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High level of lncRNA H19 expression is associated with shorter survival in esophageal squamous cell cancer patients

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ABSTRACT

Aim: Long non-coding RNA (lncRNA) is currently considered to play an important regulatory role in various diseases, including tumors, at present a hot topic in research. As a non-coding transcription product of imprinted gene, LncRNA H19 is expressed as a parent imprinted maternal allele without protein-coding ability. Increasing evidence indicates that LncH19 may be a new tumor marker for early clinical diagnosis and prognosis judgment. In this study, LncH19 expression was investigated by RNA *in situ* hybridization for further exploring the clinicopathological role of its expression in esophageal squamous cell cancer (ESCC).

Methods: 121 tumor samples and seven cases of adjacent non-tumor tissues from esophageal cancer patients were detected by RNA *in situ* hybridization (ISH) and the ISH staining was graded with modified Allred scoring.

Results: While no LncH19 expression in the tumor adjacent to normal epithelia was disclosed with the technology, significantly higher levels of LncH19 expression were detected in the tumors obtained from the patients who died within one year after surgery, compared to the expression in those tumors from the patients who survived longer than five years after the same treatment regimen ($P = 0.001$). In addition, LncH19 expression was verified to correlate with a larger tumor size ($P = 0.002$) and a higher UICC stage ($P = 0.001$).

Conclusion: Our LncH19 ISH data verify the involvement of LncH19 in ESCC. Higher levels of LncH19 expression were not only detected in tumors with larger size and in clinical late stage, but also significantly associated with shorter survival, strongly indicating its clinical significance in the malignant progression of ESCC and useful value as a poor prognostic factor for the patients.

1. Introduction

Esophageal cancer is one of the most lethal cancers. It is the sixth leading cause of cancer death and the eighth most common cancer in the world, mainly due to the extreme difficulty in diagnosing it early, to its aggressive nature and low survival rate. The five-year survival rate is about 15%–25% [1]. Worse yet, the incidence of esophageal cancer is increasing world-wide, and the incidence of related risk factors is on the rise as well [2]. There are two main types of esophageal cancer, i.e. squamous cell carcinoma and adenocarcinoma. The type and incidence of esophageal cancer vary from region to region. Among them, esophageal squamous cell carcinoma (ESCC) is the predominant histological type in several geographical areas in the world [1]. China is among the highest risk areas for ESCC. Particularly, Western Anyang in

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Henan province, China, figures as one of the highest incidence areas.

Early esophageal cancer generally does not emit obvious clinical symptoms. Although a great deal of effort has been made in screening of esophageal cancer for early diagnosis and therapy, practical and convenient technologies in this area are still in the development [3]. Furthermore, chemotherapy is an important treatment of esophageal cancer, but the emergence of multi-drug resistance during treatment becomes a serious challenge. In view of this, it is imperative to explore new reliable biomarkers and therapeutic targets for early diagnosis and better treatment of esophageal cancer.

Long noncoding RNAs (LncRNA) are heterogeneous transcripts with a length of more than 200 nucleotides, which have a limited potential to encode proteins. Multiple evidence shows that lncRNA is a tumor progression driver in a variety of cancer types. The reports show that it can either promote or inhibit tumor development, invasion and metastasis by interacting with large molecules such as DNA, chromatin, proteins, and RNA [4–6].

The H19 gene is located on the chromosome 11p15.5 and 135 kb towards the telomere with adjacent gene IGF2 imprinting and regulation. It is one of the few genes proved to be imprinted in human and expressed only from the maternal allele [14,15]. Recent research has highlighted an important role of H19 in the complex process of tumor growth [7–13]. There are studies revealing that H19 is up-regulated in various human malignancies, suggesting that it is a potential oncogene [9,16,17]. Luo et al. found that the expression level of H19 in bladder cancer was significantly increased *in vivo* and *in vitro*, and demonstrated that upregulated H19 increased bladder cancer cell proliferation by ID2 over-expression [16]. Ren and his colleagues disclosed that H19 promoted the stemness of cancer stem cells and the chemoresistance of colorectal cancer cells *in vitro* and *in vivo* with down-regulation and overexpression of H19 technologies. In addition, it also indicated a possibility of H19 activation of β-catenin pathway as a competing endogenous RNA sponge for miR-141 and thus inhibiting the stemness of colorectal cancer cells [18].

