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Original Article

Association of higher transient receptor potential melastatin 8 expression with higher tumor histologic grades, lymph node metastasis, risk factors, and worse survival in patients with head and neck squamous cell carcinoma

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KEYWORDS
Arecoline;
Head and neck squamous cell carcinoma;

Abstract *Background/purpose:* Transient receptor potential melastatin 8 (TRPM8), a thermosensitive ion channel known for its role in cold sensation and menthol response, has emerged as a potential regulator in various cancers. This study aimed to investigate expression trends of TRPM8 in head and neck squamous cell carcinoma (HNSCC) cases and oral squamous cell carcinoma (OSCC) cell lines and its association with clinicopathological features.

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Lymph node metastasis;
Oral squamous cell carcinoma;
Transient receptor potential melastatin 8

Materials and methods: The noncancerous matched tissues and HNSCC paired tissue samples from 84 HNSCC patients were utilized to evaluate the association of *TRPM8* with HNSCC clinicopathological features. *TRPM8* expression was examined in HNSCC patient tissues and OSCC cell lines treated with arecoline.
Results: Kaplan-Meier survival analysis of TCGA data revealed high *TRPM8* expression correlated with unfavorable outcomes and higher tumor histologic grades. *TRPM8* mRNA expression was upregulated in HNSCC cell lines and patients' tissue samples. Arecoline treatment led to significantly increased *TRPM8* mRNA and protein expression in OSCC cell lines. Lymph node metastasis showed a significant association with upregulated *TRPM8* expression in combined OSCC and oropharyngeal squamous cell carcinoma (OPSCC) cases. *TRPM8* mRNA expression was upregulated in HNSCC and OSCC patients with alcohol drinking and cigarette smoking habits, but not in betel quid chewing.
Conclusion: These findings reveal the involvement of *TRPM8* in HNSCC's malignant development and metastasis, suggesting that high expression of TRMP8 may be mutually causal with addiction to tobacco, alcohol, and betel nut in HNSCC patients. Further investigations are needed to determine the underlying pathways of *TRPM8* in HNSCC's development and progression.

Introduction

Head and neck squamous cell carcinoma (HNSCC) is a group of cancers that originate from the oral, pharyngeal, and laryngeal mucosal epithelium. The most common types of HNSCC are oral squamous cell carcinoma (OSCC), oral pharyngeal squamous cell carcinoma (OPSCC), and laryngeal squamous cell carcinoma (LSCC). It is a highly aggressive and life-threatening disease with high mortality rates.[1] With nearly 890,000 new cases and 450,000 deaths reported each year, HNSCC ranks as the sixth most common cancer globally.[2–4]

The incidence of HNSCC has shown a concerning increase in recent years, impacting the lives of a significant number of people in Taiwan. From 1980 to 2014, the age-standardized incidence rate (ASIR) of HNSCC rose by 5.4% per year among males and 3.1% among females.[5,6] In particular, the prevalence of oral cancer in Taiwan is notably high, with an ASIR of 27.19 cases per 100,000 person-years for males and 2.82 per 10,000 for females in 2019.[7] Furthermore, the economic burden of HNSCC in Taiwan is substantial, with total direct medical costs in 2014 and 2015 for male patients being 11.54 times higher compared to females.[8] Alcohol drinking, betel quid chewing, and cigarette smoking are three major risk factors for HNSCC in Taiwan. Taiwanese males' usage of alcohol, betel quid, and cigarettes may contribute to the elevated incidence of HNSCC in this community.[7,9,10] Betel (areca) quid chewing is significantly associated with poor prognosis in patients with oral cancer. Alkaloids present in betel (areca) nut, such as arecoline, could contribute to oral carcinogenesis by interfering with p53 activity, suppressing DNA repair processes, and causing DNA damage in epithelial cells.[11,12]

Transient receptor potential melastatin 8 (TRPM8) is a member of the transient receptor potential (TRP) ion channel family, primarily known for its role in sensing cold temperatures and responding to the cooling agent menthol.[13] While it is highly expressed in prostate tissue, TRPM8 was also detected in oral mucosal, submucosal, and muscular nerve endings.[14,15] The overexpression of TRPM8 in malignant tissues and the involvement of TRPM8 channels in cancer growth and metastasis further support its pivotal role in promoting proliferation and invasiveness, which yield significant implications for cancer diagnosis, prognosis, and treatment.[16,17] A review highlighted the potential diagnostic value of TRP channel expressions in identifying malignant transformation in HNSCC, which hold promise as prognostic markers, aiding physicians in selecting more appropriate and effective management strategies for patients.[16] Although the role of TRPM8 in HNSCC is not yet fully understood, there is emerging evidence indicating its potential involvement in cancer development and progression. Nevertheless, betel quid chewing and arecoline's effects on TRPM8 in HNSCC have not been well researched. Thus, more research is needed to examine the expression patterns of TRPM8 in HNSCC in the context of betel quid chewing and its impact on diagnostic and prognostic parameters such as metastasis and survival outcomes.

Therefore, this current study aimed to analyze the expression trends of TRPM8 in vitro and in vivo. TRPM8 expression in normal and HNSCC cell lines, and arecoline-treated OSCC cell lines was measured. Moreover, we analyzed the *TRPM8* mRNA expression of the noncancerous matched tissues (NCMT) and HNSCC paired tissue samples in association with clinical and pathological features.

Materials and methods

Cell culture

Smulow-Glickman (SG) human gingival epithelial cell line and the human HSNSCC cell lines OECM-1, TW206, SAS, SCC9, FaDu, and A253 were used in the in vitro study.

