RBBP7 is a prognostic biomarker in patients with esophageal squamous cell carcinoma

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Abstract. Retinoblastoma-binding protein 7 (RBBP7) is an important component of several complexes that regulate chromatin metabolism. It is overexpressed in certain cancer types and serves conflicting roles in tumor progression. In the present study, the expression and roles of RBBP7 were explored in esophageal squamous cell carcinoma (ESCC). Immunohistochemical staining was used to detect RBBP7 expression in ESCC tissues. The mRNA sequencing profiles from the Cancer Genome Atlas and Genotype-Tissue Expression databases were mined to analyze the mRNA expression of RBBP7 in tissues. Proliferation, clone formation, apoptosis and Transwell invasion/migration assays were performed to explore the roles of RBBP7 in ESCC. RBBP7 was highly expressed in ESCC tissues. The protein and mRNA expression levels of RBBP7 were significantly elevated in tumor tissues compared with paired adjacent normal tissues. RBBP7 overexpression was associated with a poor overall survival in patients with ESCC. Furthermore, higher RBBP7 expression was significantly correlated with poor tumor differentiation, advanced regional lymph node involvement, and pathological TNM staging. Knockdown of RBBP7 in ESCC cells did not affect tumor apoptosis or tumor growth. However, the overexpression of RBBP7 significantly enhanced the invasion and migration of ESCC cells, whereas the knockdown of RBBP7 resulted in significantly decreased invasion and migration. The present study indicated that RBBP7 is a novel biomarker and prognosticator for patients with ESCC. Furthermore, RBBP7 serves crucial roles in promoting ESCC invasion and migration.

Introduction

Esophageal cancer is the eighth most common cause of cancer-related death in women and the fifth most common in men worldwide (1). Despite the elaborate diagnostic protocols and complex therapeutic strategies developed to date, the prognosis of patients with esophageal cancer remains unsatisfactory (2-4). The 5-year survival rate of patients with esophageal cancer is just 20-30% (5,6). A major issue is that the basic molecular mechanisms driving the development and progression of esophageal cancer are not fully understood. Until now, only a few tumor biomarkers or therapeutic targets have been identified for the clinical management of esophageal cancer. Hence, novel biomarkers and therapeutic targets are needed to improve the treatment of patients with esophageal cancer.

RBBP7 overexpression was associated with a poor overall survival in patients with ESCC. Furthermore, higher RBBP7 expression was significantly correlated with poor tumor differentiation, advanced regional lymph node involvement, and pathological TNM staging. Knockdown of RBBP7 in ESCC cells did not affect tumor apoptosis or tumor growth. However, the overexpression of RBBP7 significantly enhanced the invasion and migration of ESCC cells, whereas the knockdown of RBBP7 resulted in significantly decreased invasion and migration. The present study indicated that RBBP7 is a novel biomarker and prognosticator for patients with ESCC. Furthermore, RBBP7 serves crucial roles in promoting ESCC invasion and migration.
to cancer progression and metastasis (18, 19). Until now, the expression and roles of RBBP7 in esophageal squamous cell carcinoma (ESCC) have remained unclear. Here we investigated the expression, clinical significance, and functions of RBBP7 in ESCC. We identified that RBBP7 is a prognostic factor for ESCC and plays vital roles in tumor migration and invasion.

Materials and methods

Clinical samples. We analyzed 126 formalin-fixed, paraffin-embedded (FFPE) ESCC specimens, of which 72 had adjacent non-tumor tissues, from patients undergoing resection at The Affiliated Hospital of Qindao University (Qindao, China), between April 2001 and June 2005. Retrospective clinicopathological data for these patients were also obtained, including age, sex, smoking history, alcohol consumption history, tumor size, lymph node involvement, tumor-node-metastasis (TNM) stage, and pathological grade (according to the 8th edition of the AJCC TNM staging guidelines). Overall survival (OS) was defined as the time from the date of surgery to the date of death. Disease-free survival (DFS) was defined as the time from the date of surgery to the date of recurrence or progression. The ages of these patients ranged from 39 to 95 years (average, 69 years). Eighty-seven patients had a history of smoking, and 72 patients had a history of alcohol consumption. The numbers of tissues staged as T1, T2, T3, and T4 were 10 (79.4%), 15 (11.90%), 86 (68.25%), and 15 (11.90%), respectively. The numbers of patients classified as G1, G2, and G3 were 22 (17.46%), 51 (40.48%), and 43 (34.13%), respectively. Eighty patients had regional lymph node metastasis. The numbers of patients with stage I, II, and III disease were 26 (20.63%), 40 (31.75%), and 60 (47.62%), respectively.

