Original Article

m6A reading protein RBMX as a biomarker for prognosis and tumor progression in esophageal cancer

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Background: As a member of m6A methylated binding protein, RNA binding motif protein X-linked (RBMX) has been reported to be associated with tumor invasion, metastasis and prognosis. However, the prognostic significance of RBMX expression in esophageal cancer (ESCA) remains unclear.

Methods: Based on the TIMER database, GEPIA database, cBioPortal database, CIBERSORT deconvolution algorithm, String-DB database, LinkedOmics database, etc., the RBMX expression level, mRNA expression level, prognostic relationship, genetic mutation, immune cell infiltration level, protein interaction network, differential co-expression genes and functional enrichment in esophageal carcinoma were analyzed. Immunohistochemistry was used to detect the expression of RBMX in 53 cases of esophageal carcinoma and adjacent esophageal tissues.

Results: The RBMX expression in ESCA tissue was significantly higher than that in the normal tissues. The overall survival (OS) of patients with high RBMX expression was significantly lower than that of patients with low expression (P=0.04). The protein encoded by the *RBMX* gene appeared to copy number amplification, mutation and deep deletion. The expression level of RBMX was positively correlated with the levels of follicular helper T cells, eosinophils and initial B cells (P<0.05). Genes significantly and positively correlated with RBMX expression included HNRNPA1L2, SFRS13A, HNRNPA1, etc., which were mainly enriched in biological processes (BPs) such as cell division, mRNA splicing, RNA binding and mRNA 3'-UTR binding.

Conclusions: RBMX may be as a biomarker of poor prognosis of ESCA. RBMX is closely related to the survival and prognosis, genetic mutation and immune cell infiltration of patients with ESCA.

Keywords: Bioinformatics analysis; esophageal cancer (ESCA); m6A; RNA binding motif protein X-linked (RBMX); prognosis

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Introduction

Esophageal cancer (ESCA), as one of the malignant gastrointestinal malignant tumors of the esophageal epithelium, exhibits specific geographical distribution differences. According to the global cancer statistics in 2020, ESCA ranked seventh in the world in terms of incidence and sixth in terms of death (1), which constitutes a serious threat to the life and health of patients. ESCA does not

manifest any clinical symptoms in the early stage; however, patients have been already in the advanced stage when emaciation, dysphagia and other symptoms appear, and postoperative outcomes are poor. Therefore, early detection and treatment of ESCA can significantly reduce the disease burden in the high incidence area of ESCA such as Henan, Shanxi and Shandong in China, which is significant for the secondary prevention of ESCA.

m6A methylation is regulated by methyltransferase, demethylase and reading protein. Among the existing regulatory factors related to m6A methylation modification, m6A methyltransferases include METTL3, METTL14, WTAP, KIAA1429, RBM15/RBM15B, METTL5 and ZCCHC4. m6A demethylase includes FTO, ALKBH5. m6A reading proteins include YTHDFs, IGF2BPs, HNRNPs and so on. Recent studies have found that METTL3, WTAP, FTO, YTHDF1, IGF2BP1, IGF2BP2 and IGF2BP3 are highly expressed in ESCA (2-8). METTL14, ALKBH5 and YTHDC2 are all expressed in low level in ESCA (9-11), which further affects the occurrence and development of ESCA.

The m6A reading protein, RNA binding motif protein X-linked (RBMX), is a universally expressed nuclear RNA-binding protein encoded by genes on the X chromosome (12). As a member of the HNRNP family of m6A reading protein nuclear heterogeneous ribonucleoprotein, RBMX is also known as hnRNP-G, HNRNPG, HNRPG, MRXS11, RBMXP1, RBMXRT, RNMX (13). Studies in recent years have shown that the expression of RBMX is related to human malignant tumors, with varying degrees of dysregulation in many types of tumors, e.g., up-regulated expression in cholangiocarcinoma, colorectal adenocarcinoma, ESCA, head and neck squamous cell carcinoma, hepatocellular carcinoma (HCC), lung cancer and other tumors, and down-regulated expression in bladder urothelial carcinoma, breast cancer, thyroid carcinoma, endometrial carcinoma of the uterine body and other tumors (14,15). It is also related to tumor invasion, metastasis and prognosis (16-20). However, the role of RBMX in ESCA and its possible mechanism remains unclear. In this study, bioinformatics was used to analyze the expression of m6A reading protein RBMX in ESCA tissues and the relationship with prognosis, and tissue microarray technology was used to detect the expression of RBMX in ESCA and adjacent tissue for exploration of its possible mechanism in regulating ESCA, aiming to offer new ideas for clinical diagnosis, treatment and prognosis of ESCA. We present this article in accordance with the REMARK reporting checklist (available at https://tcr.amegroups.com/article/view/10.21037/tcr-23-84/rc).

Highlight box

Key findings

- In this study, by combining bioinformatics methods with tissue chip technology, we found that RNA binding motif protein X-linked (RBMX) may be a prognostic biomarker of esophageal cancer.

What is known and what is new?

- It has been reported that RBMX is abnormally expressed in multiple tumors and is associated with tumor invasion, metastasis and prognosis.
- Since the role of RBMX in esophageal cancer and its possible mechanism are still unclear, this study further analyzed the relationship between RBMX expression, prognostic correlation, immune cell infiltration level, protein interaction and methylation level in esophageal cancer tissues, and found that RBMX is highly expressed in esophageal cancer tissues. Moreover, esophageal cancer patients with high expression of RBMX have a trend of poor prognosis.

What is the implication, and what should change now?

- In order to further clarify the function and role of RBMX in esophageal cancer, molecular biology experiments will be conducted in the future to further explore.

Methods

Data acquisition

(I) TIMER2.0 database (http://timer.cistrome.org/);
(II) GEPIA database (GEPIA2, http://gepia2.cancer-pku.cn/#index);
(III) cBioPortal database (http://www.cbioportal.org/);
(IV) UALCAN database (http://ualcan.path.uab.edu/index.html);
(V) MEXPRESS database (http://mexpress.be/);
(VI) STRING database (https://string-db.org/);
(VII) LinkedOmics Database (http://www.Linkedomics.org/).

Specimen origin

All the esophageal and adjacent normal esophageal tissues needed for the preparation of tissue chips were purchased from Shanghai Outdo Biotechnology Co., Ltd. (Model HEsoS105Su01, Shanghai, China), including 53 patients with ESCA who received surgical treatment from

November 30, 2005 to January 25, 2011, among whom 50 were ESCA tissues and adjacent normal esophageal tissues. There were 3 cases of single ESCA tissue, all of which were random retrospective tissue samples. Inclusion criteria: (I) patients did not receive preoperative chemotherapy, radiotherapy, targeted drug therapy or other antitumor therapy; (II) complete pathological data and follow-up data were available; (III) ESCA tissues and the accompanying paracancer esophageal tissues were well preserved. Exclusion criteria: (I) patients receiving radiotherapy, chemotherapy or other therapies before surgery; (II) patients who were not followed up or did not receive follow-up. According to the arrangement of tissue chips, the adjacent normal esophageal tissues (50 sites) were used as control, and the tissue chips were stored in a refrigerator at 4 ℃. This study was approved by the Ethics Committee of Shanghai Outdo Biotechnology Co., Ltd. (No. SHYJS-CP-1707008). All patients signed informed consent. The study was conducted in accordance with the Declaration of Helsinki (as revised in 2013).