To investigate the specific effects of betel nut on the expression of TRPM8 in OSCC cells, we used arecoline to treat OSCC cell lines in the following experiment. SAS and OECM-1 cells were cultured in DMEM/F12 and RPMI-1640 medium, respectively, which were supplemented with 10% fetal bovine serum (FBS) and 1% penicillin and streptomycin. All the cell lines were cultured in a 37 °C and 5% CO_2 incubator. Cell culture conditions were performed as described previously.[12] Arecoline (Sigma-Aldrich, St. Louis, MO, USA) was dissolved in culture medium, and different doses of arecoline (25, 50, and 100 μg/mL) were used to treat the cells. The control group received treatment with phosphate-buffered saline (PBS) alone. After 24 h treatment with arecoline, the cells were harvested, and subsequent mRNA and protein extraction procedures were carried out for later reverse transcription-quantitative polymerase chain reaction (RT-qPCR) and western blotting assays.

Subjects

Paired tumors and noncancerous matched tissues (NCMT) samples from 84 cases of HNSCC, diagnosed from 10 August 2007 to 16 September 2019, acquired from Changhua Christian Hospital Tissue Bank, were included in this study (Institutional Ethic Committee approved the study: 200501, 29 March 2022). Upon collection, the tissue samples were submerged in liquid nitrogen until further analysis. A minimum of 70% tumor cells presenting in selected frozen sections of HNSCC samples were required for the investigation.

RNA extraction from tissues

The experimental procedure was based on earlier research.[18] Extraction and purification of total RNA using a TRI Reagent RNA isolation kit (Molecular Research Center, Cincinnati, OH, USA), the protocol was modified following the instructions provided by the manufacturer. The quality and concentration of RNA were assessed using by NanoVue Plus spectrophotometer (General Electric Company, Boston, MA, USA), and electrophoresis in 1% agarose gel. The extracted samples were stored at −20 °C until further use.

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

The experimental procedure and analysis method refers to the previous research.[19] The RNA samples underwent DNase I treatment to eliminate DNA contamination. To analyze *TRPM8* expression, quantitative PCR (qPCR) was performed by a StepOnePlus™ Real-Time PCR System and PowerUp SYBR Green Master Mix (Applied Biosystems, Waltham, MA, USA). Glyceraldehyde-3-phosphate dehydrogenase (GAPDH) was used as an internal control. To evaluate *TRPM8* expression in the NCMT and HNSCC samples -ΔCt (Ct *GAPDH*−Ct*TRPM8*) value was calculated, the -ΔCt value reflects the relative expression level of *TRPM8* normalized to *GAPDH*. Furthermore, the degree of upregulation or downregulation of *TRPM8* in HNSCC was determined by calculating the −ΔΔCt (−ΔCt HNSCC−ΔCt NCMT), which indicates the fold change in *TRPM8* expression between HNSCC and NCMT, providing insights into its altered expression pattern in the malignant tissues. The forward and reverse primer sequences used in this study were designed based on the published sequences.[20,21]

TRPM8: Forward: 5′- GGAGAACAATGACCAGGTCT-3′,
Reverse: 5′-GGTGTCGTTGGCTTTTGTGT-3′
GAPDH: Forward: 5′-TGGTATCGTGGAAGGACTCATGAC-3′,
Reverse: 5′-ATGCCAGTGAGCTTCCCGTTCAGC-3′

Western blot analysis

The experimental protocol was described in previous research.[22] Cell lysates after denaturing in the 5x SDS sample loading buffer at 95 °C/5 min were loaded on 10% SDS-PAGE gels using approximately 30 μg of total protein in each lane. SDS-PAGE gels were transferred to Polyvinylidene difluoride (PVDF) membranes and blocked with 5% non-fat dry milk in Tris-buffered saline containing 0.1% Tween 20 for 1 h at room temperature. Subsequently, primary antibodies against TRPM8 (1:500, #ACC-049, Alomone Labs, Jerusalem, Israel) and GAPDH (1:10,000, NB300-221, Novusbiologicals, Centennial, CO, USA) were used to incubate the membranes overnight at 4 °C. The membranes were incubated for 1 h at room temperature with secondary antibodies: anti-rabbit IgG, HRP-conjugated antibody (1:30,000, Jackson ImmunoResearch, West Grove, PA, USA), and anti-mouse IgG, HRP-conjugated antibody (1:30,000, Jackson ImmunoResearch). The results were quantified using ImageJ software (National Institutes of Health, Bethesda, MD, USA), and signals were normalized to GAPDH as a loading control.

Statistical analysis

GraphPad Prism (v9, GraphPad Software Inc., La Jolla, CA, USA) was used to perform analyses of the odds ratio, Fisher's exact test, logistic regression, and 95% confidence interval (95% CI). Welch's *t*-test and the Wilcoxon signed-rank test were used for unpaired parametric and paired nonparametric comparisons, respectively. Survival analysis was performed using Kaplan−Meier survival curves and the log-rank test. The difference in comparisons was defined as statistically significant when the *P*-value was less than 0.05.

Results

Association of higher *TRPM8 gene* expression with higher tumor histologic grades and worse survival of HNSCC patients.

