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RESEARCH ARTICLE

Long non-coding RNA HOTTIP is correlated with progression and prognosis in tongue squamous cell carcinoma

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Abstract Long non-coding RNAs (lncRNAs) have been demonstrated to be a critical role in cancer progression and prognosis. However, little is known about the pathological role of lncRNA HOXA transcript at the distal tip (HOTTIP) in tongue squamous cell carcinoma (TSCC) patients. The aim of this study is to measure the expression of lncRNA HOTTIP in TSCC patients and to explore the clinical significance of the lncRNA HOTTIP. The expression of lncRNA HOTTIP was measured in 86 TSCC tissues and 14 adjacent non-malignant tissues using qRT-PCR. In our study, results indicated that lncRNA HOTTIP was highly expressed in TSCC compared with adjacent non-malignant tissues ($P<0.001$) and positively correlated with T stage (T1–2 vs. T3–4, $P=0.023$), clinical stage (I–II stages vs. III–IV stages, $P=0.018$), and distant metastasis (absent vs. present, $P=0.031$) in TSCC patients. Furthermore, we also found that lncRNA HOTTIP overexpression was an unfavorable prognostic factor in TSCC patients ($P<0.001$), regardless of T stage, distant metastasis, and clinical stage. Finally, overexpression of lncRNA HOTTIP was supposed to be an independent poor prognostic factor for TSCC patients through multivariate analysis ($P=0.023$). In conclusion, increased lncRNA HOTTIP expression may be serve as an unfavorable prognosis predictor for TSCC patients. Nevertheless, further investigation with a larger sample size is needed to support our results.

Keywords lncRNA · HOTTIP · Tongue squamous cell carcinoma · Prognosis

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Introduction

Tongue cancer is one of the most common oral cancers, especially in users of chewable tobacco and alcohol. In the USA alone, 13,590 new cases and 2150 deaths has been estimated from tongue cancer in 2014 [1]. Tongue squamous cell carcinoma (TSCC) is an aggressive malignance with rapid growth rate and high chance of regional and distant metastasis. Despite the great advances achieved in surgery and in chemoradiotherapy technology recently, patients who have had TSCC have a high risk of developing secondary or recurrent tumors in the surrounding area. Moreover, in patients with lymph nodes spread, the 5-year overall survival rate does not exceed 50 % [2, 3]. Therefore, cancer screening and early detection have major importance in the survival of TSCC patients. Identification of novel and improved markers is of great clinical value for the diagnosis and treatment of TSCC.

In recent years, many studies highlighted the role of a number of long non-coding RNAs (lncRNAs) in carcinogenesis and suggested that these genes might be used as biomarkers in cancer [4–6]. lncRNA refers to RNA molecules with size over 200 bp long and without protein coding functions [7], which is

gaining prominence because of their emerging roles in the regulation of critical cellular functions, including transcriptional, posttranscriptional, and epigenetic mechanisms of gene regulation [4, 8–10]. The HOXA transcript at the distal tip (HOTTIP) lncRNA, located at the 5′ end of the HOXA cluster, was recently functionally characterized [11]. Recently, it was reported by Quagliata et al. that HOTTIP is a negative prognostic factor in hepatocellular carcinoma (HCC) patients, and increased HOTTIP expression was associated with enhanced HCC metastasis [12]. In addition, Jiang and his colleagues reported that HOTTIP overexpression is involved in the formation of chemical and ultraviolet radiation-induced skin cancer [13]. To date, however, little is known about the significance of HOTTIP expression and TSCC prognosis.

In the present study, we investigated the expression level of lncRNA HOTTIP in human TSCC tissues and then explored the association between HOTTIP expression and clinicopathological characteristics. Our results suggest that HOTTIP may represent a novel indicator of poor prognosis and may be a potential target for the diagnosis and gene therapy of TSCCs.

Materials and methods

Sample collection

86 freshly frozen tongue cancer samples and 14 adjacent non-malignant samples were obtained from the Department of Stomatology, The First Affiliated Hospital of Jinan University, between May 2009 and August 2012. All samples had been collected before any kind of therapeutic measures, and fresh samples were immediately preserved in liquid nitrogen. None of the patients received treatment prior to radical surgical treatment. The median duration of follow-up time was 38 months (range, 23–60 months). A written informed consent was obtained from all participants. The study protocol was approved by the Ethics Committee of the First Affiliated Hospital of Jinan University in accordance with the Declaration of Helsinki (2000). The histopathological diagnosis of all samples was, respectively, diagnosed by two pathologists. The clinical staging was based on the 7th edition of the AJCC Cancer Staging Manual.

