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Original Article

The relation between NEAT1 expression level and survival rate in patients with oral squamous cell carcinoma

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Radiotherapy;
Survival rate

Abstract *Background/purpose:* Numerous studies have shown that long noncoding RNAs (lncRNAs) are involved in cancer progression and chemotherapy resistance. Nuclear enriched abundant transcript 1 (NEAT1) is an lncRNA. It affects tumor cell progression and drug resistance in various tumors. However, the relation of NEAT1 and survival rate in oral squamous cell carcinoma (OSCC) requires further study.

Materials and methods: One normal gingival epithelium cell line, SG, three oral cancer cell lines (HSC3, OEC-M1, and SAS), 34 paired non-cancerous matched tissues (NCMT), and OSCC tissues were used in this study. Tri-reagent was used for total RNA extraction. NEAT1 expression was assessed by reverse transcription-quantitative PCR (RT-qPCR).

Results: NEAT1 expression in oral cancer cell lines was lower than that in normal cells and was significantly downregulated in OSCC. NEAT1 upregulation reduced the survival rate of patients

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with OSCC. NEAT1 upregulation also reduced the survival rate of OSCC patients treated with chemotherapy and radiotherapy.

Conclusion: These results indicate that NEAT1 expression is a valuable biomarker for the prediction and prognosis of oral cancer.

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Introduction

Oral squamous cell carcinoma (OSCC) accounts for 90–95% of oral malignancies and arises from the oral mucosal and mucosal lips. In Taiwan, OSCC is the fourth most common malignancy in men.^{1,2} In South Asia, betel quid chewing is a significant risk factor for OSCC,³ and a study showed that the incidence of betel quid chewing leading to OSCC was higher than that of smoking and alcohol consumption.⁴

Long non-coding RNAs (lncRNAs), a type of transcript with over 200 nucleotides (nt) and without protein-coding potential. They have been shown to be vital regulators in various gene expression and biological processes, including cell cycle progression and tumorigenesis. lncRNAs influence cell growth, differentiation, and apoptosis into normal or malignant tissues.^{5,6} Numerous studies have shown that lncRNAs are involved in cancer progression⁷ and drug resistance.⁸ Furthermore, lncRNA could act as competing endogenous RNAs (ceRNAs) to affect the expression of miRNAs, leading to the alteration of target mRNA expression. Hundreds of lncRNAs have been found to be dysregulated among various malignancies and have emerged as oncogenes or tumor-suppressors.⁹ Notably, dysregulation of many lncRNAs is associated with OSCC and affects various aspects of cellular homeostasis, including proliferation, survival, migration, and genomic stability.^{10,11}

lncRNAs could be considered biomarkers for OSCC diagnosis and prognosis of therapy, as well as potential therapeutic targets.¹⁰ With the continuous exploration and clarification of the structure and function of lncRNAs, the dysregulation of lncRNAs has become a non-negligible regulatory element in the development of OSCC. Several lncRNAs have been reported to regulate OSCC progression, including MALAT1, CCAT1, MEG3, UCA1, HOTAIR, and NEAT1.¹¹ lncRNA nuclear paraspeckle assembly transcript 1 (NEAT1) has been reported as a vital nuclear component that leads to the breakdown of paraspeckles when it is knocked down.¹² The anomalous expression of NEAT1 has been reported in several human malignancies and exerts oncogenic activity in breast cancer,¹³ hepatocellular carcinomas,¹⁴ thyroid carcinoma,¹⁵ prostate cancer,¹⁶ and pancreatic cancer.¹⁷

Overexpression of NEAT1 could be seen in most solid malignancies; however, the fundamental functions of NEAT1 in OSCC are not clear,¹⁸ and additional datasets are needed for further investigation. In the present study, we investigated NEAT1 expression in the cancer tissue and adjacent normal tissue, as well as the clinicopathological features among participants.