Laboratory reagent

The rabbit anti-human RBMX monoclonal antibody was purchased from Abcam Company (Cambridge, UK), and the final suitable concentration of the working titer of immunohistochemistry was 1:250. The immunohistochemical two-step assay [PV-6001 rabbit two-step detection kit, the second antibody was enzyme-labeled goat anti-rabbit immunoglobulin G (IgG) polymer, Beijing Zhongshan Jinqiao Biotechnology Co., Ltd., Beijing, China]; 3,3'-diaminobenzidine (DAB) chromogenic liquid (ZLI-9018) and imported goat serum (ZLI-9022) were purchased from Beijing Zhongshan Jinqiao Biotechnology Co., Ltd. Xylene reagent, anhydrous ethanol reagent, ethanol and hematoxylin dyes with different concentration gradients were prepared. The experiment was completed in the Immunohistochemistry Room of the Central Laboratory of Xinjiang Medical University.

Follow-up of study population

The included patients were followed up by regular inpatient, outpatient review or telephone follow-up. No follow-up was lost in 53 patients. The operation time of the patients was from November 2005 to January 2011, and the follow-up time was from March 2014, and the follow-up time was from 3 to 8 years. The relevant data were obtained by Shanghai Outdo Biotechnology Co., Ltd.

Correlation analysis of RBMX expression and prognosis in patients with ESCA

Based on TIMER2.0 (21) database, differential expression of RBMX between tumor tissues and adjacent normal tissues in The Cancer Genome Atlas (TCGA) database was searched by Gene_DE under "Exploration" module, and the Wilcoxon test was performed for these tumor types. $P<0.05$ was considered to be different in expression between tumor tissue and normal tissue. The box graph was used to show the distribution of gene expression levels. Based on the GEPIA2.0 (22) database, the expression of RBMX in ESCA tissues in the TCGA database was searched through the "Profile" section of the "Expression DIY", and the expression of RBMX in ESCA tissues and paracarcinoma esophageal tissues was compared. $P<0.05$ was considered statistically significant. The correlation between RBMX expression and prognosis of ESCA patients was analyzed based on the "Survival Plots" options in the GEPIA2.0 database. With the median RBMX expression value as the cut-off value, ESCA patients were divided into high expression and low expression, the hazard ratio (HR) and P value were calculated, Kaplan-Meier method and Logrank test were used to draw the survival curve, including the overall survival (OS) curve and disease-free survival (DFS) curve, and $P<0.05$ was considered to be a statistically significant difference.

Correlation study on RBMX expression and DNA methylation

Based on the cBioPortal database (23), firstly, three groups of ESCA tissue samples from TCGA database and source data from Firehose were selected in the "Query" option, a total of 926 patients with ESCA were collected. Secondly, enter the gene name in the "Enter genes" position and click "Submit Query"; finally, methylation data of HM27 and HM450 were selected in the Plot module to analyze the transcriptome expression changes of RBMX in the three groups of ESCA tissue samples. The UALCAN database and the MEXPRESS database were used to analyze the methylation level of RBMX promoter region and the methylation level of CpG island in RBMX promoter region.

Genetic mutation analysis

Based on the cBioPortal database, two ESCA datasets: Esophageal Adenocarcinoma (TCGA, Pan-Cancer Atlas) and Esophageal Carcinoma (TCGA, Nature 2017), were

used to analyze whether there were mutations in the protein encoded by RBMX gene in ESCA tissues by the "mutations" module. Further, the SangerBox database was used to analyze whether the mutation of RBMX had any effect on its expression in ESCA in the module of "Gene mutation and expression analysis", and the results were shown by violin dot plot.

Relationship between the expression level of RBMX and the infiltration level of tumor immune cells

Based on the TIMER database, "Gene" module was selected, "RBMX" was input in "Gene Symbol", and "ESCA" was selected for "Cancer Types". The relationship between the expression level of RBMX and immune cell infiltration was analyzed, and the richness of invasive immune cells in gene expression profile was analyzed by deconvolution method. B cells, $CD8^+$ T cells, $CD4^+$ T cells, macrophages, neutrophils and dendritic cells in gene modules were included. The results were represented by scatter plots, and $P<0.05$ was considered statistically significant.

Protein-protein interaction (PPI) network construction of RBMX and related protein interaction analysis

Based on the STRING database (24), enter the gene name in the "Search" TAB to construct the PPI network of RBMX, including the number of network nodes, PPI enrichment $P<0.05$ and protein correlation coefficient in the network, and the overall score >0.4 was considered to be statistically significant. The "Similar Genes Detection" module of GEPIA database was further used to analyze the top 10 genes strongly related to RBMX expression.

Screening of differentially co-expressed genes and functional enrichment analysis of RBMX in ESCA

Based on the LinkedOmics database (25), tumor type "TCGA-ESCA" was selected on the analysis interface, including 184 patients with ESCA. Data type was selected as "RNAseq", target gene was input as "RBMX", and statistical method was selected as "Pearson Correlation test". The different co-expressed genes of RBMX in patients with ESCA were analyzed in the TCGA database, and the volcano map of different genes and the heat map of up-regulated and down-regulated genes were obtained. Enrichment analysis of Gene Ontology (GO) and Kyoto Encyclopedia of Genes and Genomes (KEGG) pathways was conducted in the Link Interpreter module, including biological process (BP), cellular component (CC) and molecular function (MF). KEGG database was used to further explore related signaling pathways, and $P<0.05$ was considered statistically significant.

Immunohistochemical staining of RBMX

The prepared tissue microarrays were baked in wax in an oven at 60 ℃ for 1 h. The endogenous peroxidase was inactivated by dewaxing with xylene and hydration with different ethanol concentrations. The dewaxed and hydrated tissue microarrays were placed on a high-temperature resistant plastic slicing rack and soaked in a high-temperature resistant plastic dyeing box containing sufficient ethylene diamine tetraacetie acid (EDTA) antigen repair solution (pH =9.0), preheated in a microwave oven at 95–100 ℃ for 3–5 min, and then heated with medium-high fire for 10–15 min. After repairing, they were cooled at room temperature, washed with phosphate buffered saline (PBS) (3 times, 5 min each time), and sealed with goat serum at room temperature for 30 minutes. The excess serum was shaken off the microarrays, which were dropped with the primary antibody diluted at 1:250, and incubated at 4 ℃ overnight. The next day, after being placed at room temperature for 40 min and washed, they were dropped with the second antibody, incubated at 37 ℃ for 30 min, and washed with PBS (3 times, 5 min each time); they were dropped with the prepared DAB chromogenic solution to react for 2 min, and the chromogenic reaction under the microscope was observed, and they were re-dyed with hematoxylin for 1 min, and then soaked in 1% hydrochloric acid ethanol differentiation solution for at least 2 s. The tissue microarrays were drowned in ethanol concentrations of 85%, 95%, 95%, 100%, 100%, xylene I and xylene II for 10 s. After all became transparent, they were sealed with neutral gum, cut into sections after drying, and stained under the microscope.

