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RESEARCH ARTICLE

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Expression of the long noncoding RNA RP11-169D4.1-001 in Hypopharyngeal Squamous cell carcinoma tissue and its clinical significance

Zhisen Shen[1,2] | Linrong Wu[1,2] | Wenjuan Hao[1,2] | Qun Li[1,2] | Chongchang Zhou[1,2]

[1]Department of Otorhinolaryngology Head and Neck Surgery, Ningbo Medical Center Lihuili Hospital, Ningbo University, Ningbo, China

[2]Laboratory of Otorhinolaryngology Head and Neck Surgery, Ningbo Medical Center Lihuili Hospital, Ningbo University, Ningbo, China

Correspondence
Zhisen Shen, Department of Otorhinolaryngology Head and Neck Surgery, Ningbo Medical Center Lihuili Hospital, Ningbo University, Ningbo, China.
Email: szs7216@163.com

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Abstract

Background: Increased research efforts have demonstrated that lncRNAs are associated with multiple head and neck tumors and play important roles in cancer. We previously found that RP11-169D4.1-001 plays a tumor-suppressive role in laryngeal cancer, but its function in human hypopharyngeal squamous cell carcinoma (HSCC) remains unknown. Thus, this research aimed to analyze the relationship between RP11-169D4.1-001 expression and HSCC clinicopathological features.

Methods: Real-time quantitative reverse transcription-polymerase chain reaction (qRT-PCR) was used to detect the expression of RP11-169D4.1-001 in 70 pairs of HSCC and adjacent normal tissues.

Results: The expression level of RP11-169D4.1-001 in HSCC tissues was significantly lower than that in adjacent normal tissues (P = .001). The expression of RP11-169D4.1-001 had no significant relationship with tumor differentiation, stage, smoking, drinking, age, tumor location, or treatment. RP11-169D4.1-001 expression was associated with T category (P = .008) and lymph node metastasis (P = .001). Survival data were assessed by Kaplan-Meier curves. Patients with high RP11-169D4.1-001 expression were found to have a shorter overall survival than patients with low RP11-169D4.1-001 expression. Multivariate analysis also indicated that target RNA was an independent factor for prognosis. The ROC curve was constructed to clarify the diagnostic value of RP11-169D4.1-001.

Conclusions: RP11-169D4.1-001 may serve as a new biomarker and potential drug target and can be used as a new biomarker and a potential drug target for the detection and treatment of hypopharyngeal cancer, respectively. Furthermore, RP11-169D4.1-001 expression may be an independent prognostic factor affecting the survival of hypopharyngeal cancer patients.

KEYWORDS
biomarker, diagnosis, HSCC, noncoding RNA, RP11-169D4.1-001

1 | INTRODUCTION

The long noncoding RNA (lncRNA) described herein, RP11-169D4.1-001, is expressed in eukaryotic cells, exceeds 200 nucleotides in length, and has no protein-coding functions.[1] In recent years, many experiments have proven that lncRNAs play important roles in tumor occurrence and progression. lncRNAs can be used for early tumor diagnosis, prognosis evaluation, and novel treatment, but their specific mechanism of action remains unclear.[2,3] Hypopharyngeal squamous cell carcinoma (HSCC) accounts for only 3%-5% of all head and neck malignancies,[4] and the annual incidence rates of HSCC are approximately 2 ~ 5/100 000 worldwide and approximately 2 ~ 4/100 000 in China. Among the malignant tumors diagnosed annually worldwide, 2.4% are hypopharyngeal cancer, and the incidence of hypopharyngeal cancer has increased in recent years.[5]

Clinically, squamous cell carcinoma is the main type of hypopharyngeal cancer, which has hidden sites of onset and poor biological characteristics and is prone to cervical lymph node metastasis. Despite advances in surgical and nonsurgical treatment, the overall fraction of hypopharyngeal cancer patients who survive has not been enhanced, and the illness is continually associated with a poor prognosis. In the initial treatment of hypopharyngeal carcinoma, 60%-80% of patients exhibit lymph node metastasis on the same side of the neck,[6] and 40% of patients exhibit contralateral neck lymph node metastasis.[7] The larynx and posterior ring space are easily invadable in the early disease stages, and hypopharyngeal carcinoma is often diagnosed in the late stages, a factor underlying the poor prognosis for head and neck cancer patients. The 5-year survival rate for these patients remains at approximately 40%.[8] Patients with this cancer often exhibit no specific symptoms in the early stages of disease, often reach the late stages of clinical treatment, and thus miss out on valuable treatment opportunities. Furthermore, because the larynx and pharynx play key roles in the functions of pronunciation, breathing, and eating, among other activities, surgical resection treatment will inevitably severely damage the abovementioned functions and greatly reduce the patient's quality of life.[9]