Materials and methods

Microarray lncRNA analysis

Tissues were obtained from primary OSCC patients from untreated patients at the National Yang-Ming University Hospital. For each cancer tissue, a paired non-cancerous matched tissue (NCMT) sample was collected from the adjacent region at the same time. The size of each sample was approximately 0.1 cm³. All samples were placed in RNALater within 15 min after excision and stored in liquid nitrogen until RNA extraction. In this study, three paired tissues were collected for microarray analysis. The genes and lncRNAs were screened using an Agilent SurePrint G3 Human V3 GE 8 × 60 K Array. Total RNA was extracted using TRI Reagent® (Molecular Research Center, Inc., Cincinnati, OH, USA). The 0.2 µg of total RNA was labeled and amplified using a Low Input Quick Amp Labeling Kit, One-Color (Agilent Technologies, Santa Clara, CA, USA). The labeled cRNAs were purified using an RNeasy Mini Kit (QIAGEN, Hilden, Germany). Based on the manufacturer's instructions, each slide was hybridized with 600 ng Cy3-labeled cRNA using a Gene Expression Hybridization Kit (Agilent Technologies) and washed with the Gene Expression Wash Buffer Kit (Agilent Technologies). An Agilent Microarray Scanner (Agilent Technologies) and Feature Extraction software 10.7 (Agilent Technologies) were applied to scan each slide with the dye channel set to green and a scan resolution of 3 µm, 20 bit. The raw data were normalized using the Quantile algorithm, Gene Spring Software 11.0 (Agilent Technologies).

Oral cancer cell line culture

Gingival epithelial Smulow–Glickman (SG) cells and the OSCC cell lines HSC3, OEC-M1, and SAS were cultured in a medium containing 10% fetal bovine serum (FBS) and 1% Antibiotic-Antimycotic, as described previously.¹⁹ The cells were harvested to determine the NEAT1 expression levels.

Subjects

The 34 paired NCMT and OSCC tissues were provided by the Changhua Christian Hospital tissue bank. The study was approved by the Institutional Review Board (200501). Tissue samples were immediately frozen in liquid nitrogen until further use. A selection process was performed on frozen sections to obtain OSCC samples with more than 70% tumor

cells, which were required for the analysis. All but two patients with OSCC were male. The clinicopathological parameters, including age, lesion site, differentiation, TNM, pathological stage, treatment, and survival status, are shown in Table 1.

RT-qPCR

RNA extraction and RT-PCR were performed as described previously.²⁰ Total RNA was prepared from OSCC and NCMTs using a Tri-reagent RNA isolation kit (Molecular Research Center, Cincinnati, OH, USA). RNA was treated with DNase I

(Stratagene, La Jolla, CA, USA) to remove contaminating DNA. Two micrograms of total RNA were reverse transcribed into complementary DNA (cDNA) using a random primer and reverse transcriptase (Stratagene). Total RNA (0.5 µg of total RNA was used to analyze NEAT1 expression by quantitative polymerase chain reaction (qPCR). The primer sequences used in this study are as follows: *NEAT-1* forward CTTCCTCCCTTAACTTATCCATTAC, reverse CTCTTCCTCCACCATTACCAACAATAC; and *glyceraldehyde-3-phosphate dehydrogenase (GAPDH)* forward TGGTATCGTGGAAG-GACTCATGAC, reverse ATGCCAGTGAGCTTCCCGTTCAGC. PowerUp™ SYBR™ Green Master Mix (Applied Biosystems™, Waltham, MA, USA) was used for PCR. Amplification of these genes was performed with 40 cycles at 95 °C for 15 s, and 60–61.7 °C for 1 min. Amplification of GAPDH served as an internal control. Three independent PCRs were performed to validate the reproducibility of the analysis. Cases exhibiting inconsistent results were excluded from the final analysis. NEAT1 expression in NCMT and OSCC is shown by $-\Delta Ct (Ct_{GAPDH} - Ct_{NEAT1})$. The fold change (log2) expression of NEAT1 was used for receiver operating characteristic (ROC) analysis to determine the cut-off score, and NEAT1 low expression and high expression groups were separated.

Statistics

Statistical data were obtained from three independent experiments. Statistically significant differences were determined using one-way ANOVA, Fisher's exact test, paired t-test, binary logistic regression analysis, and log-rank test with Prism 5.0 (GraphPad Software, Inc., La Jolla, CA, USA). ROC analysis was performed to determine the diagnostic power. Differences between the variants were considered significant at $p < 0.05$.

