Journal of Dental Sciences 19 (2024) 2074–2081

Available online at www.sciencedirect.com

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Original Article

The association between the expression level of nuclear paraspeckle assembly transcript 1 and the survival rate of head and neck cancer patients after treatment

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Received 19 March 2024; Final revision received 2 May 2024
Available online 26 May 2024

KEYWORDS
Nuclear paraspeckle assembly transcript 1;
Long non-coding RNA;
Head and neck squamous cell carcinoma;
Survival outcomes;
Oral cancer

Abstract *Background/purpose:* The long non-coding RNA (lncRNA) *nuclear paraspeckle assembly transcript 1* (*NEAT1*) exhibits diverse and complicated functions in cancer progression. Despite reports suggesting both tumor-suppressive and oncogenic effects in various cancers, its specific role in head and neck squamous cell carcinoma (HNSCC) remains unclear. This study aimed to investigate the association between *NEAT1* expression levels and survival outcomes in HNSCC patients.

Materials and methods: Paired tissue samples of tumor and non-cancerous matching tissues (NCMT) from 92 HNSCC patients were collected. *NEAT1* expression was analyzed using RT-qPCR. Clinical characteristics, treatment received, and survival rates of the patients were assessed to determine the correlation with *NEAT1* expression and explore its association with alcohol, betel quid, and cigarette use. Additionally, we examined the effect of arecoline on

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https://doi.org/10.1016/j.jds.2024.05.001

NEAT1 expression in normal human oral keratinocytes (NHOK) and fibroblasts (NHOF).
Results: The study revealed a significant downregulation of *NEAT1* expression in oral cancer tissues compared to NCMT. Meanwhile, arecoline increased *NEAT1* expression in NHOK and NHOF cells. However, patients with downregulated *NEAT1* expression exhibited higher overall survival rates, particularly in those who did not receive chemotherapy or radiotherapy.
Conclusion: NEAT1 expression levels are associated with survival outcomes in HNSCC patients, with upregulated expression indicating a worse prognosis, suggesting this lncRNA might contribute to cancer aggressiveness, especially in the absence of active treatment. These findings indicate *NEAT1* may serve as a potential prognostic biomarker in HNSCC, but further research is required to elucidate its role in cancer progression and its potential as a therapeutic target.

Introduction

Head and neck squamous cell carcinoma (HNSCC) poses a significant public health threat, claiming numerous lives globally. HNSCC refers to a broad category of malignancies originating from the squamous cells lining the mucosal surfaces of the head and neck region, including the oral cavity, pharynx, and larynx, encompassing tumors affecting the upper aerodigestive tract. With its aggressive nature and high mortality rates, roughly 890,000 new cases of HNSCC have been reported annually, making HNSCC the sixth most frequent cancer in the world and accounting for 450,000 deaths each year.[1–4] Therefore, understanding the intricate mechanisms underlying its development and progression is crucial for improving patient outcomes. Unraveling the intricate mechanisms fueling its development and progression holds the key to improving patient outcomes, and within this pursuit, the realm of long non-coding RNA (lncRNA) offers promising avenues for exploration.[5]

Certain lncRNAs have been found to prevent tumor growth, whereas other lncRNAs act as oncogenes, promoting the formation and metastasis of cancer.[6] Among these, lncRNA *nuclear paraspeckle assembly transcript 1* (*NEAT1*) has emerged as a fascinating player, showcasing oncogenic activity in various cancers.[7] Its association with oncogenic activity across various cancers, including breast, liver, thyroid, prostate, and pancreas, has ignited substantial research interest.[8–12] Our prior study using microarray analysis revealed that overexpression of *NEAT1* is associated with poor survival rate in oral squamous cell carcinoma (OSCC).[13] Additionally, other previous study reported that *NEAT1* is involved in the carcinogenic development of laryngeal squamous cell carcinoma (LSCC) and contributes to the disease's aggressiveness.[14] However, the landscape of *NEAT1*'s involvement in HNSCC on a general scale still remains unknown.

