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Original Article

Decreased expression of lncRNA GAS5 predicts poor survival and aggressive phenotype in esophageal squamous cell carcinoma

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Abstract: lncRNA GAS5 has been found to be involved in tumor progression of many tumor entities. However, the implication of lncRNA GAS5 in esophageal squamous cell carcinoma (ESCC) is still undefined. The purpose of this study is to investigate the clinicopathological and prognostic role of lncRNA GAS5 in ESCC patients. The level of lncRNA GAS5 expression was mesured by real-time PCR (qRT-PCR) in 97 pairs of ESCC tissues and adjacent non-tumor tissues. The association between lncRNA GAS5 expression and clinicopathological parameters was evaluated. Overall survival was analyzed by using the Kaplan-Meier method with log-rank test. Independent prognostic factors were determined by multivariate analysis with the Cox proportional hazard model. The results showed that lncRNA GAS5 was significantly down-regulated in ESCC tissues than in adjacent non-tumor tissues. Moreover, lncRNA GAS5 expression level was markedly associated with tumor size, differentiation, lymph node metastasis and TNM stage. In addition, lncRNA GAS5 level was significantly correlated overall survival in ESCC patients. Patients with low lncRNA GAS5 expression had worse overall survival that those with high lncRNA GAS5 expression. Multivariate analysis demonstrated that lncRNA GAS5 level was an independent prognostic factor in ESCC patients. Ectopic expression of lncRNA GAS5 inhibited cell invasion in vitro as well as tumor metastasis in vivo. Taken together, our results revealed that lncRNA GAS5 plays an critical role in ESCC progression, and could be used as a potential molecular biomarker for predicting the outcome of patients.

Keywords: Long noncoding RNA, GAS5, ESCC, progression, prognosis

Introduction

Esophageal cancer is one of the most common human malignant diseases worldwide, especially in southern and eastern Africa, parts of south America, western and northern China, and Japan [1]. According to the histopathology, esophageal cancer can be classified into the esophageal squamous cell carcinoma (ESCC) and the adenocarcinoma [2]. The ESCC is the major type of esophageal cancers in China, which accounts for about 90% of all esophageal cancers [2, 3]. Although great progress has been made in multiple strategies of diagnosis and therapeutics in recent decades for this disease, the prognosis of ESCC patients is still very poor [4]. Therefore, it is urgently needed for us to identify novel biomarkers for early diagnosis and targeted treatment strategy so as to improve patient's outcome of ESCC.

With the development of recent genome sequencing, It has been found that the human genome is comprised of less than 2% protein coding genes while more than 90% of the genome is transcribed into non-coding RNAs (ncRNA) [5]. NcRNAs are generally divided into three categories, including housekeeping RNAs, small non-coding RNAs, and long non-coding RNAs [6]. Long non-coding RNAs (lncRNAs) are RNA molecules that longer than 200 nucleotides in length and are not translated into proteins [7]. More and more studies indicated that lncRNAs play important role in multiple biological processes, such as transcriptional regulation, cell growth and differentiation, cell invasion, tumorigenesis and tumor metastasis [5, 8, 9]. They also play critical roles in the development and progression of cancers [10]. lncRNAs have been found to be new tumor biomarkers for early cancer diag-

nosis and prognosis. Increasing studies have revealed a number of lncRNAs that are aberrantly expressed in different tumor types. For instance, Kogo et al. reported that HOTAIR expression was increased in colorectal cancer tissues than that in adjacent noncancerous tissues, and higher HOTAIR expression significantly correlated with the liver metastasis in colorectal cancer patients [11]. Lai et al. found higher expression level of MALAT1 in both hepatocellular carcinoma tissue samples and cell lines, and increased expression of MALAT1 associated with a significantly higher risk of tumor recurrence [12]. Yang et al. reported that lncRNA H19 was significantly upregulated in gastric cancer tissues and cells compared with normal controls. Moreover, ectopic expression of H19 could promote cell growth [13]. lncRNA GAS5 (growth arrest-specific transcript 5) is originally isolated from NIH 3T3 cells using subtraction hybridization [14]. This gene is located at 1q25, a chromosomal locus which has been associated with lymphoma [15]. Recent studies showed that lncRNA GAS5 played important role in several tumors. For example, Maarabouni et al. indicated that lncRNA GAS5 was significantly down-regulated in breast cancer samples compared to adjacent normal breast epithelial tissues, and had a critical role in mammalian apoptosis and cell population growth [16]. Sun et al. reported that the expression of lncRNA GAS5 was markedly decreased in gastric cancer, and low expression of lncRNA GAS5 was associated adverse disease-free survival and overall survival of patients with gastric cancer. In addition, ectopic expression of lncRNA GAS5 could inhibit gastric cancer cell proliferation and meanwhile induce apoptosis both in vitro and in vivo [17]. However, the clinical and prognostic significance of lncRNA GAS5 expression in ESCC has not been reported yet. In this study, we aimed to investigate the expression of lncRNA GAS5 in ESCC and further explore the clinical significance and prognostic implication of GAS5 in ESCC.

