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RESEARCH PAPER

Serine-arginine splicing factor 2 promotes oesophageal cancer progression by regulating alternative splicing of interferon regulatory factor 3

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ABSTRACT

Objective: Often, alternative splicing is used by cancer cells to produce or increase proteins that promote growth and survival through alternative splicing. Although RNA-binding proteins are known to regulate alternative splicing events associated with tumorigenesis, their role in oesophageal cancer (EC) has rarely been explored.

Methods: We analysed the expression pattern of several relatively well characterized splicing regulators on 183 samples from TCGA cohort of oesophageal cancer; the effectiveness of the knockdown of SRSF2 was subsequently verified by immunoblotting; we measured the ability of cells treated with lenti-sh-SRSF2/lenti-sh2-SRSF2 to invade through an extracellular matrix coating by transwell invasion assay; using RNA-seq data to identify its potential target genes; we performed qRT-PCR to detect the changes of exon 2 usage in lenti-sh-SRSF2 transduced KYSE30 cells to determine the possible effect of SRSF2 on splicing regulation of IRF3; RNA Electrophoretic mobility shift assay (RNA-EMSA) was performed by the incubation of purified SRSF2 protein and biotinylated RNA probes; we performed luciferase assay to confirm the effect of SRSF2 on IFN1 promoter activity.

Results: We found upregulation of SRSF2 is correlated with the development of EC; Knock-down of SRSF2 inhibits EC cell proliferation, migration, and invasion; SRSF2 regulates the splicing pattern of IRF3 in EC cells; SRSF2 interacts with exon 2 of IRF3 to regulate its exclusion; SRSF2 inhibits the transcription of IFN1 in EC cells.

Conclusion: This study identified a novel regulatory axis involved in EC from the various aspects of splicing regulation.

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KEYWORDS
SRSF2; oesophageal cancer; alternative splicing; IRF3; IFN1; proliferation

1. Background

Oesophageal cancer (EC) is one of the most common tumours in the world, The number of newly diagnosed oesophageal cancer cases was 473,000 (95% uncertainty interval [95% UI] 459,000–485,000) and the number of deaths was 436,000 (425,000–448,000). Age-standardized incidence was 5.9 (5.7–6.1)/100,000 population and age-average mortality was 5.5 (5.3–5.6)/100,000. Oesophageal cancer caused 9.78 million (9.53–10.03) DALYs, with an age-related rate of 120 (117–123)/100,000 population. There are no early clinical symptoms, a poor outcome, and a high malignancy potential with this disease [1,2]. EC is also one of the most serious malignant digestive neoplasms, affecting more males than females [2,3], the incidence and mortality of males (9.3 and 8.2, respectively) were 2–3 times higher than those of females (3.6 and 3.2, respectively). The incidence of EC has increased rapidly in recent years, and thus has become a major health concern [4]. When patients are diagnosed with EC at stage III or IV, they have a lower chance of survival due to less effective therapeutic treatments [4,5]. The aim of this study will be to investigate the molecular mechanisms of EC and to develop novel diagnostic and molecular biomarkers, as well as potent prognostic targets for effective treatment.

Alternative splicing (AS) is a vital mechanism for regulating gene expression that cells can produce numerous protein isoforms from finite genes [6,7]. In human tissues, more than tens of millions of AS events occur in more than 91% of exon genes [8]. In previous studies, AS has been

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shown to play a significant role in the physiology, development, and the development of different diseases in humans. It is estimated that 16% of single base-pair mutations that lead to genetic diseases could cause AS defects. Moreover, the differential expression of AS genes in cancer tissue has been reported recently.

For instance, Bcl-x is a Bcl−2 gene family and has two isoforms, Bcl-xS and Bcl-xL with opposing functions in apoptosis regulation in mammalian cells. Only the anti-apoptotic Bcl-xL, which plays a mandatory role in carcinogenesis, as it is increased in 60% of small cell lung cancer and invasive breast carcinomas [9–11]. A splice variant related to MUC1, MUC1/B, has also been associated with better prognosis and more favourable tumour behaviour in EC [4]. Another example is RUNX1a, the short isoform, it could reduce CEBPα gene expression, thereby promoting ESCC progression [12].

On the other hand, many scientists have found that some RNA binding proteins (RBPs) participate in the modulation of tumorigenesis-associated AS events [6,13]. A number of proteins, such as serine/arginine-rich proteins (SRs) and heterogeneous nuclear ribonucleoproteins (hnRNPs), play important roles in this process [6,13]. SRSF2 (Serine-Arginine Splicing Factor 2) is an SR protein. Its mutations are very widespread in myelodysplastic syndromes, acute myeloblastic leukaemia, and chronic myelomonocytic leukaemia [14–16]. In addition to affecting multiple transcripts, mutations in SRSF2 are associated with leukaemogenesis [15], however it is unclear if this gene is involved in chest tumour as well. In addition, studies have shown that most of the oncogenic splicing events in ESCC seem to be associated with increased expression of subtypes associated with increased proliferation, altered cell junctions, and increased cell migration [17]. These splicing events are characteristics of the transition from normal oesophageal tissue to oesophageal cancer and subsequent tumour progression and growth, resulting in many of these splicing variants being proposed as viable biomarkers for the diagnosis and prognosis of oesophageal cancer [18]. Although previous studies have shown that the progression of oesophageal cancer is closely related to AS, there are few studies on the specific mechanism of AS. Our study aims to find a new biomarker that affects the prognosis of oesophageal cancer, and to explore the key targets of alternative splicing to promote the progression and prognosis of oesophageal cancer, providing a new possibility for precise targeted therapy. In this study, we found that SRSF2 was highly expressed in human EC, and the upregulation of SRSF2 was associated with shortened survival of EC patients. In addition, knockdown of SRSF2 resulted in decreased migration, proliferation and invasion of EC cells. As an alternative splicing factor of IRF3, SRSF2 makes IRF3 produce splicing variant IRF3 a, which antagonizes the function of normal IRF−3 [6,19,20]. Previous studies have shown that defects in IFN-β signalling are caused by the dysregulated IFN regulatory factor−3 (IRF3) pathway. Our results showed that the expression of SRSF2 affected the expression of IFN1, so we predicted that the effect of SRSF2 on IFN−1 expression was mediated by IRF3. Our study suggests that SRSF2-mediated alternative splicing regulation axis plays a key role in the occurrence and development of oesophageal cancer.

