Systematic Profiling of Survival-Associated Alternative Splicing Events in Adrenocortical Carcinoma

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Primary research

Keywords: Alternative splicing events, Splicing factors, Adrenocortical carcinoma, TCGA

DOI: https://doi.org/10.21203/rs.3.rs-33545/v1

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Abstract

**Background:** Aberrant alternative splicing (AS) is involved in many oncogenic processes and systematic analysis of survival-associated aberrant AS events has been reported in many cancers. This study aims to systematic profiling the AS signature in Adrenocortical carcinoma (ACC).

**Methods:** Data of ACC were downloaded from TCGA and TCGA SpliceSeq. Clinical information and AS events data were integrated with the same TCGA ID. Then, we performed univariate Cox analysis to identify survival-related AS events. Lasso regression and multivariate Cox analysis were used to establish prognostic model. In addition, several bioinformatics analyses were conducted to identify pathways enriched by genes of survival-associated AS events and construct splicing-factor-regulated network.

**Results:** A total of 77 patients with complete clinical information and PSI values of AS events were included in the present study. We detected 3781 AS events in 2366 genes were associated with overall survival of ACC patients. All the predictive models showed efficiency in distinguishing good and poor outcomes of ACC patients. All the AUCs of predictive models were greater than 0.7. Functional analysis genes with survival-associated AS events suggested that the POLR2H, TCEB2, PSMA1, PSMD11 and SKP2 ranked at the core. The splicing-factor-regulated network revealed the potential regulatory mechanisms of AS events in ACC.

**Conclusions:** Our systematic profiling of survival-associated AS events in ACC patients provides novel molecular alternations and contributes to decipher the underlying mechanisms of AS in oncogenesis of ACC.

**Background**

Adrenocortical carcinoma (ACC) is a rare endocrine malignancy that originates in the cortex of the adrenal gland (estimated incidence, 0.7 ~ 2.0 cases per million persons) [1, 2] with rapid progress and poor prognosis. The 5-year survival rate is less than 50% for ACC with locally advanced disease and 15% for patients with distant metastases [3–4]. Complete surgical resection of the tumor is currently the only available curative treatment option for non-metastatic ACC [5] and mitotane [6, 7] plus other chemotherapy is recommended as the treatment for patients with advanced and inoperable ACC [8]. Such poor prognosis and deficient treatments make it is imperative to screen for tumor markers of ACC.

Recently, accumulating evidence has focused on the molecular diagnosis and prognosis of ACC. Systematic analyzing of genetic and epigenetic signatures, such as mutations [9], DNA methylation [10, 11], mRNA [12] and microRNA expression [13], has contributed to the clinical diagnosis and the discovery of potential biomarkers in the patients with ACC. However, these studies, although with promising achievements, mainly focus on alterations at gene expression level while ignoring the RNA isoform diversity from a single gene regulated by alternative splicing (AS).
AS is a process that selective removal or retention of exons and/or introns by different splicing patterns to generate distinct mRNA isoforms from a single gene [14, 15]. It is a pivotal step of post-transcriptional gene expression regulation and plays a vital role in expanding protein diversity in cells [16, 17]. High-throughput sequencing technology estimated that up to 90% of human genes undergo AS [18]. Accordingly, AS can produce multiple mRNA isoforms encoding proteins with structural and/or functional differences that can have profound biological consequences. Therefore, AS is not crucial for normal physiological processes such as hematopoiesis [19], brain development [20] and skeletal muscle function [21], but also for multiple pathological states, including tumorigenesis [22]. Aberrant AS is involved in many oncogenic processes, including uncontrolled cell proliferation, evading growth suppressors, invasion and metastasis, angiogenesis and immune escape [23–25]. More importantly, dysregulation of AS is a fundamental process in cancer and research shows that AS has emerging potential therapeutic targets and biomarkers in cancer therapy [16, 22, 26]. Systematic analysis of survival-associated aberrant AS events has been reported in gastrointestinal pan-adenocarcinomas [27], breast cancer [28], ovarian cancer [29] and lung cancer [30]. However, there is no comprehensive study on the clinical significance and prognostic value of AS in ACC. Here, we performed a systematic analysis of survival-associated aberrant AS in ACC using The Cancer Genome Atlas (TCGA) RNA-seq data and TCGA SpliceSeq PSI data and evaluated the potential functions in tumor biology.