The use of these specimens and the patient information were approved by the Ethics Committee of The Affiliated Hospital of Qindao University. All patients provided written consent for the use of their specimens and disease information for future investigations according to the guidance of the ethics committee.

Immunohistochemistry (IHC). IHC was used to detect RBBP7 expression in esophageal tumor tissues. In brief, esophageal tumor tissue was serially sectioned at 5-μm thickness, deparaffinized in xylene, and rehydrated through a series of graded alcohol concentrations. The samples were boiled in a pressure cooker for 3 min to retrieve the antigens. Endogenous peroxidases were blocked using 3% hydrogen peroxidase treatment for 10 min. The sections were then incubated with anti-RBBP7 primary antibodies (20365-1-AP; Wuhan Sanying Biototechnology, Wuhan, China) overnight at 4°C. Detection was performed with a peroxidase-conjugated secondary antibody at 37°C for 30 min and a diaminobenzidine (DAB) substrate kit was used to perform the chromogenic reaction. The sections were counterstained with hematoxylin. The slides were then dehydrated through graded alcohol concentrations and covered with coverslips. The staining intensity and percentage of RBBP7-positive tumor cells were assessed. The intensity of the staining was evaluated using the following criteria: 0, negative; 1, low; 2, medium; and 3, high. The extent of staining was scored as 0, 0% stained; 1, 1-25% stained; 2, 26-50% stained; and 3, 51-100% stained. The final scores were calculated by multiplying the scores of the intensity by those of the extent and dividing the samples into four grades: 0, absent (-); 1-2, weak (+); 3-5, moderate (++); and 6-9, strong (+++).

Cell culture. Human ESCC cell lines TE1, KYSE30, and KYSE150 were bought from Creative Bioarray (New York, USA) and Eca109 was obtained from the Committee of Type Culture Collection of the Chinese Academy of Sciences (Shanghai, China). All cells were cultured with antibiotics at 37°C in a humidified 5% CO2 incubator. Cells were cytogenetically tested and authenticated before freezing. All cell lines were cultured in Dulbecco's modified Eagle's medium (DMEM)/F-12K/RPMI-1640 medium supplemented with 10% fetal bovine serum (FBS).

Plasmid construction and stable cell line establishment. The complete coding sequence of the human RBBP7 gene was cloned into vector pLV-EF1-MCS-IRES-Bsd (Biosettia, San Diego, CA, USA). Lentiviruses were produced in 293T cells for the stable transfection of the cell lines, and an empty vector was used as the control. Tumor cells (1x10⁶) were infected with 100 μl of lentivirus supernatant (1x10⁹TU/ml). Forty-eight hour later, blastidicin (InvivoGen, San Diego, CA, USA) was added for selection.

Short interfering RNA (shRNA) sequences were designed using Biosettia’s shRNA designer (http://biosettia.com/support/shrnas-designer/). Three recommended sequences for RBBP7 were synthesized and cloned into the pLV-hU6-EF1α-puro vector (Biosettia). Scrambled sequences were used as controls. The lentiviruses were then produced in 293T cells. The most efficient of the three stable cell lines was used for the relevant assays.

Western blot analysis. Whole-cell extracts were prepared by lysing cells with radioimmunoprecipitation assay (RIPA) lysis buffer with a proteinase inhibitor cocktail (Sigma-Aldrich; Merck KGaA, Darmstadt, Germany). Protein concentrations were quantified using a Pierce Protein Assay kit (Thermo Fisher Scientific, Inc., Waltham, MA, USA). Protein lysates (20 mg) were separated by SDS-PAGE, and target proteins were detected by western blot analysis with antibodies against RBBP7 (1:1,000), and β-actin (1:5,000) as a control.

