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ORIGINAL ARTICLE

Upregulation of long noncoding RNA ANRIL correlates with tumor progression and poor prognosis in esophageal squamous cell carcinoma

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Summary

Purpose: *Esophageal cancer (EC) is the 9th most common carcinoma worldwide with poor prognosis. Specific biomarkers can help predicting the development of esophageal squamous cell carcinoma (ESCC), which can improve the assessment of prognosis. This study aimed to explore long noncoding RNA (lncRNA) ANRIL expression and its potential value in ESCC prognosis.*

Methods: *Quantitative reverse transcriptase-polymerase chain reaction (qRT-PCR) was utilized to detect lncRNA ANRIL expression in 50 pairs of ESCC and matched normal samples in order to explore the role of lncRNA ANRIL in ESCC. Moreover, the association was investigated between clinical characteristics of ESCC and the expression level of ANRIL.*

Results: *Disease-free survival (DFS) and overall survival (OS) were significantly shorter in ESCC patients with higher expression level of lncRNA ANRIL. ESCC tissues examined showed an obvious increment in ANRIL expression when compared to normal tissues. Furthermore, ANRIL was positively related to lymph nodes metastasis, TNM stage and tumor clinical stage. Moreover, upregulated ANRIL expression was remarkably associated with shorter survival in ESCC patients,which was also an independent prognostic factor for both OS and DFS.*

Conclusions: *This study suggested that lncRNA ANRIL could be a potential oncogene of ESCC. ANRIL expression might be served as another potential therapeutic target and prognostic biomarker for ESCC.*

Key words: *ANRIL, esophageal squamous cell carcinoma, long noncoding RNA, prognosis*

Introduction

Esophageal cancer (EC) remains a disease with poor prognosis and is the 9th most common carcinoma worldwide [1]. In China, EC ranked 5th in incidence and 4th in mortality out of all cancer types, among which ESCC was the primary pathological subgroup [2]. However, most ESCC patients develop advanced metastatic disease with 5-year OS rate lower than 10%. Despite the intensive research in the diagnosis and therapy over the past 30 years, the low survival rate is still one of the greatest challenges. Therefore, there is an urgent need to figure out novel biomarkers which could greatly contribute to the improvement of prognosis assessment [3].

Previous studies demonstrated that lncRNAs were fundamental in cancer proliferation or migration. For instance, lncRNACASC2 inhibited the proliferation and migration of hepatocellular cancer

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[4]. Upregulated lnc-UBC1 promoted the migration and invasion of colorectal carcinoma cells [5], while lncRNAH19 was associated with metastasis and poor prognosis of bladder carcinoma [6].

Recently, lncRNA ANRIL was found to exist in various human tissues and cancer cells [7-9]. However, the function of lncRNA ANRI ESCC remained unclear. This study aimed to explore the expression level of lncRNA ANRIL and evaluate its relevance with clinical features and prognosis in ESCC oncogenesis.

Methods

Patients and tissues

Paired cancer samples and adjoining normal tissues were obtained from 50 ESCC patients who were subjected to esophagectomy between July 2012 and July 2017 at the First People's Hospital of Wujiang District. No radiotherapy or chemotherapy were administered before surgery. Clinical data were acquired from clinical records. Tissues got from surgery were stored immediately at -80°C. All tissues were analyzed by an experienced pathologist. This study conformed to requirements of the Ethics Committee of the First People's Hospital of Wujiang District. All patients were followed up every 6 months up to 5 years. OS was the time interval from the primary surgery day to the day of the last follow-up examination or death. DFS was the length of time without disease reappearance.

RNA extraction and RT-PCR

Total RNA, obtained from samples with TRIzol reagent (Invitrogen; Carlsbad, CA, USA), was reverse-transcribed to cDNAs using reverse Transcription Kit (TaKaRa Biotechnology Co., Ltd., Dalian, China). β-actin

Table 1. Correlation between lncRNA ANRIL expression and clinicopathological characteristics in ESCC patients