**Methods**

**Data collection and collation**

RNA-seq data and matched clinical data of ACC cohort were downloaded from TCGA GDC data portal (https://portal.gdc.cancer.gov/repository). There are seven types of AS events, including alternate acceptor site (AA), alternate donor site (AD), alternate promoter (AP), alternate terminator (AT), exon skip (ES), mutually exclusive exons (ME), and retained intron (RI) [31]. To quantify AS events, percent spliced in (PSI) (ranging from zero to one) values for the AS events of ACC were used [18,32]. Seven splice event types with a PSI value of more than 75% in ACC samples were downloaded from TCGA SpliceSeq (https://bioinformatics.mdanderson.org/TCGASpliceSeq/PSI download.jsp).

When obtained the data of PSI values, we removed the AS events with mean value < 0.05 or standard diversion < 0.01. We integrated clinical information and AS events data with the same TCGA ID.

**Survival analysis**

A total of 77 ACC patients with overall survival (OS) >90 days were included in this study. UpSet, a visualization technique [33], was used to quantitative analysis of intersecting sets between different types of AS. We performed univariate Cox analysis on all of AS events to calculate the association between the PSI value of each AS events and the OS of ACC patients. Those AS events with P-values < 0.05 were identified as prognosis-related AS events. Then, the selected AS events were screened by the least absolute shrinkage and selection operator (Lasso) regression. Lasso regression is a statistical method that determines the best factors to use for prediction models [34,35] by using the “glmnet” and
“survival” packages in R. We established prediction model for ACC patients by using the multivariate Cox analysis with the “survival” package in R. To further assess the predictive accuracy and sensitivity of the prediction model, we performed the receiver operating characteristic (ROC) curves analysis with the survivalROC package in R. Univariate and multivariate Cox regression were performed to determine the splicing-based prognostic signature as an independent prognostic factor.

**Bioinformatics analyses for the genes of survival-associated AS events**

To explore the molecular characteristics for the genes with survival-associated AS events, we used “STRING” version 11.0 ([http://string-db.org/cgi/input.pl](http://string-db.org/cgi/input.pl)) [36] to conduct bioinformatics analyses, which including protein-protein interaction (PPI), Gene ontology (GO), and the Kyoto Encyclopedia of Genes and Genomes (KEGG) analysis.

**Splicing factors and AS regulatory network**

To explore the regulation of splicing factors (SFs) on prognosis-related AS events, we obtained 404 SFs which was previously reported [37]. The level 3 mRNA-seq data of SFs genes were curated from the TCGA dataset. Univariate Cox analysis was performed on the prognosis-related AS events and SFs and then constructed the f SF-AS regulatory network according to the following conditions: P value less than 0.05 and Pearson correlation coefficient more than 0.65. The regulation network was plotted via Cytoscape version 3.7.1.

**Statistical analysis**

R version 3.6.1 was used for all statistical analysis. For all statistical methods, p values less than 0.05 were considered significant.

**Results**

**General information of ACC patients in the TCGA cohort**

As shown in Table 1, 92 tumors with clinical information was downloaded from TCGA. In our study cohort, the median age at diagnosis was 47.16 years old, patient age ranged from 14 year to 83 years. The overall female-to-male ratio was 3:1.6. Most patients were white (84.8%). Stage I disease was found in 9 patients (9.8%), stage II in 44 (47.8%), stage III in 19 (20.7%), stage IV in 18 (19.6%). ACC was the first diagnosed malignancy in 86 patients.

**Overview of AS events and survival associated AS events in TCGA ACC cohort**

Comprehensive AS events in seven splicing types, including AA, AD, AP, AT, ES, ME, and RI were summarized for ACC. A total of 34,420 AS events in 8994 genes were detected from TCGA SpliceSeq dataset, indicating that one gene may undergo multiple AS events simultaneously. In detail, 2707 AAs in
1960 genes, 2382 ADs in 1688 genes, 6342 APs in 2575 genes, 8201 ATs in 3575 genes, 12,269 ESs in 5337 genes, 124 MEs in 122 genes and 2395 RIs in 1605 genes (Fig. 1A).

