ORIGINAL ARTICLE

TMPO-AS1 is an independent prognostic factor for patients with laryngeal squamous cell carcinoma

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SUMMARY

OBJECTIVE: *Long noncoding RNA (lncRNAs) are frequently abnormally expressed in tumors and involved in the occurrence and progression of human cancer. Recently, a disease-related lncRNA, TMPO antisense RNA 1 (TMPO-AS1), was identified to be dysregulated in several tumors. Hence, we aimed to demonstrate whether TMPO-AS1 could be a promising prognostic marker for patients with laryngeal squamous cell carcinoma (LSCC).*

METHODS: *RT-PCR was performed to test TMPO-AS1 expressions in 187 LSCC specimens compared with matched normal specimens. Chi-squared tests were used to determine the associations between TMPO-AS1 expressions and the clinicopathological characteristics of LSCC patients. Then, the clinical outcome of LSCC patients who had lower or higher TMPO-AS1 expression was analyzed using Kaplan-Meier assays. Finally, a Cox proportional hazards model was carried out to evaluate the prognostic values of TMPO-AS1 and other clinical features.*

RESULTS: *We found that TMPO-AS1 was distinctly upregulated in human LSCC tissues compared with corresponding normal specimens ($p < 0.01$). Higher expressions of TMPO-AS1 were observed to be positively associated with the clinical stage ($p = 0.020$) and lymph node metastasis ($p = 0.027$). A clinical study in 187 patients revealed that patients with TMPO-AS1 low expressions had poorer survival than those with TMPO-AS1 high expressions ($p = 0.0012$). In addition, the result of multivariate assays demonstrated TMPO-AS1 expression is an independent predictor for the overall survival of LSCC patients.*

CONCLUSIONS: *TMPO-AS1 might be considered a novel molecule involved in LSCC progression, which provides a possible prognostic biomarker.*

KEYWORDS: *Larynx. Carcinoma, squamous cell. Laryngeal neoplasms. Biomarkers. Gene expression regulation, neoplastic.*

INTRODUCTION

Laryngeal squamous cell carcinoma (LSCC) is one of the most common head and neck tumors in the world, accounting for > 15 % of head and neck squamous cell carcinoma[s1]. According to a tumor report, the incidence of LSCC in China, particularly in the Guangdong Province, has been rising gradually[2]. Up to date, the clinical application of surgery or radiotherapy has made early-stage LSCC curable. However, the five-year overall

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survival rates of most patients with advanced tumor remain unsatisfactory, despite therapeutic advances[3,4]. The high mortality rate of LSCC may be attributed to distant metastasis. Therefore, the identification of novel diagnostic and prognostic markers is urgently needed.

Long noncoding RNAs (lncRNAs) are a class of non-protein-coding RNAs longer than 210 nucleotides that are typically recognized as mRNAs-like transcripts[5]. Previously, most lncRNAs were originally considered transcriptional "noises," but growing research in epigenetics has confirmed they play crucial regulatory functions in various gene expressions[6,7]. In recent years, the potential function of lncRNAs as promoters or inhibitors in a wide variety of tumor processes, such as growth, apoptosis, and metastasis has been described[8,9]. For instance, lncRNA MIAT, a highly expressed lncRNA in gastric cancer, was shown to be positively associated with advanced TNM stages and distant metastasis and promote tumor-cells metastasis by modulating the miRNA-141/DDX5 axis[10]. LncRNA LOC554202, a well-studied lncRNA whose overexpression was frequently reported in several tumors, was suggested as an oncogenic factor in LSCC progression because its upregulation promoted LSCC cell growth and invasion via sponging miRNA-31[11]. Up to date, a large number of functional lncRNAs had been identified and their functions were also studied *in vitro* and *in vivo*[12]. However, only a few lncRNAs have been functionally characterized in LSCC.

TMPO antisense RNA 1 (TMPO-AS1), located at 12q23.1, was first identified as an abnormally expressed lncRNA in lung adenocarcinoma by Li et al.[13]. The potential of TMPO-AS1 as a possible prognostic biomarker was also preliminarily explored using bioinformatics analysis. Then, functional assays by Qin et al.[14] confirmed TMPO-AS1 acted as a tumor promoter in lung cancer. Recently, TMPO-AS1 was also demonstrated to be overexpressed in prostate cancer and predicted advanced clinical stages as well as poor clinical outcome[15]. However, the expression and effects of TMPO-AS1 in other tumors remain unknown. In this study, we asked whether there were abnormalities of this lncRNA in LSCC.