Transwell assay. Migration and invasion assays were performed with 8.0-mm pore inserts in a 24-well Transwell chamber. For this assay, 1x10⁵ cells were isolated and added to the top chamber with DMEM/F-12K/RPMI-1640. The invasion assay was performed using 1/6 diluted Matrigel-coated filters (BD Biosciences, Franklin Lakes, NJ, USA). DMEM/ F-12K/RPMI-1640 with 10% FBS was added to the bottom chamber and the cells were allowed to incubate for 24 h. Cells that had migrated to the bottom of the filter were stained with a three-step staining system (Thermo Fisher Scientific, Inc.). All experiments were repeated independently at least three times.

Colony formation assay. The plate colony formation assay was performed by seeding 1,000 cells/well into 6-well plates in triplicate. Fresh whole medium was changed every 4 days. Two weeks after seeding, the medium was discarded and the cells were stained using 0.1% crystal violet in methanol (Sigma-Aldrich; Merck KGaA).
Cell counting kit-8 (CCK-8) cell viability assay. Two thousand cells per well were seeded into 96-well plates and incubated. An aliquot of 10 µl of CCK-8 was added to each well and incubated for 2 h. The absorbance was measured at 450 nm to calculate the number of viable cells in each well. Each measurement was performed in triplicate and the experiments were repeated twice.

The cancer genome atlas (TGCA) data analysis. The TGCA data of 198 patients with esophageal cancer were downloaded from the TCGA website. Among them, 192 patients had complete RNA sequencing data. The Genotype-Tissue Expression (GTEx) data of 286 normal esophageal tissues were downloaded from the GTEx website. The normalized counts of the RNA expression of RBBP7 were extracted and analyzed. OS and DFS were analyzed at TCGA.

Statistical analysis. Statistical analyses were performed using the IBM SPSS Statistics Program. The Wilcoxon signed ranks test was used to compare the difference between tumor tissues and their paired adjacent non-tumor tissues. χ² tests or Student's t-tests were used to analyze the association.
between RBBP7 expression and clinicopathological features. Kaplan-Meier curves were constructed and analyzed for the relevant variables. The log-rank test was used to analyze the differences in survival times among the patient subgroups. All probability values had a statistical power level of 90% and a 2-sided level of 5%. P<0.05 was considered to indicate a statistically significant difference.

Results

RBBP7 is overexpressed in ESCC. We classified a pattern of RBBP7 expression ranging from absent (scored as 0) to low, median, and high positive (scored as 1+, 2+, and 3+, respectively) (Fig. 1A). The results showed that RBBP7 was localized mainly in the cell nucleus and frequently overexpressed in ESCC. Among 126 ESCC specimens analyzed, the numbers of specimens scored as 0, 1, 2, and 3 were 24 (19.0%), 36 (28.6%), 48 (38.1%), and 18 (14.3%) (Fig. 1B).

The differential expression of RBBP7 between ESCC tissues and normal esophageal tissues was statistically analyzed. As shown in Fig. 2A, RBBP7 was highly expressed in ESCC tissues as compared with the paired non-tumor tissues. We discovered that in 72 pairs of ESCC tissues and adjacent non-tumor tissues, RBBP7 protein was detected in the majority of the ESCC tissues but not in the non-tumor tissues. Statistical analysis confirmed that the abundance of RBBP7 protein in the ESCC tissues was significantly higher than that in the corresponding non-tumor tissues (Z=-4.636, P<0.0001, Wilcoxon signed ranks test) (Fig. 2B).

Subsequently, we compared the mRNA expression of RBBP7 between esophageal carcinoma tissues and normal esophageal tissues. The whole-genome mRNA sequencing profiles of 13 paired esophageal carcinoma tissues and the corresponding normal tissues were downloaded from TCGA database. In concordance with our IHC results, the mRNA expression of RBBP7 was significantly elevated in esophageal carcinoma tissues as compared with paired non-tumor tissues (27.71±7.58 vs. 38.93±15.06; P=0.0167; paired t-test) (Fig. 2C).