The data used in this analysis was derived from The Cancer Genome Atlas (TCGA) head and neck cancer dataset in UCSC Xena Functional Genomics Explorer (https://xenabrowser.net/heatmap/).[23] The Kaplan-Meier plot (Fig. 1A) illustrated the association between *TRPM8* gene expression and HNSCC patient survival probability. The plot showed that the higher *TRPM8* expression curve consistently remains below the lower expression curve, indicating that lower *TRPM8* gene expression is associated with better survival outcomes. In addition, Fig. 1B illustrated the comparison of *TRPM8* gene expression RNASeq Illumina-HiSeq levels among different HNSCC tumor histologic

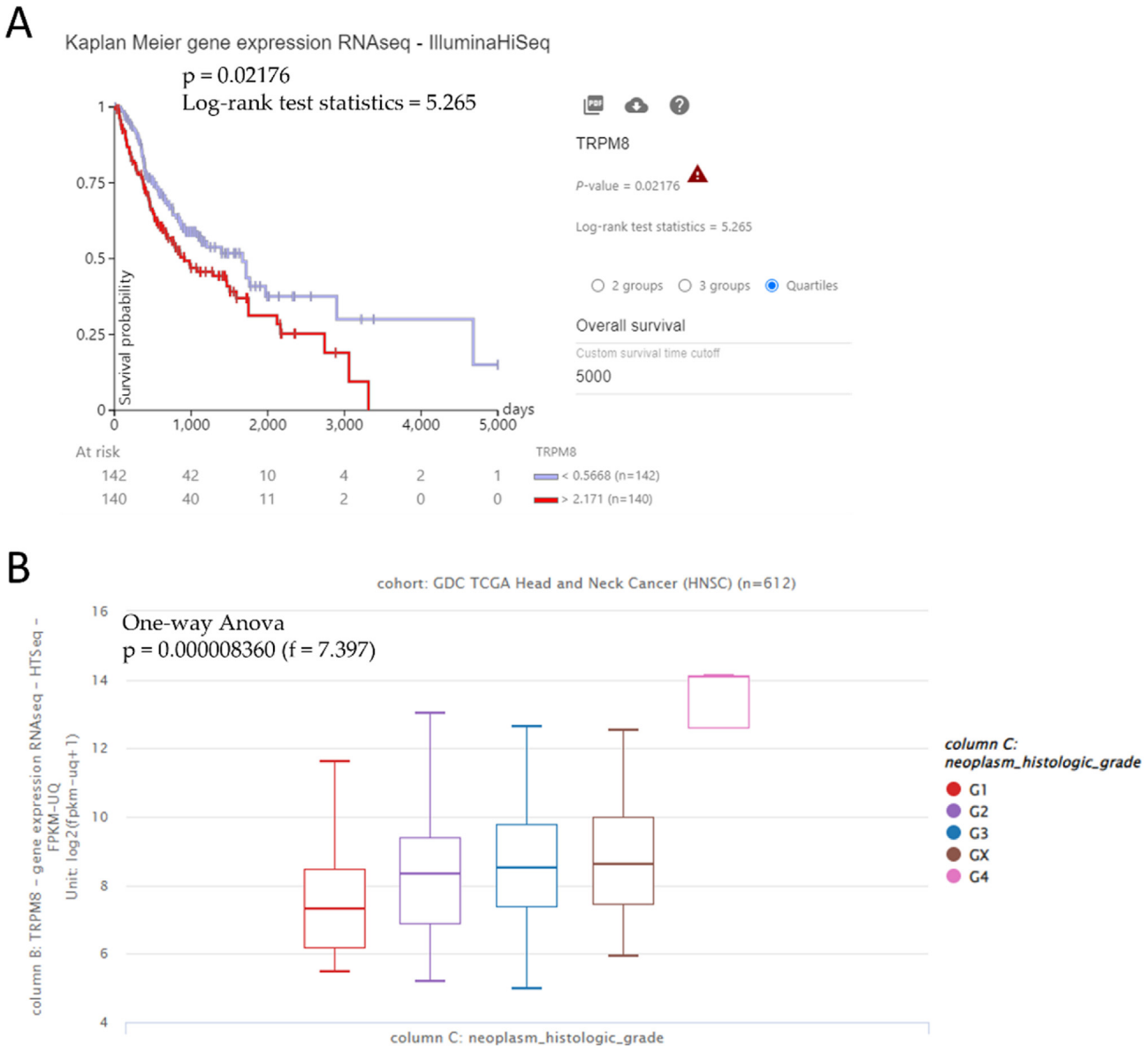

Figure 1 The *TRPM8* gene expression data from TCGA head and neck cancer dataset in UCSC Xena Functional Genomics Explorer. **A.** Kaplan-Meier plot of HNSCC survival probability based on *TRPM8* gene expression RNASeq Illumina-HiSeg. The red curve is the top 25% and the violet curve is the bottom 25% of *TRPM8* expression. **B.** Comparison of the tumor histologic grade of HNSCC in relation to *TRPM8* gene expression RNASeq Illumina-HiSeg. The x-axis, tumor histologic grades; the y-axis, *TRPM8* gene expression RNASeq Illumina-HiSeg levels. Each box on the chart represents the mean *TRPM8* expression value for a specific histologic grade. G2 and G3 exhibit higher *TRPM8* gene expression compared to G1.

grades. The median values of *TRPM8* expression levels are as follows: Grade 1(G1) = 7.35, Grade 2 (G2) = 8.35, Grade 3 (G3) = 8.52, Grade X (GX) = 8.62, and Grade 4 (G4) = 14.1. G2 and G3 demonstrate higher *TRPM8* gene expression compared to G1, while G4 is significantly higher than in the other lower histologic grades.

Upregulation of TRPM8 mRNA and protein expressions in arecoline-treated OSCC cell lines

Fig. 2A showed *TRPM8* mRNA expression was modest in the normal gingival epithelial SG cell line but moderate to highly elevated in all HNSCC and OSCC cell lines except A253.

