Introduction

Head and neck cancer collectively is one of the most common cancers worldwide. Oral squamous cell carcinoma (OSCC) is the most common subtype of head and neck cancer. SRSF3 is a proto-oncogene and is overexpressed in patients with OSCC. However, the relationship between SRSF3 expression and the clinical outcomes of patients with head and neck cancer remains unclear. By using the eBioPortal for Cancer Genomics, a public online tool originally developed at Memorial Sloan Kettering Cancer Center (New York, NY, USA), it was revealed that patients with head and neck cancer with an underexpression of SRSF3 showed better overall and disease-/progression-free survival rates. Moreover, 227 genes were found to be significantly coexpressed with SRSF3 in head and neck cancer. Then, in combination with the analysis of a previous splice-array dataset that included significantly changed genes after the silencing of SRSF3, four potential target genes of SRSF3 were identified. RBMX and HNRNPL were further confirmed to be target genes of SRSF3. Moreover, the underexpression of RBMX was determined to be significantly associated with a favorable overall survival rate among patients, while patients with an underexpression of both SRSF3 and RBMX is a subgroup of individuals with better prognoses than all other patients. These results suggest that the underexpression of SRSF3 and that of its target RBMX can be used as potential biomarkers to predict favorable overall survival among head and neck cancer patients.

Keywords: head and neck cancer, RBMX, SRSF3

Materials and Methods

Cell culture and RNA interference

CAL 27 cells were grown in Dulbecco’s modified Eagle medium (DMEM) supplemented with 10% fetal bovine serum (FBS; Gibco Laboratories, Gaithersburg, MD, USA) and 1% antibiotic-antimycotic solution (Invitrogen, Carlsbad, CA, USA). Separately, SCC-9 cells were cultured in a mixed medium of DMEM and Ham’s F12 medium (1:1) supplemented with 10% FBS and 1% antibiotic-antimycotic solution and 400 ng/mL of hydrocortisone. Human SRSF3 small interfering RNA (siRNA) was synthesized by GenePharma (Shanghai, China). Cells were transfected with 20 nM of siRNA using lipofectamine 3,000 reagent (Invitrogen) according to the manufacturer’s instructions. Forty-eight h later, cells underwent a second transfection and, 96 h after that, total protein and RNA samples were collected. The sequences of siRNAs used in this study are as follows: 5’AGAGCUAGAUGGAAGAACATA3’ (SRSF3) and 5’ACUCUAIUCUG-CAGGCUGAC3’ (nonspecific; NS).

Plasmid and transfection

The T7-tagged SRSF3 expression plasmid (T7-SRSF3) was kindly provided by Dr. Zheng Zhi-Ming (National Cancer Institute, Bethesda, MD, USA). CAL 27 or SCC-9 cells were transfected with plasmid using lipofectamine 3,000 reagent according to the manufacturer’s instructions.

Western blot

Total protein samples were loaded onto 10% sodium dodecyl sulfate-polyacrylamide gel electrophoresis gel and transferred to a nitrocellulose membrane. The membrane was blocked with 5% nonfat milk and analyzed by the following antibodies: mouse anti-SRSF3 (Santa Cruz Biotechnology, Dallas, TX, USA) and mouse anti-glyceraldehyde 3-phosphate dehydrogenase (GAPDH; Santa Cruz Biotechnology).
Reverse transcriptase polymerase chain reaction (RT-PCR)

Total RNA samples were purified using the Total RNA Miniprep Kit (AxyPrep, Madison, WI, USA), treated with DNaseI (Invitrogen), and then reverse-transcribed into complementary DNA using Moloney Murine Leukemia Virus Reverse Transcriptase (Promega, USA). PCR was performed using the following primers: 5′-TGAGTCCTTCACGATACCAAA-3′ and 5′-GACTTCCACCATATCAGTATCACACACTGCCA-3′ for SNHG12, 5′-GAGCATGGCACAGTGACCGT-3′ and 5′-GACCGTAGGACTCAGTATCACACACTGCCA-3′ for CDC45, 5′-GTTTTGTTGGGGTTGCTGC-3′ and 5′-GTGCTTTCCGCTGCCTTCT-3′ for GAPDH. 