Immunohistochemical results interpretation method and study endpoint

Under the microscope, RBMX positive staining is brownish yellow or tan. The tissues corresponding to each site of paired cancer and adjacent cancer were randomly selected to observe different representative areas. A total of 3–5 visual fields were observed under high magnification. The Image-

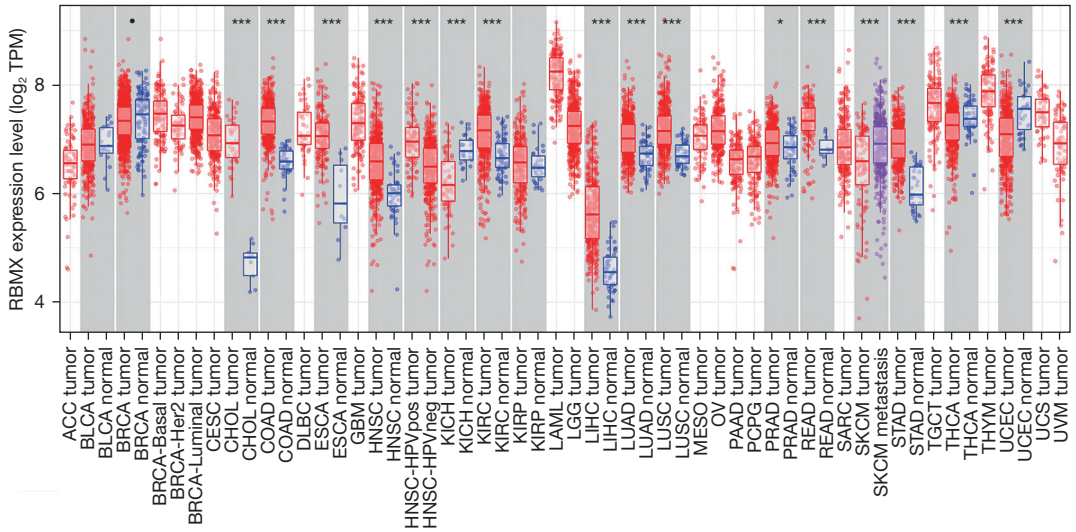

Figure 1 RBMX expression in various TCGA tumor and esophageal cancer tissues. The database: TIMER2.0 database (http://timer.cistrome.org/); GEPIA database (GEPIA2, http://gepia2.cancer-pku.cn/#index). Statistical test method: differential expression of RBMX gene between different tumors and adjacent normal tissues was analyzed using Wilcoxon test. RBMX was significantly high expressed in multiple cancers, and compared with adjacent tissues, the expression level of RBMX in the esophageal cancer tissue in TCGA-ESCA was statistically higher (P<0.001). In the figure, red represents esophageal cancer samples, blue represents normal samples and purple represents metastasis. P value significant codes: ***, P<0.001; *, P<0.05; •, P<0.1. RBMX, RNA binding motif protein X-linked; TPM, transcripts per million; TCGA, The Cancer Genome Atlas; GEPIA, Gene Expression Profiling Interactive Analysis; ESCA, esophageal carcinoma.

Pro Plus6.0 software (MEDIA CYBERNETICS Image Technology Inc., Bethesda, USA) was used to analyze the integrated optical density (IOD) value of RBMX expression and take the average value. The primary endpoints of our study were the expression of RBMX in ESCA tissues and OS, which is the time from surgery to death or the date of last follow-up.

Statistical analysis

For statistical analysis, the IOD values of cancer and paracancer group were arranged from high to low, and the study group was divided into high expression group and low expression group with the median as the cutting point. SPSS26.0 and GraphPad Prism8.0 statistical software were used to analyze the experimental results. The expression of RBMX protein in esophageal carcinoma tissues and adjacent tissues was compared by paired *T*-test. The expression of RBMX protein and its correlation with clinicopathologic parameters were compared by chi-square test. Using R 4.1.2 language programming, Kaplan-Meier survival analysis was used to calculate OS under different expressions of RBMX, and survival curves were drawn. Then, Logrank test was used to verify the correlation between RBMX expression and prognosis. P<0.05 was considered statistically significant.

Results

Analysis of differential expression of RBMX in pan-cancer and ESCA tissues

Based on the TIMER2.0 database, the expression of RBMX in 39 tumors of TCGA was analyzed. The results showed that RBMX was significantly highly expressed in cholangiocarcinoma, colorectal adenocarcinoma, ESCA, head and neck squamous cell carcinoma, HCC, lung cancer and other tumors. Compared with adjacent tissues, the expression level of RBMX in the ESCA tissue in TCGA-ESCA was statistically higher (*Figure 1*) (P<0.001). GEPIA2 database was used to further verify the expression of RBMX in 195 TCGA ESCA tissues, including 182 ESCA samples and 13 normal control samples and to compare RBMX expression in ESCA tissues and adjacent esophageal tissues. The results showed that the expression level of RBMX in ESCA tissues was significantly higher than that in normal tissues (Figure S1), with a statistically significant difference (P<0.05).

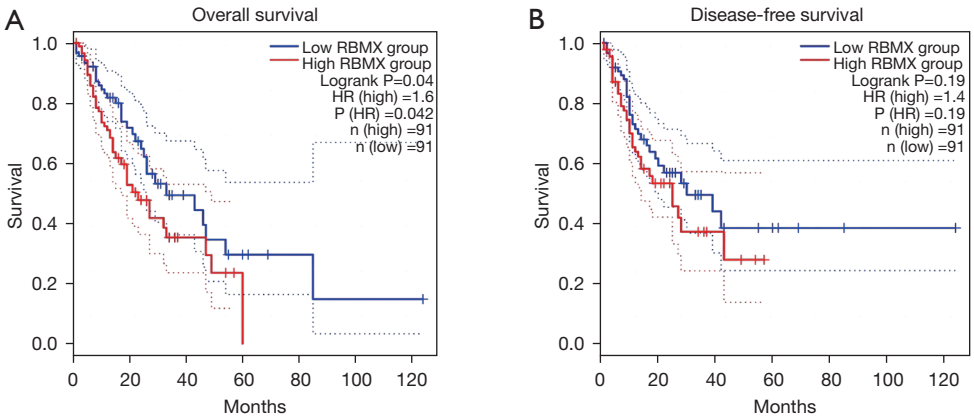

Figure 2 Relationship between RBMX expression and prognosis of patients with esophageal cancer. The database: GEPIA database (GEPIA2, http://gepia2.cancer-pku.cn/#index). (A) The OS time of esophageal cancer patients with high RBMX expression [Logrank P=0.04, HR(high) =1.6, P(HR) =0.042] was significantly lower than that of patients with low expression. (B) The DFS of esophageal cancer patients with high RBMX expression [Logrank P=0.19, HR(high) =1.4, and P(HR) =0.19] was lower than that of patients with low expression. RBMX, RNA binding motif protein X-linked; HR, hazard ratio; GEPIA, Gene Expression Profiling Interactive Analysis; OS, overall survival; DFS, disease-free survival.

Prognostic value of RBMX expression in patients with ESCA

GEPIA2 database was used to analyze the relationship between OS and DFS of patients with high and low RBMX expression. The Kaplan-Meier method and Logrank test were used to plot the survival curve. The results showed that the OS time of ESCA patients with high RBMX expression [Logrank P=0.04, HR(high) =1.6, P(HR) =0.042] was significantly lower than that of patients with low expression (*Figure 2A*). Moreover, the survival time of patients with low RBMX expression was longer, suggesting that high RBMX expression was associated with a poor prognosis. The DFS of ESCA patients with high RBMX expression [Logrank P=0.19, HR(high) =1.4, and P(HR) =0.19] was lower than that of patients with low expression (*Figure 2B*); high RBMX expression was significantly correlated with OS in patients with ESCA, indicating that RBMX could be used as one of the indicators of poor prognosis in patients with ESCA. The difference was statistically significant (P<0.05).