Arecoline is one of the main carcinogenic components found in betel nut. RT-qPCR quantification of *TRPM8* mRNA expression in the SAS cell line showed a significant increase in *TRPM8* mRNA expression at doses of 25 μg/mL and 50 μg/mL compared to the control group (Fig. 2B). Furthermore, Western blot analysis of TRPM8 protein expression in the OECM-1 and SAS cell lines following arecoline treatments demonstrated a significant increase in TRPM8 protein expression at doses of 25 μg/mL and 50 μg/mL compared to the control group (Fig. 2C and D).

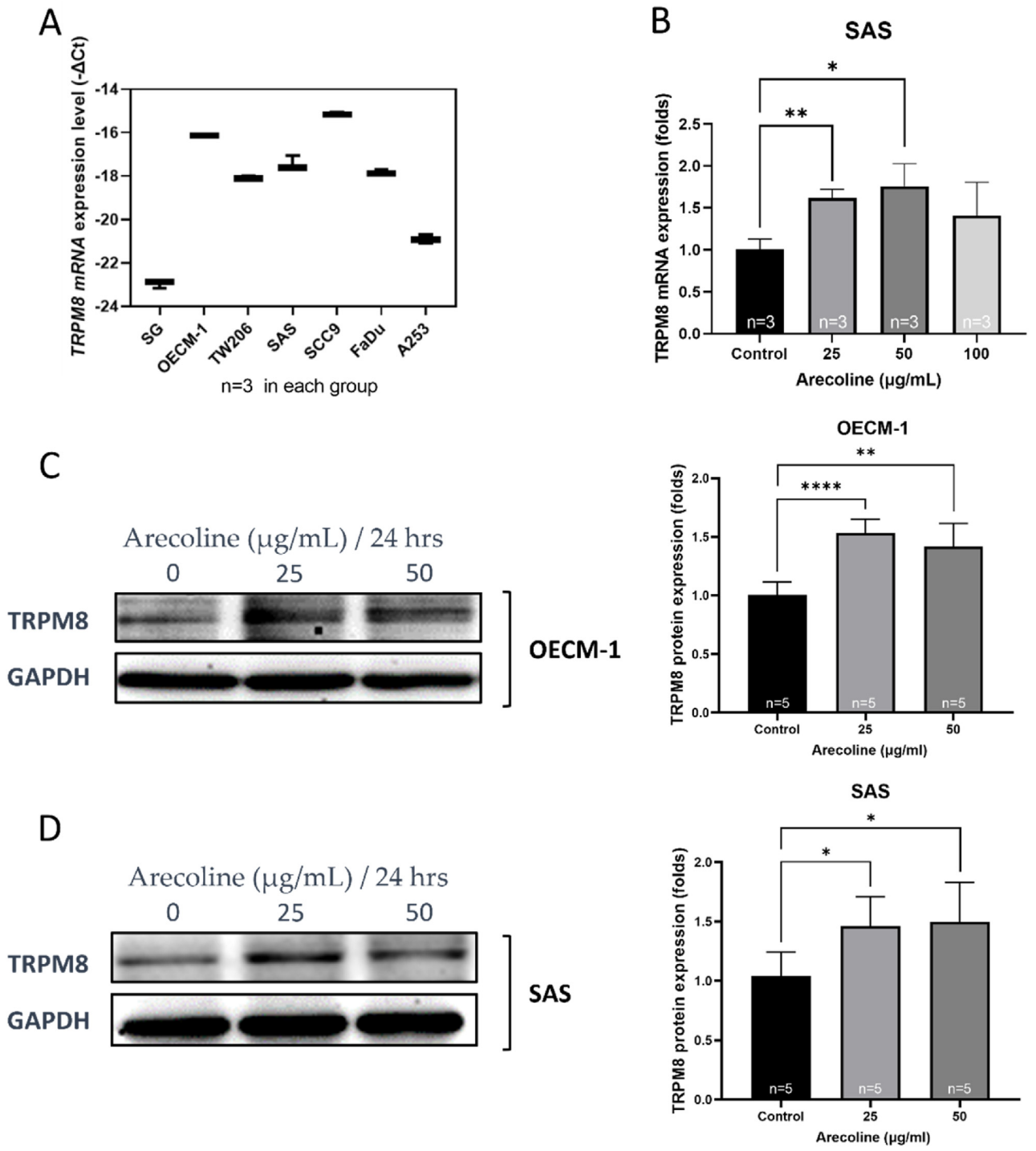

Figure 2 Upregulation of *TRPM8* mRNA and protein expressions in arecoline-treated OSCC cell lines. (**A., B.**) RT-qPCR analysis results of *TRPM8* mRNA expression. **A.** *TRPM8* mRNA expression levels in the normal gingival epithelial (SG) and HNSCC cell lines. Due to the large difference in the expression level of *TRPM8* mRNA, it is represented by -ΔCt. **B.** *TRPM8* mRNA expression in 0 μg/mL (control), 25 μg/mL, 50 g/mL, and 100 μg/mL arecoline-treated SAS cell line. Western blot results of TRPM8 protein expression in 0 μg/mL (control), 25 μg/mL, and 50 μg/mL arecoline-treated OECM-1 **(C)** and SAS **(D)** cell lines. The charts on the right show the quantification of protein expression levels, normalized to the loading control (GAPDH), under each dose. Data are expressed as mean ± SD. n, sample size; $^{*}P < 0.05$, $^{**}P < 0.01$, $^{****}P < 0.0001$. Welch's *t*-test.

