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ORIGINAL RESEARCH

Expression and significance of RRBP1 in esophageal carcinoma

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Objective: This study was to investigate the expression and clinical significance of RRBP1 in esophageal carcinoma.

Materials and methods: RRBP1 expression was detected in 120 esophageal carcinoma and matched adjacent normal tissues, and the relationship of RRBP1 with clinicopathological characteristics and prognosis was analyzed.

Results: RRBP1 was highly expressed in esophageal carcinoma tissues compared with matched adjacent normal tissues ($P<0.05$). Moreover, RRBP1 expression was associated with T stage, lymph node metastasis, and TNM stage in esophageal carcinoma ($P<0.05$). Survival analysis revealed that RRBP1, T stage, lymph node metastasis, and TNM stage were significantly associated with patients' prognosis.

Conclusion: RRBP1 is highly expressed in esophageal carcinoma and can serve as a potential biomarker to predict patients' prognosis.

Keywords: RRBP1, prognosis, esophageal carcinoma, survival analysis

Introduction

Esophageal carcinoma is one of the most common malignant tumors in China, which accounts for the sixth most common cause of cancer-related death in the world.[1,2] Surgical resection is the main treatment for esophageal cancer patients; however, the 5-year survival rate of esophageal cancer patients after surgery is still less than 25%.[3,4] Currently, the incidence of esophageal carcinoma is still increasing in China.[1,5,6] The early diagnosis of esophageal carcinoma is a tough challenge.[7,8] Thus, it would be meaningful to explore novel molecular biomarkers associated with the early diagnosis and prognosis of esophageal cancer.

RRBP1 is an endoplasmic reticulum membrane protein, which plays a critical role in the transportation and secretion of nascent proteins.[9] Recently, RRBP1 over-expression has been frequently observed in lung cancer, breast cancer, and colorectal cancer.[10–12] Moreover, RRBP1 correlates with shorter survival and can serve as a valuable prognostic factor in Her-2-positive breast cancer patients.[13] RRBP1 over-expression contributes to the progression of colorectal cancer and is useful for predicting patients' prognosis.[14] Thus, this evidence suggests that RRBP1 may be a key oncogene involved in tumor formation and progression. However, the expression and clinical significance of RRBP1 have never been reported in esophageal carcinoma.

In this study, we detected the expression of RRBP1 in 120 cases of esophageal carcinoma and matched adjacent normal tissues, and analyzed the correlation between RRBP1

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expression and clinicopathological features. Moreover, whether RRBP1 could be a potential prognostic biomarker in patients with esophageal carcinoma was further assessed.

Materials and methods

Patients and samples

One hundred and twenty esophageal carcinoma (without chemotherapy and radiotherapy before surgery) specimens were collected from patients presenting to Cangzhou Central Hospital during 2010–2014. Matched adjacent normal tissues were collected 3 cm from esophageal carcinoma tissue. Patients included 57 males and 63 females with a mean age of 58 years (range, 32–74 years). Clinical pathological characteristics including age, gender, history of smoking, tumor location, T stage, lymph node metastasis, and TNM stage were obtained from hospital records. Follow-up time was from the day of surgery. No patient was lost during follow-up and the follow-up duration ranged from 1 to 65 months (mean, 38.1 months). All the samples were diagnosed as squamous cell carcinoma. The pathological diagnosis was confirmed by two pathologists in Cangzhou Central Hospital.

Quantitative real-time polymerase chain reaction (q-RT-PCR)

All tissues were frozen in liquid nitrogen. RNA was extracted by RNAisoTM PLUS (Thermo Fisher Scientific, Waltham, MA, USA) and reverse transcribed into cDNA by cDNA Synthesis Kit (TaKaRa Corp, Dalian, China). Quantitative analysis of RRBP1 was performed using 7500 SYBR Green Fast Real-Time PCR System (Thermo Fisher Scientific,). The reaction conditions were 95°C for 10 min, followed 95° for 15 s for 40 cycles and 60°C for 60 s. The primer sequences of RRBP1 were 5′-TGAATCCTCCAAAGACCACA-3′ and 5′-CTTTCCCTCTCGCGTCTCT-3′. The primer sequences of GAPDH were 5′-CTGAACGGGAAGCTCACTGG-3′ and 5′-TGAGGTCCACCACCCTGTTG-3′. The experiments were repeated three times under the same conditions.