Real-time quantitative RT-PCR (qRT-PCR)

Real-time qRT-PCR was performed with All-in-One qPCR Mix (SYBR Green method; GeneCopoeia, Rockville, MD, USA) in a real-time PCR machine (QuantStudio 6 Flex System; Applied Biosystems, Foster City, CA, USA). The primers used for qRT-PCR were the same as those used in RT-PCR. The relative expression of target genes (HNRNP L, SNHG12, CDC45, or RBMX) was calculated using the 2-ΔΔCt method.

Patients

In a previous study, 77 patients diagnosed with OSCC were included [12]. SRSF3 has two isoforms generated by the alternative splicing of exon 4. The expression of whole SRSF3 protein in tissues was analyzed by immunohistochemistry, where SRSF3 was found to be overexpressed in OSCC tissues (clone number 7B4 against exon 3 of human SRSF3; Invitrogen). In the present study, survival data for 51 patients in the cohort were collected. All experimental protocols were approved by the Ethics Committee (No. 13, 2011 year) at the School and Hospital of Stomatology at Wuhan University.

Data analysis in the eBiportal for Cancer Genomics

A Cancer Genome Atlas Program (TCGA) dataset of head and neck squamous cell carcinoma, called TCGA Provisional, which includes 518 patients in the eBiportal online program (www.ebiportal.org), was selected. The whole transcriptional expression levels of genes in patients were measured by messenger RNA (mRNA) sequencing. Underexpression of SRSF3 or RBMX was identified by the number of standard deviations (SDs) from the mean expression level. To analyze the effects of both SRSF3 and RBMX underexpression on the survival of patients, the overall survival data were downloaded and analyzed with Graphpad Prism (Graphpad Software, La Jolla, CA, USA).

Results

Underexpression of SRSF3 correlates with a good prognosis in head and neck cancer patients

This study analyzed the relationship between the expression of SRSF3 and overall and disease-/progression-free survival rates of head and neck cancer patients in the TCGA Provisional dataset (n = 518) with the online tool eBiportal. Among other findings, the underexpression of SRSF3 significantly correlated with good overall and disease-/progression-free survival rates in head and neck cancer patients (Fig. 1). Previously, SRSF3 expression had been analyzed in a cohort of OSCC patients by immunohistochemistry, where SRSF3 was found to be overexpressed in OSCC tissues as compared with in normal tissues. To verify the results of the eBiportal analysis, in the present study, available survival information of patients in the cohort was collected (Table 1) and the relationship between the expression of SRSF3 and clinical outcomes was analyzed. In the present cohort, a lower expression of SRSF3 was confirmed to be significantly associated with good overall survival (Fig. 2), suggesting that the underexpression of SRSF3 can serve as a biomarker for a good prognosis in head and neck cancer patients.

The association between the expression of SRSF3 and clinical outcomes of other cancer types was also analyzed. Interestingly, the underexpression of SRSF3 similarly correlated with good overall and disease-/progression-free survival rates in pancreatic adenocarcinoma patients (Fig. S1), indicating that the underexpression of SRSF3 may be used as a potential biomarker for a good prognosis in pancreatic adenocarcinoma.
The underexpression of SRSF3 is defined as an SRSF3-specific staining score that is less than 0.5 SDs from the mean value. The underexpression of SRSF3 was quantified by using the ImageJ software program (National Institutes of Health, Bethesda, MD, USA). The underexpression of SRSF3 in patients with or without underexpression of SRSF3 was assessed in a cohort of 51 OSCC patients in a previous study. (A) Representative immunohistochemical staining images of SRSF3 in patients with or without underexpression of SRSF3. (B) Kaplan-Meier curves of overall survival for patients with underexpression (n = 19) or without underexpression (n = 32) of SRSF3. SRSF3-specific staining was quantified by using the ImageJ software program. (National Institutes of Health, Bethesda, MD, USA). The underexpression of SRSF3 is defined as an SRSF3-specific staining score that is less than 0.5 SDs from the mean value.