Results

Expression of NEAT1 in OSCC cell lines and paired OSCC tissues

In total, 26,083 Entrez Genes (unique) and 30,606 lncRNAs (unique) were profiled in this study using an Agilent SurePrint G3 Human V3 GE 8 × 60 K Array. A total of 2361 targets were considered differentially expressed genes and lncRNAs (fold change ≥ 2.0) by limma. The fold changes in NEAT1 expression in the three paired OSCC tissues were 0.228, 0.217, and 0.024. The microarray data revealed that NEAT1 expression was downregulated in OSCC tissues (Fig. 1A). RT-qPCR analysis was performed on OSCC cell lines and 34 OSCC/NCMT pairs. HSC3, OECM-1, and SAS cells showed NEAT1 expression at a much lower level than the SG cells (Fig. 1B). Therefore, NEAT1 seems to have consistently lower expression in OSCC cells than in normal epithelial cells. Fig. 1C shows representative NEAT1 expression in OSCC samples and corresponding NCMT samples. The NEAT1 expression ($Ct_{GAPDH} - Ct_{NEAT1} = -\Delta Ct$) in 34 OSCC and NCMT pairs was -0.9738 ± 2.215 for OSCC and -0.2117 ± 2.472 for NCMT, which indicated a significant difference between these two groups. Overall, low NEAT1 expression in OSCC cell lines and OSCC tissues was demonstrated by lncRNA microarray and RT-qPCR.

Table 1 Clinical characteristics of OSCC subjects.

OSCC ($n = 34$, mean of age = 55.68 ± 10.21)

Gender	
male	32
female	2
Site	
border of tongue	6
cheek mucosa	9
lower gum	6
others	13
Differentiation	
No record	3
well	8
moderate	23
poor	0
TNM	
No record	7
T0	0
T1	5
T2	5
T3	1
T4	16
TNM	
no record	8
$n = 0$	12
$N = 1$	5
$N = 2$	8
$N = 3$	1
Stage	
no record	5
stage BBB	2
stage I	3
stage II	2
stage III	4
stage IV	18
Chemotherapy	
no	10
yes	24
Radiotherapy	
no	12
yes	22
Survival status	
live	17
die	17

OSCC, oral squamous cell carcinoma.

Stage BBB, the stage is not described in the medical record.

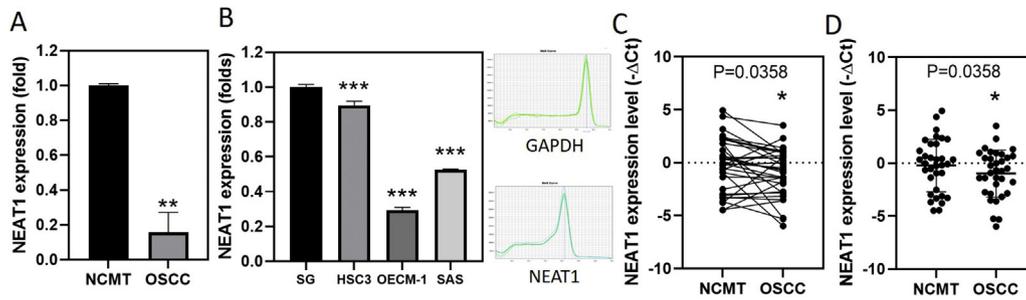


Figure 1 NEAT1 down-regulation in OSCC cell lines and OSCC tissues. (A) The NEAT1 expression fold change in three NCMT and OSCC paired tissues were analyzed by lncRNA microarray. (B) NEAT1 expression fold change in normal (SG) and OSCC (HSC3, OECM-1, and SAS) cell lines were analyzed by RT-qPCR. The melting curves of GAPDH and NEAT1 on the right side. (C) The NEAT1 up-regulation or down-regulation ($-\Delta Ct$) trend (C) and NEAT1 expression level ($-\Delta Ct$), mean \pm SD (D) in 34 NCMT and OSCC paired tissues were analyzed by RT-qPCR. A C and D, paired-t test; B, One-way ANOVA. *, $P < 0.05$; **, $P < 0.01$; ***, $P < 0.005$.

Correction of NEAT1 expression and clinical parameters in OSCC subjects

The ages of the patients ranged from 31 to 81 years (mean 55.68 ± 10.21 years). The most common primary site was the cheek mucosa (9/34 cases) (Table 1). Some case histories of the patient’s records were missed or stage BBB. Regarding the histopathological grade, 23.53% (8/31) of the OSCCs were well-differentiated. A total of 53.85% (14/26 cases) of OSCC patients presented with lymph node metastasis. A total of 62.07% (18/29 cases) of patients had stage IV tumors. The median values for relative NEAT1 expression were used as cut-offs. NEAT1 expression was not associated with any other clinical parameters tested in the subjects (Table 2).