We investigated the expression patterns of *NEAT1* in cancer tissues compared to their healthy counterparts in Taiwanese HNSCC patients. Moreover, we examined the associations between *NEAT1* levels and clinicopathological features, revealing its potential impact on tumor progression and patient prognosis. The intricate nature of *NEAT1*'s involvement in HNSCC calls for more research to fully understand its complicated role in various head and neck cancer subtypes and cell lines under diverse physiological conditions.

Materials and methods

Subjects

92 pairs of non-cancerous matched tissues (NCMT) and tissues from individuals with HNSCC were provided from the tissue bank at Changhua Christian Hospital. The study received ethical approval from the Institutional Review Board (Approval number: 200501). Tissue samples were collected and immediately frozen in liquid nitrogen for further examination. A thorough selection method was used on frozen sections to guarantee that HNSCC samples contained more than 70% tumor cells, as required for the study.[13] Table 1 provides comprehensive clinicopathological data, including age, gender, lesion location, differentiation, tumor, node, metastasis (TNM) stage, pathological stage, treatment methods, and survival status. Additionally, alcohol consumption, betel quid, and cigarette usage in connection with *NEAT1* RNA expression in HNSCC patients are included in Table 3.

Cell culture

Normal human oral keratinocytes (NHOK) and normal human oral fibroblasts (NHOF) were cultured in keratinocyte serum-free medium (Gibco BRL, Gaithersburg, MD, USA) and Dulbecco's Modified Eagle Medium (DMEM) (Gibco BRL) plus 10% fetal bovine serum (FBS), respectively, following the earlier protocols.[15] The cell lines were cultured in an incubator at 37 °C and 5% CO_2. To investigate the effects of arecoline, a major alkaloid presents in betel quid, on the expression of *NEAT1* in the NHOK and NHOF cells, we used different doses of arecoline (50 and 100 μg/ml) to treat the cells for 24 h before harvesting and extracting their RNA.

RNA extraction from tissues and cells

Total RNA was extracted and purified using a TRI Reagent RNA isolation kit (Molecular Research Center, Cincinnati,

Table 1 Subjects and clinical characteristics of HNSCC.

HNSCC (n = 92, mean of age = 57.03 ± 10.33)	
Gender	
Male	88
Female	4
Type	
Oral cancer	35
Oropharynx	22
Laryngopharynx	6
Larynx	29
Differentiation	
No record	5
Well	9
Moderately	74
Poorly	4
TNM	
No record	16
T0	0
T1	22
T2	21
T3	7
T4	26
TNM	
No record	18
N = 0	34
N > 0	40
Stage	
No record	16
Stage I	15
Stage II	5
Stage III	11
Stage IV	45
Chemotherapy	
No	31
Yes	61
Radiotherapy	
No	36
Yes	56
Survival status	
Live	36
Die	56

HNSCC, head and neck squamous cell carcinoma.
TNM, tumor, node, metastasis stage.

Table 2 *NEAT1* RNA expression in HNSCC.

HNSCC (n = 92)	Cut off at the median of 1.2-fold			
	Down (<1.2)	Up (≥1.2)	*P*	OR
Age(mean = 57)				
<57	30	16	0.2877	1.575
≥57	25	21		
Type				
Oral cancer	26	9	0.0262	2,789
Oropharynx	11	11		
Laryngopharynx	2	4		
Larynx	16	13		
Differentiation				
No record	4	1		
Well	6	3	0.6047	1,467
Moderately	42	32		
Poorly	3	1		
TNM, T				
No record	11	5		
T0+T1+T2	23	20	0.3745	0.6571
T3+T4	21	12		
TNM, N				
No record	10	8		
N = 0	23	11	0.2667	1.711
N > 0	22	18		
Stage				
No record	11	5		
Stage I + II + III	18	13	0.9801	1.012
Stage IV	26	19		

HNSCC, head head and neck squamous cell carcinoma.
TNM, tumor, node, metastasis stage.
OR, odds ratio.
The relative *NEAT1* RNA expression median value of 1.2-fold was used as the cut-off value to determine the up- or down-regulation expression group
Down, down-regulation.
Up, up-regulation.
Fisher's exact test was used to analyze the data.