**Fig. 2** Patients with the underexpression of SRSF3 showed better overall survival than those without underexpression of SRSF3 in a cohort of 51 OSCC patients in a previous study. (A) Representative immunohistochemical staining images of SRSF3 in patients with or without underexpression of SRSF3. (B) Kaplan-Meier curves of overall survival for patients with underexpression (n = 19) or without underexpression (n = 32) of SRSF3. SRSF3-specific staining was quantified by using the ImageJ software program.

The coexpression of SRSF3 with CDC45, SNHG12, RBMX, or HNRNPL in head and neck cancer is shown in Table 2. The coexpression of these genes (CDC45, SNHG12, RBMX, and HNRNPL) was found to be positively related with SRSF3. (B) Coexpression of SRSF3 with CDC45, SNHG12, RBMX, or HNRNPL in head and neck cancer from the cBioPortal online program. (C) Clustered heat maps of the data of CDC45, SNHG12, RBMX, or HNRNPL expression in the splice-array dataset.

**Table 2** Coexpression of CDC45, SNHG12, RBMX, or HNRNPL with SRSF3 in head and neck cancer and downregulation of these genes upon SRSF3 knockdown

<table>
<thead>
<tr>
<th>Gene symbol</th>
<th>Entrez gene ID</th>
<th>Correlation with SRSF3 in head and neck cancer</th>
<th>Expression upon SRSF3 knockdown (SRSF3 vs. NS)</th>
<th>Gene name</th>
</tr>
</thead>
<tbody>
<tr>
<td>RBMX</td>
<td>27316</td>
<td>0.67</td>
<td>-1.46</td>
<td>RNA-binding-motif protein, X-linked</td>
</tr>
<tr>
<td>CDC45</td>
<td>8318</td>
<td>0.58</td>
<td>-1.56</td>
<td>Cell division cycle 45</td>
</tr>
<tr>
<td>HNRNPL</td>
<td>3191</td>
<td>0.57</td>
<td>-1.98</td>
<td>Heterogeneous nuclear ribonucleoprotein L</td>
</tr>
<tr>
<td>SNHG12</td>
<td>85028</td>
<td>0.55</td>
<td>-1.90</td>
<td>Small nuclear RNA host gene 12</td>
</tr>
</tbody>
</table>

Specific targets of SRSF3 in head and neck cancer

The target genes of SRSF3 during oncogenesis remain largely unknown. To identify specific target genes of SRSF3 in head and neck cancer, the genes coexpressed with SRSF3 in the TCGA Provisional dataset were searched for. Two hundred seventy-seven genes were coexpressed with SRSF3 (r > 0.5 or r < −0.5; P < 0.05; Fig. 3A), which may be target genes of SRSF3 in head and neck cancer. Previously, a splice-array analysis had been performed to search for genes with alternative splicing events that were significantly changed between SRSF3 knockdown and control groups in U2OS cells (an osteosarcoma cell line) (Gene Expression Omnibus accession no. GSE22149). By analyzing this database, 2,287 potential SRSF3 target genes were identified. Upon comparing these two gene lists, four genes (CDC45, SNHG12, RBMX, and HNRNPL) were found to be positively related with SRSF3. These results suggest that the expression levels of RBMX and HNRNPL are truly regulated by SRSF3 in head and neck cancer. Association of SRSF3 and RBMX expression levels with overall survival

Next, the question of whether these target genes could be used to predict the prognosis of patients with head and neck cancer was considered. For this, the TCGA Provisional dataset in cBioPortal was reviewed again. Patients with an underexpression of CDC45, SNHG12, or HNRNPL showed no significantly longer overall survival time as compared with other patients (Fig. S4). However, it was found that patients with an underexpression of RBMX had a significantly longer overall survival time than did those without an underexpression of RBMX (HR: 0.605, 95% confidence interval [CI] 0.394-0.93, P = 0.0218; Fig. 6A). Furthermore, patients with an underexpression of RBMX had a significantly longer overall survival time than did those with a better prognosis than all other patients (HR: 0.46, 95% CI: 0.2515-0.8458; P = 0.0124; Fig. 6B) or those with an underexpression of only SRSF3 (HR: 0.39, 95% CI: 0.1466-1.019, log-rank test P-value = 0.05, Gehan-Breslow-Wilcoxon test P-value = 0.03; Fig. 6C). These results suggest that the expression levels of RBMX and HNRNPL are truly regulated by SRSF3 in head and neck cancer.