Western blot analysis

All tissues were frozen in liquid nitrogen. Proteins were extracted by protease inhibitors and quantified by the Pierce BCA Protein Assay Kit (Thermo Fisher Scientific). An amount of 50 μg per sample was resolved on 5% sodium dodecyl sulfate polyacrylamide gel electrophoresis and transferred onto polyvinylidene fluoride membranes. After blocking in 5% fat-free milk at room temperature, membranes were incubated with RRBP1 (Epitomics, Inc., Burlingame, CA, USA) (diluted 1:1000) and GAPDH (Zhongshan Corp, Beijing, China) (diluted 1:1000) antibodies overnight at 4°C. Then, membranes were incubated with horseradish peroxidase-conjugated secondary antibodies for 1 h. The signals were measured by enhanced chemiluminescence detection reagents.

Immunohistochemical (IHC) staining

Sections (2 μm thick) were deparaffinized with xylene and rehydrated in graded ethanol. Endogenous peroxidase was wiped off with 3% hydrogen peroxide and antigenicity was repaired by 0.01 mol/L sodium citrate buffer (pH 6.0). All sections were incubated with rabbit monoclonal RRBP1 antibody (Epitomics, Inc.) (diluted 1:200) at room temperature for 2 h. After incubation with secondary biotinylated antibody, sections were stained with diaminobenzidine (DAB) and hematoxylin.

The staining of RRBP1 was analyzed by semi-quantitative method. The staining intensity was scored as blank (0), weak (1), moderate (2), and strong (3). The percentage of positive cells was scored as <5% (0), ≥5% –<25% (1), 25% –50% (2), and >50% (3). The scores were calculated by multiplying these two values (ranging from 0 to 9). These scores (≥4) were defined as RRBP1 high-expression, and others were defined as RRBP1 low-expression (<4). All IHC scores were assessed by two pathologists independently without the clinical information.

Statistical analysis

All data were analyzed with SPSS software (version 19.0; IBM Corporation, Armonk, NY, USA). IHC results were analyzed by chi-square test. Survival analysis was performed by the Kaplan–Meier method and log-rank test. Multivariate analysis was assessed by Cox's proportional hazards model. The comparison of two-sample mean was evaluated using independent samples *t*-test. *P*-value of <0.05 was defined as statistically significant.

Ethics statement

This study was approved by the Cangzhou Central Hospital Ethnics Committee. All patients signed informed consent and agreed to the use of their tissue samples in this study.

Results

RRBP1 is highly expressed in esophageal carcinoma

First, we detected the expression of RRBP1 in 120 esophageal carcinoma specimens and matched adjacent normal tissues by qRT-PCR and Western blot assays. qRT-PCR results indicated that RRBP1 mRNA level was significantly higher in esophageal carcinoma tissues compared with matched adjacent

normal tissues (Figure 1A, *P*=0.000). Meanwhile, Western blot results revealed that RRBP1 protein was highly expressed in esophageal carcinoma tissues compared with matched adjacent normal tissues (Figure 1B, *P*=0.000). These data indicated that RRBP1 was highly expressed in esophageal carcinoma.

RRBP1 expression correlates with clinical pathological characteristics in esophageal carcinoma

Subsequently, we detected the expression of RRBP1 in 120 esophageal carcinoma specimens and matched adjacent normal tissues by IHC. As shown in Figure 2, positive expression of RRBP1 was located in cell cytoplasm and easily observed in esophageal carcinoma tissues, but was hardly detected in normal esophageal tissues. The high-expression rates of RRBP1 in esophageal carcinoma and normal esophageal tissues were 59.2% and 11.7%, respectively, and the difference was statistically significant (Table 1, *P*=0.000). Moreover, RRBP1 expression was associated with T stage, lymph node metastasis, and TNM stage in esophageal carcinoma (Table 2, *P*<0.05), but was not associated with age, gender, history of smoking, and tumor location (Table 2, *P*>0.05).