Currently, the role of lncRNAs in hypopharyngeal cancer is largely unknown. While how to diagnose hypopharyngeal cancer early and how to preserve these functions during treatment are concerns, specific early diagnostic markers and new treatments that can be applied clinically are lacking, and studying the occurrence and developmental mechanisms of laryngeal and hypopharyngeal cancer can provide a theoretical basis and a new direction for solving this problem. In this study, we further studied the expression of RP11-169D4.1-001 in hypopharyngeal cancer tissue. We aimed to analyze the relationship between RP11-169D4.1-001 expression and clinicopathological features to determine whether this lncRNA can serve as a biomarker of hypopharyngeal cancer.

2 | MATERIALS AND METHODS

2.1 | Patient sample collection

Seventy HSCC tissue samples and the corresponding matched normal tissues (dissected at >0.5 cm from the margin of the neoplastic lesion) were surgically resected from April 2010 to December 2015. All patients were from Ningbo Medical Center of Lihuili Hospital, and all hypopharyngeal carcinoma samples were independently examined by two or more pathologists to confirm cancerous and paratumoral normal tissues. Tumor stage was determined according to the American Joint Committee on Cancer (AJCC) tumor-node-metastasis (TNM) staging criteria. All specimens were harvested from males aged 42-82 years (median age, 61 years), and there were no females in this study. Before admission, none of the patients underwent biological treatment, radiotherapy, or chemotherapy; had a history of hepatitis B infection; or had a family history of hereditary disease. In addition, B-scan ultrasonography, computed tomography (CT), and other tests were used to confirm that no malignant tumors other than those of primary hypopharyngeal origin were present. Finally, the study adhered to the principle of informed consent and was approved by the Scientific Research Ethics Committee. Tumor specimens and adjacent normal tissues were immediately transferred to liquid nitrogen and stored in a −80°C cryogenic freezer upon dissection.

2.2 | Total RNA extraction

TRIzol reagent (Invitrogen) was used to extract total RNA from the HSCC and adjacent normal tissue samples, and a NanoDrop spectrophotometer and an Agilent 2100 Bioanalyzer (Agilent Technologies) were used to measure the RNA concentration and purity, respectively. The amount of RNA was calculated based on 1 $OD_{260\ nm}$ = 40 μg of RNA, and an A260/A280 ratio ranging from 1.8 to 2.1 was used to qualify RNA that could be used in subsequent experiments.[10]

2.3 | Quantitative real-time polymerase chain reaction

Sequences of the lncRNA and the housekeeping gene GAPDH were obtained from the NCBI database (http://www.ncbi.nlm.nih.gov/), and the corresponding PCR primers were prefabricated by Invitrogen. The primer sequences were as follows: GAPDH: sense, 5′-ACCCACTCCTC-CACCTTTGAC-3′, and antisense, 5′-TGTTGCTGTAGC-CAAATTCGTT-3′; and RP11-169D4.1-001: sense, 5′-TCTCACTAAGGTAGAACTGATGGGC-3′, and antisense, 5′-GACTCCTCAGGGAAAATGGAAACT-3′. Total RNA from the sample was reverse-transcribed into cDNA using the Promega GoScript Reverse Transcription (RT) System Kit, purchased from Invitrogen Corporation, according to the manufacturer's instructions. After reverse transcription, the cDNA was placed on ice and diluted with 80 μL of DEPC-H_2O; the sample was inverted and stored at −20°C.

The qPCR parameters for amplifying the lncRNA were as follows: 95°C for 10 minutes, followed by 45 cycles of 95°C for 15 seconds, 56°C for 30 seconds, and 72°C for 30 seconds. Reactions were performed according to standard protocol using GoTaq qPCR Master Mix (Promega) on a Stratagene M×3005P Real-time PCR machine. The threshold cycle (Ct) is used to determine the number of PCR cycles when a particular amplification threshold is reached and is used to reflect the amount of template. We used the housekeeping gene GAPDH to normalize the level of lncRNA. The ΔCt method was used to quantitatively analyze the level of lncRNA by ΔCt = Ct (target lncRNA) - Ct (GAPDH). The ΔCt value is one of the detection results. The $2^{-\Delta\Delta Ct}$ method was used to calculate the relative expression of RP11-169D4.1-001. Each sample was analyzed in biological triplicate.