However, H19 was initially proposed to have a tumor suppressive property based on its ability to inhibit tumorigenicity [19]. Some publications showed that loss of imprinting (LOI) of H19 could be identified not only in childhood tumors such as Wilms' tumor and hepatoblastoma but also in lung cancer and choriocarcinoma and other common adulthood cancers, indicating a complex role of H19 in tumors [14,20–22].

So far, only a few studies on the expression of H19 in ESCC have been published, in which Gao T et al. found that H19 hypermethylation was related to the LOI of IGF2, indicating a role of H19 in the occurrence, development and metastasis of ESCC [23]. Deli Tan et al. studied 64 pairs of ESCC samples and the adjacent nontumor tissues with QRT-PCR and discovered that H19 levels were significantly upregulated. Furthermore, down-regulation of H19 *in vivo* and *in vitro* experiments revealed that high expression of H19 could promote the development and metastasis of esophageal cancer [24].

To further characterize the clinicopathological role of H19 expression in human ESCC, we analyzed its expression in a panel of two groups of clinical samples. In one group, the patients died within one year after operation, and in another group, all the patients survived at least five years after similar surgery. The genders, age and clinical stage of the patients in these two groups were balanced before the experiment. In the current study, *in situ* hybridization (ISH) of H19 RNA was applied, in consideration of its detection accuracy and morphological localization, which is superior to polymerize polymerase chain reaction (PCR) method.

2. Materials and methods

2.1. Tumor specimens

Tumor samples were collected from 121 esophageal cancer patients who were diagnosed histologically at the Anyang tumor hospital, Henan, China. All of the samples were obtained during surgery and processed according to a routine standard operating procedure. Histological assessment was routinely performed by dedicated pathologists at the Departments of Anyang Tumor Hospital and First Affiliated Hospital of Zhengzhou University, China. Tissue sections (4 μm) from the paraffin sections were prepared for H19 ISH.

2.2. RNA in situ hybridization (RNA ISH) with DIG-labeled probe

Oligonucleotide probe *in situ* hybridization: the nucleotide probe was obtained from the 5′end of the digoxin - ddUTP end-transferase labeled primer. Digoxin-ddUTP terminal transferase was obtained from the DIG 5 Prime End Labeling Kit, which was purchased from Roche (Indianapolis, IN, USA). The probe sequence was as follows: 5′-DIG-GCTGT TCCGA TGGTG TCTTT GATGT TGGGC TGATG-DIG-3′.

Paraffin sections of 4 μm thickness were fixed in a fixed solution for eight hours. Wax was soaked and embedded in tissue slices after gradient alcohol dehydration. The paraffin sections were then oven-dried at 60 °C for two hours. After dewaxing in xylene, the paraffin sections were rehydrated through an alcohol series and washed in DEPC before being boiled in the repair solution for 15 min and cooled naturally. The sections were then incubated with proteinase K solution (20ug/ml) at 37 °C for 30 min. Thereafter, the sections were washed with PBS three times, each time for five minutes. Each section was then covered with pre-hybridization solution (30% Formamide, 4x SSC, 1 mmol/L EDTA, 5x Denhardts, 50mmol/LNaH2PO4 pH 7.0) at room temperature for one hour before being incubated with a H19 lncRNA probe hybridization solution (30% Formamide, 4x SSC, 1 mmol/L EDTA, 5x Denhardts, 50mmol/LNaH2PO4 pH 7.0, 8 ng/ul H19 lncRNA probe) at 37 °C overnight. After rinsing them three times during 10 min with washing buffer, the sections were then covered with blocking serum BSA (3%) at room temperature for 30 min. Digoxigenin labeled peroxidase (anti-DIG-HRP, Jackson) was added at 37 °C for 40 min. After washing the sections with PBS four times, DAB solution was added for colorization, and the length of colorization was optimized under the microscope before experiment. The sections were washed with pure water to stop the development and counterstained with hematoxylin before being dehydrated with gradient alcohol and mounted with coverslips.