High-expression of RRBP1 predicts an unfavorable survival rate in esophageal carcinoma patients

Then, we further analyzed the correlation between RRBP1 expression and patients' survival by Kaplan–Meier method and Cox's proportional hazards model. Kaplan–Meier analysis revealed that the median survival time of patients with RRBP1 high-expression was 43 months, which was significantly shorter compared with those with RRBP1 low-expression (56 months) (Table 3, Figure 3A, *P*=0.006). Moreover, T stage, lymph node metastasis, and TNM stage rather than age, gender, and history of smoking were confirmed to be associated with patients' survival (Table 3, Figure 3B–D, *P*<0.05). Furthermore, multivariate Cox regression analysis showed RRBP1 high-expression was significantly associated

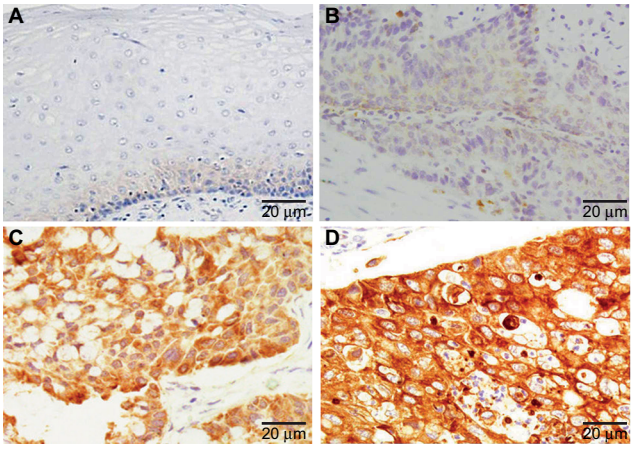

Figure 2 RRBP1 expression was detected in esophageal carcinoma and matched adjacent normal tissues by immunohistochemical staining.
Notes: (**A**) Adjacent normal tissues; (**B**) weak staining of RRBP1 in esophageal carcinoma; (**C**) moderate staining of RRBP1 in esophageal carcinoma; (**D**) strong staining of RRBP1 in esophageal carcinoma.

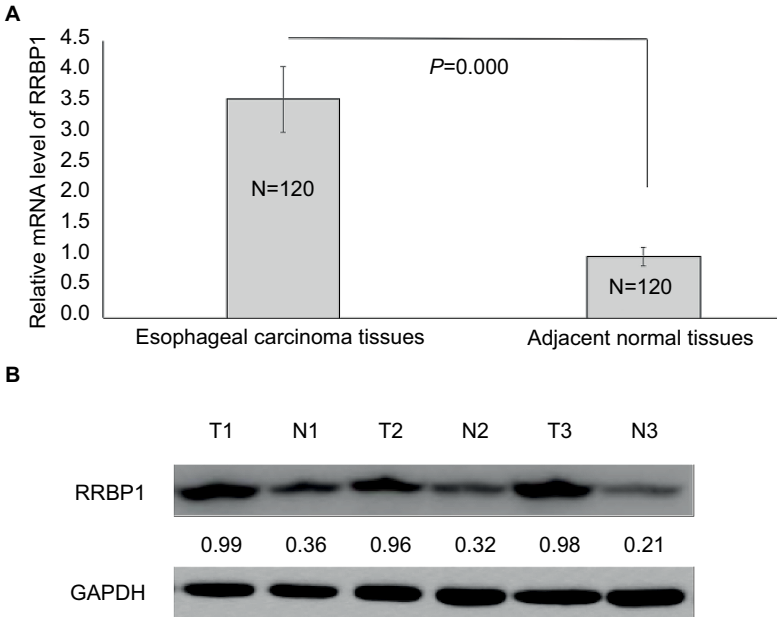

Figure 1 RRBP1 expression.
Notes: The expression of RRBP was detected in esophageal carcinoma and matched adjacent normal tissues by qRT-PCR (**A**) and Western blot (**B**). T, esophageal carcinoma tissue; N, matched adjacent normal esophageal tissue.

Table 1 RRBP1 expression in esophageal carcinoma and normal esophageal tissues by immunohistochemical staining

Types	N	RRBP1		*P*-value
		Low-expression (%)	High-expression (%)	
Esophageal carcinoma tissues	120	49 (40.8)	71 (59.2)	0.000
Normal esophageal tissues	120	106 (88.3)	14 (11.7)	

Table 2 RRBP1 expression correlation with clinicopathological characteristics in esophageal carcinoma

Clinicopathological characteristics	N	RRBP1		*P*-value
		Low-expression	High-expression	
Age (years)				
≤58	58	27	31	0.266
>58	62	22	40	
Gender				
Male	57	24	33	0.714
Female	63	25	38	
History of smoking				
Negative	55	20	35	0.853
Positive	65	29	36	
Tumor location				
Upper esophagus	56	27	29	0.14
Middle-lower esophagus	64	22	42	
T stage				
T1–T2	37	30	7	0.000
T3–T4	83	19	64	
Lymph node metastasis				
Negative	83	42	41	0.001
Positive	37	7	30	
TNM stages				
I–II	37	30	7	0.000
III–IV	83	19	64	