Univariate Cox analysis revealed that 3781 AS events in 2366 genes were associated with ACC OS rates (P<0.05). In detail, as shown in Fig. 1B, 199 AAs in 186 genes, 248 ADs in 221 genes, 679 APs in 423 genes, 1224 ATs in 724 genes, 1184 ESs in 937 genes, 8 MEs in 8 genes and 239 RIs in 209 genes were identified as survival-associated AS events. The UpSet plot (Fig. 1C) vividly revealed that one gene could undergo multiple AS events simultaneously.

Molecular characteristics of survival-associated AS events

The distributions of AS events significantly associated with patient survival are shown in Fig. 2A. We displayed the top 20 (if available) most significant survival-associated AS events for each AS type in Fig. 2B-H. To explore the molecular characteristics of genes with the top 50 most significant survival-associated AS events (if available), we performed several bioinformatics analyses. Firstly, we established a PPI network to reveal the relationships among these genes. As shown in Fig. 3A-B, POLR2H, TCEB2, PSMA1, PSMD11 and SKP2 ranked at the core in the network. According to the functional enrichments of these genes, “intracellular membrane-bounded organelle”, “mitochondrion”, “membrane-bounded organelle”, “organelle part” and “intracellular organelle part” were the five most significant cellular component terms (GO) (Fig. 4A). For biological process terms (GO), “metabolic process”, “cellular metabolic process”, “nitrogen compound metabolic process”, “intracellular transport” and “organic substance metabolic process” were the five most significant enrichments (Fig. 4B). There were no significant pathway enrichments observed in molecular function (GO). Finally, we observed that “Thermogenesis” was the only significant pathway (FDR=0.032) correlated with these genes in KEGG pathway analysis.

Prognostic predictors for ACC patients

We used the Lasso regression and multivariate Cox regression analysis to generate prognostic models (PMs) for seven AS types and for all types: PM-AA, PM-AD, PM-AP, PM-AT, PM-ES, PM-ME, PM-RI, and PM-ALL (Fig. 5 and Table 2) following univariate Cox. Then, we divided ACC patients into low and high risk groups based on median values to analyze the efficacy of prognostic models by using Kaplan-Meier (K-M) method. As shown in Fig. 6A-H, all the prognostic models could predict good and poor outcomes of ACC patients. ROC curves validated the efficiency of these prognostic models (Fig. 6I). To further elucidate the independent prognostic significance of PM-ALL, univariate and multivariate Cox regression analyses were performed. After adjusting for the clinical factors, the PM-ALL remained an independent prognostic factor for ACC patients, with an HR of 1.012 (95%CI: 1.003-1.020, P=0.007) (Table 3).

Network of survival-associated AS genes and SFs expression

SFs are RNA-binding proteins that recognize cis-regulatory elements within the pre-mRNA to influence exon selection and splice site choice (38). SF alternations are a hallmark of cancer. Therefore, we
explored the interaction networks of survival-associated AS genes and SFs. Firstly, we found 20 SFs related to survival that could be used as independent prognostic factors by using K-M method and Cox regression analysis (Table 4). Next, correlation analyses between the expression of these 20 SFs and the PSI values of survival-associated AS events were performed by using Pearson’s correlation analysis (cor > 0.6, P < 0.001). Correlation plots were then generated using Cytoscape 3.7.1. The results showed that the expression of 19 survival-related SFs (triangular nodes) were correlated with 206 survival-associated AS events (Fig. 7). Overall, 97 AS events were correlated with favorable OS (rea ovals) and 109 AS events were correlated with poor OS (green ovals).

Discussion

Aberrant AS is involved in the development process of cancer [23–25]. TCGA RNA sequencing data and TCGA SpliceSeq data have enabled investigation of AS patterns in many different kinds of cancers, such as breast cancer (28), ovarian cancer [29]. Growing evidence has shown that AS has emerging potential therapeutic targets and biomarkers in cancer therapy [16, 22, 26]. However, comprehensive information concerning dysregulation AS in ACC, which is an endocrine malignancy with rapid progress and poor prognosis and lacks selective and efficacious treatment options, is lacking.

In this study, we first found that 34,420 AS events in 8994 genes in the TCGA ACC cohort, among which 3781 AS events in 2366 genes were related to survival (P < 0.05). Among these survival-associated AS events, some splice variants may play critical roles in oncogenic processes, such as the AA variant of ZFAND6, the AD variant of ZSCAN18, the AP variant of CMC2, the ES variant of CIRBP, the ME variant of THNSL2 and the RI variant of CIRBP. Many splicing isoforms of these genes have been found to be related to survival in various types of tumors, such as papillary thyroid cancer [39], ovarian cancer [29], esophageal adenocarcinoma and esophageal squamous cell carcinoma [40].