2.3. RNA ISH score

The sections stained with H19 *in situ* hybridization were scored by two pathologists from the Department of Pathology of the First Affiliated Hospital of Zhengzhou University according to Allred scoring system. The evaluators were blinded to the clinical data of patients.

The expression level of H19 in esophageal cancer samples was evaluated by semi-quantitative manner, including the staining intensity and staining range of the samples. According to the strength of *in situ* hybridization staining, it was divided into 0, 1, 2 and 3 points, where 0 means negative. The staining range was determined according to the percentage of positive cells in tumor cells. For the scoring rules of staining intensity, staining intensity was 0, 1, 2 and 3, respectively, and staining results were no positive cells, weak staining, moderate staining, and strong staining. About the scoring rules of staining range: all cells in the sample are negative with a score of 0, the positive rate less than 25% with a score of 1, the positive rate between 25% and 50% with a score of 2, and the positive rate greater than 50% with a score of 3. The results of *in situ* hybridization were shown in Table 1. The final overall evaluation was divided into the product of dyeing intensity score and dyeing range score, in which the sample groups were 0 points (−), 1–4 points (+) of weakly positive, 5–8 points (+ +) of moderately positive, and 9 points (+ + +) of strongly positive.

Table 1
The criteria of allred scoring system used for evaluating H19 expression in esophageal cancer in our study.

The criteria of intensity scoring system				
intensity score	0	1	2	3
Staining intensity	negative	Weakly positive	Moderate positive	Strongly positive

The criteria of percentage scoring system				
Percentage score	0	1	2	3
Stained cells (%)	0	< 25%	25%-50%	> 50%
Total score	0	1-4	5-8	9
	(−)	(+)	(++)	(+++)

Notes: The total score was divided into the product of dyeing intensity score and dyeing range score. It ranged from 0 to 9.

2.4. Statistical analysis

An independent sample student's *t*-test or a one-way ANOVA was used to compare the metrological data sets that conform to the normal distribution, while a non-parametric test was used to compare the metrological data sets that do not conform to the normal distribution. Comparison between data of multiple groups of classification variables, we used the chi-square test. The multivariate Logistic regression model was used to examine the risk factors of esophageal cancer. Kaplan-Meier and log-rank tests were used to calculate and compare the survival period, and Cox proportional regression model was used to predict independent prognostic factors of esophageal cancer. All p values were two-sided. Differences were considered significant when $P < 0.05$. Analyses were conducted in SPSS version 17.0 (SPSS Lnc, Chicago, IL, USA).

3. Results

A total of 121 cases of ESCC and seven cases of adjacent non-tumor tissues were retrieved from the files at the Departments of Epidemiology and Pathology, Anyang Tumor Hospital, Henan, China, among which one part of the cancer patients died within one year after surgery while another part of the cancer patients survived at least five years after surgery. The adjacent normal tissues were resected 5–10 cm from the tumor margin. Histologically, positive H19 expression was revealed in all the cancer tissues, while the corresponding adjacent normal tissues were generally negative for H19 expression (Fig. 1). The criteria of allred scoring system used for evaluating H19 expression in our study are shown in Table 1. Clinical data, including age, gender, tumor size, tumor differentiation degree, lymph node metastasis, etc., and their H19 expression correlations are shown in Table 2.

Among the 121 ESCC samples, 38 cases (31.40%) were strongly positive, 64 cases (52.89%) were moderately positive and 19 cases (15.70%) were weakly positive, while no negative expression of H19 was disclosed in these tumor samples. The results showed that there was a statistically significant difference in the expression level of H19 between the patients who died one year after surgery and those who survived longer than five years after surgery ($P = 0.001$, one-way ANOVA, Table 1, Fig. 2). H19 expression was significantly correlated with tumor size ($P = 0.002$, one-way ANOVA as shown in Table 2) and UICC stage ($P = 0.001$, chi-square test, Table 2, Fig. 2). In this study, other clinical features such as gender and age were not significantly correlated with H19 expression (Table 2).