Characteristics	*Patients, n*	*Expression of lncRNA ANRIL*		*p value*
		High expression	*Low expression*	
Total	50	21	29	
Age(years)				0.66
≤60	22	10	12	
>60	28	11	17	
Gender				0.60
Male	24	11	13	
Female	26	10	16	
Smoking status				0.35
No	20	10	10	
Yes	30	11	19	
Drinking status				0.70
No	27	12	15	
Yes	23	9	14	
Tumor location				0.23
Upper and middle 1/3	36	17	19	
Lower 1/3	14	4	10	
T stage				0.03
T1-2	18	4	14	
T3-4	32	17	15	
Lymph node metastasis				0.00
Negative	25	3	22	
Positive	25	18	7	
Clinical stage				0.00
I-II	20	3	17	
III-IV	30	18	12	
Differentiation				0.63
High	23	11	12	
Moderate	17	7	10	
Low	10	3	7	

T1: tumor invades lamina propria, muscularis mucosa or submucosa; T2: tumor invades muscularis propria; T3: tumor invades adventitia; T4: tumor invades adjacent structures

served as normal control. RT-qPCR reactions were conducted on an ABI 7900 fast Real-time PCR system (ABI, CA, USA). The method of $2^{-\Delta\Delta Cq}$ was used to calculate the relevant expression fold changes of mRNAs.

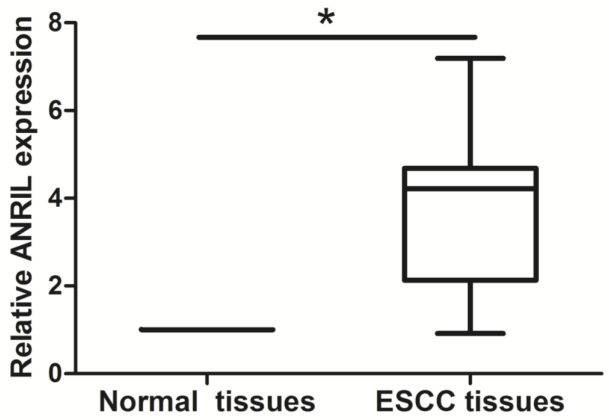

Figure 1. The expression levels of lncRNA ANRIL was monitored in human ESSC tissues and corresponding normal tissues by RT-qPCR (n=50). *Compared with normal tissues, p<0.001.)

Statistics

Mann-Whitney U test, chi-square test, Kaplan-Meier method, log-rank test, univariate and multivariate Cox analyses were used as appropriate. SPSS.21 software package (SPSS Inc., Chicago, IL, USA) was used for data analyses. P<0.05 was considered as statistically significant.

Results

Upregulated ANRIL in ESCC tissues

We assessed the expression of ANRIL in 50paired ESCC and normal tissues by RT-PCR. The results showed that ANRIL levels were remarkably higher in tumor tissues compared with normal tissues (Figure 1).

The correlation between clinicopathological variables and ANRIL expression

The median value of ANRIL was used to divide the patient cohort into high and low ANRIL

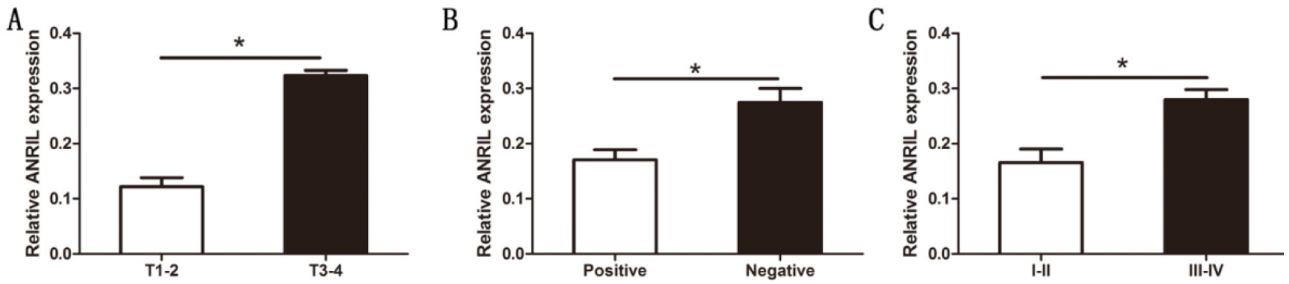

Figure 2. The association between ANRIL expression level and T stage, clinical tumor stage and lymph node metastasis. **(A)**: ANRIL expression was higher in tumor stage 3-4 (T3-4) patients than that in tumor stage 1-2 (T1-2) patients. **(B)**: ANRIL expression was higher in positive lymph node metastasis patients than that in negative lymph node metastasis patients. **(C)**: ANRIL expression was higher in TNM III-IV patients than that in stage I-II patients. *(p<0.05)