Higher *TRPM8 mRNA* expression in HNSCC tissues than in noncancerous matched tissues

This study comprised a total of 84 patients diagnosed with HNSCC, including 32 cases of OSCC, 22 cases of OPSCC, 27 cases of LSCC, and 3 cases of hypopharyngeal squamous cell carcinoma (HPSCC). Detailed epidemiology, clinical, and pathological data for each case were listed in Table 1.

TRPM8 mRNA expression levels were investigated in HNSCC tumor tissues and NCMT. The analysis included subgroups of OSCC, OPSCC, and LSCC. The results demonstrated that *TRPM8* mRNA expression was significantly upregulated in HNSCC compared to NCMT ($P < 0.05$) (Fig. 3A). Moreover, a similar upregulation trend was observed in OSCC compared to NCMT, although the difference was not statistically significant ($P = 0.07$) (Fig. 3B). In contrast, no significant difference in *TRPM8* mRNA expression was observed between OPSCC and LSCC compared to NCMT (Fig. 3C and D).

Association of higher TRPM8 mRNA expression with lymph node metastasis as well as alcohol drinking, tobacco smoking, and betel quid chewing habits of HNSCC patients

A comprehensive summary and analysis of clinical and pathological parameters are provided in Table 2. Importantly, a substantial portion of the HNSCC patients in this cohort had a history of alcohol consumption (56/84), tobacco smoking (64/84), and/or betel nut chewing habits (52/84). These lifestyle factors have been widely recognized as major risk factors contributing to the development of head and neck cancer.[24–26] Moreover, a significant association between lymph node metastasis and the upregulation of *TRPM8* expression in the combined data of OSCC and OPSCC cases was observed ($P < 0.05$).

HNSCC and OSCC tissues from patients drinking alcohol exhibited a significant increase in *TRPM8* mRNA expression compared to NCMT (Fig. 4A and D). Similarly, cigarette smoking was significantly associated with elevated *TRPM8* mRNA expression in both HNSCC and OSCC (Fig. 4C and F). Although HNSCC and OSCC tissues from betel-quid-chewing patients showed increases in *TRPM8* expression compared to NCMT, the difference did not reach statistical significance ($P = 0.17$ and 0.07, respectively) (Fig. 4B and E).

Table 1 Subjects and clinical characteristics.

	HNSCC (84)	OSCC (32)	OPSCC (22)	LSCC (27)	HPSCC (3)
Gender					
male	80	30	20	27	3
female	4	2	2	0	0
Age					
<57	40	18	8	12	2
≧57	44	14	14	15	1
Alcohol					
no	28	14	4	9	1
yes	56	18	18	18	2
Betel nut					
no	32	14	4	12	2
yes	52	18	18	15	1
Cigarette					
no	20	10	2	7	1
yes	64	22	20	20	2
Differentiation					
no record	5	3	0	1	1
Well	8	7	1	0	0
moderate	67	22	17	26	2
poor	4	0	4	0	0
TNM, T					
no record	7	6	0	1	0
T0+T1+T2	34	8	10	14	2
T3+T4	43	18	12	12	1
TNM, N					
no record	7	6	0	1	0
N = 0	26	7	5	12	2
N > 0	51	19	17	14	1
Stage					
no record	4	3	0	1	0
stage BBB	3	3	0	0	0
stage I + II + III	23	6	6	9	2
stage IV	54	20	16	17	1
Radiotherapy-survival status					
live	20	10	4	4	2
dead	35	10	11	13	1
Chemotherapy-survival status					
live	22	9	7	5	1
dead	38	13	12	12	1
Survival status					
live	35	16	7	10	2
dead	49	16	15	17	1

HNSCC: Head and neck squamous cell carcinoma; OSCC: Oral squamous cell carcinoma; OPSCC: Oropharyngeal squamous cell carcinoma; LSCC: Laryngeal squamous cell carcinoma; HPSCC: Hypopharyngeal squamous cell carcinoma.

Discussion

Previous studies also found highly expressed *TRPM8* in two OSCC cell lines HSC3 and HSC4, which derived from the human tongue, and in various other cancer cell lines, including esophagus, gastric, pancreatic, lung, breast, and skin cancer.[20,27–30] In this present study, *TRPM8* mRNA was moderate-to-highly expressed in various HNSCC and OSCC cell lines but had low expression in SG cells. The results indicate that up-regulation of *TRPM8* has potential involvement in the molecular pathways of these cancers. Furthermore, our treatment with arecoline resulted in a significant increase in *TRPM8* mRNA and protein expression in the SAS and OECM-1 cell lines. Previous studies discovered *TRPM8*'s diverse roles in different types of tumors. Lan et al. (2019) found that TRPM8 plays a role in the proliferation and immune evasion of esophageal cancer.[27] Okamoto et al. (2012) also reported that TRPM8 channels were found to be involved in OSCC cell invasion, whereas using a TRPM8 antagonist restricted the capacity of the cells to migrate and invade.[20] Yamamura et al. (2008), in contrast, found that menthol, a TRPM8 agonist, activated calcium influx in

Table 2 Comparisons of the association between clinicopathological parameters and *TRPM8* mRNA expression in HNSCC.