Materials and methods

Patients and tissue samples

A total of 97 paired human ESCC and adjacent non-tumor tissues were obtained from patients who underwent surgery at Tianyou Hospital of Wuhan University of Science and Technology between June, 2010 and March, 2013. The specimens were snap frozen in liquid nitrogen and stored until further use. None of the patients had received any chemotherapy or radiotherapy prior to surgery. All patients were followed up regularly. A comprehensive set of clinicopathological factors (including age, gender, tumor size, tumor depth, differentiation, T stage, lymph node invasion, and peritoneal dissemination) were obtained. Overall survival time was calculated from the date of the surgery to the date of death or last contact. This study was approved by the Medical ethics committee of Institutional Review Board of Wuhan University of Science and Technology. Written informed consent was obtained from all participants, and tissue specimens were obtained and handled according to ethical and legal standards.

Real-time PCR analysis

Total RNA was isolated from tissues by using Trizol reagent (Invitrogen) according to the manufacturer's protocol. RNA was reverse transcribed into cDNA using the Primer-Script one step RT-PCR kit (Promega, Madison, WI, USA). The PCR amplification were performed for 40 cycles of 94°C for 30 s, 60°C for 30 s, and 72°C for 30 s with 1.0 μl of cDNA using the SYBR Premix Dimmer Eraser kit (Takala, Dalian, China) on a ABI7900 system (Applied Biosystems, Foster City, USA). GAPDH was used as a reference, and lncRNA GAS5 level were normalized to GAPDH. The primers (Invitrogen) were designed as follows: for human lncRNA GAS5, the forward primer was 5'-CCATGGATGACTTGCTTGGG-3' and the reverse primer was 5'-TGCATGCTTGCTTGTTGTGG-3'; for human GAPDH, the forward primer was 5'-CCCACTCCTCCACCTTTGAC-3' and the reverse primer was 5'-ATGAGGTCCACCACCCTGTT-3'. Relative quantification of RNA expression was calculated by using the $2^{-\Delta\Delta CT}$ method. Each sample was tested in triplicate.

Construction and transfection of expression vector for lncRNA GAS5

The lncRNA GAS5 sequences were synthesized and subcloned into the pcDNA3.1 (Invitrogen, Shanghai, China) vector. The pcDNA constructs or the empty vector were transfected into KYSE-70 cells cultured on six-well plates according to the manufacturer's instructions. The empty vector was used as the control. The expression level of lncRNA GAS5 was detected

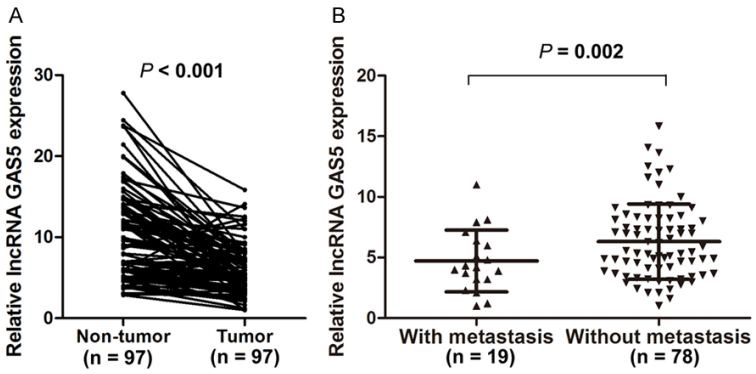

Figure 1. lncRNA GAS5 is significantly down-regulated in ESCC. A. Relative expression level of lncRNA GAS5 in ESCC tissues and adjacent normal tissues (*P < 0.001). B. Relative expression level of lncRNA GAS5 in ESCC tissues with and without distant metastasis (*P = 0.002).

by qRT-PCR. We obtained stably transfected clones by G418 selection (Promega). A stable transfectant of the pcDNA3.1 empty vector was used as a control. For transfection, complexes of Lipofectamine 2000 (Invitrogen Corp, Carlsbad, USA) and one of the plasmids mentioned above was prepared according to the manufacturer's instructions. The level of lncRNA GAS5 expression after transfection was assayed by real-time PCR.