OH, USA), with the process adjusted according to the manufacturer's instructions. The RNA concentration was determined by using NanoVue Plus spectrophotometer (General Electric Company, Boston, MA, USA), and running electrophoresis in 1% agarose gel to confirm the RNA purity. RNA samples were later processed with DNase I (Stratagene, La Jolla, CA, USA) to eliminate contaminant DNA. Subsequently, two micrograms of total RNA were converted into complementary DNA (cDNA) by using reverse transcriptase and a random primer.

Reverse transcription-quantitative polymerase chain reaction (RT-qPCR)

To analyze *NEAT1* RNA expression, quantitative PCR (qPCR) was performed by a StepOnePlus™ Real-Time PCR System (Applied Biosystems, Waltham, MA, USA). Glyceraldehyde-3-phosphate dehydrogenase (GAPDH) was used as an internal control. The primer sequences utilized in this investigation are shown below:

NEAT1: *Forward:* 5'-CTTCCTCCCTTTAACTTATCCATTCAC-3',
Reverse: 5'-CTCTTCCTCCACCATTACCAACAATAC-3';
GAPDH: *Forward:* 5'-TGGTATCGTGGAAGGACTCATGAC-3',
Reverse: 5'-ATGCCAGTGAGCTTCCCGTTCAGC-3'.

These forward and reverse primer sequences were designed based on the sequences used in the previously published study.[16,17] The PowerUp SYBR Green Master Mix (Applied Biosystems) was used for PCR, and genes were amplified using 40 cycles of 95 °C for 15 s and 60 °C for 1 min. To ensure analysis repeatability, triplicate PCRs were performed for each sample. Samples with conflicting findings were removed from the final analysis. *NEAT1* RNA expression in the NCMT and HNSCC samples was measured

Table 3 Effect of alcohol, betel quid, and cigarette on *NEAT1* RNA expression in HNSCC and OSCC.

	Down (<1.2)	Up (≥ 1.2)	*P*	OR
HNSCC (n = 92)				
Alcohol				
No	22	13	0.6374	1.231
Yes	33	24		
Betel quid				
No	22	16	0.7567	0.8750
Yes	33	21		
Cigarette				
No	16	9	0.6143	1.276
Yes	39	28		
OSCC (n = 35)				
Alcohol				
No	11	5	0.4917	0.5867
Yes	15	4		
Betel quid				
No	11	3	0.6357	1.467
Yes	15	6		
Cigarette				
No	8	3	0.8864	0.8889
Yes	18	6		

HNSCC, head and neck squamous cell carcinoma.
TNM, tumor, node, metastasis stage.
OSCC, oral squamous cell carcinoma.
OR, odds ratio.
The relative *NEAT1* RNA expression median value of 1.2-fold was used as the cut-off value to determine the up- or down-regulation expression group.
Down, down-regulation.
Up, up-regulation.

by -ΔCt (Ct*GAPDH*—Ct*NEAT1*), the -ΔCt value represents the relative expression level of *NEAT1* normalized to *GAPDH*. The median value of 1.2-fold for relative RNA expression of *NEAT1* between HNSCC and NCMT was used as a cut-off value to determine if one subject belonged to the *NEAT1* up- or down-regulation expression group.

Statistical analysis

Data were analyzed using GraphPad Prism (v9, GraphPad Software Inc., La Jolla, CA, USA) to calculate odds ratios, Fisher's exact test, unpaired t-test, Mann—Whitney U test, and logistic regression. Survival analysis was carried out utilizing Kaplan—Meier survival curves, with significance determined by the log-rank test. Differences were considered statistically significant at a *P*-value <0.05.