	HNSCC (84)					OSCC (32)					OPSCC (22)					LSCC (27)					OSCC + OPSCC				
	N > T	N < T	P	OR	95% CI	N > T	N < T	P	OR	95% CI	N > T	N < T	P	OR	95% CI	N > T	N < T	P	OR	95% CI	N > T	N < T	P	OR	95% CI
Gender																									
male	42	38	0.0404	∞	1 to ∞	12	18	0.2579	∞	0.2788 to ∞	14	6	0.0497	∞	0.8629 to ∞	14	13				26	24	0.0452	∞	0.9244 to ∞
female	0	4				0	2				0	2				0	0				0	4			
Age																									
<57	18	22	0.3822	0.6818	0.2798 to 1.609	6	12	0.5809	0.6667	0.1637 to 2.627	5	3	0.9332	0.9259	0.1778 to 4.695	5	7	0.3434	0.4762	0.1130 to 2.132	11	15	0.4078	0.6356	0.2123 to 1.793
≥57	24	20				6	8				9	5				9	6				15	13			
Alcohol																									
no	14	14	>0.9999	1	0.4198 to 2.382	7	7	0.1977	2.6	0.6639 to 12.64	2	2	0.5308	0.5	0.0678 to 3.922	5	4	0.7854	1.25	0.2186 to 5.171	9	9	0.8473	1.118	0.3334 to 3.379
yes	28	28				5	13				12	6				9	9				17	19			
Betel nut																									
no	13	19	0.1776	0.5426	0.2282 to 1.352	5	9	0.854	0.873	0.1963 to 3.341	2	2	0.5308	0.5	0.0678 to 3.922	5	7	0.3434	0.4762	0.113 to 2.132	7	11	0.3356	0.5694	0.1846 to 1.840
yes	29	23				7	11				12	6				9	6				19	17			
Cigarette																									
no	9	11	0.6084	0.7686	0.2868 to 2.037	5	5	0.3248	2.143	0.4320 to 11.32	0	2	0.0497	0	0 to 1.1159	4	3	0.7448	1.333	0.278 to 6.323	5	7	0.6104	0.7143	0.2081 to 2.667
yes	33	31				7	15				14	6				10	10				21	21			
Differentiation													1	0											
no record	1	4				1	2						13	8		0	1	N.A	N.A	N.A	1	2			
well	3	5	0.3899	0.5211	0.1312 to 2.237	2	5	0.5579	0.5578	0.09970 to 3.221	1	0				0	0				3	5			
moderate	36	31				9	13				11	6	0.4391	∞	0.06349 to ∞	14	12				20	19	0.4778	0.5727	0.1395 to 2.686
poor	2	2				0	0				2	2				0	0				2	2			
TNM, T																									
no record	4	3				4	2				0	0				0	1				4	2			
T0+T1+T2	16	18	0.7206	0.8485	0.3607 to 2.039	2	6	0.6709	0.6667	0.112 to 3.766	6	4	0.7462	0.7647	0.1456 to 4.015	7	7	0.6709	0.7143	0.1568 to 3.397	8	10	0.8811	0.9143	0.2995 to 2.797
T3+T4	22	21				6	12				8	4				7	5				14	16			
TNM, N																									
no record	4	3				4	2				0	0				0	1				4	2			
N = 0	15	11	0.2959	1.66	0.6542 to 4.549	4	3	0.077	5	0.8640 to 24.96	5	0	0.0545	9.842	0.4711 to 205.6	5	7	0.2488	0.3968	0.0970 to 1.888	9	3	***0.0192***	5.308	1.245 to 20.02
N > 0	23	28				4	15				9	8				9	5				13	23			
Stage																									
no record	1	3				1	2				0	0				0	1				1	2			
stage BBB	3	0				3	0				0	0				0	0				3	0			
stage I + II + III	13	10	0.4114	1.508	0.556 to 3.789	3	3	0.2446	3	0.5327 to 15.63	5	1	0.2396	3.889	0.4704 to 51.49	4	5	0.4841	0.56	0.1291 to 3.308	8	4	0.0944	3.143	0.7972 to 10.54
stage IV	25	29				5	15				9	7				10	7				14	22			
Radiotherapy-survival status																									
live	10	10	0.2525	0.5217	0.1759 to 1.650	3	7	0.1775	0.2857	0.05698 to 1.720	3	1	0.9299	1.125	0.1149 to 19.01	3	1	0.6223	1.875	0.2133 to 28.67	6	8	0.1632	0.375	0.09662 to 1.631
dead	23	12				6	4				8	3				8	5				14	7			
Chemotherapy-survival status																									
live	9	13	0.1422	0.4515	0.1468 to 1.245	2	7	0.2513	0.3333	0.0565 to 2.083	3	4	0.3106	0.375	0.07011 to 2.432	3	2	0.7933	0.75	0.1134 to 5.667	5	11	0.1211	0.3571	0.09812 to 1.266
dead	23	15				6	7				8	4				8	4				14	11			
Survival status																									
live	15	20	0.2685	0.6111	0.2691 to 1.477	6	10	>0.9999	1	0.2555 to 3.913	3	4	0.1663	0.3043	0.0518 to 1.790	5	5	0.8826	0.8889	0.1618 to 48.10	9	14	0.2533	0.5294	0.1827 to 1.533
dead	27	22				6	10				11	4				9	8				17	14			

HNSCC: Head and neck squamous cell carcinoma; OSCC: Oral squamous cell carcinoma; OPSCC: Oropharyngeal squamous cell carcinoma; LSCC: Laryngeal squamous cell carcinoma; T: Tumor; N: Noncancerous matched tissue; OR: Odds ratio; CI: Confidence interval.