The above results indicate that the expression level of H19 is related to the survival time of patients and the malignancy degree of esophagus cancer. As a result, based on the current data, we made (created) a multivariate Logistic regression model to test the risk factors of esophageal cancer so as to verify our hypothesis. The logistic regression model analysis indicated that UICC stage, lymph node metastasis and H19 expression intensity were the main factors that significantly affected survival ($P < 0.05$) as shown in Table 3. For instance, the lower the UICC stage of esophageal cancer patients, the greater the chance for a survival rate longer than five years. And, those patients who had lymph node metastasis during surgery were more likely to die within one year compared to those without lymph node metastasis; patients with a strong positive H19 expression were more likely to die within one year than those with a weak positive or moderate positive H19 expression.

We used Kaplan-Meier and log-rank tests to compare the relationship between H19 expression and survival in 121 patients with esophageal cancer. The results (Fig. 3) showed that H19 expression was a risk factor for shorter survival in esophageal cancer patients ($P < 0.001$). The Cox proportional hazards model indicated that UICC stage, t lymph node metastasis and H19 expression intensity were all independent influencing factors for the prognosis of esophageal squamous cell carcinoma patients ($P < 0.001$) as shown in Table 4.

4. Discussion

Long non-coding RNA (lncRNA) has been shown to play an important role in the development and progression of cancer [6]. H19 is widely studied as LncRNA, which is actively involved in various stages of tumors and is expressed in almost all tumors. However, the role of H19 in tumorigenesis and progression has long been controversial. Various literature reports with contradictory data indicated that H19 can be either tumor suppressor or tumor promoter.

As far as our knowledge goes, LncRNA H19 showed tumor driver function in multiple carcinomas, and it is rarely expressed in non-cancerous tissues [10], which is consistent with our experiment result. H19 has also been shown to enhance the survival ability of tumors under malignant conditions in liver cancer and bladder cancer cell lines [11,25]. Meanwhile, it is well known that p53 is the main tumor suppressor gene in cancer. A lot of studies demonstrated that p53 not only inhibits the promoter activity of the H19 gene [26–29], but it also inhibits the expression of H19 *in vivo* by inducing DNA demethylation in the upstream imprinting control region (ICR) of the H19 gene [28]. Besides, H19 responds to various stress conditions, such as decreased P53 and hypoxia, by activating a tumorigenic cell survival program [11,29,30]. Moreover, H19-derived mir-675 was recently found to play an important role in inhibiting p53 and p53-dependent protein expression in bladder cancer cells [25], implying that the interaction between H19 and P53 is a significant step in the development of cancer. Also, recent studies by Shoshani and colleagues have shown that H19 inhibits polyploid-mediated growth arrest, followed by further mutations in the oncogene that lead to tumor progression [30,31]. However, it is worth mentioning that H19 may have different functions at different development stages [32]. H19 is significant in the embryonic stage, where it acts as a promoter, promoting differentiation, and thus in this stage, it may contradict its seeming tumorigenic effect as seen in adults [33,34].

Current clinical practice commonly uses six types of serum biochemical markers for the diagnosis of esophageal cancer: SCC (squamous cell carcinoma associated antigen), CEA, P53-Ab, CF21-1 (CYFRA21-1), vascular endothelial growth factor-C and microRNA (miRNA) [35]. Although each of these biomarkers is associated with esophageal carcinoma, they are not very accurate and should only be drawn on as supporting diagnosis. The conclusive diagnosis of esophageal cancer often requires an endoscopic biopsy. Such an invasive examination often brings great pain to the patients and is expensive and inappropriate for early detection. Therefore, it is valuable to find new and accurate serological markers and convert them into effective clinical tools. LncRNA is a hotspot of tumor-related research and a component of epigenetic, transcriptional and post-transcriptional regulation. Among LncRNA, H19 has been studied in depth. On the basis of previous studies, it is conceivable that overexpression of H19 is