**Table 3** Specific target genes of SRSF3 in head and neck cancer

<table>
<thead>
<tr>
<th>Gene name</th>
<th>Entrez gene ID</th>
<th>Spearman’s correlation</th>
<th>Fold Change</th>
<th>P-value</th>
</tr>
</thead>
<tbody>
<tr>
<td>RBMX</td>
<td>27316</td>
<td>-1.46</td>
<td>1.60E-02</td>
<td></td>
</tr>
<tr>
<td>CDC45</td>
<td>8318</td>
<td>-1.56</td>
<td>8.16E-03</td>
<td></td>
</tr>
<tr>
<td>HNRNPL</td>
<td>3191</td>
<td>-1.98</td>
<td>5.16E-03</td>
<td></td>
</tr>
<tr>
<td>SNHG12</td>
<td>85028</td>
<td>-1.90</td>
<td>2.27E-02</td>
<td></td>
</tr>
</tbody>
</table>

**Fig. 3** Specific target genes of SRSF3 in head and neck cancer. (A) Two hundred twenty-seven genes were coexpressed with SRSF3 in the TCGA Provisional dataset of head and neck cancer (threshold: r > 0.5 or r < −0.5; P < 0.05) in the cBioPortal online program. Previously, a splice-array analysis was performed to search for genes with alternative splicing events that were significantly altered between an SRSF3-knockdown group and a control group in U2OS cells (an osteosarcoma cell line) (Gene Expression Omnibus accession no. GSE22149). By analyzing this database, 2,287 potential SRSF3 target genes were identified. Upon comparing these two gene lists, four genes (CDC45, SNHG12, RBMX, and HNRNPL) were found to be positively related with SRSF3. These results suggest that the expression levels of RBMX and HNRNPL are truly regulated by SRSF3 in head and neck cancer. Association of SRSF3 and RBMX expression levels with overall survival

Next, the question of whether these target genes could be used to predict the prognosis of patients with head and neck cancer was considered. For this, the TCGA Provisional dataset in cBioPortal was reviewed again. Patients with an underexpression of CDC45, SNHG12, or HNRNPL showed no significantly longer overall survival time as compared with other patients (Fig. S4). However, it was found that patients with an underexpression of RBMX had a significantly longer overall survival time than did those without an underexpression of RBMX (HR: 0.605, 95% confidence interval [CI] 0.394-0.93, P = 0.0218; Fig. 6A). Furthermore, patients with an underexpression of RBMX had a significantly longer overall survival time than did those with a better prognosis than all other patients (HR: 0.46, 95% CI: 0.2515-0.8458; P = 0.0124; Fig. 6B) or those with an underexpression of only SRSF3 (HR: 0.39, 95% CI: 0.1466-1.019, log-rank test P-value = 0.05, Gehan-Breslow-Wilcoxon test P-value = 0.03; Fig. 6C). These results suggest that the expression levels of RBMX and HNRNPL are truly regulated by SRSF3 in head and neck cancer.
suggest that RBMX could be a suitable biomarker along with SRSF3 for predicting the outcomes of head and neck cancer patients.