2.4 | Statistical analysis

The data were analyzed with SPSS software 18.0 (SPSS Inc.). Values of $P < .05$ were deemed statistically significant. The paired-sample t test was used to assess differences between two experimental groups. The relationships between clinicopathological characteristics (age, primary location, differentiation type, clinical stage, smoking history, etc) and RP11-169D4.1-001 were analyzed by the chi-square test or Fisher's exact test, and the data in the study are expressed as the means ± standard deviations to determine high and low expression levels based on the mean of the HSCC tissue expression. The Kaplan-Meier method was used to create survival curves for patients with high or low RP11-169D4.1-001 expression and used multivariate regression analysis to define the determinating factors for prognosis. Diagnostic value was determined by an evaluation of receiver operating characteristic (ROC) curves.

3 | RESULTS

3.1 | RP11-169D4.1-001 expression levels in hypopharyngeal carcinoma and adjacent normal tissues

In total, 70 male patients were enrolled in the study. None of the 70 patients diagnosed with squamous cell cancer included in this analysis exhibited distant metastasis, but there may have been cervical lymph node metastasis. Furthermore, 13 patients were nonsmokers, while 57 patients had a history of smoking, and all patients were diagnosed for the first time. The expression of RP11-169D4.1-001 was significantly lower in the tumor samples compared to that in adjacent noncancerous mucosal samples ($P = .001$; Figure 1).

3.2 | Relationship between the expression level of RP11-169D4.1-001 and clinicopathological factors in patients with hypopharyngeal carcinoma

As shown in Table 1, the expression of RP11-169D4.1-001 had no significant relationship with tumor differentiation, stage, smoking, drinking, age, tumor location, or treatment. RP11-169D4.1-001

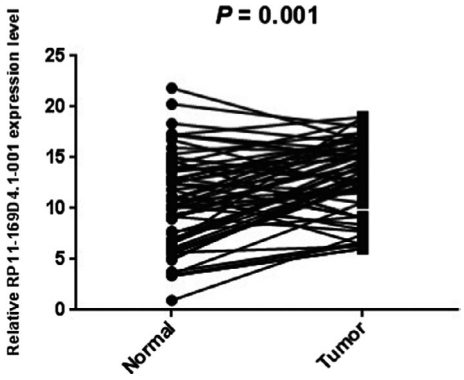

FIGURE 1 RP11-169D4.1-001 was expressed at significantly lower levels in tumor tissues than in matched normal mucosal tissues (n = 70, $P = .001$)

TABLE 1 Associations between RP11-169D4.1-001 expression and HSCC patient clinicopathological characteristics

		RP11-169D4.1-001 Expression Level		
Characteristics	**Cases**	**Low (%)**	**High (%)**	***P* value**
Age (y)				
<60	43	36	7	.534
≥60	27	21	6	
Primary location				
Pyriform sinuses	51	42	9	.739
Else	19	15	4	
Differentiation				
Well and moderate	46	36	10	.520
Poor	24	21	3	
Clinical stage				
I-II	25	20	5	.819
III-IV	45	37	8	
Smoking history				
Yes	57	45	12	.437
No	13	12	1	
Drinking history				
Yes	52	42	10	1.000
No	18	15	3	
T category				
T1-T2	45	37	8	.008
T3-T4	25	20	5	
Lymph node metastasis				
N0	18	10	8	.001
N+	52	47	5	
Treatment				
Only surgery	8	5	3	.161
Other	62	52	10	

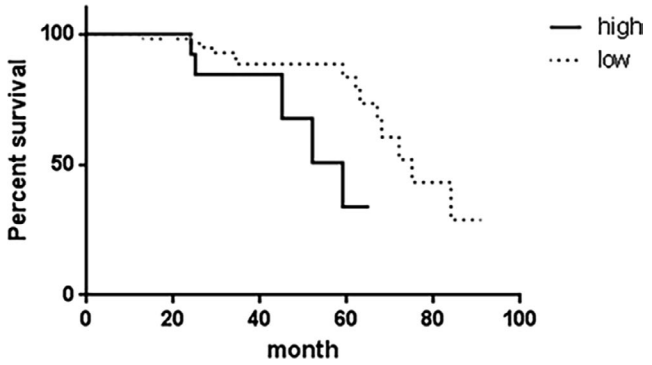

FIGURE 2 Kaplan-Meier analysis showed that patients with high RP11-169D4.1-001 expression (n = 13) had a shorter overall survival than patients with low RP11-169D4.1-001 expression (n = 57)

expression was associated with T category (P = .008) and lymph node metastasis (P = .001).