Next, given the molecular function of AS events is partly described by the downstream functional impact, we conducted PPI network analysis. We found POLR2H, TCEB2, PSMA1, PSMD11 and SKP2 ranked at the core in the network. As THE HUAMAN GENE DATABASE GeneCards displays that POLR2H encodes an essential and highly conserved subunit of RNA polymerase II that is shared by the other two eukaryotic DNA-directed RNA polymerases, I and III and alternative splicing of POLR2H generates multiple transcript variants. To our knowledge the related study of POLR2H was rare, but it has been shown to be associated with the occurrence and progression of prostate cancer [41]. TCEB2 (also known Elongin B, ELOB) encodes the protein elongin B, which is a subunit of the transcription factor B (SIII) [42, 43]. Deng et al. reported that TCEB2 Confers Resistance to VEGF-targeted Therapy in ovarian cancer [44]. As many problems of ACC are still unresolved, such as disease prevention and earlier detection, these new discovered core genes may provide new insights for us.

In addition, we proposed the predictive model for each splice type and all available splice types of AS events using Lasso regression and multivariate Cox regression. All the predictive models showed efficiency in distinguishing good and poor outcomes of ACC patients. All the AUCs of predictive models...
were greater than 0.7, suggesting good power and potential in application of prognosis prediction for ACC patients. However, the efficiency should be verified by another independent cohort.

Alterations in activity of regulatory SFs are an important mechanism of aberrant AS in cancer [45]. Therefore, we identified 20 survival-associated SFs in ACC patients, such as KHSRP, SRSF7, SRSF2 and HNRNPA2B1. Serine/arginine (SR) proteins and heterogenous ribonuclear proteins (hnRNPs) are the two key families of SFs. Many studies have confirmed that SR and hnRNPs involve in tumorigenesis, such as isoforms PKM2 or TP53β of SRSF3 alters cell metabolism and induces cellular senescence [46, 47]. SRSF7 is upregulated in lung cancer, and its knockdown impacts cell proliferation [48]. In glioblastoma, hnRNPA2/B1 mediates its tumorigenic effect through alternative splicing of key oncogenes and tumor suppressors [49]. Moreover, splicing correlation network revealed that multiple AS events were correlated with SFs expression in ACC. These findings might provide new insight into the mechanisms underlying the development and progression of ACC.

Conclusions

The current study conducted a comprehensive analysis base of survival-associated AS events in ACC patients, which might contribute to uncover the function of AS events in ACC. Moreover, the identification of prognostic SFs and construction of the correlation networks between SFs and AS events will pave the way for in-depth exploration of splicing-related mechanisms in the oncogenic process of ACC. This comprehensive analysis AS events and SFs also provided valuable therapeutic targets that require further validation.

Declarations

Ethics approval and consent to participate

The study was approved by the Institutional Review Board of the Affiliated Hospital of Southwest Medical University, and was performed following the TCGA publication guidelines.

Consent for publication

Not applicable.

Availability of data and materials

Not applicable.

Competing interests

The authors declare that they have no competing interests.

Funding
Authors' contributions

YX, XF and QW conceived and designed the study. XF, WQ, FT, MG and ZJ collected the data. XF, QW, YL and XT performed the analysis and graphics. XF, QW, YL, FT, XT, MG and ZJ wrote the manuscript. YX revised the manuscript. All authors have read and approved the final manuscript.

Acknowledgements

We thank the TCGA dataset and TCGA SpliceSeq for sharing large amounts of data.

Abbreviations

AS, Alternative splicing; ACC, Adrenocortical carcinoma; PSI, Percent spliced in; AA, Alternate acceptor; AD, Alternate donor; AP, Alternate promoter; AT, Alternate terminator; ES, Exon skip; ME, Exclusive exons; RI, Retained intron; ROC, receiver operating characteristic; PPI, protein-protein interaction; GO, Gene ontology; KEGG, Kyoto Encyclopedia of Genes and Genomes; SFs, splicing factors; PM, Prognostic models.