Correction of NEAT1 expression and survival rate in OSCC subjects

The association between NEAT1 expression, survival rate, chemotherapy survival rate, and radiotherapy survival rate was investigated. ROC analysis was used to determine the cutoff score for NEAT1 expression. The cutoff score was equal to the median of NEAT1 expression, meaning that NEAT1 expression was normally distributed in this study (Fig. 2A). The area under the ROC curve (AUC) was 0.7647 ($0.7 \leq AUC \leq 0.8$ indicates the data had acceptable discrimination). NEAT1 expression was significantly high in patients with OSCC ($P = 0.0053$, OR = 10.56), radiotherapy-die-OSCC patients ($P = 0.0274$, OR = 11.67), and chemotherapy-die-OSCC patients ($P = 0.0104$, OR = 14.00) (Table 3). We conducted a binary logistic regression analysis to analyze the correlation of living status between high NEAT1 expression and cancer therapy. Table 4 provided reveals the high neat expression is an independent survival factor to living status ($p < 0.01$) instead of cancer therapy methods ($p > 0.05$). High NEAT1 expression was significantly correlated with living status. In addition, the survival curve of the OSCC patients ($n = 34$, $P = 0.0027$), radiotherapy-OSCC patients ($n = 22$, $P = 0.0204$), and chemotherapy-OSCC patients ($n = 24$,

Table 2 NEAT1 expression and clinical characteristics of OSCC subjects.

OSCC (n = 34)				
	Low	High	P	OR
Age (mean = 56)				
<56	9	8	>0.9999	1.266
≥56	8	9		
Site				
border of tongue	3	3		
cheek mucosa	5	4	>0.9999	1.270
lower gum	3	3		
others	6	7		
Differentiation				
no record	3	0		
well	6	2	0.0971	5.625
moderate	8	15		
poor	0	0		
TNM, T				
no record	6	1		
T0+T1+T2	4	6	>0.9999	0.9524
T3+T4	7	10		
TNM, N				
no record	6	2		
N = 0	6	6	0.6922	1.8000
N > 0	5	9		
Stage				
no record	5	0		
stage BBB	1	1		
stage I + II + III	3	6	0.6924	0.6250
stage IV	8	10		

OSCC, oral squamous cell carcinoma. Stage BBB, the stage is not described in the medical record. OR, odds ratio.

$P = 0.0125$) were verified using the log-rank (Mantel–Cox) test, and the results are shown in Table 2 (Fig. 2B–D). Overall, the low expression of NEAT1 significantly reduced the survival rate of patients with OSCC, even when they received radiotherapy or chemotherapy.