3.3 | Relationship between RP11-169D4.1-001 expression level and survival time

Kaplan-Meier analysis demonstrated that patients with high RP11-169D4.1-001 expression had a shorter overall survival than patients with low RP11-169D4.1-001 expression (Figure 2). A log-rank test confirmed that the results were statistically significant (P < .05). Importantly, multivariate analysis also indicated that RP11-169D4.1-001 was an independent factor for prognosis (Table 2). The results indicate that the expression of RP11-169D4.1-001 may be involved in the development of HSCC and may affect patient survival.

3.4 | Diagnostic efficiency of the target gene

Receiver operating characteristic curves were constructed using paracancerous tissues as controls (Figure 3). The area under the RP11-169D4.1-001 curve was 0.66 (95% CI = 0.568 ~ 0.749, P < .05), the cutoff point was ΔCt = 13.82, and the sensitivity and specificity were 0.54 and 0.7, respectively.

4 | DISCUSSION

This research aimed to analyze the relationship between RP11-169D4.1-001 expression and HSCC clinicopathological features. We used q-RTPCR to detect the expression of RP11-169D4.1-001 in 70 pairs of HSCC and adjacent normal tissues. We found that the expression level of RP11-169D4.1-001 in HSCC tissues was significantly lower than that in adjacent normal tissues. A variety of treatments for hypopharyngeal or laryngeal tumors have been developed and compared, including surgery, radiotherapy (CRT), radiation therapy (RT), or a combination of these various treatments.[11] In this study, the patients underwent the most appropriate surgical procedure, 8 patients underwent only surgery, and 62 patients were supplemented with radiotherapy and/or radiotherapy. The expression of RP11-169D4.1-001 had no significant relationship with tumor differentiation, stage, smoking, drinking, age, tumor location, and treatment. RP11-169D4.1-001 expression was associated with T category and lymph node metastasis. The survival time of 70 patients was based on the time of first diagnosis. The end of the follow-up period was June 30, 2018. Kaplan-Meier analysis showed that patients with high RP11-169D4.1-001 expression had shorter survival rates than patients with low RP11-169D4.1-001 expression. Sensitivity reflected the true-positive rate, while specificity reflected the true-negative rate. The closer AUC was to 1, the better the diagnostic effect; AUC had a lower accuracy at 0.5 ~ 0.7; a certain accuracy at 0.7 ~ 0.9; and a higher accuracy above 0.9.[12] Through ROC curve analysis, we found that RP11-169D4.1-001 had a lower diagnostic value and lower sensitivity and specificity in HSCC. Of course, this finding may be due to the limited number of samples studied, and we are not completely sure that RP11-169D4.1-001 has diagnostic value in HSCC. Studies have shown that combined

TABLE 2 Multivariate analysis of prognostic factors for overall survival in HSCC patients

Variables	Hazard ratio	95% CI	*P* value
Age	0.991	0.926-1.061	.804
Primary location (pyriform sinuses/other)	0.879	0.280-2.757	.825
Differentiation (poor/well & moderate)	0.900	0.237-3.424	.878
Clinical stage (I-II/III-IV)	1.032	0.357-2.980	.954
Smoking history (no/yes)	1.650	0.346-7.873	.530
Drinking history (no/yes)	0.989	0.250-3.918	.988
T category (T1-T2/ T3-T4)	0.695	0.232-2.082	.516
Lymph node metastasis (no/yes)	1.805	0.482-6.765	.381
Treatment (only surgery/else)	1.265	0.238-6.732	.783
RP11-169D4.1-001 levels (high/low)	0.258	0.071-0.946	.41

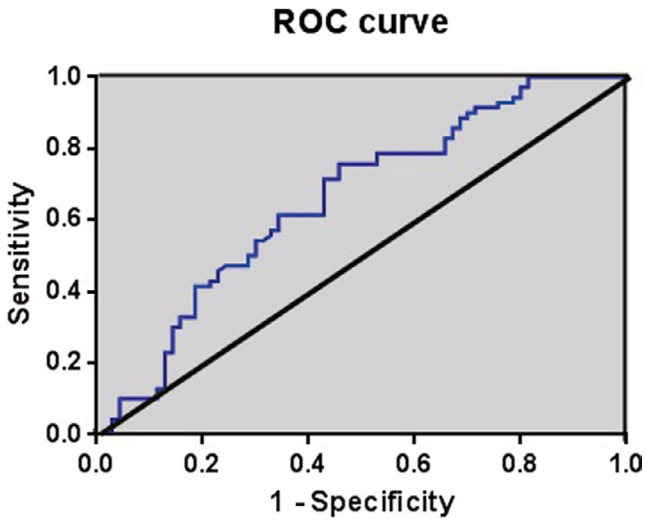

FIGURE 3 ROC curve analysis

biomarkers can improve the diagnostic accuracy of cancer.[13-15] Our next experiment can consider combining RP11-169D4.1-001 with other biological indicators to improve the diagnosis rate of hypopharyngeal cancer. The maximum Youden index was used as a cutoff point. In our study, the cutoff point was ΔCt = 13.82. Based on the results of the study, we can conclude that when the value of RP11-169D4.1-001 in the tissue is low than 13.82, we consider that it may have hypopharyngeal cancer.