Furthermore, we compared the mRNA expression of RBBP7 between esophageal carcinoma tissues from TCGA database (n=181) and normal esophageal tissues from the GTEx database (n=286). The results of this comparison also showed that the mRNA expression of RBBP7 was significantly higher in the esophageal carcinoma tissues (P<0.05; unpaired t-test) (Fig. 2D).

### Table I. Association between RBBP7 expression and clinicopathological features of patients with ESCC.

| Variable                  | Total (n=126) | Low (n=70) | High (n=54) | χ² | P-value |
|---------------------------|---------------|------------|-------------|----|---------|
| Sex                       |               |            |             |    |         |
| Male                      | 105           | 51         | 54          | 0.229 | 0.407   |
| Female                    | 21            | 9          | 12          |     |         |
| Age (years)               |               |            |             |    |         |
| <65                       | 45            | 19         | 26          | 0.457 | 0.237   |
| ≥65                       | 81            | 41         | 40          |     |         |
| Smoker                    |               |            |             |    |         |
| Yes                       | 87            | 38         | 49          | 0.247 | 0.129   |
| No                        | 39            | 22         | 17          |     |         |
| Alcoholic                 |               |            |             |    |         |
| Yes                       | 72            | 33         | 39          | 0.719 | 0.388   |
| No                        | 54            | 27         | 27          |     |         |
| T staging                 |               |            |             |    |         |
| T1-T2                     | 101           | 46         | 55          | 0.379 | 0.238   |
| T3-T4                     | 25            | 14         | 11          |     |         |
| Nodal involvement         |               |            |             |    |         |
| Yes                       | 80            | 45         | 35          | 6.544 | 0.016*  |
| No                        | 46            | 15         | 31          |     |         |
| Differentiation           |               |            |             |    |         |
| G1-G2                     | 83            | 46         | 37          | 5.936 | 0.023*  |
| G3                        | 43            | 14         | 29          |     |         |
| TNM staging               |               |            |             |    |         |
| I/II                      | 66            | 41         | 25          | 11.686 | 0.001* |
| III                       | 60            | 19         | 41          |     |         |

*P<0.05 (χ² tests). RBBP7, retinoblastoma-binding protein 7; ESCC, esophageal squamous cell carcinoma; TNM, tumor-node-metastasis.
RBBP7 is a biomarker of advanced nodal stage and TNM stage. To investigate the clinicopathological significance of RBBP7 in ESCC, we analyzed the potential correlations between RBBP7 expression and the clinicopathological characteristics of ESCC patients. As shown in Table I, a high expression of RBBP7 was positively correlated with poor differentiation ($\chi^2=5.936, P=0.023$, Chi-squared test), regional lymph node involvement ($\chi^2=6.544, P=0.016$, Chi-squared test), and advanced pTNM staging ($\chi^2=11.686, P=0.001$, Chi-squared test). No significant correlations were observed between RBBP7 expression and sex, age, smoking history, alcohol consumption history, or T staging.

Subsequently we analyzed the mRNA expression of RBBP7 and the corresponding clinical data of esophageal carcinoma patients from TCGA database. We found that a high mRNA expression of RBBP7 was significantly correlated with poor differentiation ($t=-2.153, P=0.035$, unpaired t-test), regional lymph node involvement ($t=-2.477, P=0.024$, unpaired t-test), and advanced pTNM staging ($t=-2.218, P=0.028$, unpaired t-test) (Table II).

Next, we analyzed the mRNA expression of RBBP7 and the corresponding follow-up data of esophageal carcinoma patients from TCGA database to assess the prognostic significance of RBBP7 mRNA expression. In concordance with the immunohistochemical analysis, the median OS and DFS of patients with a higher mRNA expression of RBBP7 were significantly poorer than those of patients with a lower mRNA expression of RBBP7 (19.71 vs. 32.42 months and 13.01 vs. 24.05 months, respectively; log-rank test) (Fig. 3C and D).