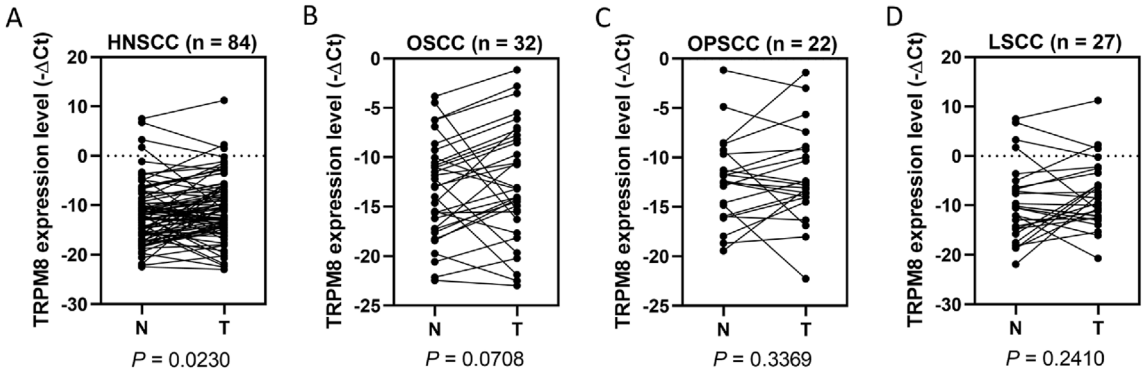

Figure 3 Association of *TRPM8* mRNA expression between tumor tissue from NCMT (N) and tumor (T). **A.** HNSCC, n = 84, $P = 0.023$. **B.** OSCC, n = 32, $P = 0.07$. **C.** OPSCC, n = 22, $P = 0.3369$. **D.** LSCC, n = 0.2410, $P = 0.2410$. n, sample size. *$P < 0.05$. Wilcoxon Matched Pairs Signed-Rank Test was used to analyze the data.