2. Results

2.1 Upregulation of SRSF2 is correlated with the development of EC

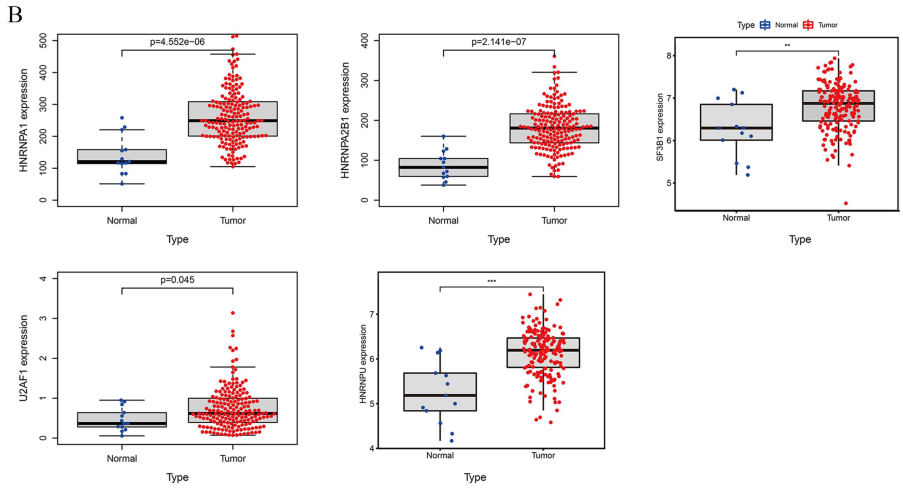
A new era of EC research is emerging as clinically relevant genomic and molecular information has been gradually elucidated [21–27]. Herein, we analysed the expression pattern of several relatively well characterized splicing regulators, such as U2AF1, SRSF2, SF3B1, hnRNP A1, hnRNP A2 and hnRNP U, on 196 samples from The Cancer Genome Atlas (TCGA) cohort of oesophageal cancer. We performed differential expression analysis and survival analysis on these splicing factors. Among them, the expression levels of SRSF2 and hnRNP family showed significant differences in 183 oesophageal cancer tissues and 13 normal oesophageal tissue samples. The hnRNP family has been shown to be associated with the development and prognosis of oesophageal cancer, so we chose SRSF2 for subsequent research (Figure 1b). SRSF2 level was increased in EC patients compared with the normal controls (4.874 ± 0.203 versus 7.042 ± 0.265, $p = 2.141e-07$; Figure 1a, d). Moreover, Kaplan-Meier analysis of TCGA survival data for EC patients demonstrated that patients with higher SRSF2 levels had lower disease-free survival compared with those with lower SRSF2 expression (median 763 versus 1361 days, $p = 0.0203$; Figure 1e). There was no significant correlation between the expression of other splicing factors and the survival prognosis of oesophageal cancer (Figure 1c). These results indicate that increased SRSF2 expression is associated with DFS (disease-free survival) decreased in EC patients.

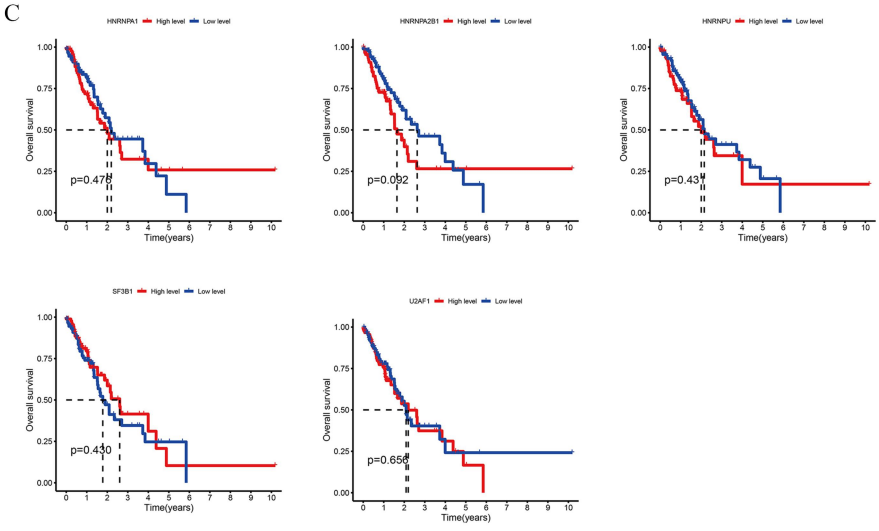
2.2 Knock-down of SRSF2 inhibits EC cell proliferation, migration, and invasion

To illustrate the effect of SRSF2 upregulation on EC cell behaviour, The expression of SRSF2 in various oesophageal cancer cell lines was queried on depmap.org. Finally, 7 of the 24 oesophageal cancer cell lines with high expression of SRSF2 were selected for further study. According to the results of PCR and Western blot analysis, we selected cell lines KYSE30 and KYSE510, in which the expression level of SRSF2 was relatively higher than others (Figure 2a, b). A lentivirus construct containing shRNA specific to SRSF2 was transfected into KYSE30 cells and KYSE510 cells, and the efficiency of SRSF2 knock-down was subsequently verified by immunoblotting analysis (Figure 2c). The intracellular SRSF2 protein level was reduced by 3-fold in KYSE30 and KYSE510 cells transduced with lentivirus carrying SRSF2 shRNAs (lenti-sh-SRSF2) than the control lentivirus (lenti-control) (Figure 2c). Consequently, knock-down of SRSF2 in KYSE30 and KYSE510 cells led to a reduced ability to proliferate (Figure 2d). Next, we also investigated whether decreased SRSF2 expression has an inhibitory effect on KYSE30 and KYSE510 cells' migration and invasion. As expected, the wound healing assay showed that cell migration was reduced in SRSF2 knock-down KYSE30 and KYSE510 cells compared with the control (Figure 2e). Moreover, the transwell invasion assay

A

Characteristics of the 183 Patients.	
Age at study entry	62.75±21.25
Patient country of procurement — no. (%)	
Asian	46 (25.14%)
White	112 (61.20%)
Black And African American	5 (2.73%)
Not Reported	20 (10.93%)
Male sex — no. (%)	156 (85.25%)
Normal cytogenetic profile — no./totalno. (%)	11 (6.01%)
Cancer cytogenetic profile — no./totalno. (%)	160 (87.43%)
Not Reported	12 (6.56%)
Pathologic stage — no. (%)	
Stage 1	18 (9.84%)
Stage 2	78 (42.62%)
Stage 3	55 (30.05%)
Stage 4	9 (4.92%)
Not Reported	23 (8.57%)





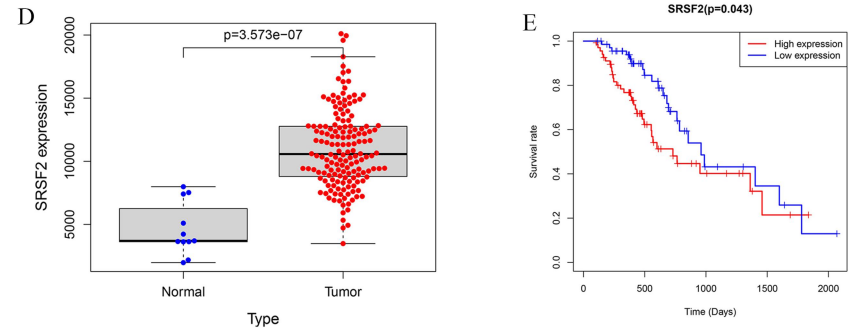


Figure 1. SRSF2 is upregulated in EC tissues. (a), EC patients' information from TCGA database. (b), Relative U2AF1, SF3B1, hnRNP A1, hnRNP A2 and hnRNP U mRNA level in EC and normal tissues from TCGA database. (c), Survival analysis of U2AF1, SF3B1, hnRNP A1, hnRNP A2 and hnRNP U. (d), Relative SRSF2 mRNA level in EC and normal tissues from TCGA database. (e), Statistical analysis of survival probability of SRSF2 high- (n = 11) and low-expressing (n = 52) EC patients using Kaplan – Meier analysis. Statistical analysis is described in the Materials and Methods. * $p < 0.05$; ** $p < 0.01$.