RBBP7 promoted the migration and invasion of ESCC cells. To explore the roles of RBBP7 in tumor progression, we established stable cell lines in which RBBP7 was overexpressed or knocked down. RBBP7 was knocked down in two ESCC cell lines (TE1 and Eca109) (Fig. 4A) and overexpressed in two other ESCC cell lines (KYSE30 and KYSE150) (Fig. 4B). The roles of RBBP7 in tumor growth, survival, migration, and migration were explored in vitro. The results of CCK-8 assays showed that RBBP7 knockdown had no effect on tumor viability (Fig. 5A). Colony formation assays showed that RBBP7 inhibition did not change the colony formation rates (TE1: 21.0±1.7 vs. 23.5±0.87; P=0.266; Eca109: 18.4±0.95 vs. 19.0±0.58; P=0.619; unpaired t-tests) (Fig. 4C). RBBP7 knockdown did not change the rate of apoptosis under normal culture conditions (TE1: 5.4±0.4 vs. 5.3±0.2; P=0.231; Eca109: 4.1±0.2 vs. 5.2±0.3; P=0.352; unpaired t-tests) (Fig. 4D). These data suggested that RBBP7 has no roles in maintaining ESCC cell growth and survival.

However, the changes in RBBP7 expression showed significant effects in the cell migration and invasion assays.

### Table II. Association between RBBP7 mRNA expression and clinicopathological features of patients with ESCA from TCGA.

| Variable               | Total | RBBP7 mRNA (RPKM) | t value | P-value |
|------------------------|-------|-------------------|---------|---------|
| Sex                    |       |                   |         |         |
| Male                   | 166   | 37.71±17.83       | -0.883  | 0.635   |
| Female                 | 31    | 41.03±17.04       |         |         |
| Age (years)            |       |                   |         |         |
| <65                    | 108   | 38.85±19.17       | 0.607   | 0.148   |
| ≥65                    | 76    | 37.23±15.49       |         |         |
| T staging              |       |                   |         |         |
| T1-T2                  | 73    | 35.24±14.57       | -1.152  | 0.259   |
| T3-T4                  | 91    | 38.36±19.14       |         |         |
| Nodal staging          |       |                   |         |         |
| N0                     | 80    | 33.39±11.70       | -2.477  | 0.024*  |
| N1-N3                  | 84    | 39.96±20.43       |         |         |
| Differentiation        |       |                   |         |         |
| G1-G2                  | 95    | 35.00±15.37       | -2.153  | 0.035*  |
| G3                     | 48    | 42.74±22.42       |         |         |
| TNM staging            |       |                   |         |         |
| I-II                   | 96    | 33.87±13.97       | -2.218  | 0.028*  |
| III                    | 64    | 39.74±20.61       |         |         |

Independent t-test; *P<0.05. ESCA, esophageal carcinoma; RBBP7, retinoblastoma-binding protein 7; TCGA, The Cancer Genome Atlas; TNM, tumor-node-metastasis.
Figure 3. Clinical significance of RBBP7 in ESCC. (A and B) Kaplan-Meier analysis of overall survival of the 126 patients with ESCC according to different RBBP7 levels. (C and D) Kaplan-Meier analysis of overall survival and disease-free survival of the 182 patients with esophageal cancer according to different RBBP7 mRNA levels. Log-rank tests were used in (A-D). RBBP7, retinoblastoma-binding protein 7; ESCC, esophageal squamous cell carcinoma; IHC, immunohistochemistry; TCGA, The Cancer Genome Atlas.

Figure 4. RBBP7 has no effect on tumor growth and survival processes in ESCC. (A) TE1 and Eca109 cells were transfected with lentiviruses to knock down RBBP7 expression. Western blot assays were performed to verify the stable cell lines. (B) KYSE150 and YSE30 cells were transfected with lentiviruses to upregulate RBBP7 expression. Western blot assays were performed to verify the stable cell lines. (C) A plate colony formation assay was used to detect the colony formation rate. (D) Flow cytometry analysis was used to detect the cell apoptosis rate. Unpaired t tests were used in (C) and (D); data are shown as the mean values ± standard deviation. Scale bar, 1 cm. RBBP7, retinoblastoma-binding protein 7; ESCC, esophageal squamous cell carcinoma; ns, not significant; PI, propidium iodide.
As shown in Fig. 5B and C, RBBP7 knockdown significantly inhibited the migration and invasion of TE1 and Eca109 cells. Conversely, RBBP7 overexpression strongly enhanced the migration and invasion of KYSE150 and KYSE30 cells. Taken together, these results suggested that RBBP7 has vital roles in the migration and invasion of ESCC cells.