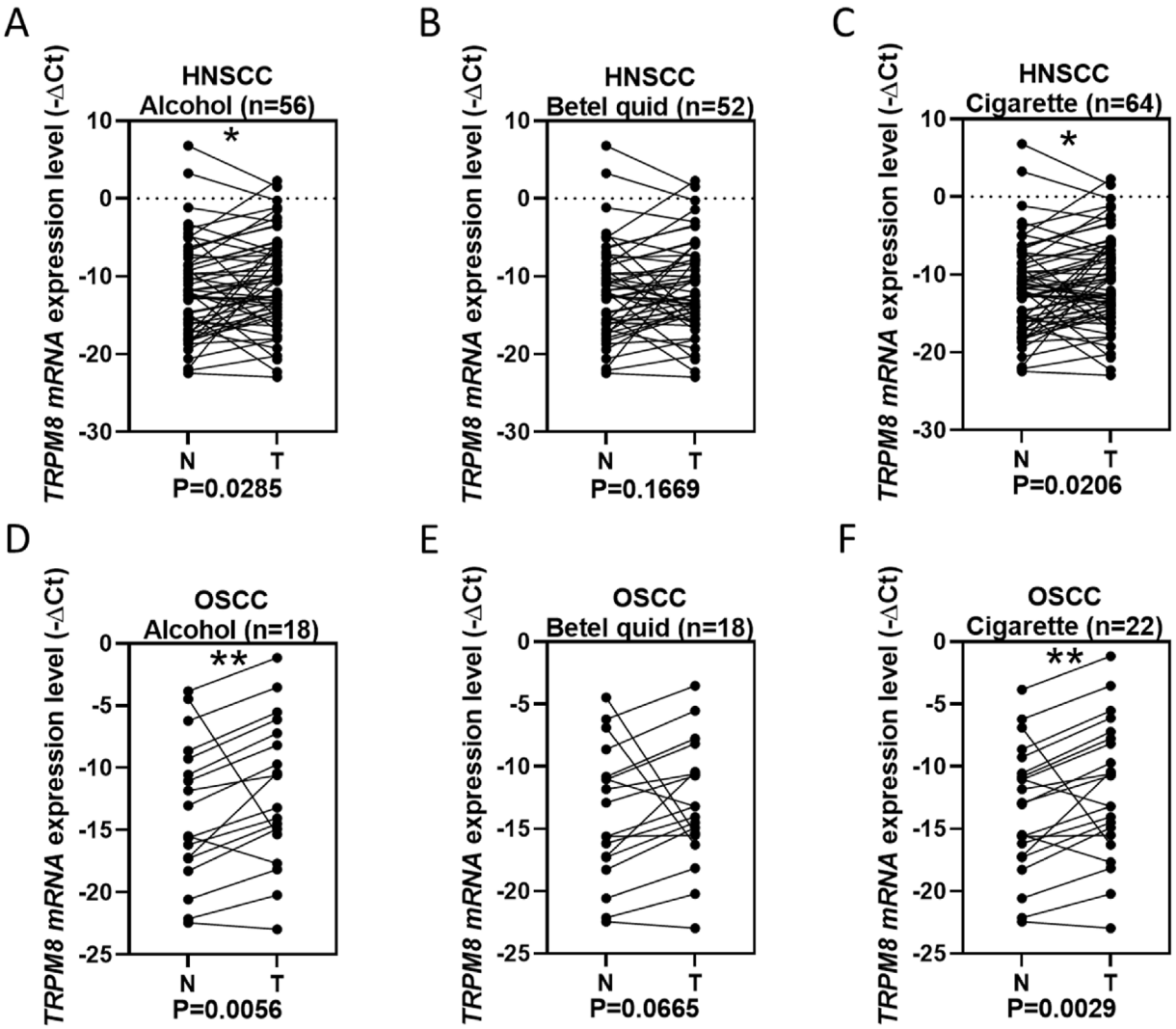

Figure 4 Association between *TRPM8* mRNA expression (−ΔCt) trend and alcohol drinking, betel quid chewing, and cigarette smoking in HNSCC and OSCC. **(A, D)** HNSCC and OSCC tissues from patients drinking alcohol had *TRPM8* mRNA expression significantly increased compared to NCMT. (**B, E)** Although HNSCC and OSCC tissues from patients chewing betel quid had *TRPM8* expression increased, it was not statistically significant. **(C, F)** Both HNSCC and OSCC *TRPM8* mRNA expression was significantly increased compared to NCMT in cigarette smoking patients. n, sample size. *$P < 0.05$, **$P < 0.01$. Wilcoxon Matched Pairs Signed-Rank Test was used to analyze the data.

melanoma cells and suppressed cell viability.[31] This emphasizes the complex nature of its involvement in tumorigenesis. Whether TRPM8 plays a tumor-promoting or tumor-suppressing role remains unclear.

In the clinical analysis, we found that *TRPM8* mRNA expression was significantly upregulated in HNSCC compared to NCMT, suggesting its involvement in HNSCC development. Aligning with our data, previous studies have investigated the expression of *TRPM8* in various types of cancer, including prostate, pancreatic, and bladder cancer, and reported *TRPM8* expression elevated in malignant tissues compared to non-malignant tissues.[32,33]

A comprehensive pan-cancer analysis discovered that TRPM8 levels were increased in various kinds of malignancies and associated with the patient's unfavorable prognosis.[34] Our analysis of TCGA data revealed that higher expression levels in tumor histologic grades G2, G3, and G4 suggest a possible role of TRPM8 in the progression or aggressiveness of tumors in HNSCC. Survival analysis also showed that higher *TRPM8* gene expression was associated with worse survival outcomes, indicating a potential prognostic significance of *TRPM8* in HNSCC. Similarly, a study on urothelial carcinoma of the bladder found that overexpression of *TRPM8* correlated with higher histological grade and tumor stage, as well as poor prognosis.[35] From our data, the demographic and clinicopathological characteristics of the patients were summarized, providing valuable insights into the diverse nature of HNSCC. Notably, a significant association between lymph node metastasis and the upregulation of *TRPM8* expression in the combined data of OSCC and OPSCC cases was observed. This suggests that TRPM8 may play a potential role in the metastatic process of these cancers. Additionally, we observed an upregulation trend in *TRPM8* mRNA expression in OSCC compared to NCMT, but the difference was not statistically significant, possibly due to the small sample size. Consequently, it is important to interpret these findings with caution and acknowledge the need for further investigation with a larger sample size to not only determine the role of *TRPM8* in OSCC but also to re-evaluate the association of *TRPM8* with prognostic factors such as TNM, histologic grade, tumor stage, and survival outcomes.

Interestingly, our study revealed significant associations between *TRPM8* mRNA expression and certain lifestyle factors in HNSCC. Specifically, patients who reported drinking alcohol and smoking cigarettes exhibited significantly higher *TRPM8* mRNA expression in HNSCC tissue compared to NCMT. This finding suggests a potential link between alcohol drinking, cigarette smoking, and *TRPM8* dysregulation in HNSCC development. Furthermore, the upregulation of *TRPM8* mRNA expression in alcohol-drinking and cigarette-smoking OSCC patients was even more consistent. These results suggest that alcohol and tobacco use may have a more pronounced effect on *TRPM8* expression in OSCC. In contrast, HNSCC and OSCC tissues from patients who reported betel quid chewing also showed increased *TRPM8* expression compared to NCMT but did not reach statistical significance. This can be attributed to the small sample size of betel-nut-chewing patients included in our study. Nevertheless, whether these risk factors have antagonistic, additive, or synergistic effects on the upregulation of *TRPM8* expression remains unclear. However, we have confirmed that arecoline upregulated the mRNA and protein expression of *TRPM8* in vitro. Betel quid chewing is a prevalent habit in the Indian Subcontinent, East-Southeast Asia, Taiwan, and some Pacific Island populations and has been implicated in the development of OSCC.[36] Hence, future research with a larger sample size is needed to comprehensively investigate the relationship between betel nut chewing and *TRPM8* expression in HNSCC, as well as to determine whether people with high *TRPM8* expression are prone to abusing alcohol, betel quid, and tobacco, resulting in an increased risk of HNSCC, or whether their exposure to these risk factors induces *TRPM8* expression, causing an increased risk of HNSCC. In this current study, unfortunately, TRPM8 protein level evaluations, such as immunohistochemical staining, in HNSCC tissues were not performed due to the small amount and limited availability of collected tissue samples. Subsequent studies should include protein expression analysis to complement the mRNA expression findings and establish a more complete picture of TRPM8's role in HNSCC.

In conclusion, our data showed the upregulation of *TRPM8* mRNA expression in HNSCC and OSCC tissues from patients with alcohol drinking, tobacco smoking, and betel nut chewing habits. TRPM8 plays a significant role in the malignant development and metastasis of HNSCC, and in the arecoline-modulated progression of oral cancers, suggesting its potential as a prognostic marker. However, the absence of TRPM8 protein expression data in HNSCC patient tissues presents a limitation. Larger sample sizes, functional studies, and protein-level analyses are necessary to determine the precise role and underlying mechanistic pathways of TRPM8 in HNSCC's development and progression, as well as its potential as a therapeutic target for HNSCC.

Declaration of competing interest

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

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