Results

Demographic and clinical characteristics of HNSCC patients

A total of 92 patients with HNSCC were included in this study, with an average age of 57.03 ± 10.33 years. The majority are male (88); only four patients are female. Most patients had oral cancer (35), followed by oropharyngeal cancer (22), and then laryngeal cancer (29) and laryngopharyngeal cancer (6). Detailed demographic and clinical information was summarized in Table 1.

NEAT1 RNA expression in HNSCC subtypes and *NEAT1* RNA was down-regulated in OSCC

The comparison of *NEAT1* RNA expression levels between non-cancerous matched tissue (NCMT) and HNSCC tissue, as depicted in Fig. 1A, revealed no statistically significant differences. Based on the provided data in Table 2, most oral cancer cases (26 of 35) have *NEAT1* RNA expression being decreased (expression fold change less than 1.2 compared to NCMT). While significant downregulation of *NEAT1* RNA was observed in oral cancer, the remaining 67 cases of other HNSCC (oropharyngeal, laryngopharyngeal, and laryngeal cancers) did not exhibit this trend (Fig. 1B). The distribution of "down" (29 cases) and "up" (28 cases) groups remained statistically comparable in these other HNSCC subtypes (Table 2).

NEAT1 RNA expression was not significantly associated with alcohol, cigarette use, or betel quid chewing in HNSCC patients but was upregulated in NHOK and NHOF cells under arecoline treatment in vitro

The expression of *NEAT1 RNA* is downregulated in the oral cancer subgroup, contrary to other head and neck cancer sites. Therefore, the association between risk factors for oral cancer such as alcohol consumption, betel quid chewing, cigarette use habits, and *NEAT1* RNA expression

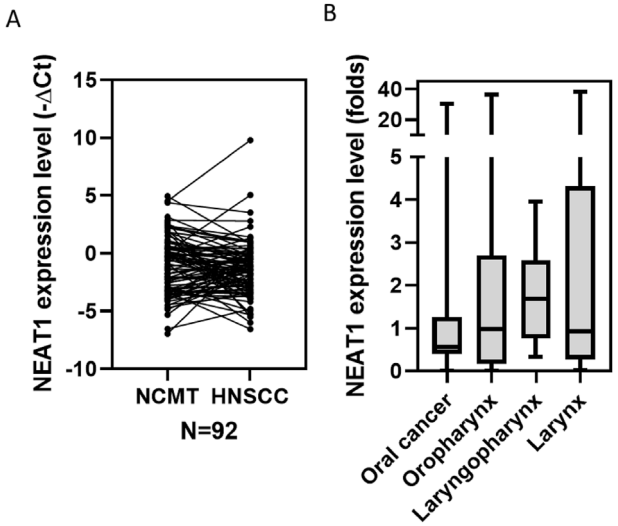

Figure 1 Expression levels of *NEAT1* RNA in HNSCC. **A.** Comparison of *NEAT1* RNA expression levels between non-cancerous matched tissue (NCMT) and HNSCC tissue ($P = 0.5107$). Mann—Whitney U test was used to analyze the data. **B.** *NEAT1* RNA expression levels across different HNSCC subtypes.

was compared. Table 3 includes data on alcohol consumption, betel quid, and cigarette usage in HNSCC patients with *NEAT1 RNA* expression levels. The data shows no significant association between those lifestyle habits and *NEAT1* RNA expression. Conversely, Fig. 2 illustrates arecoline treatment produced *NEAT1* RNA upregulation in NHOK and NHOF cells. While arecoline treatment at both 50 and 100 μg/ml caused a significant increase in *NEAT1 RNA* expression in the NHOK cell line (Fig. 2A), only the 100 μg/ml dose can induce significant upregulation of *NEAT1* RNA expression in NHOF (Fig. 2B).