Analysis of correlation between RBMX expression and DNA methylation

The changes in the genomic level of RBMX were analyzed by the cBioPortal database, the UALCAN database and the MEXPRESS database. The results showed that there was no correlation between the expression of RBMX and the methylation level in the selected 3 groups of ESCA studies, including 1,185 samples of ESCA (Figure S2), the difference was not statistically significant (Spearman r=–0.06, P=0.251; Pearson r=–0.04, P=0.470). The methylation level of RBMX promoter in ESCA tissue was lower than that of RBMX gene in normal tissue, and the methylation level of CpG island in RBMX gene promoter region in ESCA tissue was also significantly lower than that in normal tissue (Figure S3). These results indicated that the high expression of RBMX in ESCA may not be regulated by DNA methylation modification.

Genetic mutation analysis of RBMX in ESCA

Based on the cBioPortal database, two ESCA datasets: Esophageal Adenocarcinoma (TCGA, Pan-Cancer Atlas) and Esophageal Carcinoma (TCGA, Nature 2017), were used to analyze whether the proteins encoded by the *RBMX* gene in ESCA tissues were mutated. The results suggested that the *RBMX* gene had genetic changes such as structural variation, mutation and chromosome deletion in different tumors. The mutation of RBMX in ESCA was further analyzed through the "Mutations" module. The results showed that RBMX gene had missense mutation and other genetic changes in ESCA (*Figure 3*). The SangerBox database was further used for gene mutation and expression analysis, and the results showed that RBMX had higher expression in ESCA after mutation (Figure S4).

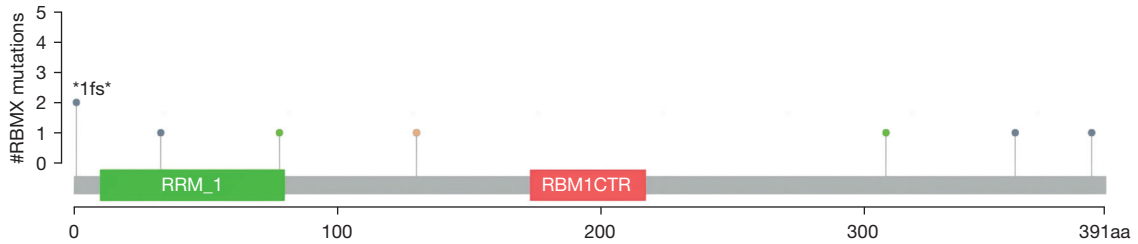

Figure 3 Genetic mutation of RBMX in esophageal cancer. The database: cBioPortal database (http://www.cbioportal.org/). *RBMX* gene had genetic changes such as structural variation, mutation and chromosome deletion in different tumors and *RBMX* gene had missense mutation and other genetic changes in esophageal cancer. In the figure, green dots represent missense, blue dots represent truncating, and yellow dots represent splicing. The numbers in the X-axis represent the position of the amino acid sequence (0–391aa) encoding the RBMX protein; the green and red boxes represent the two domains of the RBMX protein. "*1fs*" represents a type of change in the protein encoded by the *RBMX* gene. RBMX, RNA binding motif protein X-linked.

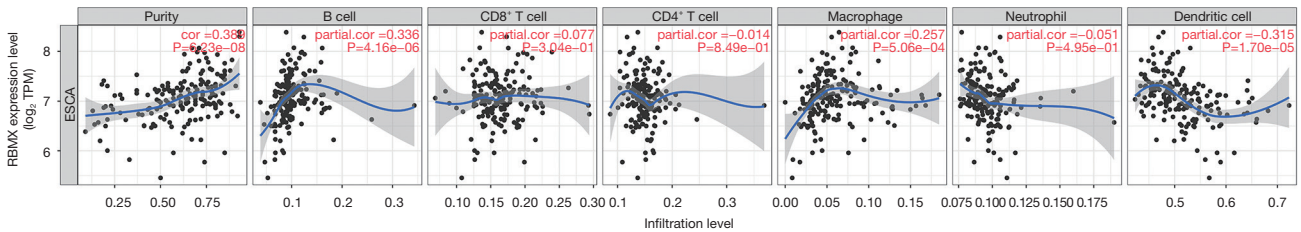

Figure 4 Relationship between RBMX expression and immune cell infiltration levels in esophageal cancer. Tool of analysis: TIMER database (http://timer.cistrome.org/). In esophageal cancer, the expression level of RBMX was negatively correlated with the infiltration degree of dendritic cells (r=–0.315, P=1.70e-05). It was positively correlated with the infiltration degree of B cells (r=0.336, P=4.16e-06) and macrophages (r=0.257, P=5.06e-04). It was positively correlated with purity (r=0.389, P=6.23e-08), and the difference was statistically significant. There was no correlation with $CD4^+$ T cells (r=–0.014, P=8.49e-01), $CD8^+$ T cells (r=0.077, P=3.04e-01), neutrophils (r=–0.051, P=4.95e-01). RBMX, RNA binding motif protein X-linked; TPM, transcripts per million; ESCA, esophageal carcinoma.

Relationship between the expression level of RBMX and the infiltration level of tumor immune cells

The relationship between RBMX expression level and the infiltration of 6 kinds of immune cells was analyzed by the TIMER database. The results showed that the high expression of RBMX was negatively correlated with the infiltration degree of dendritic cells (r=–0.315, P=1.70e-05). It was positively correlated with the infiltration degree of B cells (r=0.336, P=4.16e-06) and macrophages (r=0.257, P=5.06e-04). It was positively correlated with purity (r=0.389, P=6.23e-08), and the difference was statistically significant. There was no correlation with $CD4^+$ T cells (r=–0.014, P=8.49e-01), $CD8^+$ T cells (r=0.077, P=3.04e-01), neutrophils (r=–0.051, P=4.95e-01) (*Figure 4*). It indicated that RBMX might further regulate the occurrence and development of ESCA by affecting the infiltration of immune cells.

PPI network construction and related protein interaction analysis of RBMX

The PPI network of RBMX was constructed by STRING, having 11 nodes, 55 edges, an average node degree of 10, and a PPI enrichment P value of 1.0e-16. PPI-enriched interacting proteins were HNRNPA1, HNRNPR, TRA2B, HNRNPM, HNRNPK, HNRNPC, PTBP1, HNRNPA2B1, HNRNPH1, and HNRNPL (*Figure 5A*). To further study the molecular mechanism of RBMX in tumors, 10 targeted binding proteins of RBMX and 10 genes related to RBMX expression were screened for a series of correlation analysis. The RBMX expression levels were significantly positively correlated with DHX9 (r=0.78, *Figure 5B*), HNRNPR (r=0.75, *Figure 5C*), HNRNPA1 (r=0.71, *Figure 5D*) and six other related genes (P<0.001) (Figure S5).