Discussion

A number of studies to date have demonstrated that SRSF3 plays important roles in many aspects of carcinogenesis. However, the association of SRSF3 with the clinical outcomes of cancer patients remains poorly understood. It was previously found that SRSF3 is overexpressed in triple-negative breast cancer [16], and a higher expression of SRSF3 was associated with a shorter survival time and poorer prognosis [17]. However, Torres et al. found that the silencing of SRSF3 in the colorectal cancer cell line KM12 promotes cell proliferation and metastasis. Furthermore, these authors determined that the loss of SRSF3 is associated with poor survival in colorectal cancer [15]. In contrast with their results, in a TCGA dataset of colorectal cancer (TCGA Provisional), no significant correlation between the underexpression of SRSF3 and overall or disease-/progression-free survival in colorectal cancer patients was identified (Fig. S2). Moreover, Kuranaga et al. reported that SRSF3 is overexpressed in colon cancer tissues and the silencing of SRSF3 induced a marked growth inhibition in colon cancer cells [18]. Kurokawa et al. showed that the knockdown of SRSF3 could significantly reduce cell proliferation in OSCC cells [13]. Therefore, more evidence may be required in order to better understand the function of SRSF3 in colon/colorectal cancer.

Previously, it was demonstrated that SRSF3 is significantly overexpressed in OSCC and positively associated with high-grade cancer and lymphatic metastasis [12]. The silencing of SRSF3 could significantly reduce cell proliferation in OSCC cells [13]. In line with these findings, here, it was further demonstrated that the underexpression of SRSF3 correlates with a good prognosis in head and neck cancer patients (Fig. 1A, B) and this result was confirmed in a study cohort of patients with OSCC. Therefore, SRSF3 may be a valuable biomarker for the prediction of prognosis of patients with OSCC. Anti-SRSF3 treatment may benefit patients with high levels of SRSF3 expression.

Several methods may be used to inhibit SRSF3 expression. A number of studies including the present one have applied specific siRNAs targeting different regions of SRSF3 mRNA to suppress its expression [11,20]. Lu et al. reported that caffeine treatment could reduce SRSF3 expression [21] and also found that digoxin could inhibit SRSF3 expression [22]. In a different previous study, exon 4 of human SRSF3 was deemed an alternative exon [13]. SRSF3 exon 4 has an in-frame stop codon. Transcripts with exon

Fig. 4 The knockdown of SRSF3 decreased the expression levels of SNHG12, CDC45, RBMX, and HNRNPL. (A, B) CAL 27 or SCC-9 cells were treated with SRSF3-specific siRNA or NS siRNA twice during a period of 48 h. RT-PCR analysis showed that the knockdown of SRSF3 down-regulated the expression levels of SNHG12, CDC45, RBMX, and HNRNPL. (C, D) The presented histograms summarize the effects of the knockdown of SRSF3 on the expression levels of SNHG12, CDC45, RBMX, and HNRNPL in CAL 27 and SCC-9 cells. Data are represented as means ± standard errors, n = 3, *P < 0.05. (E, F) Real-time qRT-PCR was used to analyze the expression levels of SNHG12, CDC45, RBMX, and HNRNPL in CAL 27 or SCC-9 cells with or without SRSF3 knockdown. Data are represented as means ± standard errors, n = 3, *P < 0.05. (G) The knockdown efficiency of SRSF3 was analyzed by western blot. GAPDH served as a loading control.

Fig. 5 The overexpression of SRSF3 increased the expression levels of RBMX and HNRNPL. (A, B) CAL 27 or SCC-9 cells were transfected with SRSF3 expression plasmid or vector control plasmid (pEGFP-N1), respectively. RT-PCR analysis showed that the overexpression of SRSF3 increased the expression levels of RBMX and HNRNPL. (C) The presented histograms summarize the effects of SRSF3 overexpression on the expression levels of RBMX and HNRNPL. Data are represented as means ± standard errors, n = 3, *P < 0.05. (D) The overexpression of T7-tagged SRSF3 (T7-SRSF3) was analyzed by western blot. GAPDH served as a loading control.