Upregulation of *NEAT1* is associated with reduced survival probability in HNSCC patients

Table 4 demonstrates the association between *NEAT1* RNA expression, treatment received, and survival status in HNSCC patients. Overall, patients with down-regulated *NEAT1* RNA expression had greater survival rates, regardless of treatment received. Significant higher survival with down-regulation of *NEAT1* is also observed in patients who did not receive chemotherapy ($P = 0.0468$). Similar pattern, but the difference in survival rate between the *NEAT1* RNA down- and up-regulation groups is more pronounced in patients who did not receive radiotherapy ($P = 0.0055$). In addition, this trend can be seen in chemotherapy-received patients but has no statistical significance ($P = 0.1342$), while no difference was found in the survival rate in the radiotherapy group.

In agreement with data from Table 4, the Kaplan—Meier plots in Fig. 3 illustrate the survival probability of HNSCC patients in relation to *NEAT1* RNA expression. A reduced survival probability was observed in the HNSCC patients with upregulated *NEAT1* RNA expression (Fig. 3A). On one hand, there was no significant difference in survival probability among up- and down-regulated *NEAT1* RNA expression groups in the patients who received chemotherapy and radiotherapy treatment (Fig. 3B—D). On the other hand, non-receiving chemotherapy and/or radiotherapy treatment patients with a lower survival probability expressed considerably higher levels of the *NEAT1* gene (Fig. 3C—E). That indicates higher levels of *NEAT1* RNA expression were associated with a worse survival probability, especially in HNSCC patients who did not get chemotherapy and radiotherapy.

Table 4 Association between NEAT1 RNA expression and treatment with survival probability in HNSCC.

HNSCC (n = 92)	Cut off at the fold median of 1.2-fold			
	Down (<1.2)	Up (≥1.2)	*P*	OR
Survival status				
Live	27	9	0.0170	3.000
Die	28	28		
No radiotherapy treatment	23	14	0.7026	1.181
Live	14	2	0.0055	9.333
Die	9	12		
Radiotherapy treatment	32	23		
Live	13	7	0.4384	1.564
Die	19	16		
No chemotherapy treatment	20	11	0.5092	1.351
Live	11	2	0.0468	5.500
Die	9	9		
Chemotherapy treatment	35	26		
Live	16	7	0.1342	2.286
Die	19	19		

HNSCC, head and neck squamous cell carcinoma.
TNM, tumor, node, metastasis stage.
OR, odds ratio.
The relative NEAT1 RNA expression median value of 1.2-fold was used as the cut-off value to determine the up- or down-regulation expression group.
Down, down-regulation.
Up, up-regulation.
Fisher's exact test was used to analyze the data.

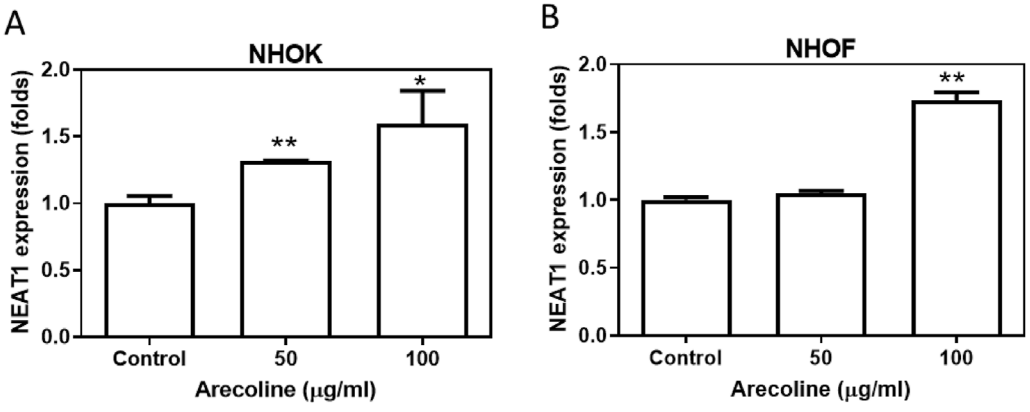

Figure 2 Arecoline treatment induced *NEAT1* RNA upregulation in normal cells. **A.** Arecoline treatment caused a significant increase in *NEAT1* RNA expression in normal human oral keratinocytes (NHOK). **B.** Arecoline treatment at 100 μg/ml significantly increased *NEAT1* RNA expression in normal human oral fibroblasts (NHOF). *NEAT1* RNA expression levels in NHOK and NHOF cells were determined by RT-qPCR at 24 h after treatment. All experiments were performed in triplicate. Data are expressed as mean ± SD. *$P < 0.05$, **$P < 0.01$. Unpaired t-test was used to analyze the data.