METHODS

Patients and Specimens

LSCC tissues and adjacent normal tissues from 187 patients with LSCC who had undergone medicinal resection were collected between 2011 and 2014 from the Taian City Central Hospital. All of the specimens were checked by two pathologists. All specimens were obtained at the time of surgery and immediately snap-frozen in liquid for the subsequent experiments. None of these patients underwent local or systemic therapies before the operations. This study was approved by the Medical Ethics Committee of the Taian City Central Hospital and informed consent was obtained from all participants. The demographic and clinicopathological data are listed in Table 1.

Quantitative PCR analysis

For the extraction of total RNA from LSCC specimens and normal samples, Trizol reagent was purchased from Life Technologies (Haidian, Beijing, China) and used based on the company's protocol. The synthesization of cDNA was performed using the RevertAid First Strand cDNA Synthesis Kit (Thermo Scientific, Pudong, Shanghai, China). The levels of lncRNA were examined by applying qRT-PCR, which was performed using Power SYBR Green PCR Master Mix (Biosystems, Nanjing, Jiangsu, China). The RT-PCR assays were carried out for 40 cycles with the successive arrangements: 94°C for 10 min, 55°C for 30 s, and 72°C for 20 s. GAPDH was applied as

TABLE 1. RELATIONSHIP BETWEEN LNCRNA TMPO-AS1 EXPRESSION LEVELS AND CLINICOPATHOLOGICAL PARAMETERS OF LSCC PATIENTS.

Clinicopathological features	Number of cases	TMPO-AS1 expression		p
		High	Low	
Age				0.270
<55 years	96	45	51	
≥55 years	91	50	41	
Gender				0.437
Male	125	61	64	
Female	62	34	28	
Tobacco exposure				0.127
Smoker	100	56	44	
Nonsmoker	87	39	48	
Differentiation				0.304
Well	115	55	60	
Moderately/poorly	72	40	32	
Clinical stage				0.020
I/II	125	56	69	
III/IV	62	39	23	
Lymph node metastasis				0.027
Negative	13 9	64	75	
Positive	48	31	17	

TABLE II. UNIVARIATE AND MULTIVARIATE ANALYSIS OF OVERALL SURVIVAL IN LSCC PATIENTS

Variable	Univariate analysis			Multivariate analysis		
	HR	95% CI	p	HR	95% CI	p
Age	1.786	0.662-2.433	0.155	-	-	-
Gender	1.553	0.754-2.412	0.118	-	-	-
Tobacco exposure	1.449	0.824-2.325	0.155	-	-	-
Differentiation	1.728	1.028-2.58	0.114	-	-	-
Clinical stage	2.892	1.375-5.018	0.011	2.683	1.217-4.653	0.021
Lymph node metastasis	3.127	1.472-5.213	0.008	2.884	1.215-4.886	0.013
TMPO-AS1 expression	3.035	1.365-4.735	0.013	2.885	1.217-4.357	0.017