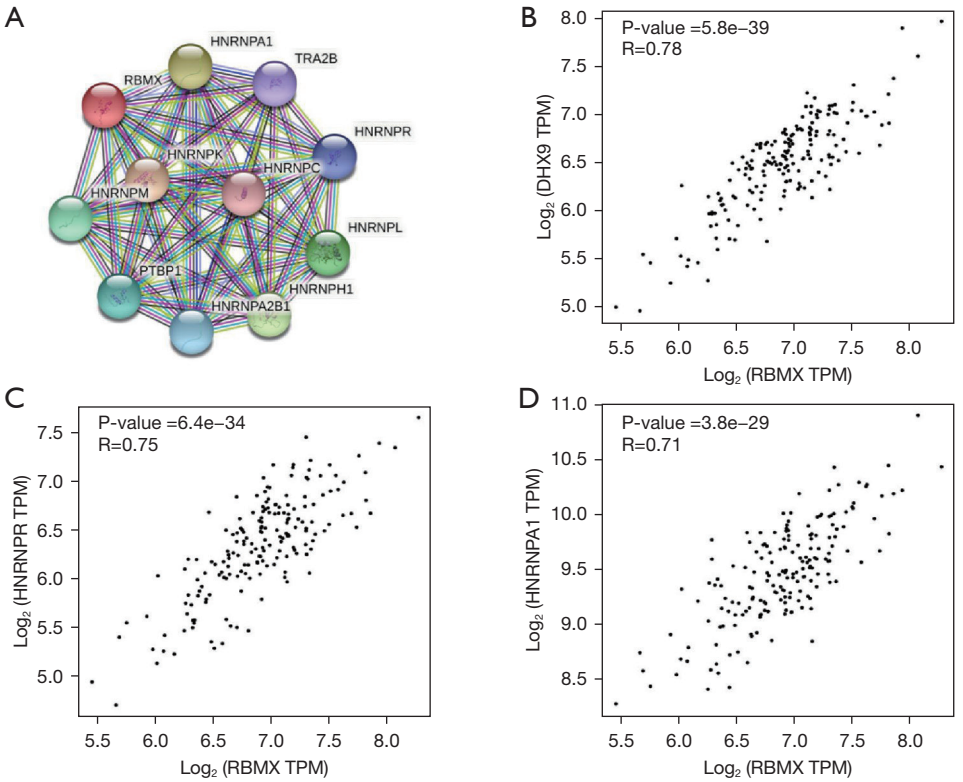

Figure 5 PPI protein interaction network of *RBMX* gene (A) and RBMX-related genes (B-D). The database: STRING database (https://string-db.org/). (A) PPI-enriched interacting proteins were HNRNPA1, HNRNPR, TRA2B, HNRNPM, HNRNPK, HNRNPC, PTBP1, HNRNPA2B1, HNRNPH1, and HNRNPL. (B-D) The RBMX expression levels were significantly positively correlated with DHX9 (r=0.78), HNRNPR (r=0.75), HNRNPA1 (r=0.71) and six other related genes (P<0.001) (Figure S6). TPM, transcripts per million; RBMX, RNA binding motif protein X-linked; PPI, protein-protein interaction.

Differential co-expression gene screening and functional enrichment analysis of RBMX in ESCA

Differential co-expressed genes of RBMX in ESCA were analyzed based on the LinkedOmics database to screen out the top 50 genes with significant differences (P<0.05) and the closest association for plotting a heat map ("Z-score" is used to show how many times the standard deviation of the data is increased or decreased compared with the mean; "group" represents the group with high or low expression of *RBMX* gene). The heat map showed that genes significantly positively correlated with RBMX expression in ESCA included HNRNPA1L2, SFRS13A, SFRS3, HNRNPA1, SFRS1, etc. (*Figure 6*), among which HNRNPA1, HNRNPC, and HNRNPL were also involved in the protein interaction network. GO enrichment and KEGG analysis were performed on the top 50 differentially co-expressed genes that were screened most closely with RBMX co-expressed genes based on DAVID database. The results showed that these related genes were mainly enriched in cell division, mRNA splicing, mRNA transport, RNA processing, RNA binding, protein binding, mRNA 3'-UTR binding and other BPs; KEGG was mainly enriched in the mRNA splicing pathway, suggesting that RBMX might regulate the occurrence and development of ESCA by regulating related molecules, affecting biosynthesis, regulating RNA splicing and other related signaling pathways (Figure S6).

Expression of RBMX protein in ESCA and adjacent esophageal tissues

Immunohistochemical results showed that RBMX was mainly expressed positively in the nucleus of ESCA cells. Paired *t*-test was used to analyze the expression of RBMX in ESCA tissues and corresponding adjacent

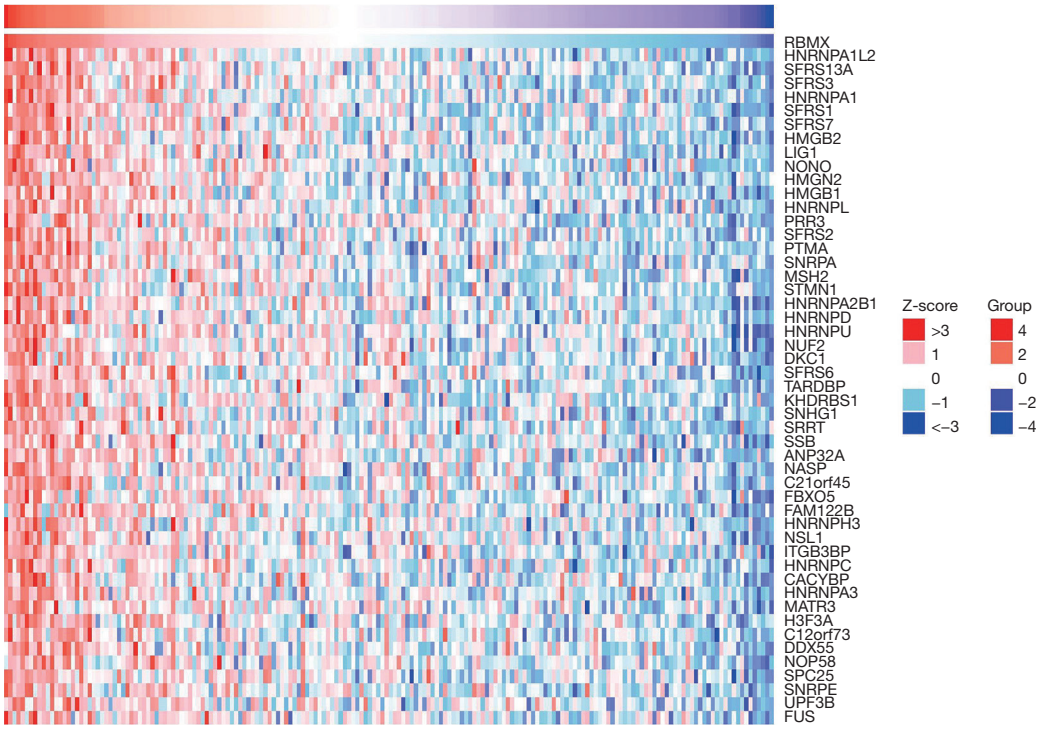

Figure 6 RBMX differentially co-expresses top 50 genes in esophageal cancer. The database: LinkedOmics Database (http://www.Linkedomics.org/). The heat map showed that genes significantly positively correlated with RBMX expression in esophageal cancer included HNRNPA1L2, SFRS13A, SFRS3, HNRNPA1, SFRS1, etc. (Figure S6), among which HNRNPA1, HNRNPC, and HNRNPL were also involved in the protein interaction network. GO enrichment and KEGG analysis results showed that these related genes were mainly enriched in cell division, mRNA splicing, mRNA transport, RNA processing, RNA binding, protein binding, mRNA 3'-UTR binding and other biological processes; KEGG was mainly enriched in the mRNA splicing pathway (Figure S6). In the figure, the X-axis represents the samples of esophageal cancer, and the Y-axis represents the coexpressed genes from top to bottom that are highly correlated with RBMX. "Group", represents the group with high or low expression of *RBMX* gene; "Z-score", used to show how many times the standard deviation of the data is increased or decreased compared with the mean. RBMX, RNA binding motif protein X-linked; GO, Gene Ontology; KEGG, Kyoto Encyclopedia of Genes and Genomes.

tissues. The results showed that the expression of RBMX in ESCA tissues was significantly higher than that in the corresponding adjacent tissues, with statistically significant difference (t=7.023, P<0.001, *Figure 7*, *Table 1*).