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Tables

Table 1. General characteristics of ACC patients in TCGA Cohort
| Clinical characteristics | Total (92) | Percentage (%) |
|--------------------------|------------|----------------|
| Age at diagnosis (y)     | 47.16      |                |
|                          | (14-83)    |                |
| Gender                   |            |                |
| Female                   | 60         | 65.2           |
| Male                     | 32         | 34.8           |
| Race                     |            |                |
| White                    | 78         | 84.8           |
| Asian                    | 2          | 2.2            |
| Black or AA              | 1          | 1.1            |
| Unknown                  | 11         | 12             |
| T                        |            |                |
| T1                       | 9          | 9.8            |
| T2                       | 49         | 53.3           |
| T3                       | 11         | 12             |
| T4                       | 21         | 22.8           |
| Unknown                  | 2          | 2.2            |
| N                        |            |                |
| N0                       | 80         | 87             |
| N1                       | 10         | 10.9           |
| Unknown                  | 2          | 2.2            |
| M                        |            |                |
| M0                       | 72         | 78.3           |
| M1                       | 18         | 19.6           |
| Unknown                  | 2          | 2.2            |
| Stage                    |            |                |
| I                        | 9          | 9.8            |
| II                       | 44         | 47.8           |
| III                      | 19         | 20.7           |
| IV                       | 18         | 19.6           |
| Unknown                  | 2          | 2.2            |
| Prior_malignancy         |            |                |
| Yes                      | 6          | 6.5            |
| No                       | 86         | 93.5           |
| Synchronous_malignancy   |            |                |
| No                       | 86         | 93.5           |
| Unknown                  | 6          | 6.5            |
| Treatment_type           |            |                |
| Chemotherapy             | 53         | 57.6           |
| Radiotherapy             | 39         | 42.4           |