Quantitative real-time PCR

Expressions of lncRNA HOTTIP in TSCC and non-malignant tissues were detected. Total RNA was extracted from cells using Trizol reagent (Invitrogen, San Diego, CA, USA) according to the manufacturer's protocol. The quantitative real-time PCR (qRT-PCR) was carried out using a Roche LightCycler (Roche, Basel, Switzerland) and SYBR Green reaction mix (Qiagen, Germany) to detect the level of lncRNA HOTTIP, with β-actin as a normalizing control. The PCR primers for lncRNA HOTTIP or β-actin were as follows: lncRNA HOTTIP forward: 5′-GTGGGGCCCAGACCCGC-3′; lncRNA HOTTIP reverse: 5′-AATGATAGGGACACAT CGGGGAACT-3′; β-actin forward: 5′-GAAATCGTGCGT GACATTAA-3′; β-actin reverse: 5′-AAGGAAGGCTGG AAGAGTG-3′. The relative expression of lncRNA HOTTIP was calculated and normalized using the delta-delta CT ($2^{-\Delta\Delta Ct}$) method relative to β-actin. Independent experiments were done in triplicate.

Statistical analysis

Statistical analyses were performed using SPSS Statistics 13.0 (IBM Chicago, IL, USA). The unpaired *t* test was applied to test the differential expression of lncRNA HOTTIP in cancer tissues compared to adjacent non-malignant tissues. The chi-square test was applied to the examination of the relationship between lncRNA HOTTIP expression levels and clinicopathological characteristics. Overall survival was defined as the interval from the date of diagnosis to tongue cancer-related death. Survival curves were plotted using the Kaplan-Meier method and the log-rank test. The significance of survival variables was analyzed using the Cox multivariate proportional hazards model. *P* value of less than 0.05 was considered statistically significant.

Results

LncRNA HOTTIP is highly expressed in TSCC

In order to assess the role of lncRNA HOTTIP in TSCC, we performed qRT-PCR to examine the status of lncRNA HOTTIP expression in all 86 tongue cancer samples and 14 adjacent non-malignant samples. Compared with 14 adjacent non-malignant tissues in matched pair study, 14 tongue cancer tissues showed increased expression levels of lncRNA HOTTIP ($P<0.001$, Fig. 1).

Relationship between lncRNA HOTTIP expression and clinicopathological characteristics in TSCC patients

In the 86 TSCC cases, there were 52 males and 34 females with age ranging from 26 to 73 years. We further investigated the association between lncRNA HOTTIP expression and clinicopathological characteristics of TSCC patients. Based on a previous study [14], TSCC tissue samples were classified into low-expression group ($n=42$) and high-expression group ($n=44$), according to the median expression level of all TSCC samples (median ΔCT value 7.04). The association between lncRNA HOTTIP expression levels and clinicopathological characteristics in patients with TSCC was showed in Table 1.

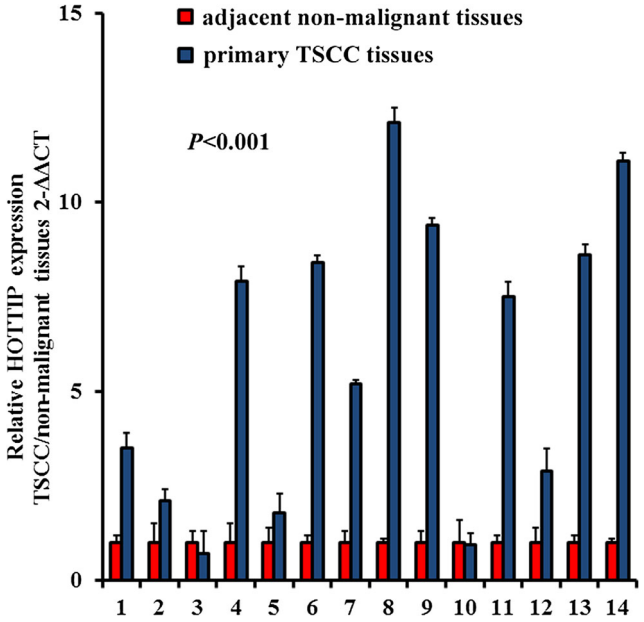

Fig. 1 Expression of lncRNA HOTTIP is increased in TSCC tissues compared with non-malignant tissues through qRT-PCR