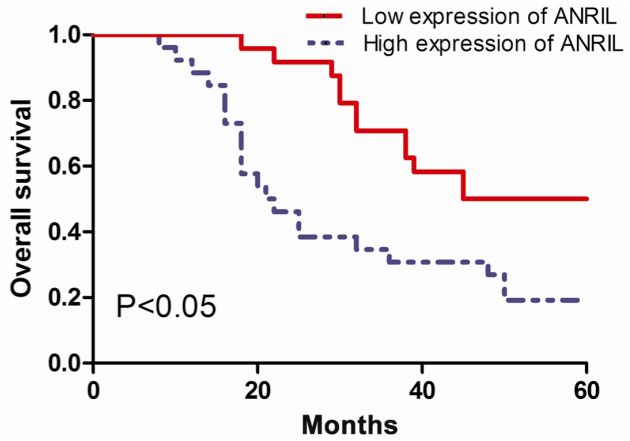

Figure 3. Kaplan-Meier survival curves of ESCC patients after esophagectomy. The overall survival of patients in the lncRNA ANRIL high-expression group was significantly worse compared to patients in the low-expression group (n=50, log-rank test, p<0.05).

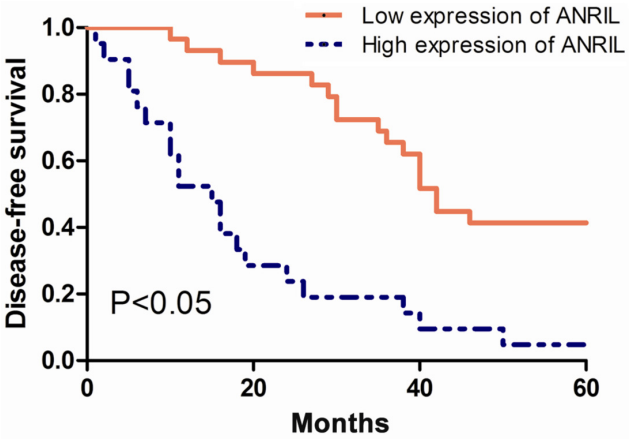

Figure 4. Kaplan-Meier survival curves of ESCC patients after esophagectomy. The disease-free survival of patients in the lncRNA ANRIL high-expression group was significantly worse compared to patients in the low-expression group (n=50, log-rank test, p<0.05).

Table 2. Univariate and multivariate analyses for overall survival in patients with ESCC

Risk factors	*Category*	*Univariate analysis*		*Multivariate analysis*	
		HR (95%CI)	*p*	*HR (95%CI)*	*p*
ANRIL expression	High (n=21)/Low (n=29)	0.292 (0.111-0.768)	0.013	0.271 (0.128-0.574)	0.001
Age (years)	>60 (n=28)/ ≤60 (n=22)	1.130 (0.471-2.713)	0.785		
Gender	Male (n=24)/ Female (n=26)	1.370 (0.508-3.698)	0.534		
Smoking status	Yes (n=30)/ No (n=20)	1.519 (0.487-4.740)	0.472		
Drinking status	Yes(n=23)/ No (n=27)	1.167 (0.478-2.852)	0.734		
Tumor location	Lower 1/3 (n=14)/ Upper and middle 1/3 (n=36)	1.279 (0.448-3.655)	0.646		
T stage	T3-4 (n=32)/ T1-2 (n=18)	2.340 (0.825-6.636)	0.110		
Lymph node metastasis	Positive (n=25)/ Negative (n=25)	1.104 (0.437-2.790)	0.835		
Clinical stage	III-IV (n=30)/ I-II (n=20)	2.440 (0.941-6.331)	0.067	2.997 (1.299-6.911)	0.010
Differentiation	Low (n=10)/Moderate/High(n=40)	0.977 (0.312-3.061)	0.968		

Table 3. Univariate and multivariate analyses for disease-free survival in patients with ESCC