Correlation between RBMX expression and clinicopathologic parameters in patients with ESCA

All 53 patients with ESCA met the criteria of our study design, including 35 males and 18 females, aged from 37 to 78 years, with squamous cell carcinoma as the pathological type. The follow-up time was from 4 to 96 months. The clinicopathological features of the included patients also included pathological grade (3 cases of grade I, 40 cases of grades II, and 10 cases of grade III), tumor stage (1 case of T1, 10 cases of T2, 34 cases of T3, 1 case of T4a, 7 cases were not classified), and lymph node stage (27 cases of N0, 13 cases of N1, 8 cases of N2, 3 cases of N3, and 2 cases were not classified), survival status (death or survival), survival time (4 to 96 months), and follow-up time (2006 to 2014). Chi-square test was used to compare the expression of RBMX protein and its correlation with clinicopathological parameters. The results showed that there was no significant correlation between the expression of RBMX in ESCA and the age, gender, histopathologic morphology, pathological grade, tumor stage, lymph node metastasis and other factors, and the statistical significance was not significant, as shown in *Table 2*.

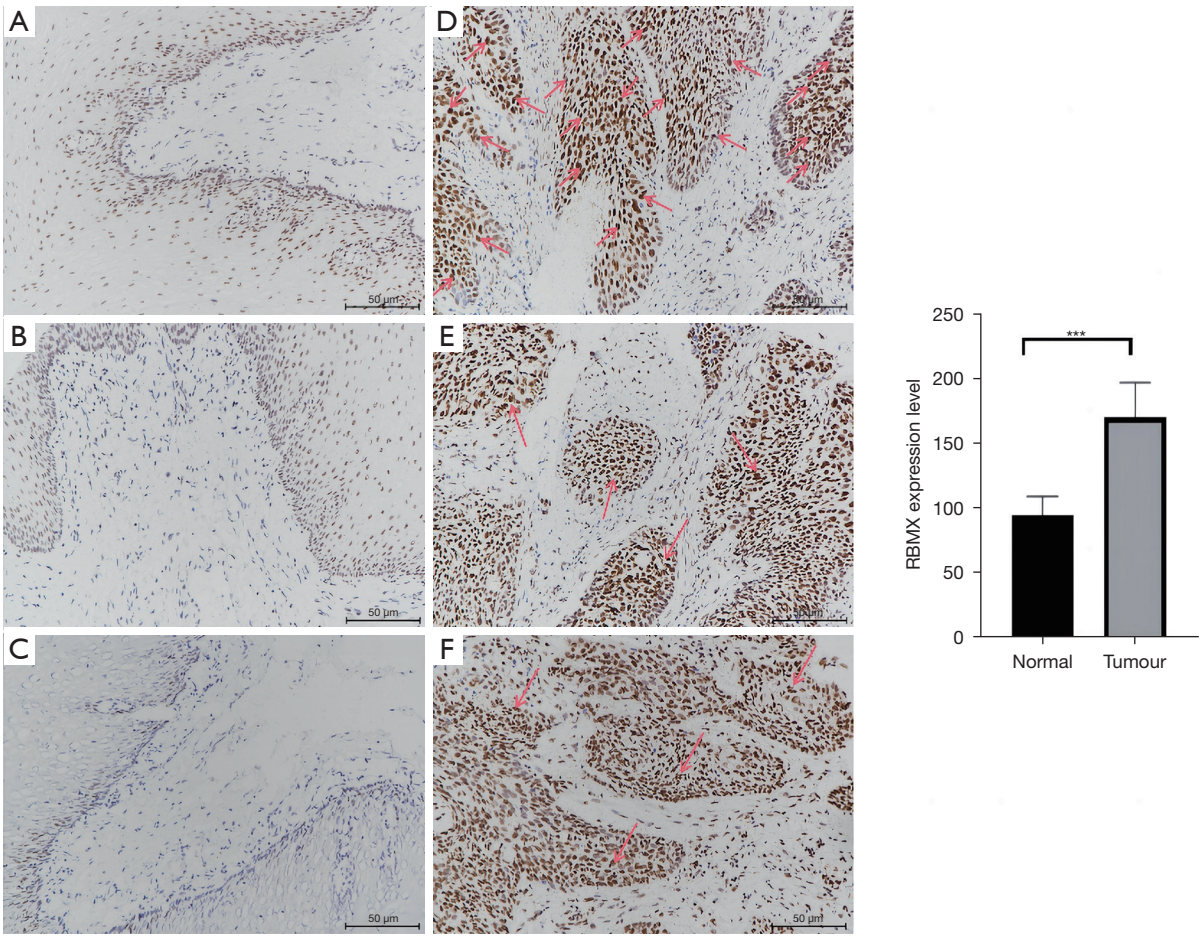

Figure 7 RBMX detection results in typical esophageal cancer and adjacent esophageal tissue. Tool of analysis: Image-Pro Plus6.0 (MEDIA CYBERNETICS Image Technology Inc., Bethesda, USA). Immunohistochemical results showed that RBMX was mainly expressed positively in the nucleus of esophageal cancer cells. The expression of RBMX in esophageal cancer tissues was significantly higher than that in the corresponding adjacent tissues, with a statistically significant difference (P<0.001) (immunohistochemistry, 200×). (A-C) Negative RBMX expressions in adjacent esophageal tissue; (D-F) positive RBMX expressions in esophageal cancer (the brown cells and regions indicated by the red arrows represent RBMX expression sites and cancer nest areas). P value significant codes: ***, P<0.001. RBMX, RNA binding motif protein X-linked.

Table 1 Expression of RBMX in esophageal carcinoma and adjacent tissues

Organization type	Site number (n)	IOD mean	*t*	P
Esophageal cancer tissues	50	164.68	7.023	<0.001
Adjacent tissues	50	93.9		

RBMX, RNA binding motif protein X-linked; IOD, integrated optical density.

Table 2 Correlation between RBMX expression and clinicopathologic parameters of patients with esophageal cancer

Clinical features	Level	IOD high (n=27)	IOD low (n=26)	χ^2	P
Gender (%)	Male	17 (63.0)	18 (69.2)	0.232	0.63
	Female	10 (37.0)	8 (30.8)		
Age, years, median [IQR]		57 [37, 78]	60 [48, 75]	23.79	0.474
Distant metastatic site (%)	No	24 (88.9)	25 (96.2)	1.002	0.317
	Yes	3 (11.1)	1 (3.8)		
Pathological grading (%)	I	0 (0.0)	3 (11.5)	4.283	0.369
	II	22 (81.5)	18 (69.2)		
	III	5 (18.5)	5 (19.2)		
Pathological morphology (%)	Not classified	3 (11.1)	2 (7.7)	2.963	0.813
	Infiltrating ulcer type	2 (7.4)	1 (3.8)		
	Ulcerative type	13 (48.1)	14 (53.8)		
	Uplift type	6 (22.2)	7 (26.9)		
	Basin type	1 (3.7)	0 (0.0)		
	Constricted type	1 (3.7)	2 (7.7)		
	Mushroom type	1 (3.7)	0 (0.0)		
Total lymph nodes, median [IQR]		12 [1, 30]	20 [3, 35]	22.902	0.69
Positive lymph node, median [IQR]		1 [0, 16]	0 [0, 7]	9.035	0.339
T (%)	Not classified	1 (3.7)	6 (23.1)	6.072	0.194
	T1	1 (3.7)	0 (0.0)		
	T2	6 (22.2)	4 (15.4)		
	T3	18 (66.7)	16 (61.5)		
	T4a	1 (3.7)	0 (0.0)		
N (%)	Not classified	0 (0.0)	2 (7.7)	4.43	0.351
	N0	13 (48.1)	14 (53.8)		
	N1	6 (22.2)	7 (26.9)		
	N2	6 (22.2)	2 (7.7)		
	N3	2 (7.4)	1 (3.8)		
M (%)	M0	24 (88.9)	25 (96.2)	1.002	0.317
	M1	3 (11.1)	1 (3.8)		

RBMX, RNA binding motif protein X-linked; IOD, integrated optical density; IQR, interquartile range.