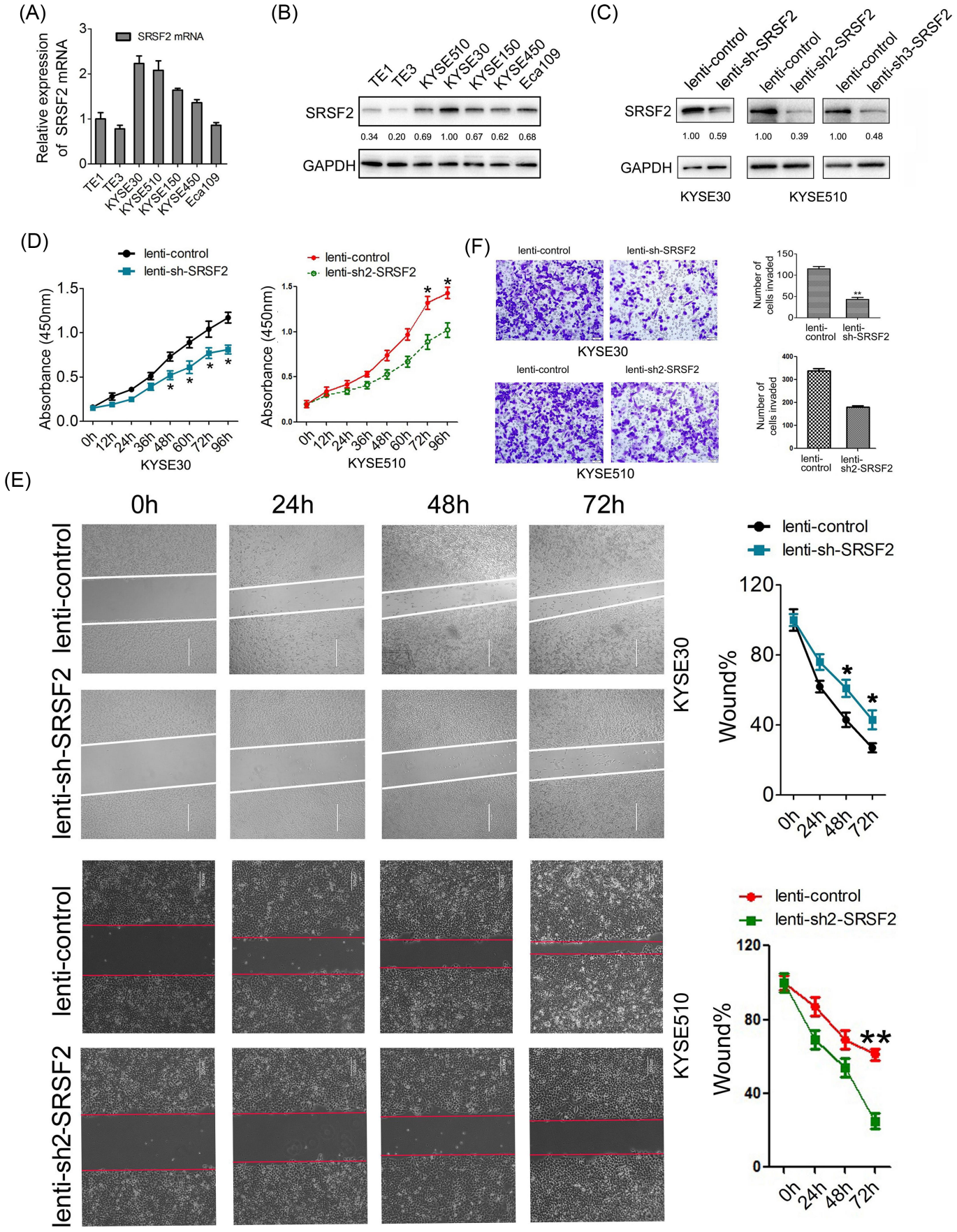


Figure 2. Knock-down of SRSF2 inhibits EC cell proliferation, migration and invasion. (a), the SRSF2 mRNA levels were detected in seven EC cell lines (TE1, TE3, KYSE30, KYSE510, KYSE150, KYSE450 and Eca109) by Qrt-PCR analysis. (b), the SRSF2 protein levels were detected seven EC cell lines (TE1, TE3, KYSE30, KYSE510, KYSE150, KYSE450 and Eca109) by immunoblotting analysis. (c), SRSF2 protein levels were detected in KYSE30 cells after transduced with lenti-control or lenti-sh-SRSF2 by immunoblotting analysis. (d), Cell proliferation assay of KYSE30 cells after transduced with lenti-control or lenti-sh-SRSF2 using CCK–8. (e), Wound healing

indicated a significant decrease in the ability of cells treated with lenti-sh-SRSF2/lenti-sh2-SRSF2 to invade through an extracellular matrix coating (Figure 2f). Taken together, all these results indicate that SRSF2 might function as an oncogene by regulating cell proliferation, migration and invasion in EC cells.