Risk factors	*Category*	*Univariate analysis*		*Multivariate analysis*	
		HR (95%CI)	*p*	*HR (95%CI)*	*p*
ANRIL expression	High(n=21)/Low(n=29)	0.321 (0.126-0.814)	0.017	0.335 (0.161-0.699)	0.004
Age (years)	>60 (n=28)/ ≤60 (n=22)	1.376 (0.590-3.211)	0.461		
Gender	Male (n=24)/ Female (n=26)	1.235 (0.477-3.201)	0.664		
Smoking status	Yes (n=30)/ No (n=20)	1.820 (0.597-5.551)	0.293		
Drinking status	Yes(n=23)/ No (n=27)	1.186 (0.500-2.811)	0.699		
Tumor location	Lower 1/3 (n=14)/ Upper and middle 1/3 (n=36)	1.248 (0.440-3.538)	0.677		
T stage	T3-4 (n=32)/ T1-2 (n=18)	2.778 (1.013-7.616)	0.047	2.438 (0.998-5.956)	0.05
Lymph node metastasis	Positive (n=25)/ Negative (n=25)	1.332 (0.537-3.304)	0.537		
TNM stage	III-IV (n=30)/ I-II (n=20)	2.188(0.892-5.366)	0.087	2.382 (1.069-5.310)	0.034
Differentiation	Low (n=10)/Moderate/High(n=40)	0.920(0.304-2.786)	0.883		

expression group. Chi-square revealed a positive correlation between ANRIL levels and lymph node metastasis, T stage and TNM stage. Besides, no association between ANRIL levels and other clinical characteristics, for instance gender, age, smoking, drinking, tumor location and differentiation, was found (Table 1). Moreover, ANRIL expression was higher in T stage 3-4, positive lymph node metastasis and clinical stage III-IV patients (Figure 2A-2C).

Prognostic value of ANRIL in ESCC

The survival analyses revealed that patients with ESCC with ANRIL downregulation exhibited significantly longer 5-year OS as well as DFS than patients without ANRIL downregulation, indicating ANRIL expression was associated with worse clinical outcome of ESCC (Figures 3 and 4). Multivariate analysis showed that independent prognostic factors for OS were ANRIL and clinical stage (Table 2). Furthermore, ANRIL expression level, T stage and clinical tumor stage were also considered as independent prognostic factors for DFS (Table 3).

Discussion

ESCC is a cancer with high prevalence and mortality, especially in China [3]. Despite successful surgical resection in early stage, many patients with ESCC develop metastasis soon after [10]. To improve ESCC survival rate, clear understanding of the mechanisms of ESCC development is required. Latest research reveals that lncRNAs play a crucial role in cell migration, proliferation and differentiation. For instance, lncRNA HOTAIR has been reported to promote metastasis in ESCC [11]. Moreover, lncRNA H19 also promotes malignant development in ESCC by inducing epithelial-to-mesenchymal transition [12]. LncRNA MALAT1 also promotes malignant development in ESCC by targeting beta-catenin via Ezh2 [13].

Previous studies revealed that lncRNA ANRIL participated in important parts of the metastatic and progression processes of many cancers. For example, ANRIL is activated by SOX2 and contributes to enhancing cell proliferation of nasopharyn-

geal carcinoma [14]. ANRIL acts as an oncogene in osteosarcoma and can be an independent factor for predicting patients' prognosis [15]. After ANRIL was knocked down in human glioma cells, the progression and metastasis were suppressed and apoptosis was enhanced [16]. Nevertheless, the role of ANRIL in ESCC was previously poorly investigated. Therefore, in our current study, the results have shown that lncRNA ANRIL expression was upregulated in ESCC tissues.

Progression and metastasis are the main causes of worse prognosis in patients with ESCC [17]. In our study, we found that ANRIL expression level was lower in ESCC patients with early tumor stage, early clinical stage or no lymph node metastasis. Moreover, ANRIL expression level was positively associated with lymph node metastasis and tumor stage. Besides, ANRIL expression was also remarkably related to OS and DFS of ESCC patients. Univariate and multivariate Cox analyses revealed that ANRIL expression level, T stage and TNM stage could be independent prognostic factors for OS and DFS. These findings suggested that ANRIL expression was remarkably related with ESCC oncogenesis.

Conclusions

This research reveals a new biomarker in the development of ESSC and indicates that lncRNA ANRIL is vital to the carcinogenesis of ESCC and can be used as a promising marker for ESCC.

Conflict of interests

The authors declare no conflict of interests.

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