AA, African American; ACC, adrenocortical carcinoma.
| Type | Gene | Exon | Coef | HR   | Lower | Upper | P-value |
|------|------|------|------|------|-------|-------|---------|
| AA   | MED11| 3.1  | 13.076| 477637 | 222.02 | 1.03E+09 | 0.0008  |
|      | ZNF69| 3.3  | -2.0847 | 0.1243 | 0.0159 | 0.9710 | 0.0468  |
|      | WASH| 3.1  | -14.886 | 3.43E-07 | 1.10E-09 | 0.0001 | 3.82E-07 |
|      | STRAD| 11.1 | -30.226 | 7.46E-14 | 8.27E-24 | 0.0006 | 0.0097  |
|      | WASH| 4P   | -26.719 | 2.49E-12 | 4.02E-22 | 0.0154 | 0.0201  |
|      | CIRBP| 9.5:9.6:9.7 | -9.0765 | 0.0001 | 4.86E-07 | 0.0268 | 0.0011  |
|      | CERS5| 6.1  | -24.995 | 1.39E-11 | 8.70E-19 | 0.0002 | 0.0031  |
|      | HMGA| 8.1  | -12.473 | 3.83E-06 | 6.14E-10 | 0.0238 | 0.0051  |
| AD   | TRAFD| 1.2  | 8.2589 | 3861.8 | 9.0125 | 165482 | 0.0075  |
|      | BEX2| 1.2  | -19.118 | 4.98E-09 | 5.85E-18 | 4.2336 | 0.0683  |
|      | NOP2| 1.2  | 2.4790 | 11.930 | 0.4690 | 303.45 | 0.1332  |
|      | PRKAG| 1.2  | 6.6491 | 772.15 | 1.8656 | 319568 | 0.0305  |
|      | RPS6| 1.2:1.3:1.4 | 17.270 | 316505 | 4060.8 | 2.47E+11 | 0.0001  |
|      | SLC35| 1.2  | 10.792 | 48659. | 151.06 | 156734 | 0.0002  |
|      | ARM6| 1.2  | 13.203 | 542091 | 625.38 | 4.7E+08 | 0.0001  |
|      | ABCC5| 7.2:7.3:7.4 | -2.4290 | 0.0881 | 0.0131 | 0.5888 | 0.0121  |
| ALL  | CIRBP| 7.2:7.3 | 13.735 | 923122 | 0.0535 | 1.59E+13 | 0.1061  |
|      | BLOC1| 1.2  | 6.2825 | 535.15 | 1.9797 | 144656 | 0.0278  |
|      | TRAFD| 1.2  | 6.2825 | 535.15 | 1.9797 | 144656 | 0.0278  |
|    |    |    |    |    |    |    |    |    |    |
|----|----|----|----|----|----|----|----|----|----|
| 1  | 54 | 35 | 89 | .5 | 76 |    |    |    |    |
| METTL 15 | 12.3 | -22.763 | 1.30E-10 | 1.31E-18 | 0.0129 | 0.0153 |    |    |    |
| HM13:12.1:12.2 | -2.0453 | 0.1293 | 41 | 83 | 1.6429 | 0.1147 |    |    |    |
| AP DUT 2.1 | -5.5269 | 0.0039 | 78 | 8.55E-05 | 0.1851 | 0.0047 |    |    |    |
| PGRM C2 2.1 | 6.2150 | 500.20 | 41 | 30.458 | 24 | 8214.6 | 1.35E-05 |    |    |
| PSMG3 3.1 | 11.172 | 71116.75 | 77 | 597.70 | 846164 | 4.61E-06 |    |    |    |
| PSMA1 3.1 | -21.707 | 3.74E-10 | 75 | 8.66E-18 | 0.0161 | 0.0155 |    |    |    |
| AT METTL 15 | -29.333 | 1.82E-10 | 13 | 1.85E-20 | 0.179E-06 | 0.0003 |    |    |    |
| DNAJC 5.2 | 9.2648 | 10559.74 | 74 | 30.507 | 365505 | 0.0018 |    |    |    |
| USP4 7 | 16.056 | 939883 | 49 | 334.09 | 2.64E+09 | 0.021 |    |    |    |
| KLHL3 11 | 2.2593 | 9.5772 | 39 | 0.5486 | 167.18 | 0.1214 |    |    |    |
| STOM L1 8.2 | -9.3207 | 8.95E-05 | 4.10E-07 | 0.0195 | 0.0006 | 95 |    |    |    |
| C1RL 2 | -28.778 | 3.17E-13 | 4.26E-19 | 2.37E-07 | 3.03E-05 |    |    |    |    |
| USMG 5 | 35.874 | 3.8E+1 | 277808 | 5.20E+24 | 0.0008 | 31 |    |    |    |
| GGCX 2 | -9.1525 | 0.0001 | 4.89E-07 | 0.0229 | 0.0008 | 54 |    |    |    |
| MMAA 5 | -21.709 | 3.73E-10 | 5.03E-14 | 2.76E-06 | 1.80E-06 |    |    |    |    |
| PSEN2 11 | -23.509 | 6.16E-11 | 1.92E-20 | 0.1980 | 0.0352 | 98 |    |    |    |
| ME THNSL 9| 10 |    |    |    |    |    |    |    |    |
| EIF6 2.3 | 17.875 | 579849 | 83.734 | 4.02E+04 | 0.0091 | 8 |    |    |    |
| TST 1.2 | 20.134 | 5.55E+05 | 8768.0 | 3.51E+05 | 0.0003 | 58 |    |    |    |
| PILRB | 8.4   | -4.2710 | 0.0139 | 0.0003 | 0.5148 | 0.0203 |
|-------|-------|---------|--------|--------|--------|--------|
|       | 9     | 66      | 79     | 64     | 05     |

AA, Alternate acceptor site; AD, Alternate donor site; AP, Alternate promoter; AT, Alternate terminator; ES, Exon skip; ME, Mutually exclusive exons; RI, Retained intron.
Table 3. Univariate and multivariate Cox analysis for PM-ALL

| Clinical factors | Univariate | P-value | Multivariate | P-value |
|------------------|------------|---------|--------------|---------|
|                  | HR         |         | HR           |         |
|                  | 95%CI      |         | 95%CI        |         |
|                  | Lower      | Upper   | Lower        | Upper   |
| Age              | 1.36       | 0.61    | 3.02         | 0.44    | 1.513   | 0.566   | 4.047   | 0.409   |
| Gend             | 0.96       | 0.42    | 2.18         | 0.92    | 0.815   | 0.313   | 2.124   | 0.675   |
| Stage            | 2.88       | 1.81    | 4.57         | 0.00    | 0.778   | 0.154   | 3.927   | 0.762   |
| T                | 3.34       | 2.07    | 5.40         | 0.00    | 3.911   | 1.422   | 10.75   | 0.008   |
| M                | 6.33       | 2.74    | 14.6         | 0.00    | 1.237   | 0.185   | 8.273   | 0.827   |
| N                | 2.08       | 0.78    | 5.57         | 0.14    | 2.388   | 0.639   | 8.931   | 0.196   |
| Treat_type       | 1.22       | 0.55    | 2.71         | 0.60    | 0.863   | 0.359   | 2.076   | 0.743   |
| Risk Score       | 1.01       | 1.00    | 1.02         | 0.00    | 1.012   | 1.003   | 1.020   | 0.007   |