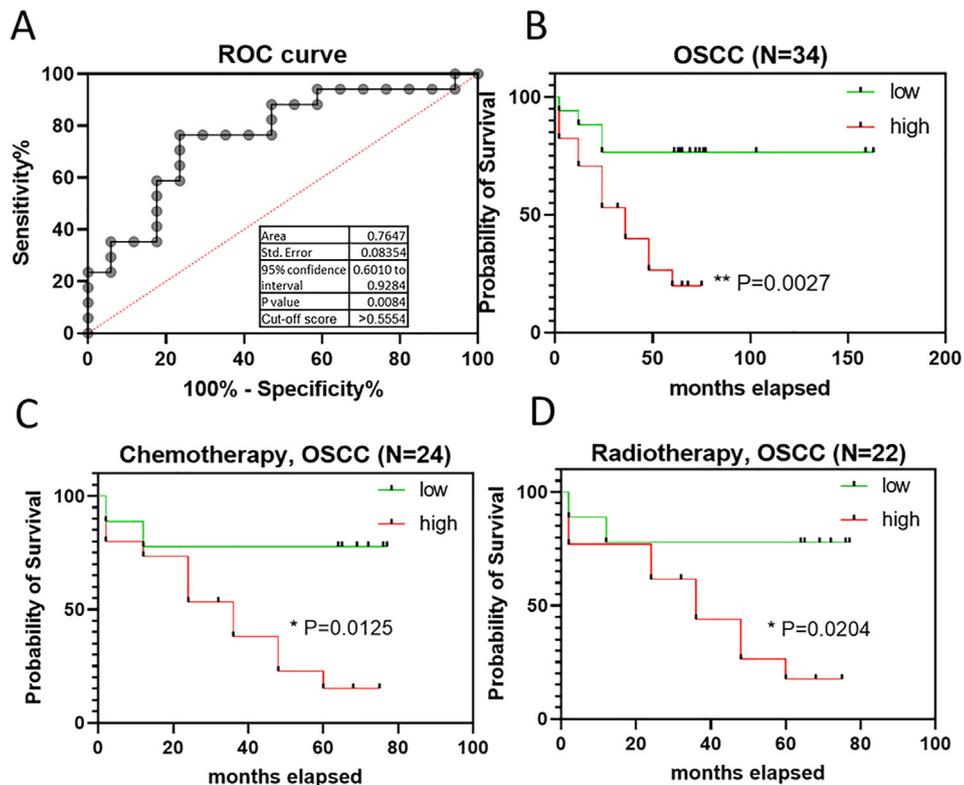


Figure 2 The relation between the NEAT1 expression and survival curve in OSCC. (A) NEAT1 expression level and survival status of OSCC patients, ROC curve, ROC analysis. (B) Low and high NEAT1 expression of OSCC patients, survival curve. Low and high NEAT1 expression of chemotherapy-OSCC patients (C), and radiotherapy-OSCC patients (D).

Table 3 NEAT1 expression and survival rate in OSCC.

OSCC (n = 34)				
	Low	High	P	OR
Survival status				
live	13	4	0.0053	10.56
die	4	13		
Radiotherapy				
no treatment	8	4	0.2818	2.889
treatment	9	13		
No radiotherapy treatment				
live	6	1	0.2222	9.00
die	2	3		
Radiotherapy treatment				
live	7	3	0.0274	11.67
die	2	10		
Chemotherapy				
no treatment	8	2	0.0570	6.667
treatment	9	15		
No chemotherapy treatment				
live	6	1	>0.9999	3.000
die	2	1		
Chemotherapy treatment				
live	7	3	0.0104	14.00
die	2	12		

OSCC, oral squamous cell carcinoma.
OR, odds ratio.

Discussion

Increasing evidence suggests that NEAT1 is dysregulated in many solid tumors. The over-expression of NEAT1 could be seen in stomach adenocarcinoma, prostate adenocarcinoma, liver hepatocellular carcinoma, and kidney cell carcinoma compared to that in the adjacent normal tissues.¹⁸ In contrast, under-expression could be seen in breast cancer, esophageal carcinomas, pheochromocytoma, and hematological malignancies.¹⁸ Compared with NMCT, NEAT1 in OSCC tissues was under-expressed in 22 cases and over-expressed in 12 cases. Using the mean expression value of NEAT1 as a cut-off value, RT-qPCR assay data indicated that NEAT1 was significantly downregulated in OSCC tissues (Fig. 1C). However, NEAT1 was significantly upregulated in OSCC tissue from patients in Shandong and Liaoning, China.^{21,22} The difference in the results may be due to the different risk factors of oral cancer. Betel quid chewing, cigarette smoking, and alcohol drinking habits are prevalent in Taiwan, but these habits are not as prevalent in Shandong and Liaoning, China.²³ Furthermore, the genetic variants rs3741384 in NEAT1 may influence the survival of patients with OSCC.²⁴ The single nucleotide polymorphism (SNP) of NEAT1 may hinder the progress of OSCC, but the NEAT1 expression level correlated with the SNP of NEAT1 has not yet been clarified. The SNP status and expression level of NEAT1 should be investigated in the future to determine whether the SNP is a key factor that complicates the patient's prognosis.

Table 4 The binary logistic regression analysis.

Variable	B	S.E.	Wald	df	Sig.	OR	Effect size
Neat expression (1 = low, 2 = high)	2.093	0.860	5.921	1	0.015	8.113	Cox & Snell R Square = 0.283, Nagelkerke R Square = 0.377
Radiotherapy (1 = no, 2 = yes)	-20.147	28420.72	0.00	1	0.999	0	
Chemotherapy (1 = no, 2 = yes)	20.461	28420.72	0.00	1	0.999	769206689	
Constant	-3.759	1.774	4.490	1	0.034	2.87	

$\chi^2 = 11.296$ ($p = 0.01$), Hosmer and Lemeshow Test = 0.508 n.s.