2.3 SRSF2 regulates the splicing pattern of IRF3 in EC cells

To further explore the pathway by which SRSF2 regulates EC proliferation, invasion, and migration, we performed the following experiments. SRSF2 is a serine/arginine-rich protein belonging to the family of SR proteins that are crucial regulators of alternative pre-mRNA splicing [28,], [29,30]. When reading the literature on alternative splicing, we discovered IRF3, a member of the interferon regulator family. IRF3 as an alternative splicing-related transcription factor, is associated with the occurrence of various tumour diseases. Previous studies have shown that IRF−3a expression is ubiquitous in oesophageal cancer cells, but its expression levels differ tissue-specifically compared to IRF−3. Furthermore, the data suggest that alternative splicing of IRF−3a expression may be further regulated by SR proteins, as well as by conditions that regulate transcription elongation [31]. IRF3 serves as an essential transcriptional activator for type I interferons (IFNα/β), and associates with CBP/p300 to control multiple downstream genes [6]. The IRF−3 gene consists of 8 exons, in which the second exon is often skipped to produce different splice isoforms, such as IRF3a in which the exon 2 is replaced by an intron, the exon 2, exon 3 and exon 6 deletion produces IRF3b and IRF3c with combined deletion of exon 2 and exon 3 (Figure 3a). To determine the possible effect of SRSF2 on splicing regulation of IRF3, we first performed qRT-PCR to detect the changes of exon 2 usage in lenti-sh-SRSF2 transduced KYSE30 cells. As shown, in KYSE30 cells, knock-down of SRSF2 led to a significant increase in IRF3 mRNA, but a dramatic reduction in IRF3a mRNA, and a slight decrease in IRF3b and IRF3c mRNA levels (Figure 3b). Western blot analysis showed that the expression of IRF3 was significantly increased, and the expression of IRF3a was significantly decreased (Figure 3c). Subsequently, to further verify the function of SRSF2 on IRF3 splicing, we used another EC cell line, Eca109, which has a relatively low endogenous SRSF2 level, to increase SRSF2 expression by pCMV6-SRSF2 transfection. Immunoblotting was performed to confirm the increase in SRSF2 protein in Eca109 cells (Figure 3d). Accordingly, an obvious increase in IRF3a but a decrease in IFR3 levels was also observed upon the upregulation of SRSF2 in Eca109 cells (Figure 3e). Western blot analysis showed that the expression of IRF−3 was significantly decreased, and the expression of IRF−3a was significantly increased (Figure 3f). These results suggested that SRSF2 could control the splicing pattern of IRF3 pre-mRNA by promoting the exclusion of exon2 in EC cells.

2.4 SRSF2 interacts with exon 2 of IRF3 to regulate its exclusion

Previous studies have identified the SRSF2 binding motifs as SSAG (S=C/G) [26–28]. In order to locate the functional SRSF2 target sites on IRF3, we screened IRF3 pre-mRNA sequences and found exon 2 includes a potential receptor sequence (GCAG) for SRSF2 at the position 50 nt downstream from the 3' splice site of exon 2 (Figure 4a). We next explored the possibility that SRSF2 binds specifically to this motif. RNA-EMSA was performed by the incubation of purified SRSF2 protein and biotinylated RNA probes containing wild type GCAG (E2_WT_probes) or mutant GCUA (E2_MUT_probes) of IRF3 exon 2 (Figure 4a). Results showed a clear shift band due to the formation of a complex with SRSF2 protein and wild type RNA probes, whereas mutations in probes led to a large decrease in SRSF2 binding (Figure 4b). This data confirmed the presence of SRSF2 binding sites in exon 2 of IRF−3 pre-mRNA.

Furthermore, to determine whether the GCAG motif is required for the function of SRSF2 after exon 2 exclusion, we constructed minigenes that contain only exon 1–3 (Figure 4c). QRT-PCR analysis was performed in Eca109 cells co-transfected with the minigenes along with pCMV6-SRSF2. The results of Figure 4d show that SRSF2 significantly promotes exon 2 with the wild type GCAG motif, while it has no obvious effect on mutant exon 2. To bind together, SRSF2 recognizes the GCAG sequence to facilitate the exclusion of exon 2 from IRF3.

2.5 SRSF2 inhibits the transcription of IFN1 in EC cells

It is reported that IRF3 could interact with p300 to initiate the transcription of the type I IFN (IFN1) gene, and IFN1 had anti-proliferative, antiviral, and immunomodulatory activities in different cell types, including tumour cells [32,33]. We first determined the effect of over-expression and knock-down of SRSF2 on endogenous IFN1 gene expression. We used KYSE30 cells, which have a relatively high endogenous SRSF2 level, to deplete SRSF2 by shRNAs, and Eca109 cells, which have a lower endogenous SRSF2 level, to restore SRSF2 by pCMV6-SRSF2 transfection. QRT-PCR analysis showed that there was ~ 5-fold induction in IFN1 mRNA levels in lenti-sh-SRSF2 transduced KYSE30 cells as compared to the control (Figure 5a). IFN1 mRNA levels were reduced by 30% in Eca109 cells when SRSF2 was expressed ectopically (Figure 5a). Moreover, we performed a luciferase assay to confirm the effect of SRSF2 on IFN1 promoter activity. As shown in Figure 5b, co-transfection of pCMV6-SRSF2 with IFN1 promoter constructs (−556 ~ +55 to the TSS of IFN1 gene) significantly reduced luciferase activity in Eca109 cells as compared with the control (Figure 5b). Rather, there was a significant induction by approximately 2-fold in IFN1 promoter

assays of KYSE30 cells after transduced with lenti-control or lenti-sh-SRSF2. The relative ratio of wound closure per field was shown in the right. (f), Transwell analysis of KYSE30 cells after transduced with lenti-control or lenti-sh-SRSF2. The relative ratio of invasive cells per field is shown in the right. For all Qrt-PCR results, the data are presented as the mean ± SEM, and the error bars represent the standard deviation obtained from three independent experiments. * $p < 0.05$; ** $p < 0.01$.