Invasion assays

Cell invasion assays were performed using KYSE-70 cells. Cell culture was performed in transwell chambers (Corning, NY, USA). The insert membranes were coated with diluted Matrigel (San Jose, CA, USA). Cells (1×10^5) were added to the upper chamber and were cultured for 48 h. Finally, the insert membranes were cut and stained with crystal violet (0.04% in water; 100 ml), and the migrated cells were counted under an inverted microscope and were photographed.

In vivo metastasis assay

All animal experiments were conducted according to the protocols approved by the Animal Care Committee of the Renmin Hospital of Wuhan University. Three-week old BALB/C athymic nude mice were obtained from Cancer Institute of the Chinese Academy of Medical Science. For *in vivo* metastasis study, 2×10^6 cells (NC and lncRNA GAS5) were injected into the mice through the lateral tail vein. After 7 weeks, mice were killed and the lungs were dissected out and paraffin embedded. Consecutive sections (4 μm) were made and subjected to hematoxylin-eosin staining. The micro-metastases in the lung were evaluated under a dissecting microscope.

Statistical analysis

All statistical analyses were performed using SPSS version 16.0 and GraphPad 5.0 software. Data were expressed as mean ± SD. The Wilcoxon signed rank test was applied to test the differential expression of lncRNA GAS5 between cancer tissues and adjacent normal tissues. Categorical data were analyzed using the two-side chi-square test. Overall survival was estimated by using the Kaplan-Meier method and compared with the log-rank test. The independent prognostic factor was analyzed by performing the Cox multivariate proportional hazards model. A P value of < 0.05 was considered statistically significant.

Results

Expression of lncRNA GAS5 is significantly down-regulated in ESCC

We firstly determined lncRNA GAS5 expression level in 97 paired human ESCC and adjacent normal tissues by real-time PCR. As shown in **Figure 1**. After normalization to GAPDH expression levels, the expression level of lncRNA GAS5 was significantly lower in tumor tissues as compared with normal tissues (P < 0.001). Moreover, expression of lncRNA GAS5 was decreased in patients with distant metastasis than those without metastasis (P = 0.002). These data indicated that abnormal GAS5 expression may be involved in ESCC pathogenesis.

Relationship between lncRNA GAS5 expression and clinicopathological factors in patients with ESCC

The median level of GAS5 expression (7.52) was used as a cutoff value to divide all 97 patients into two groups. ESCC patients who express GAS5 at levels higher than the cutoff

Table 1. The correlation between clinicopathological parameters and lncRNA GAS5 expression in 97 ESCC patients

Characteristics	n	Low expression	High expression	*P* value
Age				0.770
< 60	41	21	20	
≥ 60	56	27	29	
Gender				0.584
Male	37	17	20	
Female	60	31	29	
Tumor size				< 0.001
< 4 cm	44	13	31	
≥ 4 cm	53	35	18	
Tumor location				0.839
Upper	28	15	13	
Middle	41	19	22	
Lower	28	14	14	
Differentiation				< 0.001
Well	19	7	12	
Moderate	45	14	31	
Poor	33	27	6	
Lymph node invasion				0.318
Absent	33	14	19	
Present	64	34	30	
Distant metastasis				0.002
Absent	78	33	45	
Present	19	17	4	
TNM stage				0.040
I-II	34	12	22	
III-IV	63	36	27	

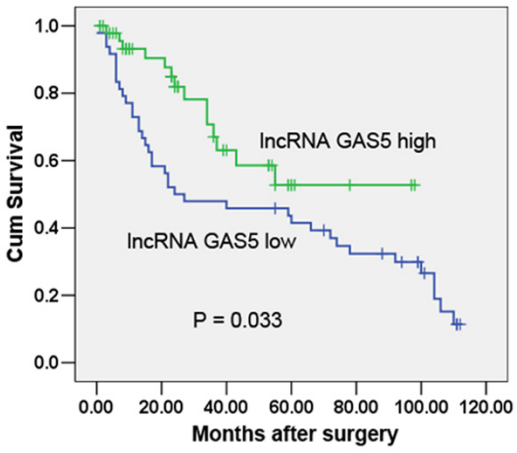

Figure 2. Kaplan-Meier curve of overall-survival in ESCC patients with high lncRNA GAS5 level (n = 49) and low lncRNA GAS5 level (n = 48) (*P* = 0.033).