Dependent variable: Living status (0 = live, 1 = die).

S.E.: standard error; df: degree of freedom; OR: odds ratio; Sig.: significance; χ^2 = Chi-square; n.s.: no significant.

The lncRNA NEAT1 may upregulate proliferation and epithelial–mesenchymal transition (EMT) and repress apoptosis by activating vascular endothelial growth factor A (VEGF-A) and the Notch signaling pathway in OSCC cell lines.²⁵ Betel quid chewing, cigarette smoking, and alcohol consumption are the major risk factors of OSCC in Asia. Arecoline treatment induced NEAT1 expression and EMT markers in the HPV + laryngeal carcinoma cell line HEP-2.²⁶ The interaction between the NEAT1 rs512715 and rs2239895 genetic polymorphisms and cigarette smoking did not have a notable effect.²⁷ Increased NEAT1 expression in patients with laryngeal squamous cell carcinoma, which is linked with alcoholism and smoking habits, was correlated with poor patient outcomes.²⁸ A similar finding was also observed in patients with type 2 diabetes mellitus patients.²⁹ People who have two or three risk habits will experience an enhanced synergism effect of OSCC. However, the single and synergistic effects of these OSCC-risk factors on NEAT1 regulation require further *in vitro* and *in vivo* studies. The oral cancer-risk habits of oral cancer patients should be recorded in more detail for NEAT1 expression analysis in the future.

NEAT1 is enriched in the nucleus but is also found in the cytoplasm, which is a significant component of paraspeckles. In a previous study, NEAT1 knockout mice lost paraspeckles in the cells, but these mice were viable and as fertile as normal mice.³⁰ However, NEAT1 displays typical characteristics of cancer drivers, especially the function of miR sponges. Several studies have shown the miR sponge role of NEAT1. For example, one study found that NEAT1 promoted colorectal cancer progression by competitively binding miR-34a with SIRT1 and enhancing the Wnt/beta-catenin signaling pathway.³¹ NEAT1 also could be a sponge for miR-98-5p, promoting the expression of oncogene *high-mobility group AT-hook 2 (HMGA2)* in prostate cancer³² and *mitogen-activated protein kinase 6 (MAPK6)* in non-small-cell lung cancer.³³ Furthermore, NEAT1 was aberrantly upregulated in docetaxel-resistant prostate cancer cells. At the same time, functional and mechanistic analyses revealed that NEAT1 knockdown improved docetaxel sensitivity of docetaxel resistant prostate cancer cells and 5-Fu resistant cervical cancer cells through de-repressing the microRNA-34a.^{34,35} NEAT1 acts as an miRNA sponge to antagonize the interactions between multiple tumor suppressor miRNAs and target mRNAs. For example, attenuation of the inhibitory effects of miR-26-EZH2 (enhancer of

zeste homolog 2, a subunit of the polycomb repressive complex) can induce oxaliplatin-resistance in gastric cancer,³⁶ miR-129-ABC transporters (ABCB1, ABCC5, and ABCG1; multi-drug resistance [MDR]-related) can induce chemo-resistance in gastric cancer,³⁷ and miR-23a-3p-FOXA1 axis can induce Taxol resistance in breast cancer.³⁸ If the anti-chemotherapy miRs and NEAT1 were affected by the RNA degradation pathway, they might be involved in PPAR α -modulated anti-oral tumorigenesis.³⁹ The wildtype p53 directly binds the promoter of NEAT1 to active NEAT1 expression, and NEAT1 may contribute to the execution of cell death after p53 induction.⁴⁰ It is possible that wildtype p53 enhances NEAT1 expression in NCMT, and the mutant P53 lost its ability to activate NEAT1 expression in OSCC tissues. Finally, whether the SNP of NEAT1 changes the miRNA sponge function to dysregulate chemoresistant and radioresistant miR expression needs further study.

Our study found that NEAT1 expression is downregulated in oral cancer tissues. After separation of the NEAT1 expression by ROC analysis cut-off, the high NEAT1 expression group had a low survival rate, even in chemotherapy- and radiotherapy-treated patients. These results indicate that NEAT1 expression is a valuable biomarker for the prediction and prognosis of oral cancer.

Declaration of competing interest

The authors have no conflict of interest to report.

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