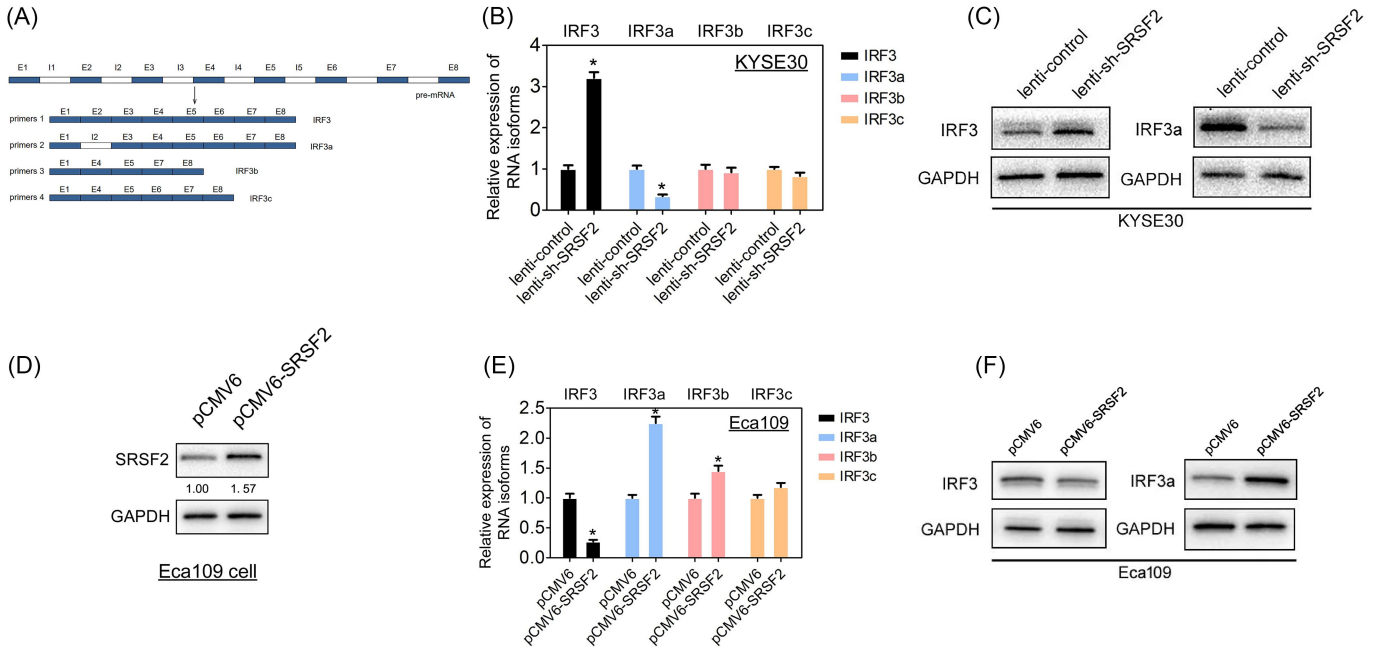


Figure 3. SRSF2 promotes the exclusion of exons 2 of IRF3. (a), schematic diagrams of IRF3(full-length IRF3), IRF3a (E2 replaced by i2), IRF3b (only E2 excluded) and IRF3c (including E2 and E3 excluded). IRF3 exons are numbered from 1 to 4 and introns are numbered from 1 to 2. Arrows indicate the positions of specific primer sets for different IRF3 splice variants determined by quantitative RT-PCR analysis. (b), Relative expression of IRF3 isoforms in KYSE30 cells transfected with lenti-control or lenti-sh-SRSF2.(c), Western blotting was used to detect the changes of IRF3 and IRF3a protein levels in cells with low SRSF2 knock-out.(d) Western blotting was used to detect the expression of SRSF2 protein in Eca109 cells transfected with pcmv6 empty vector or pCMV6-SRSF2.(e) Relative expression of IRF3 isoforms in Eca109 cells transfected with pCMV6 empty vector or pCMV6-SRSF2.(f) Western blotting was used to detect the expression of IRF3 and IRF3a protein in Eca109 cells transfected with pCMV6 empty vector or pCMV6-SRSF2. For all quantitative RT-PCR results, the data were expressed as mean ± SEM, and the error bars represented the standard deviation from three independent experiments. * $p < 0.05$; * * $p < 0.01$.

activity in KYSE30 cells transfected with shRNAs specific to SRSF2 (Figure 5b). Taken together, these results indicated that SRSF2 could inhibit the promoter activity and transcription of the IFN1 gene in EC cells.

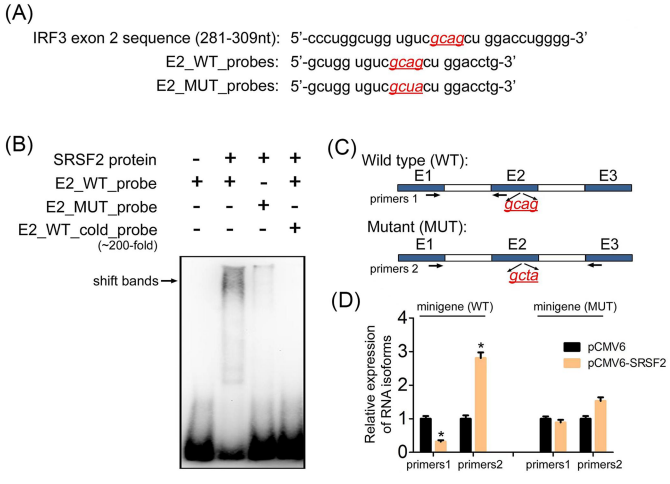


Figure 4. SRSF2 interacts with specific sequences in IRF3 exon 2. (a), Sequences of exon 2 of IRF3. The putative binding sites and mutant sites for SRSF2 are indicated in underlined italics. (b), Purified SRSF2 proteins were used for RNA electrophoretic mobility shift assay with the indicated biotinylated RNA probes or clod probes. (c), Schematic diagram showing the minigenes containing IRF3 exon 1 to 3. The putative binding sites and mutant sites for SRSF2 are indicated in underlined italics. The arrows indicate the location of specific primer sets designed for Qrt-PCR analysis of different IRF3 splicing variants. (d), QRT-PCR analysis of the splicing variants of with RNAs extracted from Eca109 cells after transfected with pCMV6 empty vector or pCMV6-SRSF2. For all Qrt-PCR results, the data are presented as the mean ± SEM, and the error bars represent the standard deviation obtained from three independent experiments. * $p < 0.05$; ** $p < 0.01$.

3. Materials and methods

3.1 Cell culture

The human EC cell lines (American Type Culture Collection (ATCC)) chosen for the experiments were cultured at 37°C in DMEM medium (Sigma) containing 10% FBS in an atmosphere incubator with 5% CO2. Media were supplemented with 100ug/ml streptomycin and 100ug/ml penicillin.

3.2 Analysis of TCGA data

The EC microarray and RNA-seq data were downloaded from The Cancer Genome Atlas database (TCGA). The gene expression data were normalized and log2 transformed. R packages (ggplot2), R packages (ggpubr) and R packages (limma) were used to merge the downloaded transcriptome data and draw images. Using the method of moments and Fisher's least significant difference (LSD) contrast method, the model of one-way analysis of variance (ANOVA) was widely used in identifying differentially expressed genes between the control and tumour group.

3.3 RNA isolation and Qrt-PCR analysis

RNA was isolated from the cells using RNeasy Kits (QIAGEN), and production cDNA was synthesized utilizing the iScript cDNA Synthesis Kit. The primer sequences were: IRF3Fwd (5'-TCGAGTTTGAGAGCTACCCG–3') and IRF3Rev (5'-TCTTGTTCACCCAGGCCA–3'). Real-time PCR reactions were duplicated and performed with the Vii7 System from ABI company. Empty carrier PCMV6 was used as a negative control.