PM, Prognostic model.
| Gene Name   | HR      | HR.95L   | HR.95H   | P-value       |
|------------|---------|----------|----------|---------------|
| KHSRP      | 1.106488 | 1.059608 | 1.155441 | 4.62E-06     |
| SRSF7      | 1.251807 | 1.124576 | 1.393432 | 4.01E-05     |
| SRSF3      | 1.178449 | 1.084695 | 1.280308 | 0.000104     |
| ILF3       | 1.134072 | 1.060237 | 1.213049 | 0.000249     |
| HNRNPA2B1  | 1.058154 | 1.024804 | 1.092589 | 0.000541     |
| HNRNPH1    | 1.161704 | 1.063532 | 1.268938 | 0.000877     |
| PNN        | 1.320241 | 1.118295 | 1.558656 | 0.001038     |
| KHDRBS1    | 1.082557 | 1.02699  | 1.141131 | 0.003172     |
| DDX39A     | 1.061124 | 1.019533 | 1.104411 | 0.003635     |
| CLASRP     | 1.348143 | 1.101165 | 1.650515 | 0.003812     |
| HNRNPR     | 1.232519 | 1.069703 | 1.420117 | 0.003827     |
| HNRNPA1    | 1.02519  | 1.007966 | 1.042708 | 0.004005     |
| DDX50      | 1.242495 | 1.070727 | 1.441819 | 0.004234     |
| SRRT       | 1.150545 | 1.0451   | 1.266629 | 0.004244     |
| DHX9       | 1.175994 | 1.047023 | 1.32085  | 0.006233     |
| SRSF2      | 1.078669 | 1.020899 | 1.139708 | 0.007008     |
| SNRPD1     | 1.197308 | 1.049665 | 1.365718 | 0.007322     |
| SNRPE      | 1.056598 | 1.014811 | 1.100106 | 0.007493     |
| ZC3H11A    | 1.27433  | 1.062298 | 1.528683 | 0.009032     |
| ILF2       | 1.021163 | 1.005041 | 1.037543 | 0.009899     |
Figure 1

Overview of AS events in ACC. A: Number of AS events and corresponding genes; B: Number of survival-associated AS events and involved genes; C: UpSet plot in ACC, showing the interactions among the seven types of survival-associated AS events. One gene could undergo more than one type of AS events. AS: Alternative splicing; ACC, Adrenocortical carcinoma; AA, Alternate acceptor site; AD, Alternate donor site; AP, Alternate promoter; AT, Alternate terminator; ES, Exon skip; ME, Mutually exclusive exons; RI, Retained intron.
**Figure 2**

Top 20 (if available) most significant survival associated AS events in ACC. A: Volcano map of AS events associated with patient survival. B-G: The top 20 survival-associated AS events for AA, AD, AP, AT, ES and RI splicing types. H: The whole survival-related AS events for ME splicing type.

**Figure 3**

The PPI network analysis of genes with survival-related AS events in ACC. A: The PPI network; B: The hub nodes in the PPI network. The top 30 hub nodes are displayed. PPI: Protein-protein interaction.
### A