Overall, no statistically significant association was observed between lncRNA HOTTIP expression levels and patient's age, gender, smoking status, drinking status, and differentiation (P=0.984, 0.538, 0.266, 0.658 and 0.620, respectively). Although high lncRNA HOTTIP expression was more common in advanced nodal stage patients compared with low lncRNA HOTTIP expression cases (28/44 vs. 18/42), this result was not statistically significant (P=0.053). However, lncRNA HOTTIP was positively associated with clinical stage (P=0.018), T stage (P=0.023), and distant metastasis status (P=0.031) in TSCC patients.

LncRNA HOTTIP expression is associated with overall survival in TSCC patients

In order to assess the prognostic value of lncRNA HOTTIP expression for TSCC, we investigated the association between lncRNA HOTTIP expression levels and overall survival (OS) through Kaplan-Meier analysis and log-rank test. In 86 TSCC cases, we observed that lncRNA HOTTIP expression was significantly associated with TSCC patients' OS (P<0.001, Fig. 2). Moreover, we also observed that lncRNA HOTTIP overexpression was an unfavorable prognostic factor in TSCC patients (P<0.001, Table 2), regardless of T stage, distant metastasis, and clinical stage. Finally, multivariate analysis showed that increased lncRNA HOTTIP expression was an independent poor prognostic factor for TSCC patients (P=0.023, Table 2).

Table 1 Associations between lncRNA HOTTIP expression and clinicopathological characteristics in TSCC

Characteristics	Number	High expression	Low expression	P
Age (year)				
<60	47	24	23	0.984
≥60	39	20	19	
Gender				
Female	34	16	18	0.538
Male	52	28	24	
Smoker				
Yes	48	22	26	0.266
No	38	22	16	
Drinker				
Yes	37	18	19	0.685
No	49	26	23	
T stage				
T1–2	53	24	29	0.023
T3–4	33	20	13	
Nodal stage				
Negative	40	16	24	0.053
Positive	46	28	18	
Distant metastasis				
Absent	78	37	41	0.031
Present	8	7	1	
Clinical stage				
I–II	38	14	24	0.018
III–IV	48	30	18	
Differentiation				
Well	35	17	18	0.620
Moderate	39	22	17	
Poor	12	5	7	

Discussion

As previous studies reported, at least 70–90 % genomic DNAs are transcribed to the RNAs that do not produce any proteins. These parts of the genomes are known as non-coding RNA (ncRNA) genes, which produce efficient RNA molecules [15–17]. LncRNAs are ncRNA transcripts longer than 200 nucleotides (nt) that are transcribed from various genomic locations, such as in the promoters, enhancers, introns, or antisense coding regions of genes, or in their own stand-alone position in the genome [18]. Based on their roles, the dysregulation of lncRNAs is involved in several diseases including cancer [19, 20]. In the present study, we examined the expression of LncRNA HOTTIP and its clinicopathological/prognostic significance in 86 specimens of primary TSCCs and 14 samples of adjacent non-malignant samples.

The key feature of all ncRNAs is that they are not translated into proteins but rather function directly at the RNA level [21,

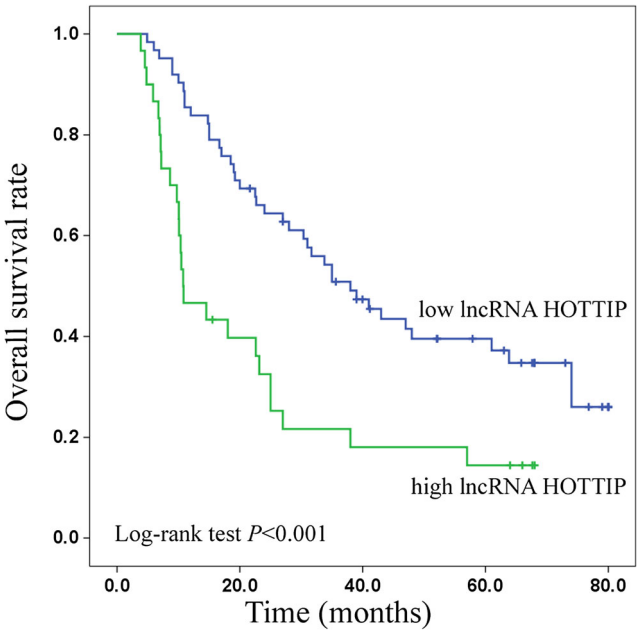

Fig. 2 Increased lncRNA HOTTIP expression predicts a poor prognosis in TSCC patients