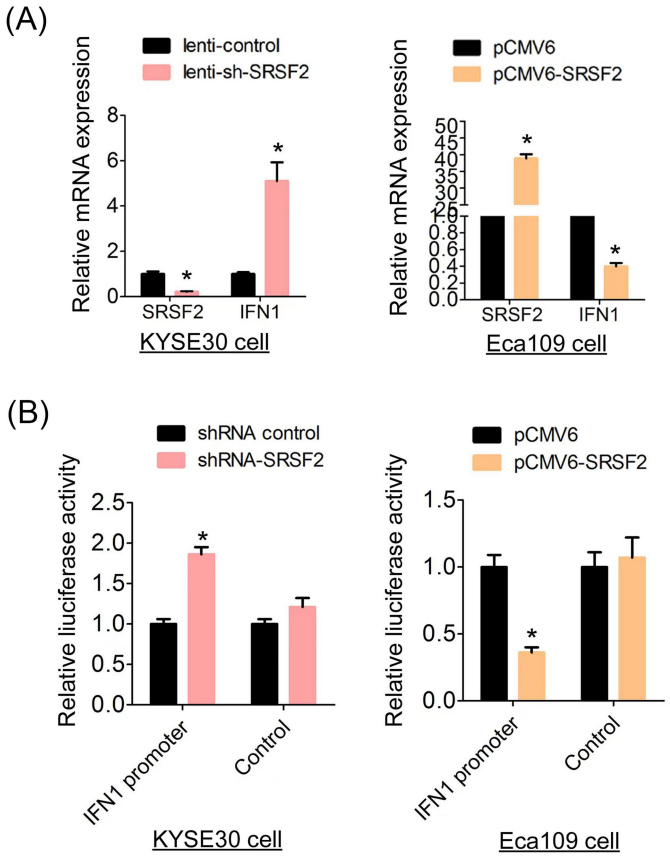


Figure 5. SRSF2 inhibits IFN1 transcription in EC cells. (a), QRT-PCR analysis of SRSF2 mRNA and IFN1 mRNA in KYSE30 cells after transduced with lenti-control or lenti-sh-SRSF2 (the left panel), or in Eca109 cells after transfected with pCMV6 empty vector or pCMV6-SRSF2 (the right panel). (b), the relative luciferase activities in KYSE30 cells after transfected with shRNA-control or shRNA specific to SRSF2 (the left panel), or in Eca109 cells after transfected with pCMV6 empty vector or pCMV6-SRSF2 (the right panel). For all quantitative results, the data are presented as the mean ± SEM, and the error bars represent the standard deviation obtained from three independent experiments. * $p < 0.05$; ** $p < 0.01$.

3.4 Oligonucleotides and constructs

The shRNA specific to SRSF2 and control shRNAs (shRNA-control) were transfected (100 nM) using Lipofectamine 2000 (Invitrogen, USA). For SRSF2 overexpression, use the pCMV6 vector (pCMV6-SRSF2) downstream of the CMV promoter. The promoter-luciferase reporter constructs were generated by cloning PCR-amplified DNA fragments of the human IFN1 promoter in the pGL3-basic vector (Promega). IFN1 promoter forward: 5'-CAGGATGGAGGAAGCAAA–3', reverse: 5'- CGCCAGTGAGAAGCAAGTA –3'. The Eca109 cells were transfected using Lipofectamine 2000 and luciferase activity was detected in the cell lysates. The results were normalized and measured as relative to light units per Renilla luciferase activity.

3.5 Lentivirus production

The shRNA specific to SRSF2 was cloned downstream of a lentivirus (pSIH1, System Biosciences, USA) to generate lenti-sh-SRSF2. The kit of matching lentivirus packaging (System Biosciences, USA) was used according to the instructions. Harvest viral particles and add them to frozen cells for storage.

3.6 Cell proliferation assay

KYSE30 cells were released by trypsinization and incubated in 64 well plates with Cell Counting Kit–8 (CCK–8, DOJINDO, Japan), and cultured for 1 h at 37°C. Proliferation rates were determined every 12 hours after transfection. The optical density was measured at 450 nm wavelength in a microplate. All experiments were performed in triplicate.

3.7 Cell migration and invasion assays

KYSE30 cells were transfected with control or shRNA on 6-well transwells. Linear scratch wounds (in triplicate) were created after 24 h of transfection. Cells were maintained in serum-free medium and images were taken every 24 h. Each group had three duplicate holes. The pre-made markers were used as fixed-point observation points to take regular photos every 4 hours to observe the scratch healing, and the time points with obvious differences in migration ability between the treatment/control group were selected for photography. Shoot the same position as 0 h, magnification (100 ×). The data of three independent repetitions were averaged. The larger the value, the faster the cell migration. T-test was used to calculate the *p* value and draw the histogram.

After 24 hours of transfection, cell invasion assay was performed using an 8 μm aperture chamber (Milipol, Switzerland). 2 × 105 KYSE30 cells were seeded in the upper chamber coated with Matrigel (Sigma-Aldrich, USA). The lower chamber was placed in DMEM medium containing 10% FBS. Invasive cells on the lower surface of the chamber were stained with 0.5% crystal violet (Sigma, USA). Leica DC 300F upright microscope was used to observe and photograph. The diameter of the visual field seen by the microscope was 1/4 of the diameter of the mask at the bottom of the chamber, and the cells in at least five random fields (Olympus, Japan) were counted.

3.8 RNA electrophoretic mobility shift assay (RNA EMSA)

These biotin-labelled E2_WT_probes, E2_MUT_probes were used from RiboBio Life Science. The RNA EMSA Kit (Pierce, USA) were incubated with various concentrations of SRSF2-Flag (Sigma-Aldrich) used to the manufacturer's protocol. The reactions were performed with 200-fold molar excess of the cold probes preincubation, then incubated by native 8% PAGE gel electrophoresis. The probes are listed in Table 1.

Table 1. Primers used for Qrt-PCR.

Name		Sequence (5'–3')
IRF3	E1 forward	TCGAGTTTGAGAGCTACCCG
	E2 reverse	TCTTGTTCACCCAGGCCA
IRF3a	E1 forward	TCGAGTTTGAGAGCTACCCG
	I2 reverse	GCACGCACCTGGAAGATT
IRF3b	E1 forward	TCGAGTTTGAGAGCTACCCG
	E3 reverse	GTTGGCAGGTCTGGCTTATC
IRF3c	E1 forward	TCGAGTTTGAGAGCTACCCG
	E4 reverse	GTACTGCCTCCACCATTGGT
IFN1 promoter	forward	CAGGATGGAGGAAGCAAA
	reverse	CGCCAGTGAGAAGCAAGTA

3.9 Immunoblotting analysis

Whole-cell lysates were subjected to immunoblotting analysis using standard methods. Proteins were separated by 10% SDS-PAGE and transferred onto PVDF membranes (Millipore Corporation, USA). Membranes were blocked with 5% non-fat dried milk for 2 h and incubated with anti-SRSF2 antibody at 4°C overnight. The membranes were incubated for 2 h with goat anti-rabbit antibody (ZSGB-bio, China) at room temperature after washing with TBST (pH 7.6, 136 mM NaCl, 20 mM Tris, 0.1% Tween−20). SRSF2 antibody was purchased from Abcam Biotechnology. The experiment was repeated at least twice with similar results.