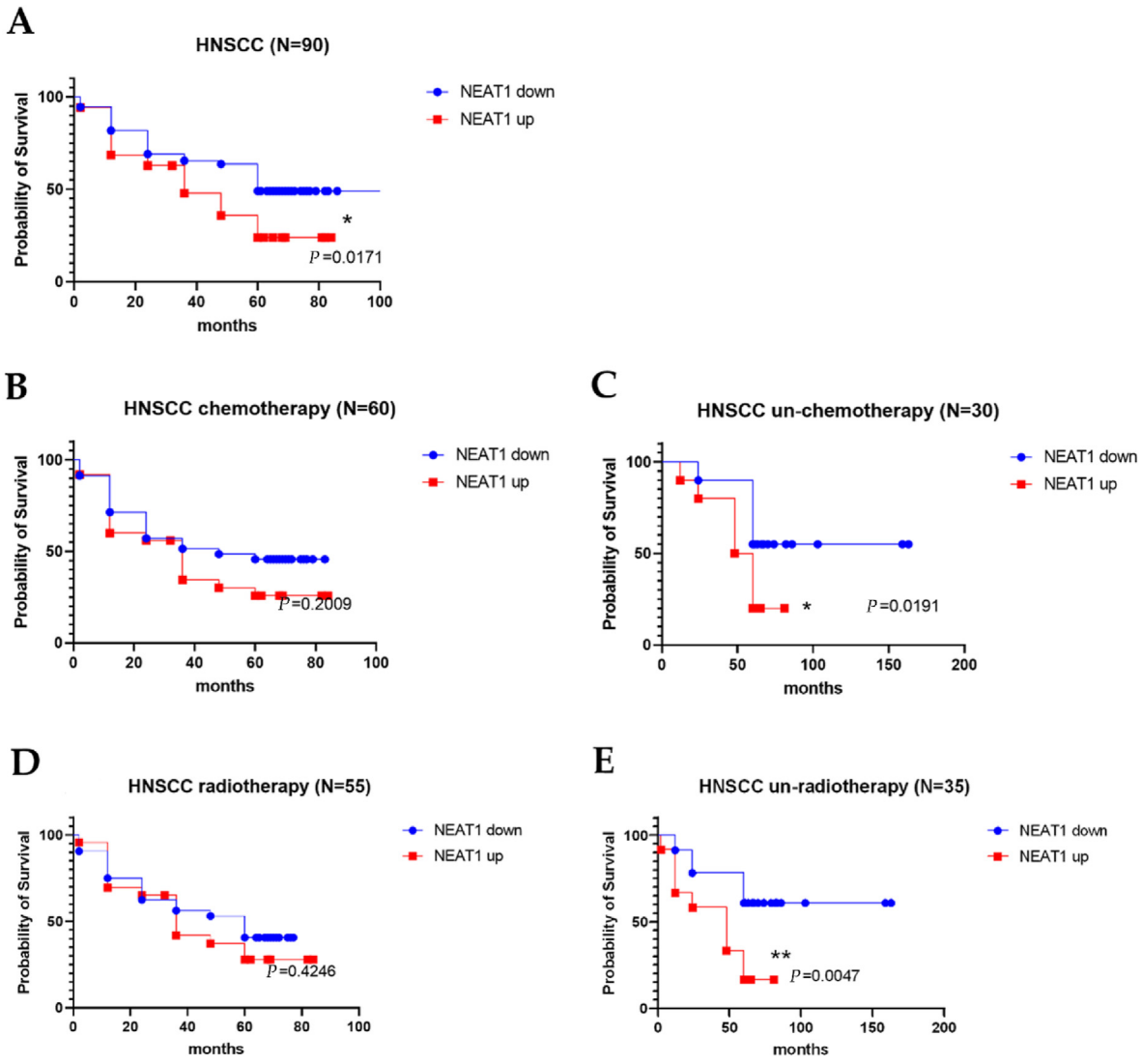

Figure 3 Kaplan–Meier plot of the survival probability of HNSCC patients in relation to *NEAT1* RNA expression. The red curve represents the up-regulation group, and the blue curve is the down-regulation group of *NEAT1* RNA expression. A reduced survival probability was observed in the HNSCC patients with increased *NEAT1* RNA expression (**A**). There was no significant difference in survival probability between "up" and "down" *NEAT1* RNA expression groups in the patients who received chemotherapy and radiotherapy treatment (**B, D**). In the non-chemotherapy and non-radiotherapy patients, the up-regulated *NEAT1* group showed a significantly lower survival probability compared to the down-regulated group (**C, E**). n, sample size. $^{*}P < 0.05$, $^{**}P < 0.01$. Kaplan–Meier survival curves and the log-rank test were used to analyze the data.

Discussion

Since its discovery, *NEAT1* has been linked to upregulation in a variety of cancers. However, there are contradictions since *NEAT1* may also act as a tumor suppressor in several cancers, including breast invasive carcinoma, esophageal carcinoma, nasopharyngeal carcinoma, and acute myeloid leukemia.[18–21] This present study found downregulation of *NEAT1* expression compared to normal tissues in most cases of oral cancer. Meanwhile, we did not observe significant alterations in *NEAT1* expression in other HNSCC subtypes, including oropharyngeal, laryngopharyngeal, and laryngeal cancers, possibly due