22]. A previous report using serial analysis of gene expression (SAGE) showed that about 60 % of the detected lncRNAs have aberrant expression in oral premalignant lesions [23]. In a recent study, Fang and his colleagues investigated the expression levels of several cancer-related lncRNAs in TSCC [24]. They reported that the expression levels of lncRNA UCA1 were significantly enhanced in TSCCs and were correlated with tumor lymph node metastasis than in paired primary tumors. Moreover, in one of Gao et al' studies, eight differentially expressed lncRNAs were identified in TSCC patients [25]. Their results showed that overexpression of lnc-MBL2-4:3 was significantly associated with the node metastasis. Further, patients with advanced T stage demonstrated a further reduction of lnc-AL355149.1-1 in the tumor tissues, and changes of lnc-MBL2-4:3 and lnc-AL355149.1-1 expression levels were noticed in the cisplatin-resistant TSCC cells.

HOTTIP, a novel lncRNA, which is located at the 5′ tip of the HOXA locus and coordinates the activation of multiple 5′ HOXA genes in vivo [11], has been identified as one of 231 lncRNAs associated with the human HOX loci [26]. In one previous study, Tsang and his colleagues reported that knockdown of HOTTIP attenuated hepatocellular carcinoma (HCC) cell proliferation in vitro and markedly abrogated tumorigenicity in vivo. In addition, knockdown of HOTTIP also inhibited migratory ability of HCC cells and significantly abrogated lung metastasis in mouse xenograft mode [27]. A similar trend was seen in our study. We also found that lncRNA HOTTIP was highly expressed in 14 TSCC tissues compared with non-malignant tissues. Furthermore, we analyzed the association between the expression of lncRNA HOTTIP and clinicopathological characteristics in 86 TSCC patients. We found that lncRNA HOTTIP was positively associated with clinical stage, tumor size, and distant metastasis in TSCC patients.

In conclusion, our findings indicated that the expression level of HOTTIP has the potential to be an independent unfavorable prognostic indicator for TSCC patients. Despite this, our investigation has some limitations that should be pointed

Table 2 Univariate and multivariate Cox regression of prognostic factors for overall survival in pancreatic cancer

Parameter	Univariate analysis			Multivariate analysis		
	HR	95 % CI	*P*	HR	95 % CI	*P*
Age (year)						
<60 vs. ≥60	0.793	0.517–1.335	0.691			
Gender						
Female vs. male	1.257	0.685–1.769	0.460			
Differentiation						
Well vs. moderate vs. poor	1.132	0.738–1.428	0.592			
T stage						
T1–2 vs. T3–4	3.362	1.584–6.192	0.001	2.482	1.236–3.846	0.035
Nodal stage						
Negative vs. positive	2.739	1.482–4.279	0.007	1.207	0.638–2.931	0.228
Distant metastasis						
Absent vs. present	3.538	1.579–7.472	0.003	1.726	0.587–3.495	0.120
Clinical stage						
I–II vs. III–IV	3.511	1.549–5.208	0.005	1.631	0.627–2.752	0.162
LncRNA HOTTIP						
Low vs. high	2.964	1.357–4.789	0.001	2.113	1.062–3.115	0.023

HR hazard ratio, *95 % CI* 95 % confidence interval

out. Firstly, it is a retrospective study, conducted on a small population. Secondly, because methodology of this research is static, we do not know when lncRNA HOTTIP is expressed or how it relates to metastatic capacity. Therefore, the functional consequences of altered HOTTIP expression, the different feature of HOTTIP expression between TSCC and other malignancies, and the underlying mechanisms of the heterogeneous expression levels need to be extensively investigated in the future.

Conflicts of interest None

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