Table 3 Univariate and multivariate analysis of prognostic variables for 5-year OS

Variables	OS		
	Univariate (P)	Multivariate	
		HR (95% CI)	P
Age	0.579	1.000 (0.949–1.054)	0.997
Gender	0.153	0.727 (0.304–1.738)	0.474
Pathological morphology	0.351	0.892 (0.651–1.222)	0.476
Pathological grading	0.265	1.199 (0.735–1.956)	0.467
T stage	0.167	1.635 (0.543–4.918)	0.382
N stage	0	1.869 (1.127–3.100)	0.015
M stage	0.029	1.130 (0.301–4.244)	0.856

OS, overall survival; HR, hazard ratio; CI, confidence interval.

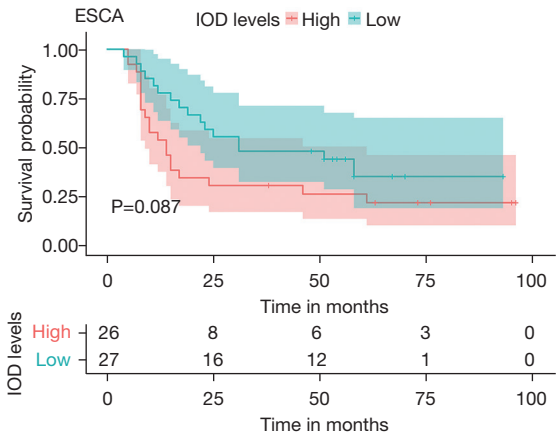

Figure 8 Kapan-Meier survival curve of positive expression and negative expression of RBMX protein in esophageal cancer tissue. Tool of analysis: R language (R 4.1.2). Survival analysis results showed that the survival rate of the group with high RBMX expression (IOD High) was significantly higher than that of the group with low RBMX expression (IOD Low). The results showed that esophageal cancer patients with high expression of RBMX had a trend of poor prognosis, which was consistent with GEPIA2 database analysis. In the figure, pink represents high expression and blue represents low expression. ESCA, esophageal carcinoma; IOD, integrated optical density; RBMX, RNA binding motif protein X-linked.

Correlation between expression level of RBMX and clinicopathological parameters and OS time of ESCA patients

As of April 2014, there were 36 deaths and 17 survival cases among 53 patients with ESCA. Twenty-one of the patients who died showed high expression of RBMX, which was statistically significant (P<0.001). The univariate analysis showed that among the parameters analyzed, typical prognostic markers such as lymph node status (P<0.001) and distant metastasis (P=0.029) were associated with OS. Results of multivariate analysis showed that lymph node status was correlated with OS of patients with statistical significance (HR =1.869, 95% confidence interval: 1.127–3.100), as shown in *Table 3*.

The relationship between the expression of RBMX protein and the prognosis of patients with ESCA

Combined with the survival analysis and prediction results of the GEPIA2 database, the survival analysis was further conducted by using R language programming and Kaplan-Meier method and Logrank test. The results showed that ESCA patients with high expression of RBMX had a trend of poor prognosis, which was consistent with GEPIA2 database analysis (*Figure 8*).

Discussion

As one of the malignant tumors of the digestive system with high morbidity and mortality, the occurrence and development of ESCA involve the participation of multiple factors and genes. Due to the atypical clinical manifestations of ESCA, effective methods for early detection of ESCA are lacking (26). Currently, ESCA is mainly treated by surgery, combined with radiotherapy and chemotherapy, but the

3- and 5-year survival rates of ESCA have not improved significantly, and the mortality rate has not dropped, at a rate of still over 30%. ESCA poses a significant threat to people's health. Improving the treatment efficiency and prognosis of ESCA is a practical problem that needs to be solved clinically. Therefore, to improve the efficacy and prognosis of ESCA, besides strengthening basic research and screening, it is more important to explore the factors influencing the prognosis of ESCA, identify potential therapeutic targets, and thereby explore effective treatment methods.

As one of the m6A reading proteins, RBMX's primary functions are splicing control, transcription (27,28) and maintenance of genome integrity (29). It is found that RBMX is a crucial switch closely related to cancer drivers (30). Song *et al.* (31) found that RBMX was highly expressed in both HCC patient tissues and HCC cell lines, indicating that RBMX significantly promoted the occurrence of HCC tumor, which was consistent with the TIMER query results. When RBMX was overexpressed, HCC cells showed increased viability, proliferation and sorafenib resistance. In addition, when RBMX/BLACAT1 was up-regulated, the autophagy level and cancer cell dryness were also improved. Relevant experimental data show that the proliferation, colony-forming ability and autophagy of HCC cells can be improved by stabilizing BLACAT1 expression by RBMX, thus promoting the development and drug resistance of HCC. Yan *et al.* (32) found that RBMX was significantly down-regulated in metastatic bladder cancer (BCa) tissue, which is associated with poor prognosis, and RBMX can inhibit the combination of the RGG motif in hnRNP A1 and the flanking sequence of pyruvate kinase M (PKM) exon 9, resulting in lower PKM2 and higher PKM1 levels. In addition, RBMX inhibits aerobic glycolysis and counteracts PKM2 overexpression induced BCa cell invasion by hnRNP A1-dependent PKM replacement splicing. It can be concluded that the splicing properties of RBMX may also be involved in the influence of RBMX on the occurrence and development of tumors. Although RBMX has been identified as a new target for cancer treatment in many tumors, there is no report on RBMX expression in ESCA and its relationship with prognosis.