Discussion

To the best of our knowledge, this is the first study to have investigated the expression, clinical significance, and roles of RBBP7 in ESCC. We found that RBBP7 was frequently overexpressed in ESCC tissues. A higher RBBP7 expression was significantly correlated with a poorer prognosis. This suggests that RBBP7 might be a cancer-related protein with a functional role in tumor progression.

RBBP7 was reported to function as a tumor-suppressor gene that inhibits tumor growth by mediating JNK signaling (15) or binding to HDAC1 and Sp1 (19). RBBP7 also facilitated stress-induced apoptosis and suppressed tumorigenicity in breast epithelial cells (16). Nevertheless, we found that RBBP7 had no effect on tumor growth and survival in ESCC. Furthermore, the overexpression of RBBP7 in ESCC tissues at both the protein and mRNA levels correlated with poorer differentiation, regional lymph node involvement, and advanced pTNM staging, suggesting that RBBP7 might promote tumor progression in patients with ESCC. We inferred that RBBP7 might have complex roles that may vary among different kinds of tumors owing to the tissue-specificity and intricate functions of the HDAC complexes that RBBP7 interacts with. This hypothesis is well supported by several published studies. In a recent study, RBBP7 overexpression was found to facilitate the epithelial-mesenchymal transition and promote migration and invasion (18). RBBP7 is a component of the Mi2/NuRD complex. Additionally, RBBP7 could be recruited by TWIST to the proximal regions of the E-cadherin promoter to repress the transcription of E-cadherin and thereby promote cell migration and invasion (20). Another report revealed that RBBP7 can be recruited to the Sp1 site of the RECK promoter region by Sp1 to activate RECK, leading to increased MMP9 activity that may promote metastasis (19). These studies have suggested that RBBP7 might play important roles in the epithelial-mesenchymal transition and contribute to cancer metastasis, providing a molecular mechanism for our findings that RBBP7 promoted the migration and invasion of ESCC cells in vitro. This potential functional role of RBBP7 in ESCC progression was further supported by our clinical analysis results, which showed that a high RBBP7 expression is significantly correlated with advanced regional lymph node involvement and pTNM staging. Together, these results suggested that RBBP7 may facilitate the metastasis of ESCC. Further studies are needed to verify this hypothesis and clarify the underlying mechanisms.
We found that the OS and DFS of patients with ESCC who had a higher expression of RBBP7 were significantly shorter than those of patients who had a lower expression of RBBP7. This might be the consequence of a higher metastasis risk in ESCC patients with higher RBBP7 expression levels. Our findings suggested that a high expression of RBBP7 could be used as a prognosticator for poor survival. Prospective clinical trials are needed to verify the prognostic roles and usefulness of RBBP7 in ESCC.

Some limitations of this study are noted. Because nearly all the patients with esophageal carcinoma in China have ESCC, we did not include the clinical data of esophageal adenocarcinoma in this study. As a result, the expression and clinical significance of RBBP7 in esophageal adenocarcinoma remain unclear.

In conclusion, our findings demonstrated that RBBP7 was highly expressed in esophageal cancer. RBBP7 promoted esophageal cancer cell migration and invasion. We identified RBBP7 as a prognostic biomarker for patients with esophageal carcinoma.

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Availability of data and materials
The datasets used and/or analyzed during the current study are available from the corresponding author on reasonable request.

Authors' contributions
Study concept and design: HL and NY. Acquisition of data: NY. Analysis and interpretation of data: PZ. Drafting of the manuscript: NY. Critical revision of the manuscript for important intellectual content: HL. Statistical analysis: LW, XH and SY. Study supervision: HL.

Ethics approval and consent to participate
The use of these specimens and the patient information were approved by the Ethics Committee of The Affiliated Hospital of Qingdao University.

Patient consent for publication
All patients provided written consent for the use of their specimens and disease information for future investigations according to the guidance of the ethics committee.

Competing interests
The authors declare that they have no competing interests.

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