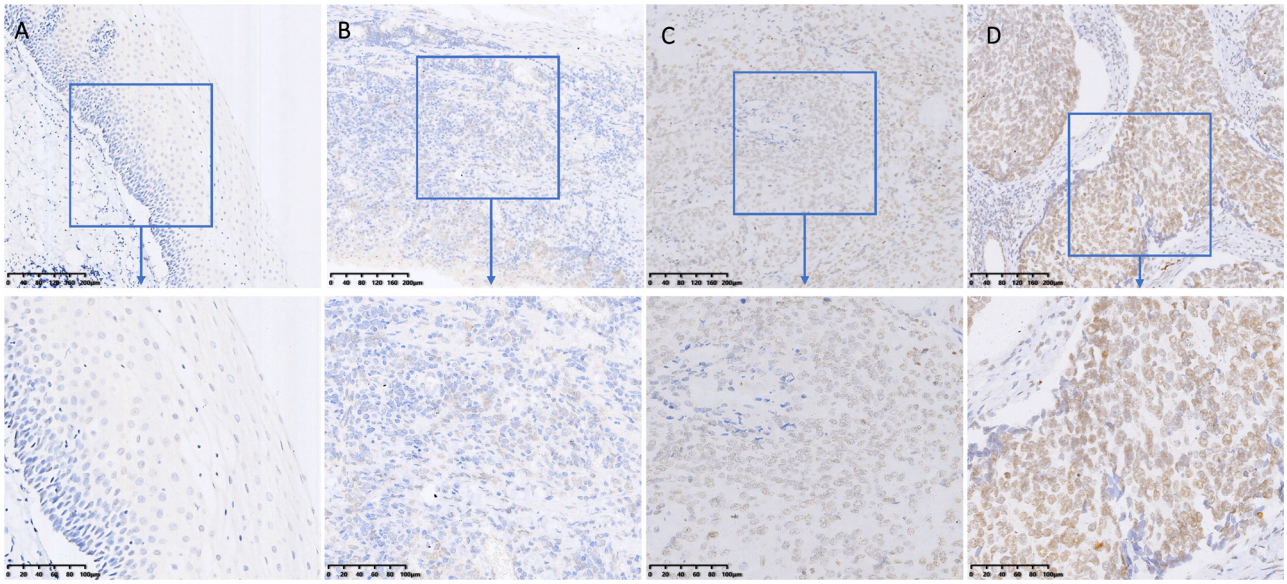

Fig. 1. LncH19 ISH results in human squamous esophagus samples. A shows negative lncH19 staining in tumor adjacent normal squamous esophageal epithelial cells, while B shows weakly positive lncH19 cytoplasmic staining in the cancer cells in a ESCC sample, C shows moderately positive lncH19 cytoplasmic staining in the cancer cells in another ESCC sample, and D shows strong lncH19 cytoplasmic staining in the cancer cells in a ESCC sample. (Magnification in the upper panel: 100×, Magnification in the lower panel: 200×).

significantly related to poor results for cancer patients. However, RNA ISH technology has not been applied in the H19 study in large scale clinical samples. The advantage of this technology is able to combine morphology and H19 cellular localization, so that disclosing rather accuracy information of H19 expression in tumors. Therefore, in the present study, we have innovatively studied the role of H19 with RNA ISH technology in two typical esophageal cancer samples from one group of ESCC patients who died within one year after surgery and another ESCC group who survived longer than five years after surgery. The LncH19 was found to be highly expressed in the tissues of ESCC under different UICC stages with different tumor sizes. We also used Kaplan-Meier and log-rank tests and discovered that high H19RNA expression in the 121 esophageal cancer samples was closely related to shorter survival ($P < 0.001$). Most of the patients who died within one year after surgery showed a higher H19 expression in their tumors, while the other patients survived longer than 5 years tended to get a lower H19 expression score. Hence, overexpression of H19 may contribute to the development and progression of esophageal cancer with poor clinicopathological characteristics and poor prognosis of patients. Indeed, further logistic regression analysis and Cox proportional

Table 2
General characteristics of the study subjects.