3.10 Statistics

ALL experiments were repeated at three times. Statistical analyses were evaluated using the two-tailed t-test, and one-way ANOVA. Statistically significance was set at $P < 0.05$. Statistical analysis was performed using SPSS program (SPSS 15.0, USA).

4. Discussion

Diverse types of cancer pathogenesis are associated with aberrant RNA splicing, but the underlying mechanism remains unknown. SRSF2 is a well-known alternative splicing factor, and it has been shown to regulate alternative splicing of CD44, Tau, RUNX1, BCL2L2, BCL2A1, TP53 and E-cadherin pre-mRNA either through promoting or inhibiting exon exclusion [34,35]. SRSF2 mutations are frequently found in patients with myelodysplastic syndromes and certain leukaemias, but its involvement in solid tumours has only begun to be examined [35]. Here we reported that the splicing regulator SRSF2 was upregulated in human oesophageal cancer (EC) patients, and its high level was associated with shortened survival in patients. We also indicated that SRSF2 could regulate the splicing pattern of IRF3 through promoting the exclusion of exon 2. This could attenuate the biogenesis of full length IRF3 mRNA in EC cells.

IRF3 is an unusual member of the IRF family and plays critical roles in immune systems [6,36,37,], [38,39]. Alternative splicing of IRF3 has been shown to be a critical mechanism for the regulation of IRF3 function. Recently, different splicing variants of IRF3, referred to as IRF3a, 3b, 3c, 3d, 3e, and 3f, were identified as deletions of exons 2, 3, or 6 or some combination thereof [6]. IRF3a is the first characterized IRF3 splicing variant, and its original exon 2 in IRF3 is displaced by intron 2. IRF3a provides an example to demonstrate how structural alteration makes a protein become an antagonist of its normal counterpart. It lacks a portion of the N-terminal DBD domain of IRF3, thus it is unable to bind to classical IRF motif elements, but it could form a heterodimer with IRF3 and inhibit IRF3 transcriptional activity [6]. In this study, we showed that SRSF2 could promote the generation of IRF3 splicing variants at the expense of IRF3 expression in EC. Therefore, we speculated that the alternative splicing of IRF3 by SRSF2 leads to the production of splicing variants, which leads to the interference of IRF3 activity in EC cells and the inhibition of downstream IFN1 gene transcription. It has been shown that intracellular IFN1 signalling affects cell differentiation, proliferation, and apoptosis. Moreover, recent studies have revealed specific IFN1-regulated genes that may contribute to IFN1-mediated suppression of cancer progression and metastasis [33].

According to our results, we speculate that there is a regulatory axis of SRSF2-IRF3-IFN1 in oesophageal cancer cells in vitro, but whether it does exist still needs further study. In the next study, we will focus on whether the effect of SRSF2 on IFN1 is through the alternative splicing of IRF3. In addition, we will collect oesophageal cancer specimens from our hospital for gene sequencing and cross-validation with RNA-seq data in the TCGA database. After getting the exact conclusion, we will carry out animal experiments to verify our conclusion more deeply. Finally, we hope that our research results can be applied to clinical practice to obtain benefits for patients with oesophageal cancer.

5. Conclusions

Cancer cells often use alternative splicing to produce or increase proteins that promote growth and survival [35], because alternative splicing generates protein diversity. While RNA-binding proteins are known to regulate tumorigenesis-associated alternative splicing events [36], their roles in oesophageal cancer (EC) have been poorly documented. Here we investigated the role of an RNA-binding protein, SRSF2 (Serine-Arginine Splicing Factor 2), in human EC. By analysing the expression pattern of several relatively well characterized splicing regulators on 183 samples from The Cancer Genome Atlas (TCGA) cohort of EC. This study identified a novel regulatory axis involved in EC from the various aspects of splicing regulation.

Abbreviations

SRSF2	Serine-Arginine Splicing Factor 2
EC	oesophageal cancer
TCGA	The Cancer Genome Atlas
IRF3	interferon regulatory factor 3
hnRNP	nuclear ribonucleoproteins
pre-Mrna	precursor messenger RNA

Disclosure statement

No potential conflict of interest was reported by the authors.

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Author's contributions

Ziqing Wei designed and performed experiments, interpreted data and wrote the manuscript. Yuyao Wang performed bioinformatics analysis; Zhijie Shang, Wenyuan Ma and Wenqing Xing perform partial experiments. Peng Lu provided help and advice. Yuxuan Wang and Huiyu Li designed the study, directed the experiments.

Availability of data and materials

All data are fully available without restriction.

References

[1] Zhao JY, Wang F, Li Y, et al. Five miRnas considered as molecular targets for predicting esophageal cancer. Med Sci Monit. 2015;21:3222–3230. doi:10.12659/MSM.895001

[2] Sohda M, Kuwano H. Current status and future prospects for esophageal cancer treatment. Ann Thorac Cardiovasc Surg. 2016;23:1–11. doi:10.5761/atcs.ra.16-00162

[3] Guo W, Wang C, Guo Y, et al. RASSF5A, a candidate tumor suppressor, is epigenetically inactivated in esophageal squamous cell carcinoma. Clin Exp Metastasis. 2015;32(1):83–98. doi:10.1007/s10585-015-9693-6

[4] Kahkhaie KR, Moaven O, Abbaszadegan MR, et al. Specific MUC1 splice variants are correlated with tumor progression in esophageal cancer. World J Surg. 2014;38(8):2052–2057. doi:10.1007/s00268-014-2523-1

[5] Kamangar F, Dores GM, Anderson WF. Patterns of cancer incidence, mortality, and prevalence across five continents: defining priorities to reduce cancer disparities in different geographic regions of the world. J Clin Oncol. 2006;24(14):2137–2150. doi:10.1200/JCO.2005.05.2308

[6] Guo R, Li Y, Ning J, et al. HnRNP A1/A2 and SF2/ASF regulate alternative splicing of interferon regulatory factor-3 and affect immunomodulatory functions in human non-small cell lung cancer cells. PLoS ONE. 2013;8(4):e62729. doi:10.1371/journal.pone.0062729

[7] Cáceres JF, Kornblihtt AR. Alternative splicing: multiple control mechanisms and involvement in human disease. Trends Genet. 2002;18(4):186–193. doi:10.1016/S0168-9525(01)02626-9

[8] Pan Q, Shai O, Lee LJ, et al. Deep surveying of alternative splicing complexity in the human transcriptome by high-throughput sequencing. Nat Genet. 2008;40(12):1413–1415. doi:10.1038/ng.259

[9] Hui L, Zhang X, Wu X, et al. Identification of alternatively spliced mRNA variants related to cancers by genome-wideEsts alignment. Oncogene. 2004;23(17):3013–3023. doi :10.1038/sj.onc.1207362

[10] Zhou F, Yang Y, Xing D. Bcl-2 and Bcl-Xl play important roles in the crosstalk between autophagy and apoptosis. FEBS J. 2011;278(3):403–413. doi:10.1111/j.1742-4658.2010.07965.x

[11] Wu L, Mao C, Ming X. Modulation of Bcl-x alternative splicing induces apoptosis of human hepatic stellate cells. Biomed Res Int. 2016;2016:1–7. doi:10.1155/2016/7478650

[12] Wu H, Zheng J, Deng J, et al. LincRNA-uc002yug.2 involves in alternative splicing of RUNX1 and serves as a predictor for esophageal cancer and prognosis. Oncogene. 2015;34(36):4723–4734. doi:10.1038/onc.2014.400

[13] Busch A, Hertel KJ. Evolution of SR protein and hnRNP splicing regulatory factors. Wiley Interdiscip Rev RNA. 2012;3(1):1–12. doi:10.1002/wrna.100

[14] Bejar R. Splicing factor mutations in cancer. Adv Exp Med Biol. 2016;907:215–228.