Firstly, In the current study, the data of several databases of ESCA were firstly analyzed by bioinformatics. Based on the TIMER2.0 database, the expression of RBMX in different tumors was analyzed. The results showed that RBMX was highly expressed in many tumors and displayed a significantly statistical difference in ESCA ($P<0.001$). The expression of RBMX in ESCA was further analyzed based on the GEPIA2 database. The expression of RBMX in ESCA was significantly higher than that in normal tissues, consistent with the results of TIMER2.0 data analysis. Then, based on the GEPIA2 database, the correlation between RBMX expression and prognosis of ESCA patients was analyzed. The results showed that the OS rate of patients with high RBMX expression was lower, which was related to a poor prognosis. The research of Guo *et al.* (33) pointed out that RBMX was further identified as the target gene of SRSF3, and the low expression of RBMX was determined to be significantly related to the good OS rate of patients. Patients with both low expressions of SRSF3 and RBMX were a subgroup of individuals with a better prognosis than all other patients. In the study of T-cell non-Hodgkin lymphoma, Schümann *et al.* (34) found that low RBMX expression could predict better chemotherapy response, OS and progression-free survival in T-cell non-Hodgkin lymphoma patients, indicating that the level of RBMX protein expression may be a factor leading to chemoresistance, thereby affecting prognosis. These results suggest that high expression of RBMX is not necessarily associated with poor prognosis of cancer, and there may be other influencing factors. To verify RBMX expression in ESCA, the expression of RBMX in ESCA and its adjacent tissues was detected by tissue microarray and immunohistochemistry. Compared with traditional pathological paraffin sections, tissue microarray technology allows the experimental conditions as consistent as possible, thereby significantly reducing the experimental cost by screening and punching multiple pathological sections and then fixing them on the same paraffin block for parallel analysis. The results showed that compared with the adjacent esophageal tissues, the expression of RBMX in ESCA tissues was significantly higher with a statistically significant difference ($P<0.001$), which was consistent with the data analysis results of GEPIA2. However, there was no correlation between the high expression of RBMX and the pathological grading, lymph node metastasis and clinical staging of ESCA, suggesting that there might be other influencing factors for high BMX expression. Then, the relationship between the expression of RBMX and the prognosis of ESCA patients was analyzed. The results showed that the prognosis of high RBMX expression was poor, which was consistent with the data analysis results of GEPIA2; however, the difference between high expression and low expression of RBMX in ESCA was not statistically significant ($P=0.087$). Due to the limited number of

samples, there might be some deviation in the statistical results. The next step will be to expand the sample size to analyze the prognosis of RBMX in ESCA.

To further explore the downstream regulation mechanism of RBMX in the occurrence and development of ESCA, the differentially co-expressed genes of RBMX in TCGA-ESCA were analyzed based on the LinkedOmics database. The results showed that the genes closely related to RBMX expression were HNRNPA1L2, SFRS13A, SFRS3, HNRNPA1, SFRS1, etc. The protein interacting with RBMX was subsequently analyzed based on the String-DB database, which showed that proteins interacting with RBMX included HNRNPA1, HNRNPR, TRA2B, HNRNPM, HNRNPK, HNRNPC, PTBP1, HNRNPA2B1, HNRNPH1 and HNRNPL. These results suggest that RBMX has complex molecular interactions in the development of ESCA. Meanwhile, based on the DAVID database, GO, and KEGG enrichment analysis of top 50 differentially co-expressed genes showed that these co-expressed genes were mainly enriched in mRNA splicing, RNA processing, cell cycle and other processes, suggesting that RBMX may regulate the development of ESCA through mRNA splicing and cell cycle pathways. In this study, based on the RNA-seq data and clinical information of ESCA tumors in the TCGA database, the TIMER database was used to evaluate the correlation between RBMX expression level and infiltration level of 6 kinds of immune cells in ESCA. The results showed that the high expression of RBMX was negatively correlated with the infiltration degree of dendritic cells (r=–0.315, P=1.70e-05). It was positively correlated with the infiltration degree of B cells (r=0.336, P=4.16e-06) and macrophages (r=0.257, P=5.06e-04). It was positively correlated with purity (r=0.389, P=6.23e-08), and the difference was statistically significant. There was no correlation with $CD4^+$ T cells (r=–0.014, P=8.49e-01), $CD8^+$ T cells (r=0.077, P=3.04e-01), neutrophils (r=–0.051, P=4.95e-01), suggesting that regulating tumor immune response-related pathways may be one of the essential mechanisms of RBMX promoting the progress of ESCA. Since RBMX is highly expressed in ESCA and is associated with prognosis, the expression of RBMX is somewhat correlated with the level of immune cell infiltration, while whether the survival time of patients has a certain relationship with immune cell infiltration remains unclear. Therefore, the role of RBMX in immune regulation and whether it can be used as a biomarker or a combination therapy target need to be further studied.

Finally, the upstream mechanism of high expression of RBMX in ESCA was explored. The occurrence of tumors was usually closely related to the abnormal expression or change of genes, and the transformation of methylation status or gene amplification may affect the progression of tumors. In this study, the cBioPortal database, the UALCAN database and the MEXPRESS database were used to analyze the correlation between RBMX expression and DNA methylation, the methylation level of RBMX promoter region and the methylation level of CpG island in RBMX promoter region, and the results showed that, The expression of RBMX in ESCA is not correlated with DNA methylation level, suggesting that the expression of RBMX in ESCA may not be regulated by DNA methylation modification, and its genomic level may be affected by other factors. Genome sequencing revealed truncated RBMX gene mutation in lung cancer patients, suggesting that RBMX is a potential tumor-inhibiting factor (35). In the study of clinical acquired drug resistance in patients with BRAF mutant melanoma, Hartman *et al.* (36) found a new phenotypic change pattern, namely the RBMX frameshift variant, in the process of establishing a preclinical model of melanoma resistance to verofinib or trametinib to deeply understand the mechanism of drug resistance, which provided a brand-new idea for the acquired drug resistance of targeted therapy. This study further analyzed the genetic mutation of RBMX in ESCA through the cBioPortal database. The results showed that 1.29%, 2.4%, and 0.18% of ESCA patients had the gene mutation, gene copy number amplification and deep deletion of RBMX, respectively, suggesting that the high RBMX expression in ESCA may be related to gene mutation, copy number amplification and deep deletion. Therefore, further study on whether these mutations change the expression of RBMX in ESCA, whether they affect the prognosis of patients with ESCA, or whether they change the function of RBMX will be helpful to explore the role of RBMX in the occurrence and development of ESCA.

In conclusion, the RBMX expression in ESCA and its relationship with prognosis, immune cell infiltration level, protein interaction and methylation level were analyzed by bioinformatics method, and the expression of RBMX in ESCA was verified by tissue microarray technology. The results showed that RBMX was highly expressed in ESCA with poor prognosis, which was consistent with the results of bioinformatics analysis, indicating that RBMX might be a prognostic biomarker of ESCA. Although the current analysis is mainly based on the expression of RBMX in

ESCA, there may be complex molecular relationships in the occurrence and development of ESCA, and it is not clear whether the identification and splicing characteristics of RBMX will affect the prognosis of ESCA. Therefore, in order to clarify the biological function and role of RBMX in ESCA and further determine the specificity of whether the expression level of RBMX can be used as a biomarker for predicting ESCA, we will carry out molecular biology experiments for verification and exploration in the future.

Conclusions

RBMX may be one of the biomarkers of poor prognosis of ESCA. RBMX is closely related to the survival and prognosis, genetic mutation and immune cell infiltration of patients with ESCA.

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Footnote

Reporting Checklist: The authors have completed the REMARK reporting checklist. Available at https://tcr.amegroups.com/article/view/10.21037/tcr-23-84/rc

Data Sharing Statement: Available at https://tcr.amegroups.com/article/view/10.21037/tcr-23-84/dss

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Conflicts of Interest: All authors have completed the ICMJE uniform disclosure form (available at https://tcr.amegroups.com/article/view/10.21037/tcr-23-84/coif). The authors have no conflicts of interest to declare.

Ethical Statement: The authors are accountable for all aspects of the work in ensuring that questions related to the accuracy or integrity of any part of the work are appropriately investigated and resolved. The study was approved by the Ethics Committee of Shanghai Outdo Biotechnology Co., Ltd. (No. SHYJS-CP-1707008) and informed consent was taken from all individual participants. The study was conducted in accordance with the Declaration of Helsinki (as revised in 2013).

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