Clinical Feature	TOTAL(N = 121)	WEAKLY POSITIVE(N = 19)	MODERATE POSITIVE(N = 64)	STRONGLY POSITIVE(N = 38)	P value
Survival					0.001a
≤1 year of death(%)	63(52.1)	6(5.0)	12(9.9)	45(37.2)	
≥5year of survival(%)	58(47.9)	20(16.5)	20(16.5)	18(14.9)	
Age(years)					0.132b
≥60(%)	60(49.6)	13(10.7)	15(12.4)	32(26.4)	
<60(%)	61(50.4)	13(10.7)	17(14.0)	31(25.6)	
Sex					0.591c
Female(%)	63(52.1)	15(12.4)	18(14.9)	30(24.8)	
Male(%)	58(47.9)	11(9.1)	14(11.6)	33(27.3)	
Size					0.002a
≥3 cm(%)	107(88.4)	18(14.9)	30(24.8)	59(48.8)	
<3 cm(%)	14(11.6)	8(6.6)	2(1.7)	4(3.3)	
UICC stage					0.001c
PT1(%)	16(13.2)	10(8.3)	2(1.7)	4(3.3)	
PT2(%)	30(24.8)	2(1.7)	12(9.9)	16(13.2)	
PT3-PT4(%)	75(62.0)	14(11.6)	18(14.9)	43(35.5)	
Histological grade					0.060c
1(%)	4(3.4)	1(0.8)	2(1.7)	1(0.8)	
2(%)	85(70.2)	19(15.7)	27(22.3)	39(32.2)	
3(%)	32(26.4)	6(5.0)	3(2.5)	23(19.0)	
lymphatic metastasis					0.110c
yes(%)	45(37.2)	7(5.8)	9(7.4)	29(24.0)	
no(%)	76(62.8)	19(15.7)	23(19.0)	34(28.1)	
Tumor location					0.519c
Lower(%)	23(19.0)	6(5.0)	5(4.1)	12(9.9)	
Middle(%)	80(66.1)	14(11.6)	24(19.8)	42(34.7)	
Upper(%)	18(14.9)	6(5.0)	3(2.5)	9(7.4)	

NOTE: a:one-way ANOVA, b: Kruskal-Wallis Test, c: chi-square test, UICC : Union for International Cancer control.

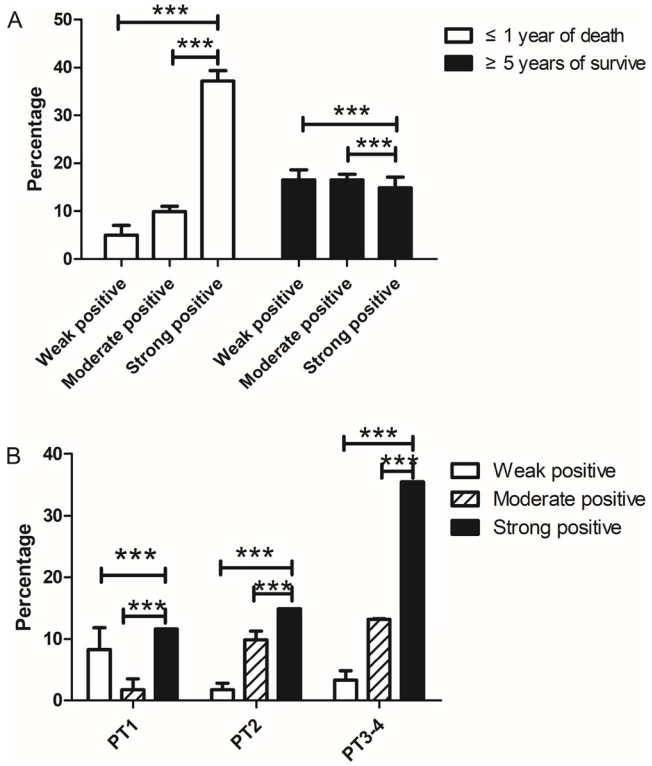

Fig. 2. Histograms of the lncH19 ISH results in ESCC samples. A shows the differences of the lncH19 ISH results in the two ESCC groups. B shows histograms of the lncH19 expression in consideration of UICC stages. ***p = 0.001.

hazards model showed that H19 overexpression, UICC stage and lymph node metastasis were all risk factors for esophageal cancer. These findings emphasize that a high expression of H19 is associated with ESCC malignancy and negatively influenced the survival of the patients.