Table 3 Patient survival: Kaplan–Meier survival analysis

Variables	N	Survival time (months, 95% CI)	*P*-value
RRBP1			
Low-expression	49	56 (51–60)	0.006
High-expression	71	43 (39–48)	
Gender			
Male	57	51 (47–56)	0.323
Female	63	47 (42–52)	
Age (years)			
≤58	58	50 (45–55)	0.963
>58	62	50 (45–54)	
History of smoking			
Negative	55	49 (44–55)	0.845
Positive	65	50 (45–54)	
Tumor location			
Upper esophagus	56	50 (45–53)	0.213
Middle-lower esophagus	64	49 (45–54)	
T stage			
T1–T2	37	58 (53–62)	0.001
T3–T4	83	43 (39–48)	
Lymph node metastasis			
Negative	83	55 (51–59)	0.000
Positive	37	36 (31–41)	
TNM stages			
I–II	37	58 (53–62)	0.033
III–IV	83	43 (39–46)	

with unfavorable survival rate in esophageal carcinoma. Except for age, gender, history of smoking and tumor location, T stage, lymph node metastasis and TNM stage were also confirmed to be correlated with patients' survival (Table 4, $P<0.05$).

Discussion

RRBP1, an endoplasmic reticulum membrane protein, is mainly located on the endoplasmic reticulum membrane and plays an important role in the transportation and secretion of nascent proteins.[9,11,15] Moreover, RRBP1 is crucial for the terminal differentiation of secretory tissues and the procollagen biosynthesis of secretory tissues.[16–19] Recently, RRBP1 has been reported to be connected to the regulation of unfolded protein response signaling molecules and the accumulation of perinuclear autophagosomes of cancer cells.[10,20,21] In addition, RRBP1 was confirmed as an oncogene highly expressed in lung cancer, breast cancer, and colorectal cancer.[10–12] RRBP1 over-expression predicts unfavorable survival rates in colorectal cancer patients.[14] However, the expression and clinical significance of RRBP1 have never been reported in esophageal carcinoma.

In this study, in order to investigate the clinical significance of RRBP1 in esophageal carcinoma, we detected the expression of RRBP1 in 120 cases of esophageal carcinoma and matched adjacent normal tissues by qRT-PCR, Western blot, and IHC assays. qRT-PCR and Western blot results both showed that RRBP1 was highly expressed in esophageal carcinoma tissues compared to matched adjacent normal tissues, suggesting that RRBP1 high-expression might contribute to the occurrence of esophageal carcinoma. Meanwhile, IHC results showed that RRBP1 high-expression was observed in 59.2% esophageal carcinoma, but only in 11.7% matched adjacent normal tissues. Thus, IHC results were consistent