Fig. 6 The underexpression of SRSF3 and RBMX is associated with a good prognosis in head and neck cancer. (A) Kaplan-Meier curves of overall survival for patients with underexpression (n = 47) or without underexpression (n = 471) of RBMX were generated from the cBioPortal program. Underexpression of RBMX is defined as an expression level that is less than 1 SD from the mean level in head and neck cancer. (B) Kaplan-Meier curves of five-year overall survival rates for patients with underexpression (n = 22) or without underexpression (n = 496) of both SRSF3 and RBMX. (C) Kaplan-Meier curves of five-year overall survival rates for patients with the underexpression (n = 22) of both SRSF3 and RBMX or the underexpression of only SRSF3 (n = 36).
4 encode truncated SRSF3 proteins or are degraded by nonsense-mediated decay. Only transcripts without exon 4 can encode full-length functional SRSF3. An exonic splicing suppressor (ESS) in exon 4 suppresses its inclusion [13]. Later, an antisense oligonucleotide to reduce the expression of full-length functional SRSF3 by blocking this ESS and increasing exon 4 inclusion was developed [14]. FaDu cells (a head and neck cancer cell line) or CAL 27 cells treated with this antisense oligonucleotide showed significantly slower growth in comparison with control cells, indicating that this method may be useful for head and neck cancer treatment.

RBMX (also called hnRNP G) is an RNA-binding-motif gene located on the X-chromosome. It is additionally a splicing factor and regulates RNA splicing [23,24] and also plays key roles in DNA damage repair [25] and chromosome segregation [26]. RBMX interacts with a noncoding RNA and regulates genome stability. The depletion of RBMX leads to chromosome segregation defects and abnormal nuclear division [27]. Therefore, RBMX is essential for cell division and proliferation.

So far, the relationship between RBMX and cancer is unclear. It has been reported that the expression of RBMX is positively correlated with the expression of proapoptotic Bax gene in human breast cancer [28], suggesting that RBMX may induce apoptosis. Therefore, more studies are required for understanding the function of RBMX during carcinogenesis. In the present study, the underexpression of RBMX was found to predict favorable overall survival in head and neck cancer (log-rank test $P=0.0218$). Moreover, a small group of patients with the underexpression of both SRSF3 and RBMX showed significantly better survival than did all other patients or patients with the underexpression of only SRSF3.

In summary, the present results suggest that the underexpression of SRSF3 and RBMX of full-length functional SRSF3 is required for understanding the function of RBMX during carcinogenesis. In the present study, the underexpression of RBMX was found to predict favorable overall survival in head and neck cancer (log-rank test $P=0.0218$). Moreover, a small group of patients with the underexpression of both SRSF3 and RBMX showed significantly better survival than did all other patients or patients with the underexpression of only SRSF3.

Acknowledgments

This work was supported by grant nos. 81271143, and 81470741 from the National Science Foundation of China. This work was also supported by the Health Commission of Hubei Province scientific research project (no. WJ2019Z014).

Supplementary Files

Supplementary Figures:

Fig. S1 The effects of SRSF3 expression on the prognostics of pan-creatic adenocarcinoma patients. Kaplan-Meier curves of overall survival (A) and disease-/progression-free survival (B) of pancreatic adenocarcinoma patients ($n=179$ from the TCGA Provisional dataset) with or without underexpression of SRSF3 in the eBioPortal. Underexpression of SRSF3 was defined as an expression value of less than 0.87 SDs from the mean value.

Fig. S2 The effects of SRSF3 expression on the prognostics of colorec-tal adenocarcinoma patients. Kaplan-Meier curves of overall survival (A) and disease-/progression-free survival (B) of colorectal adenocarcinoma patients ($n=222$ from the TCGA Provisional dataset) with or without underexpression of SRSF3 in the eBioPortal. Underexpression of SRSF3 was defined as an expression value of less than 1 SD from the mean value.

Fig. S3 Overexpression of SRSF3 had no effect on the expression levels of SNHG12 and CDC45: CAL 27 or SCC-9 cells were transfected with SRSF3 expression plasmid or vector control plasmid (pEGFP-N1), respecti-vely. RT-PCR was used to analyze the expression levels of SNHG12 (A) or CDC45 (B) in CAL 27 or SCC-9 cells.

Fig. S4 The effects of CDC45, HNRNPL, or SNHG12 expression on the prognosis of head and neck cancer patients. Kaplan-Meier curves of overall survival rates among patients with or without the underexpression of CDC45 (A), HNRNPL (B), or SNHG12 (C) in the eBioPortal. Underexpression was defined as an expression value of less than 1 SD from the mean value.

Please find supplementary files; doi: 10.2334/josnusd.18-0485

References