value were assigned to the high expression group (n = 49, GAS5 expression level ≥ cutoff point), and those with expression lower than the cutoff value were assigned to the low expression group (n = 48, GAS5 expression level < cutoff point). We next analyzed the association between the expression of lncRNA GAS5 and clinicopathological parameters of ESCC patients. As shown in **Table 1**, lncRNA GAS5 expression was significantly associated with tumor size (< 4 cm vs. T ≥ 4 cm; *P* < 0.001), differentiation (*P* < 0.001), distant metastasis (*P* = 0.002) and TNM stage (*P* = 0.040). However, there was no correlation between lncRNA GAS5 expression level and age (< 60 vs. ≥ 60, *P* = 0.770), gender (female vs. male, *P* = 0.584), tumor location (*P* = 0.839) and lymph node invasion (*P* = 0.318).

lncRNA GAS5 downregulation associates with poor prognosis in patients with ESCC

Kaplan-Meier analysis with the log-rank test was performed to determine the expression of lncRNA GAS5 on survival of ESCC patients. As shown in **Figure 2**. Patients with low level expression of lncRNA GAS5 tended to have worse overall survival than those with high level lncRNA GAS5 expression (log-rank test, *P* = 0.033). Moreover, to determine whether the expression of lncRNA GAS5 was an independent prognostic factor for ESCC, univariate and multivariate analyses were carried out. Univariate analysis demonstrated that distant metastasis (*P* = 0.047), TNM stage (*P* = 0.039) and lncRNA GAS5 expression level (*P* = 0.033) were significantly associated with overall survival of ESCC patients (**Table 2**). However, multivariate analysis using the Cox proportional hazards model for all variables that were significant in the univariate analysis showed that only lncRNA GAS5 expression level was an independent prognostic factor for patients with ESCC (*P* = 0.047, **Table 2**).

lncRNA GAS5 inhibits cell invasion in ESCC cells

As lncRNA GAS5 was significantly down-regulated in ESCC tissues with distant metastasis, we suspected that lncRNA GAS5 might be involved

Table 2. Univariate and Multivariate analysis of various potential prognostic factors in 97 ESCC patients

Characteristics	Univariate analysis		Multivariate analysis	
	HR[b] (95% CI[c])	*P*	HR[b] (95% CI[c])	*P*
Age	0.89 (0.70-1.29)	0.158	-	-
Gender	1.11 (0.81-1.73)	0.536	-	-
Differentiation	1.12 (0.72-1.49)	0.173	-	-
Tumor size	1.01 (0.81-1.32)	0.512	-	-
Tumor location	1.15 (0.83-1.42)	0.095	-	-
Lymph node invasion	1.12 (1.02-1.80)	0.112	-	-
Distant metastasis	1.48 (1.15-2.17)	0.047[a]	1.27 (1.09-1.97)	0.102
TNM stage	1.29 (1.07-2.01)	0.039[a]	1.06 (1.09-1.91)	0.127
lncRNA GAS5 level	1.91 (1.57-2.69)	0.033[a]	1.32 (1.19-2.37)	0.047[a]

[a]$P < 0.05$; [b]HR: hazard ratio; [c]CI: confidence interval.

in regulating cellular biology. We first detected the expression level of lncRNA GAS5 in six ESCC cell lines (EC9706, Eca109, TE1, TE13, KYSE-450, KYSE-70) and a normal esophageal epithelial cell Het-1A. The result showed that all the ESCC cell lines displayed lower expression of lncRNA GAS5 as compared with normal cell Het-1A (**Figure 3A**). To analyze the effect of lncRNA GAS5 on ESCC cells, KYSE-70 cells were transfected with lncRNA GAS5 expressing vector to overexpress lncRNA GAS5 (**Figure 3B**). Transwell assay indicated that the invasion ability markedly suppressed in ESCC cells after ectopic expression of lncRNA GAS5 (**Figure 3C** and **3D**). These data showed that overexpression of lncRNA GAS5 could inhibit invasion of ESCC cells.

Ectopic expression of lncRNA GAS5 inhibits tumor metastasis in vivo

We then investigated the *in vivo* tumor growth effects of lncRNA GAS5 in ESCC cells. To investigate the *in vivo* metastasis effect of lncRNA GAS5, cells were injected into the tail vein of nude mice. The results showed that mice injected with lncRNA GAS5 presented with significantly less metastatic nodules in the lung (**Figure 4A** and **4B**). These results demonstrated that ectopic expression of lncRNA GAS5 could inhibit the metastasis of ESCC cells.

