 4, the significant association of rs221636 with oral cavity cancer risk was found among nondrinkers (adjusted OR = 0.60; 95% CI = 0.44–0.81; $P = 2.30 \times 10^{-2}$ for heterogeneity test) and subjects with squamous cancer (adjusted OR = 0.68, 95% CI = 0.53–0.87; $P = 4.30 \times 10^{-2}$ 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 ($P = 1.97 \times 10^{-2}$). 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)[24,25] 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 and 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) ($P = 2.13 \times 10^{-6}$, 1.28×10^{-4}, and 1.85×10^{-6}, 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[5–9]. Specially, *let-7a* was down-expressed in the tissue of oral cavity cancer and might affect the metastasis and prognosis of oral cavity cancer[26,27]. In contrast, *Lin28* and its homolog, *Lin28B*, are often overexpressed in primary human tumors[14,15]. Recent evidence has also reported that *Lin28A/Lin28B* block *let-7* precursors from being processed to mature miRNAs, suggesting

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%
	rs3811463	1p36.11	A > G	0.146/0.134	4.94×10^{-4}	97.8%
Lin28B	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-7[a]	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%

[a]SNPs in the promoter region of *let-7* family: rs10877887 (286 bp upstream of *let-7i*) 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*[28]. Furthermore, studies show that *let-7* represses the translation of *Lin28*[29,30] and the knockdown of *Lin28* in cell culture restores levels of mature *let-7* miRNAs[14,31]. 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[4–6,13,32,33].

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* loop 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[31]. 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

	Cases (N = 384)		Controls (N = 731)			
SNP	N%		N%		Adjusted OR (95%CI)[a]	*P*[a]
rs4659441						
CC	306	79.69	555	75.92	1.00	
CT	70	18.23	138	18.88	0.92(0.66–1.28)	6.28×10^{-1}
TT	3	0.78	8	1.09	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	69.79	524	71.78	1.00	
AG	110	28.65	187	25.58	1.16(0.87–1.54)	3.14×10^{-1}
GG	0	0.00	2	0.27	-	-
Additive model	-	-	-	-	1.14(0.86–1.51)	3.66×10^{-1}
Dominant model	-	-	-	-	1.15(0.87–1.53)	3.34×10^{-1}
Recessive model	-	-	-	-	-	-
rs221636						
AA	256	66.66	418	57.18	1.00	
AT	116	30.21	266	36.39	**0.72(0.54–0.95)**	**1.86×10^{-2}**
TT	11	2.86	30	4.10	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	31.51	244	33.38	1.00	
TA	190	49.48	342	46.79	1.12(0.84–1.50)	4.30×10^{-1}
AA	68	17.71	121	16.55	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	44.79	291	39.81	1.00	
TC	165	42.94	343	46.92	0.80(0.61–1.05)	1.09×10^{-1}
CC	41	10.68	82	11.22	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	29.69	191	26.13	1.00	
TC	197	51.30	380	51.98	0.87(0.64–1.17)	3.49×10^{-1}
CC	64	16.67	153	20.93	**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}

[a]Adjusted by age, sex, smoking status and alcohol status. Significant values ($p < 0.05$) are in bold.

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

Variables	Case			Control			Adjusted OR (95%CI)[a]	P^a	$P_{heterogeneity}$
	AA	AT	TT	AA	AT	TT			
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}	$\mathbf{2.30 \times 10^{-2}}$
Ever	103	67	4	125	77	4	1.06(0.72–1.55)	7.77×10^{-1}	
Histology									
Squamous cell carcinoma	233	98	9				0.68(0.53–0.87)	2.17×10^{-3}	$\mathbf{4.30 \times 10^{-2}}$
Others[b]	23	18	2				1.23(0.73–2.06)	4.45×10^{-1}	

[a]Adjusted by age, sex, smoking status and alcohol status. Significant values ($p < 0.05$) are in bold.
[b]Including adenocarcinoma, undifferentiated carcinoma and undetermined cancer.

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 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*) were determined through miRBase 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-7* 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[24,25,34]. Additionally, the linkage disequilibrium analysis was conducted to optimize the selection of SNPs ($r^2 > 0.8$). As a result, six SNPs (Table 2) of *let-7* and *Lin28* were selected for genotyping.

Genotyping. Genomic DNA was extracted from a leukocyte pellet by proteinase 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

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

rs221636	Drinking status	Cases	Controls	Adjust OR (95%CI)	P[a]
AA	Ever	103	125	1.00	
AT	Ever	67	77	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^{-10}
TT	Never	7	26	0.20(0.08–0.49)	5.01×10^{-4}
Multiplicative interaction					1.97×10^{-2}

[a]Derived from logistic regression with an adjustment for age, sex and smoking status.

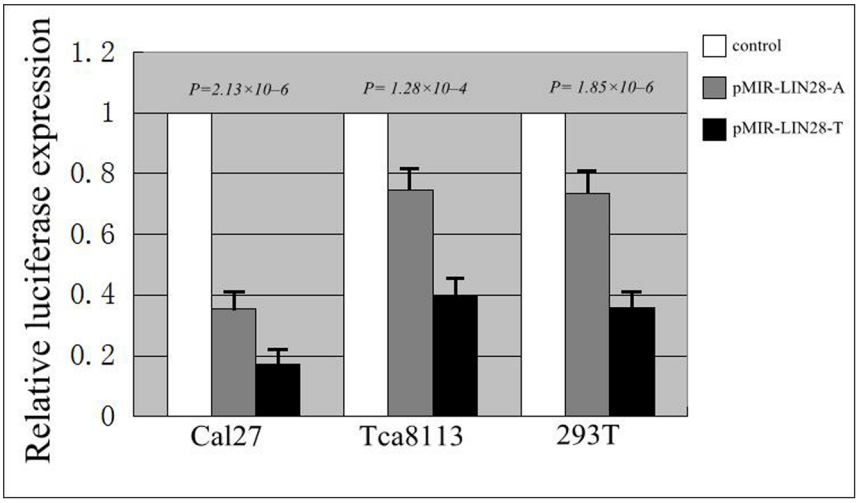

Figure 1 | In vitro target binding assays for rs221636 A/T in Cal27, Tca8113 and 293T cell lines. Each transfection was performed with pRL-SV40 plasmids as normalized controls. The mean fold change ± SD for plasmid with different alleles are shown after normalized by control plasmid in parallel transfection. *Lin28* 3′-UTR luciferase reporter plasmids (A allele or T allele) were co-transfected with chemically synthesized mature miR-548p in three cell lines. The *P* values are 2.13×10^{-6}, 1.28×10^{-4} and 1.85×10^{-6} for Cal27, Tca8113 and 293T cell lines, respectively.

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 (Applied Biosystems) 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 ug/ml streptomycin (Gibco) and incubated at 37°C in an incubator with 5% CO_2. Cells were seeded at 1×10^5 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 χ^2 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 goodness-offit χ^2 test to compare the observed genotype frequencies with the expected ones among the control subjects. The unpaired Student's t-test was used to evaluate the associations of differences in dual-luciferase reporter gene expressions.

All the statistical analyses were performed with the Statistical Analysis System software (v.9.1.3; SAS Institute, Cary, NC). 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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