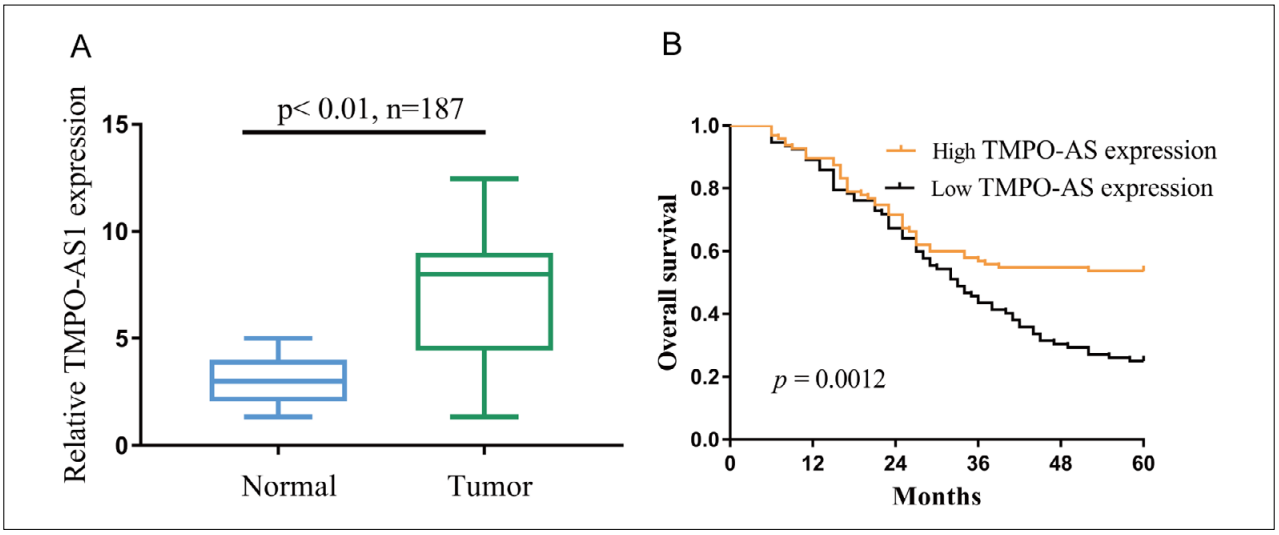

FIGURE 1. The associations between TMPO-AS1 levels and survival time in LSCC patients. (A) The expressions of TMPO-AS1 were determined by RT-PCR in 187 paired LSCC and adjacent normal tissues. (B) The associations between TMPO-AS1 levels and survival time in LSCC patients.

an endogenous control for the normalization of TMPO-AS1 expressions. The data were analyzed and expressed relative to the threshold cycle (CT) values. The primers' sequences for RT-PCR were as follows: TMPO-AS1, 5'- AGCCCACACACTACAGGCAG-3' (forward) and 5'- GCACAAAAGCAGTACGACCTA-3' (reverse); GAPDH, 5'- ACTCATGACCACAGTCCATGCC-3' (forward) and 5'- AGAGGCAGGGATGATGTTCTGA-3' (reverse).

Statistical analysis

The statistical assays were performed by SPSS software package (SPSS Inc., Chicago, IL, USA). Student's t-tests were used for the assays of the differences between the two groups. The correlations between clinicopathological parameters and TMPO-AS1 levels were determined using chi-square tests. Survival curves were plotted by the use of the Kaplan-Meier methods and possible differences in survival time were determined by the log-rank tests. The prognostic relevance of several variables to overall survival was analyzed using multivariate assays. A value of $p<0.05$ was considered statistically significant.

RESULTS

A significant upregulation of TMPO-AS1 was observed in LSCC tissues

To explore whether TMPO-AS1 was abnormally expressed in LSCC, our group used qRT-PCR to detect the TMPO-AS1 levels in 187 LSCC patients. As presented in Figure 1A, TMPO-AS1 was found to be distinctly higher in LSCC specimens compared with matched non-tumor samples ($p < 0.01$). Our data

suggested TMPO-AS1 as a new player that may display an oncogenic function in LSCC cells.

The correlation between TMPO-AS1 and clinical parameters of LSCC

Then, our group investigated the clinical significance of TMPO-AS1 in LSCC patients. The cutoff value, determined using the median expression of TMPO-AS1, was used to divide the 187 LSCC patients into two groups (High: n =95 and Low: n =92). The associations between TMPO-AS1 expressions and the clinical parameters of LSCC were summarized in Table 1; the results revealed that increased expressions of TMPO-AS1 positively correlated with the clinical stage (p = 0.020) and lymph node metastasis (p = 0.027), but not with age, gender, tobacco exposure, or differentiation (p >0.05). These observations revealed increased expressions of TMPO-AS1 predicted distinctly aggressive clinical features, suggesting that this lncRNA may promote LSCC progression.

Association of TMPO-AS1 expressions with patients' survival time

To further verify whether the overexpression of TMPO-AS1 had prognostic value in LSCC patients, our group performed a five-year follow-up analysis and collected clinical information on 5-year survivors from 122 to 187 patients. Then, Kaplan-Meier assays were applied and the results indicated that patients with higher TMPO-AS1 expressions had worse overall survival than those with lower TMPO-AS1 expressions (p= 0.0012, Figure 1B). Subsequently, univariate and multivariate analysis was used for further determining the practicability of TMPO-AS1 as a biomarker. In univariate assays, TMPO-AS1 expression, clinical stage, and lymph node metastasis were confirmed to be associated with the overall survival of LSCC patients (All p > 0.05). Moreover, in multivariate analysis, we confirmed high TMPO-AS1 expression (HR= 2.885, 95% CI: 1.217-4.357, p =0.017), together with clinical stage and lymph node metastasis, as an independent prognostic biomarker for LSCC patients.