Discussion

ESCC remains to be one of the leading causes of death, and finding new molecular targets for early diagnosis, prognosis prediction, and therapeutic targets has the potential to improve the clinical strategies and outcomes of this disease. More and more studies indicated long non-coding RNAs are involved in the progression of tumors [18]. Recent study revealed that lncRNA expression was significantly altered in ESCC tissue through screening lncRNA expression profile [19]. For instance, Gao et al. reported that long non-coding RNA 91H contributes to tumor progression and occurrence of ESCC by inhibiting IGF2 expression [20]. Zang et al. found that lncRNA TP73-AS1 expression was up-regulated in ESCC tissues and associated with tumor location and clinical stage of ESCC. In addition, knockdown of lncRNA TP73-AS1 inhibits cell proliferation and induce apoptosis via the caspase-3 dependent apoptotic pathway [21]. However, no study has elucidated the clinical significance and biological functions of lncRNA GAS5 in ESCC.

Previously, It has been found that lncRNA GAS5 is down-regulated in bladder cancer, and ectopic expression of lncRNA GAS5 can inhibit cell proliferation by regulating CDK6 [22]. Sun et al. reported that lncRNA GAS5 was markedly downregulated in gastric cancer tissues, and associated with larger tumor size and advanced pathologic stage in gastric cancer [17]. More recently, Hu et al. demonstrated that lncRNA GAS5 could suppress the migration and invasion of hepatocellular carcinoma via miR-21 [23]. These studies indicated that lncRNA GAS5 plays important role in cancer development and metastasis. In this study, we explored the clinical role of lncRNA GAS5 in ESCC patients. Our results indicated that lncRNA GAS5 expression was significantly lower in ESCC tissues compared with that of adjacent non-tumor tissues. In addition, low lncRNA GAS5 expression was associated with tumor size, tumor depth, lymph node invasion and TNM stage, suggesting that down-regulation of lncRNA GAS5 plays an important role in ESCC progression. This is in accordance with that reported in other tumor entities [24, 25]. Moreover, we found that patients with low lncRNA GAS5 expression had shorter overall survival than those with high lncRNA GAS5 expres-