| term ID       | Cellular component                                      | FDR      | Gene count |
|--------------|---------------------------------------------------------|----------|------------|
| GO:0043231   | intracellular membrane-bounded organelle                | 2.89E-08 | 178        |
| GO:0005739   | mitochondrion                                           | 9.49E-08 | 50         |
| GO:0043227   | membrane-bounded organelle                              | 1.47E-06 | 182        |
| GO:0044422   | organelle part                                          | 1.47E-06 | 157        |
| GO:0044446   | intracellular organelle part                            | 1.47E-06 | 154        |
| GO:0044428   | nuclear part                                            | 2.04E-06 | 92         |
| GO:0070013   | intracellular organelle lumen                           | 2.04E-06 | 104        |
| GO:0005634   | nucleus                                                 | 4.07E-06 | 126        |
| GO:0044429   | mitochondrial part                                      | 9.87E-06 | 34         |
| GO:0005654   | nucleoplasm                                             | 1.50E-05 | 75         |
| GO:0032991   | protein-containing complex                              | 1.50E-05 | 95         |
| GO:0031981   | nuclear lumen                                           | 2.44E-05 | 83         |
| GO:0043229   | intracellular organelle                                 | 4.09E-05 | 186        |
| GO:0044424   | intracellular part                                      | 4.27E-05 | 205        |
| GO:0005730   | nucleolus                                               | 6.93E-05 | 30         |
| GO:0043226   | organelle                                               | 0.00011  | 187        |
| GO:0005759   | mitochondrial matrix                                    | 0.00074  | 18         |
| GO:0044444   | cytoplasmian part                                       | 0.00074  | 148        |
| GO:0031967   | organelle envelope                                      | 0.0011   | 31         |
| GO:0005740   | mitochondrial envelope                                  | 0.0029   | 22         |
| GO:0000127   | transcription factor TFIIC complex                      | 0.0031   | 3          |
| GO:0005829   | cytosol                                                 | 0.0038   | 87         |
| GO:0044451   | nucleoplasm part                                        | 0.0088   | 27         |
| GO:0005737   | cytoplasm                                              | 0.0127   | 164        |
| GO:0005743   | mitochondrial inner membrane                            | 0.0132   | 15         |
| GO:1902494   | catalytic complex                                       | 0.0144   | 30         |
| GO:190234    | transferase complex                                     | 0.0153   | 20         |
| GO:0044798   | nuclear transcription factor complex                    | 0.0229   | 8          |
| GO:0044439   | peroxisomal part                                        | 0.0256   | 6          |
| GO:0005968   | Rab-protein geranylgeranyltransferase complex            | 0.0329   | 2          |
| GO:0031966   | mitochondrial membrane                                  | 0.0343   | 18         |
| GO:0044464   | cell part                                               | 0.0364   | 217        |
| GO:0005753   | mitochondrial proton-transporting ATP synthase complex  | 0.0414   | 3          |

### B

| term ID       | Biological process                                      | FDR      | Gene count |
|--------------|---------------------------------------------------------|----------|------------|
| GO:0008152   | metabolic process                                       | 0.00039  | 159        |
| GO:0044237   | cellular metabolic process                              | 0.00039  | 149        |
| GO:0006807   | nitrogen compound metabolic process                     | 0.0033   | 139        |
| GO:0046907   | intracellular transport                                 | 0.0037   | 38         |
| GO:0071704   | organic substance metabolic process                     | 0.0037   | 148        |
| GO:0044238   | primary metabolic process                               | 0.0083   | 142        |
| GO:0044248   | cellular catabolic process                              | 0.0083   | 41         |
| GO:0034641   | cellular nitrogen compound metabolic process            | 0.0169   | 92         |
| GO:1901564   | organonitrogen compound metabolic process               | 0.0169   | 94         |
| GO:0034622   | cellular protein-containing complex assembly            | 0.0172   | 25         |
| GO:0065003   | protein-containing complex assembly                     | 0.0194   | 37         |
| GO:0070031   | peroxisome organization                                 | 0.0237   | 7          |
| GO:0042791   | 5S class rRNA transcription by RNA polymerase III      | 0.0331   | 3          |
| GO:0042797   | tRNA transcription by RNA polymerase III               | 0.0331   | 3          |
| GO:0051726   | regulation of cell cycle                                | 0.0395   | 29         |
| GO:1901575   | organic substance catabolic process                     | 0.0395   | 37         |
Figure 4

GO analysis of genes with survival-related AS events in ACC. A: Cellular component; B: Biological process. GO: Gene ontology.

Figure 5

Lasso regression analysis for seven splicing types and all splicing types. A-H: AA, AD, AP, AT, ES, ME, RI, and all splicing types.
Figure 6

Kaplan-Meier and ROC curves of the eight prognostic models for ACC. A-H: Kaplan-Meier curves for each prognostic model. Red line indicates high-risk group whereas green line indicates low-risk group. I: The ROC curves of each predictive model.
Figure 7

Correlation network of survival-associated SFs and AS events in ACC. Survival-associated SFs (Triangles) were positively (red lines) or negatively (green lines) associated with the PSI value of adverse prognosis AS events (red dots) or favorable prognosis AS events (green dots).