DISCUSSIONS

In this study, for the first time, we provided strong evidence that TMPO-AS1 levels were frequently up-regulated in LSCC tissues, which suggested positive associations between the dysregulation of TMPO-AS1 and LSCC progression. Then, we analyzed whether higher levels of TMPO-AS1 were related to several clinical factors, finding that patients with upregulation of TMPO-AS1 exhibited advanced clinical stage and lymph node metastasis. It was known to us that most tumor patients with metastasis have a poor clinical outcome. Thus, we wondered whether TMPO-AS1 may influence the clinical prognosis of LSCC patients. As expected, in a clinical assay with 187 LSCC patients, we observed that in LSCC patients with increased TMPO-AS1 expressions. For further study of the clinical application of TMPO-AS1 as a prognostic biomarker, multivariate analysis was then performed and the results confirmed that TMPO-AS1 served as an independent biomarker for predicting the clinical outcome of LSCC patients.

LSCC is one of the most common malignant neoplasms. In order to improve the clinical prognosis of this tumor, sensitive biomarkers need to be identified that can be used to predict how well the body responds to a therapeutic schedule[16,17]. In recent years, critical molecular events during LSCC progression have been identified due to the development of genomics and proteomics[18]. These advances have resulted in the disclosure of new LSCC biomarkers, such as mRNAs, proteins, and ncRNAs[19-21]. These diverse markers could be detectable in plasma, marrow, and tumor specimens. Importantly, lncRNAs are considered to be ideal biomarkers for diagnosing LSCC and predicting prognosis because they are easy to detect, stable, and positively correlated with frequent tumor metastasis and clinical outcome[22].

To our best knowledge, this is the first time the possible prognostic value of TMPO-AS1 in LSCC patients has been reported. Several limitations of our experiments should be considered. Firstly, there was a relatively small sample size enrolled, which may result in statistical discrepancy. Secondly, our findings suggested TMPO-AS as a tumor promoter in LSCC. However, due to the funds and time available, functional explorations using *in vitro* and *in vivo* assays were not performed in the current study. Thirdly, the potential mechanism involved in the advanced progression of LSCC mediated by TMPO-AS1 overexpression needs to be explored.

CONCLUSIONS

Our findings highlighted the great value of TMPO-AS as a novel marker and possible therapeutic target.

Conflict of interest

The authors declare no conflicts of interest.

Author Contributions

All authors contributed to data analysis, drafting and revising the article, gave final approval of the version to be published, and agree to be accountable for all aspects of the work.

Lihua Zhang and Yu Zhang contributed the same to this article.

RESUMO

OBJETIVO: *RNAs longos não-codificantes (INcRNAs) são frequentemente expressos anormalmente em tumores e estão envolvidos na ocorrência e progressão do câncer humano. Recentemente, um INcRNA relacionado com a doença, o TMPO antisense RNA 1 (TMPO-AS1), foi identificado como desregulado em vários tumores. Por isso, procuramos demonstrar se o TMPO-AS1 poderia ser um marcador de prognóstico promissor para pacientes com carcinoma de células escamosas da laringe (LSCC).*

MÉTODOS: *RT-PCR foi realizado para medir as expressões do TMPO-AS1 em 187 espécimes de LSCC em comparação com espécimes normais correspondentes. Foram utilizados testes Qui-quadrado para determinar as associações entre as expressões do TMPO-AS1 e as características clínicas dos pacientes com LSCC. Em seguida, o desfecho clínico dos pacientes com LSCC que tinham uma expressão do TMPO-AS1 inferior ou superior foi analisado com ensaios Kaplan-Meier. Por último, o modelo de riscos proporcionais de Cox foi utilizado para avaliar o valor prognóstico do TMPO-AS1 e outras características clínicas.*

RESULTADOS: *Observamos que o TMPO-AS1 estava claramente super-regulado nos tecidos de LSCC humanos em comparação com os espécimes normais correspondentes (p<0,01). Expressões mais elevadas de TMPO-AS1 estavam positivamente associadas ao estágio clínico (p=0,020) e à metástase linfática (p=0,027). Um estudo clínico com 187 pacientes revelou que aqueles com expressões mais baixas de TMPO-AS1 tiveram uma sobrevida pior do que aqueles com expressões elevadas de TMPO-AS1 (p=0,0012). Além disso, o resultado de ensaios multivariados demonstrou que a expressão do TMPO-AS1 é um preditor independente para a sobrevida global de pacientes com LSCC.*

CONCLUSÕES: *TMPO-AS1 pode ser considerado uma molécula nova envolvida na progressão do LSCC, o que proporciona um possível biomarcador de prognóstico.*

PALAVRAS-CHAVE: *Laringe. Carcinoma de células escamosas. Neoplasias laríngeas. Biomarcadores. Regulação neoplásica da expressão gênica.*

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