to the small sample size of these cancers. Nonetheless, this discrepancy demonstrates the potential heterogeneity within the HNSCC spectrum, emphasizing the importance of conducting subgroup analyses in future studies with larger sample sizes. The observed downregulation of *NEAT1* in oral cancer, while not evident in other subtypes, prompts exploration into potential variations in tumorigenesis pathways or unique genetic alterations specific to oral cancer, demanding further investigation in subsequent investigations.

Moreover, our findings suggest that *NEAT1* expression holds promise as a potential prognostic biomarker in HNSCC. Patients exhibiting upregulated *NEAT1* demonstrated poor overall survival rates, a trend that is substantially prominent in non-chemo-radiotherapy-received groups. The intriguing aspect of our study lies in the

correlation between the upregulation of *NEAT1* RNA expression and reduced survival probability in HNSCC patients. As mentioned earlier, the downregulation of *NEAT1* was found in most cases of oral cancer, but it was contrarily associated with improving survival outcomes in overall HNSCC cases. Thus, our findings indicate that the upregulation of *NEAT1* RNA expression may serve as an unfavorable marker in HNSCC, indicating a potential link between elevated *NEAT1* levels and reduced survival probability. However, to establish the clinical utility of *NEAT1* as a prognostic marker, further validation through larger cohorts and prospective studies is imperative.