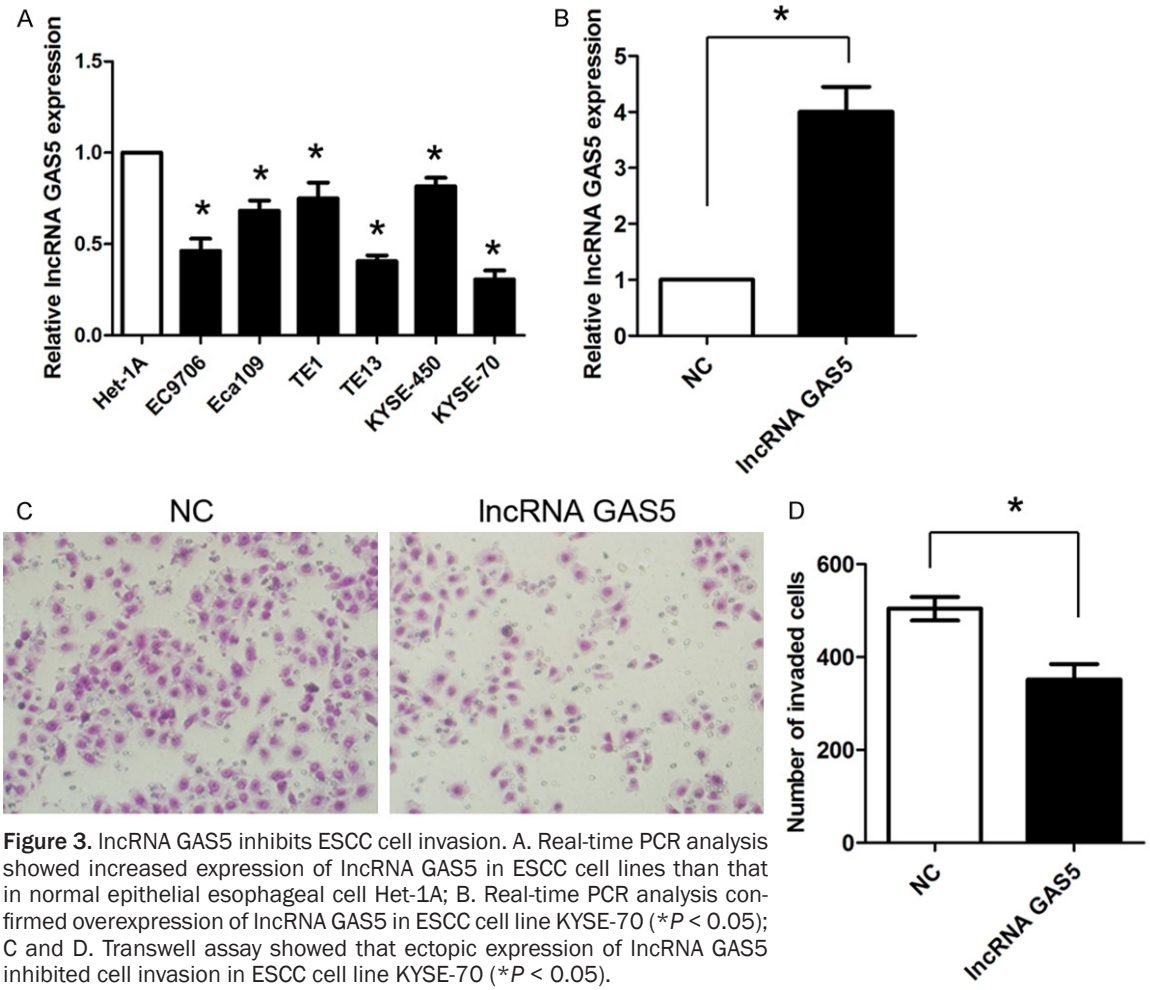

Figure 3. lncRNA GAS5 inhibits ESCC cell invasion. A. Real-time PCR analysis showed increased expression of lncRNA GAS5 in ESCC cell lines than that in normal epithelial esophageal cell Het-1A; B. Real-time PCR analysis confirmed overexpression of lncRNA GAS5 in ESCC cell line KYSE-70 (*$P < 0.05$); C and D. Transwell assay showed that ectopic expression of lncRNA GAS5 inhibited cell invasion in ESCC cell line KYSE-70 (*$P < 0.05$).

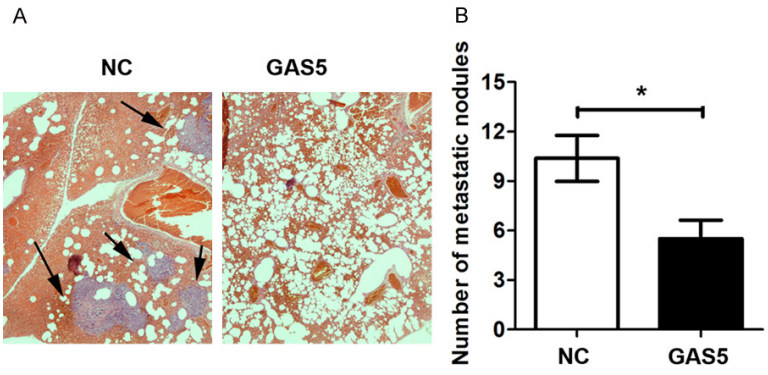

Figure 4. Ectopic expression of lncRNA GAS5 inhibits tumor metastasis *in vivo*. A and B. Micro-metastatic nodules in the lung after injecting with NC and lncRNA GAS5 cells (*$P < 0.001$).

sion. More importantly, both the univariate and multivariate survival analysis revealed that low lncRNA GAS5 expression was correlated with worse overall survival in ESCC patients, which indicated that lncRNA GAS5 was an independent prognostic marker for patients with ESCC. Finally, we determined the biological role of lncRNA GAS5 in ESCC. We chose representative ESCC cell lines and measured lncRNA GAS5 expression in these cell lines. We found that all ESCC cell lines presented with lower expression level of lncRNA GAS5 than esophageal epithelial cell Het-1A. The upregulation of lnRNA GAS5 inhibits cell invasion in KYSE-70 cells, Ectopic expression of lncRNA GAS5 inhibited tumor metastasis *in vivo*. Our data proved that lncRNA GAS5 might be an important modulator involved in ESCC progression.

In conclusion, our results offer the first evidence that lncRNA GAS5 plays important role in the progression of ESCC and that the downregulation of lncRNA GAS5 could independently predict shorter overall survival of patients, implying that lncRNA GAS5 might be a potential marker for further risk stratification in the treatment of ESCC. However, further studies are needed to explore the molecular mechanisms of lncRNA GAS5 in ESCC.

Disclosure of conflict of interest

None.

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