Head and neck malignancies represent the sixth most common malignancy worldwide,[16] and laryngeal and hypopharyngeal cancers are among the most common head and neck cancers.[17,18] While how to diagnose hypopharyngeal cancer early and how to preserve these functions during treatment are concerns, specific early diagnostic markers and new treatments that can be applied clinically are lacking, and studying the occurrence and developmental mechanisms of laryngeal and hypopharyngeal cancer can provide a theoretical basis and a new direction for solving this problem. The role of lncRNAs in the development of head and neck malignancies is currently receiving increasing attention.[19,20] We previously found that HOTAIR, NEAT1, AB209630, and other lncRNAs can be used as important prognostic markers for laryngeal carcinomas and play important roles in the multiplication, metastasis, and invasive mechanisms of tumors.[21-23] However, few studies on lncRNAs associated with hypopharyngeal cancer have been published, and the specific roles of these lncRNAs and the signaling pathways involved are unclear.

Cervical lymph node drainage is abundant, and different primary tumors in the head and neck are prone to cervical lymph node metastasis. Due to the unique lymphatic and vascular anatomy of hypopharyngeal carcinoma, the tumor is prone to cervical lymph nodes and distant metastasis and mainly invades the cervical lymph nodes (II-IV area) and the posterior pharyngeal lymph nodes.[24-27] Many studies have found that cervical lymph node metastasis is a major factor affecting the prognosis of patients with hypopharyngeal carcinoma.[28-30] Therefore, the treatment of hypopharyngeal cancer must address the underlying cervical lymph node spread and early primary tumors. In the next study, we can confirm our findings with new patients and relevant preoperative and postoperative blood samples can be collected.

RP11-169D4.1-001 can be found in the Ensembl database under database number ENST00000450804. The expression and function of RP11-169D4.1-001 in laryngeal carcinoma have been studied.[21] ChIP expression profile analysis showed that RP11-169D4.1-001 was expressed at a 5.26-fold higher level in laryngeal cancer tissue than in normal tissue (P = .002).[21] Shen[21] found the expression of RP11-169D4.1-001 in 88 laryngeal cancerous tissue specimens to be significantly lower than that in adjacent noncancerous tissue samples (P < .001) and to be significantly lower in metastatic lymph nodes than in nonmetastatic lymph nodes (P < .05). In this study, we analyzed the clinical features of 70 patients with hypopharyngeal carcinoma and the expression of RP11-169D4.1-001 in tissue samples from these patients. Previous studies have shown that smoking is a risk factor for upper gastrointestinal cancers, including laryngeal, hypopharyngeal, and esophageal cancers.[31] Therefore, the current study specifically studied the relationship between RP11-169D4.1-001 expression and cigarette smoking, revealing that this association was not statistically significant (P = .437). Thus, we surmise that silencing RP11-169D4.1-001 expression may downregulate the body's tumor suppressor signaling pathway, which promotes the progression of laryngeal and hypopharyngeal cancer, and hypothesize that overexpressing RP11-169D4.1-001 may help inhibit tumor progression. However, whether this lncRNA can be used as an important marker for the follow-up monitoring of patients with hypopharyngeal cancer requires further study. In addition, further exploring the specific role and mechanism of RP11-169D4.1-001 in the development of hypopharyngeal carcinogenesis is still necessary to provide novel diagnostic and treatment strategies for hypopharyngeal cancer.

5 | CONCLUSION

High RP11-169D4.1-001 expression is associated with the risk and prognosis of HSCC; therefore, this lncRNA may be a biomarker for assessing HSCC.

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ETHICAL APPROVAL

Experimental procedures were reviewed and approved by the Ethics Committee of Ningbo Lihuili Hospital. All participants signed written informed consent documents.

ORCID

Zhisen Shen https://orcid.org/0000-0001-6660-0488

Chongchang Zhou https://orcid.org/0000-0002-8728-6819

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