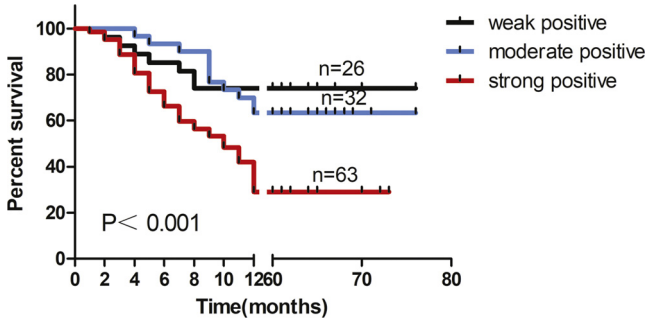

Fig. 3. Kaplan-Meier survival probabilities outcomes of patients with different expression of H19. Strong H19 expression is significantly associated with shorter overall survival in the ESCC patients. (p < 0.001).

Table 4
Multivariate Cox regression analysis of 121 patients with esophageal cancer.

	β	SE	Wald	P	RR	RR95%CI
UICC stage	0.797	0.290	7.527	0.006	2.218	1.256–3.919
size	−0.668	0.544	1.511	0.219	0.513	0.177–1.488
old	0.304	0.251	1.467	0.226	1.355	0.829–2.216
lymphatic metastasis	0.646	0.263	6.028	0.014	1.909	1.139–3.198
Expression intensity of H19	0.524	0.193	7.358	0.007	1.689	1.156–2.466

NOTE: SE: standard error; RR: relative risk; CI: confidence interval.

5. Conclusion

In this study, RNA ISH of H19 methodology was applied in a series of 121 ESCC patients with long follow-up clinical data. It was verified that H19 was negative in all the normal esophageal epithelia adjacent to the tumor, and varying positive expression was detected in all the ESCC samples. Significantly stronger H19 RNA expression was revealed in those ESCC patients who died within one year after operation, while

Table 3
Risk factors for esophageal cancer tested by logistic regression model.

	β	SE	Wals χ2	P	OR	95%CI
Age(years)						
≥60	−0.656	0.517	1.608	0.205	0.519	0.188-1.430
<60					1.00	
Sex						
Male	−0.209	0.522	0.160	0.690	0.812	0.292-2.259
Female					1.00	
Size						
≥3cm	1.069	1.264	0.714	0.398	2.911	0.244-34.705
<3cm					1.00	
Uicc stage						
PT1	−3.539	1.332	7.062	0.008	0.029	0.002-0.395
PT2	−1.065	0.577	3.400	0.065	0.345	0.111-1.069
PT3-PT4					1.00	
Histological grade						
1	0.028	1.353	0.000	0.983	1.029	0.073-14.579
2	0.693	0.614	1.276	0.259	2.001	0.601-6.664
3					1.00	
Tumor location						
Lower	1.573	0.849	3.434	0.064	4.822	0.913-25.465
Middle	1.119	0.702	2.538	0.111	3.062	0.773-12.134
Upper					1.00	
Lymph node metastasis						
Negative	−1.698	0.584	8.470	0.004	0.183	0.058-0.574
Positive					1.00	
Expression intensity of H19						
Weakly positive	−1.938	0.673	8.294	0.004	0.144	0.038-0.538
Moderate positive	−1.698	0.609	7.762	0.005	0.183	0.055-0.604
Strongly positive					1.00	

NOTE: SE: standard error; OR: odds ratio; CI: confidence interval.

those ESCC patients who survived at least five years after surgery showed less H19 RNA expression in their tumors, indicating a useful prognostic marker and therapeutic target of H19 RNA in this type of tumor. However, it is worth noting that this study only explored its possible clinicopathological role in a limited number of samples with principal component analyses, such a study and its molecular mechanism should deserve further large-scale studies.

Declaration of Competing Interest

The authors declare no conflict interest in this work.

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