[15] Garza E, Del Poeta G, Martínez-Losada C, et al. A case of SRSF2 mutation in chronic lymphocytic leukemia. Leuk Res Rep. 2016;6:11–14. doi:10.1016/j.lrr.2016.03.001

[16] Luo C, Cheng Y, Liu Y, et al. SRSF2 regulates alternative splicing to drive hepatocellular carcinoma development. Cancer Res. 2017;77(5):1168–1178. doi:10.1158/0008-5472.CAN-16-1919

[17] Mao S, Li Y, Lu Z, et al. Survival-associated alternative splicing signatures in esophageal carcinoma. Carcinogenesis. 2019;40(1):121–130. doi:10.1093/carcin/bgy123

[18] Dlamini Z, Hull R, Mbatha SZ, et al. Prognostic alternative splicing signatures in esophageal carcinoma. Cancer Manag Res. 2021;13:4509–4527. doi:10.2147/CMAR.S305464

[19] Marozin S, Altomonte J, Stadler F, et al. Inhibition of the IFN-β response in hepatocellular carcinoma by alternative spliced isoform of IFN regulatory factor-3. Mol Ther. 2008;16:1789–1797. doi:10.1038/mt.2008.201

[20] Li C, Ma L, Chen X. Interferon regulatory factor 3-CL, an isoform of IRF3, antagonizes activity of IRF3. Cell Mol Immunol. 2011;8(1):67–74. doi:10.1038/cmi.2010.55

[21] Lin DC, Hao J-J, Nagata Y, et al. Genomic and molecular characterization of esophageal squamous cell carcinoma. Nat Genet. 2014;46(5):467–473. doi:10.1038/ng.2935

[22] Dai M, Zhu X-L, Liu F, et al. Cholesterol Synthetase DHCR24 induced by insulin aggravates cancer invasion and progesterone resistance in endometrial carcinoma. Sci Rep. 2017;7(1). doi:10.1038/srep41404

[23] Zainulabadeen A, Yao P, Zare H, et al. Underexpression of specific interferon genes is associated with poor prognosis of melanoma. PLoS ONE. 2017;12(1):e0170025. doi:10.1371/journal.pone.0170025

[24] Song Y, Li L, Ou Y, et al. Identification of genomic alterations in oesophageal squamous cell cancer. Nature. 2014;509(7498):91–95. doi:10.1038/nature13176

[25] Gao YB, Chen Z-L, Li J-G, et al. Genetic landscape of esophageal squamous cell carcinoma. Nat Genet. 2014;46(10):1097–1102. doi:10.1038/ng.3076

[26] Sasaki Y, Tamura M, Koyama R, et al. Genomic characterization of esophageal squamous cell carcinoma: insights from next-generation sequencing. World J Gastroenterol. 2016;22(7):2284–2293. doi:10.3748/wjg.v22.i7.2284

[27] Hao JJ, Lin D-C, Dinh HQ, et al. Spatial intratumoral heterogeneity and temporal clonal evolution in esophageal squamous cell carcinoma. Nat Genet. 2016;48:500–1507. doi:10.1038/ng.3683

[28] Zhang J, Lieu YK, Ali AM, et al. Disease-associated mutation in SRSF2 misregulates splicing by altering RNA-bindingaffinities. Proc Natl Acad Sci, USA. 2015;112(34):E4726–4734. doi:10.1073/pnas.1514105112

[29] Daubner GM, Cléry A, Jayne S, et al. A syn-anti conformational difference allows SRSF2 to recognize guanines and cytosines equally well. Embo J. 2012;31(1):162–174. doi:10.1038/emboj.2011.367

[30] Moon H, Cho S, Loh TJ, et al. SRSF2 promotes splicing and transcription of exon 11 included isoform in Ron proto-oncogene. Biochim Biophys Acta. 2014;1839:1132–1140. doi:10.1016/j.bbagrm.2014.09.003

[31] Yong L, Hu X, Song Y, et al. Identification of novel alternative splicing variants of interferon regulatory factor 3. BBA. 2011 March;1809:166–175.

[32] Zhang K, Yin X-F, Yang Y-Q, et al. A potent in vivo antitumor efficacy of novel recombinant type I interferon. Clin Cancer Res. 2017;23:2038–2049. doi:10.1158/1078-0432.CCR-16-1386

[33] Ortiz A, Fuchs SY. Anti-metastatic functions of type 1 interferons: foundation for the adjuvant therapy ofcancer. Cytokine. 2017;89:4–11. doi:10.1016/j.cyto.2016.01.010

[34] Kędzierska H, Popławski P, Hoser G, et al. Decreased expression of SRSF2 splicing factor inhibits apoptotic pathways in RenalCancer. Int J Mol Sci. 2016;17(10):1598. doi:10.3390/ijms17101598

[35] Shahrabi S, Khosravi A, Shahjahani M, et al. Genetics and epigenetics of myelodysplastic syndromes and response to drug therapy: new insights. Oncol Rev. 2016;10:311. doi:10.4081/oncol.2016.311

[36] Prinarakis E, Chantzoura E, Thanos D, et al. S-glutathionylation of IRF3 regulates IRF3-CBP interaction and activation of the IFN beta pathway. Embo J. 2008;27:865–875. doi:10.1038/emboj.2008.28

[37] Ren W, Wang C, Wang Q, et al. Bromodomain protein Brd3 promotes Ifnb1 transcription via enhancing IRF3/p300 complex formation and recruitment to Ifnb1 promoter in macrophages. Sci Rep. 2017;7(1). doi:10.1038/srep39986

[38] Zu CX, Wu H-Y, Ma F-C, et al. Prognostic alternative splicing signatures and underlying regulatory network in esophageal carcinoma. Am J Transl Res. 2019;11:4010–4028.

[39] Shuang SM, et al. Survival-associated alternative splicing signatures in esophageal carcinoma. Carcinogenesis. 2019;40:121–130. doi:10.1093/carcin/bgy123