NEAT1 is an important lncRNA involved in various nuclear functions, particularly within paraspeckles. It influences mRNA processing, gene regulation, and contributes to diverse cellular processes.[22] The complex role of *NEAT1* in HNSCC survival remains unclear, necessitating exploration of its interactions with other genes or pathways involved in cancer progression. In nasopharyngeal carcinoma, *NEAT1* knockdown has been reported to be able to inhibit cell proliferation and migration by impeding *Wnt/β-catenin* signaling. The study also found that *NEAT1* knockdown inhibited *Wnt/β-catenin* signaling via miR-34a-5p. *NEAT1* binds directly to miR-34a-5p, diminishing its expression. In addition, *NEAT1* knockdown lowered cell migration, invasion, proliferation, and epithelial-to-mesenchymal transition (EMT) via miR-34a-5p.[23] Moreover, *NEAT1* upregulation may activate miR-124/NF-κB signaling modulation, hinting at a potential role in the tumorigenesis and progression pathways in HNSCC.[24] Conversely, downregulation of *NEAT1* could impede this activation, potentially contributing to improved survival outcomes. Additionally, high expression levels of the lncRNA *NEAT1* were associated with unfavorable overall survival outcomes in breast cancer patients, while inhibition of *NEAT1* expression led to the suppression of the EMT process via increased levels of miR-146 b-5p.[25] The downregulation of *NEAT1* could potentially disrupt this interaction, acting as a hindrance to cancer cell proliferation. A study in LSCC revealed that *NEAT1* functions as a sponge for miR-107, resulting in enhancing the expression of *cyclin-dependent kinase 6* (*CDK6*), a key member of the *CDK* family strongly associated with the progression of head and neck squamous cell carcinoma.[14,26] In lung cancer, *NEAT1* has been associated with facilitating cyclin D1 expression, thereby driving cell cycle progression.[27] Consequently, downregulation of *NEAT1* may disturb the cell cycle of tumor growth, presenting a potential target for therapeutic intervention in HNSCC. Moreover, *NEAT1*'s involvement in epithelial—mesenchymal transition, a critical process for cancer invasion and metastasis, has been documented. Downregulation of *NEAT1* might hinder EMT, restricting cancer spread within the head and neck cancers context.[28,29] *NEAT1* has been linked to the promotion of *matrix metalloproteinase* (*MMP*) expression, enzymes involved in extracellular matrix degradation, and metastasis. Silencing of *NEAT1* could potentially reduce *MMP* activity, thereby limiting the invasive potential of cancer.[30,31] Some studies suggested that *NEAT1* might play a role in suppressing the anti-tumor immune response via increasing CD8+ T cell apoptosis, which contributes to the restraint of T-cell activity in tumor elimination. Repression of *NEAT1* in HNSCC could potentially enhance the immune system's activity against cancer cells.[32] These multifaceted roles of *NEAT1* in diverse molecular pathways highlight its intricate involvement in the complex landscape of cancer progression. Hence, unraveling the underlying mechanisms related to *NEAT1* expression and its impact on survival outcomes in HNSCC is critical for achieving a thorough comprehension and suitable therapeutic strategy for these malignancies.

In other aspects, we found that there was no statistical significance between the use of cigarettes, alcohol, and betel quid and the expression of *NEAT1* in patients with head and neck cancer. However, in addition to the observed downregulation of *NEAT1* expression in oral cancer, our investigation also revealed arecoline, a major alkaloid in betel quid, could induce *NEAT1* expression in NHOK and NHOF. In other words, *NEAT1* expression exhibited an elevation in reaction against the exposure of arecoline, a well-known carcinogen, thus suggesting a potential protective mechanism against oral carcinogenesis. Hence, it is plausible that *NEAT1* may act as a tumor suppressor in oral cancer, as earlier mentioned *NEAT1*'s involvement in various tumor-suppressive pathways in other cancer types.[32] Therefore, further investigation is needed to identify under what circumstances or factors modulate *NEAT1*'s activities as a tumor oncogene or suppressor.

In conclusion, this study provides evidence for the potential prognostic significance of *NEAT1* expression in HNSCC. The observed downregulation of *NEAT1* associated with improved survival outcomes in HNSCC suggests that *NEAT1* may serve as a valuable biomarker for classification, prognosis, and treatment approach. However, further research, including larger cohort studies and investigations into the underlying mechanisms, is essential to validate the clinical utility of *NEAT1* and explore its potential as a therapeutic target in distinct cases of HNSCC.

Declaration of competing interest

The authors have no conflicts of interest relevant to this article.

Acknowledgments

Experiments and data analysis were performed in part through the use of the Medical Research Core Facilities Center, Office of Research & Development at China Medical University, Taichung, Taiwan, R.O.C.). This study was funded by the China Medical University, grant numbers CMU112-MF-41 and CMU112-S-51, and the Ministry of Science and Technology (MOST), grant numbers MOST 111-2314-B-039-027-MY3.

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