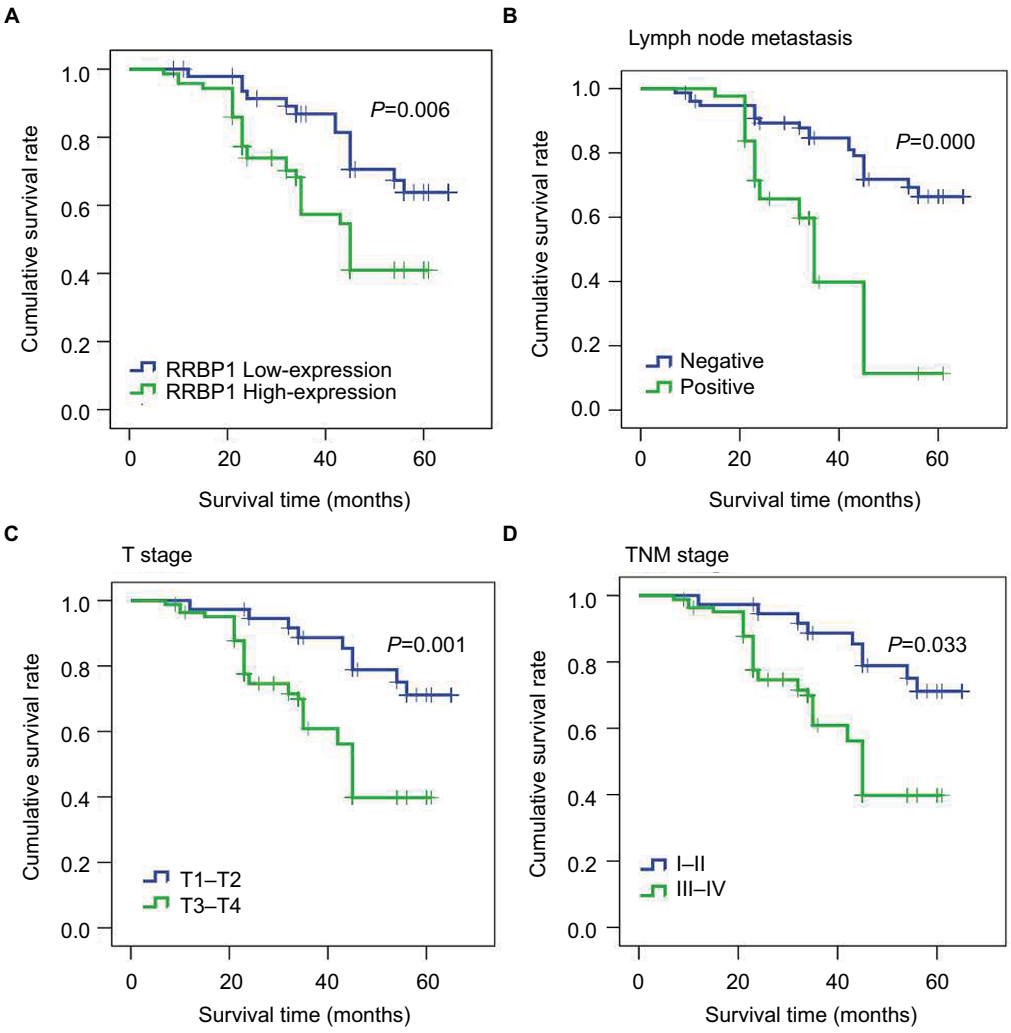

Figure 3 Kaplan–Meier survival analysis.
Notes: Results indicated that RRBP1 expression (**A**), lymph node metastasis (**B**), T stage (**C**), and TNM (**D**) stage were associated with patients' prognosis.

Table 4 Patients' survival evaluation by multivariate Cox regression analysis

Variables	Hazard ratio	95% CI	*P*-value
RRBP1 (high-expression vs low-expression)	2.441	1.267–4.702	0.008
Gender (male vs female)	1.329	0.736–2.400	0.346
Age (≤58 vs >58 years)	0.994	0.516–1.916	0.987
History of smoking (positive vs negative)	0.963	0.498–1.862	0.912
Tumor location (upper vs middle-lower)	0.929	0.538–1.629	0.921
T stage (T3–T4 vs T1–T2)	3.054	1.453–6.421	0.003
Lymph node metastasis (positive vs negative)	4.024	2.180–7.424	0.000
TNM stage (III-IV vs I-II)	3.054	1.452–6.421	0.003

with qRT-PCR and Western blot results, which further supported that RRBP1 high-expression was correlated with the occurrence of esophageal carcinoma. In addition, our data revealed that RRBP1 expression was associated with T stage, lymph node metastasis, and TNM stage in esophageal carcinoma, which suggested that RRBP1 expression might be connected to the progression of esophageal carcinoma. Survival analysis showed that patients with RRBP1 high-expression presented shorter survival rates compared with those with RRBP1 low-expression, indicating that RRBP1 might serve as a prognostic biomarker in esophageal carcinoma. It is well-known that T stage, lymph node metastasis, and TNM stage are key factors associated with the progression of esophageal carcinoma and patients' survival.[22–28] In the present study, our data also indicated that T stage, lymph node metastasis, and TNM stage were independent prognostic factors in esophageal carcinoma. Thus, our data suggested that RRBP1 high-expression might contribute to the progression of esophageal carcinoma, which results in a poorer prognosis. In addition, Liang et al reported that RRBP1 was a valuable prognostic factor in Her-2-positive breast cancer patients.[13]

Pan et al reported that RRBP1 promoted the progression of colorectal cancer and predicted prognosis.[14]

Conclusion

This paper is the first to report that RRBP1 is an oncogene highly expressed in esophageal carcinoma. Additionally, our data indicate that RRBP1 may be connected with the occurrence and progression of esophageal carcinoma, and serve as an independent prognostic factor to predict patients' prognosis. Of course, further investigations are needed to validate our findings.

Acknowledgment

Thanks to all patients who agreed to participate in this study.

Author contributions

All authors contributed toward data analysis, drafting and revising the paper and agree to be accountable for all aspects of the work.

Disclosure

The authors report no conflicts of interest in this work.

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