Abstract. Cholangiocarcinoma (CCA) is a type of malignant tumor that originates in the mucosal epithelial cells of the biliary system. It is a highly aggressive cancer that progresses rapidly, has low surgical resection rates and a high recurrence. At present, no prognostic molecular biomarker for CCA has been identified. However, CCA progression is affected by mRNA precursors that modify gene expression levels and protein structures through alternative splicing (AS) events, which create molecular indicators that may potentially be used to predict CCA outcomes. The present study aimed to construct a model to predict CCA prognosis based on AS events. Using prognostic data available from The Cancer Genome Atlas, including the percent spliced index of AS events obtained from TCGASpliceSeq in 32 CCA cases, univariate and multivariate Cox regression analyses were performed to assess the associations between AS events and the overall survival (OS) rates of patients with CCA. Additional multivariate Cox regression analyses were used to identify AS events that were significantly associated with prognosis, which were used to construct a prediction model with a prognostic index (PI). A receiver operating characteristic (ROC) curve was used to determine the predictive value of the PI, and Pearson's correlation analysis was used to determine the association between OS-related AS events and splicing factors. A total of 38,804 AS events were identified in 9,673 CCA genes, among which univariate Cox regression analysis identified 1,639 AS events associated with OS (P<0.05); multivariate Cox regression analysis narrowed this list to 23 CCA AS events (P<0.001). The final PI model was constructed to predict the survival of patients with CCA; the ROC curve demonstrated that it had a high predictive power for CCA prognosis, with a highest area under the curve of 0.986. Correlations between 23 OS-related AS events and splicing factors were also noted, and may thus, these AS events may be used to improve predictions of OS. In conclusion, AS events exhibited potential for predicting the prognosis of patients with CCA, and thus, the effects of AS events in CCA required further examination.

Introduction

Cholangiocarcinoma (CCA) is a malignant tumor that originates in the mucosal epithelial cells of the biliary system. With a high degree of malignancy, rapid progression, low surgical resection rates and high recurrence rates, CCA prognoses are unsatisfactory (1-4). Recent studies of the molecular pathological mechanisms of CCA have demonstrated that tumor formation, growth, invasion, metastasis and other processes in CCA are regulated by a variety of molecules and signal transduction pathways, such as the aPKC-ι/P-Sp1/Snail signaling pathway and the Merlin/YAP/c-Myc/mTOR signaling pathway (5,6). This information has assisted early diagnosis, prognosis predictions and drug development. However, due to the complexity of CCA development, there are currently no specific biomarkers that meet clinical standards (7-10). Therefore, there is an urgent need to identify suitable biomolecular markers that can screen for CCA and assist in prognostic evaluations.

Eukaryotic mRNA precursors are known to remove certain unused fragments during the maturation process of mRNAs, allowing the remaining fragments to be rejoined. The fragments that are removed or left in place vary in their
effects, depending on the cell type and status. Thus, a gene can encode multiple proteins in a process called alternative splicing (AS) (11,12). Previous studies have demonstrated that AS events occur ubiquitously in eukaryotes, with >95% of genes undergoing AS events (13-15). However, AS events are more common in cancer cells and affect cancer development and treatment resistance (16-24). In addition, AS events in tumor cells may be used as molecular markers that can differentially diagnose tumor types and predict prognosis (25-28), as well as serve as potential targets for cancer treatment (29-33).

Application of AS events as a means diagnosing and predicting prognosis has been demonstrated in in prostate adenocarcinoma, uteri corpus endometrial carcinoma and colorectal cancer (34-36).

CCA involves a high frequency of gene mutations and abnormal epigenetic changes, including DNA methylation, and histone and RNA modifications (37), all of which may lead to the occurrence of AS events (19,38-47). AS events modify gene expression and influence protein structure by changing coding regions, thus regulating certain biological processes. For example, the widely reported tumor suppressor genes tumor protein p53, AT-rich interaction domain 1A, PTEN and PI3K, as well as the proto-oncogenes NOTCH1 and MET proto-oncogene, receptor tyrosine kinase, cause variations in gene function through AS events, thus affecting cancer development (30). Similarly, the apoptosis-related genes BCL-X and modulator of VRAC current 1 have been demonstrated to serve opposite functions in promoting and resisting apoptosis due to the occurrence of AS events (48-51). AS can also alter the amino acid sequence of a protein, which may destroy the target for certain antitumor drugs and result in drug resistance (20,30,52). AS events therefore serve a unique role in the diagnosis and treatment of tumors.

Previous studies on the association between AS events and tumors have focused on the single-gene level, and few large-scale data mining studies based on high-throughput sequencing exist (53-55). The Cancer Genome Atlas (TCGA) database (portal.gdc.cancer.gov) contains high-throughput sequencing data and comprehensive clinical information from a large number of cancer samples. Previous studies using TCGA data have reported that AS events may be used as prognostic indicators for lung (56), ovarian (57), bladder (58) cancer and gastrointestinal pan-adenocarcinomas (59,60). However, the prognostic value of AS events has not yet been reported for CCA. In the present study, the overall survival (OS)-related AS events in CCA were systemically evaluated. These findings may facilitate development of novel genomic models for clinical cancer management, and construction of novel models based on the prognostic index (PI) to predict CCA survival.

Materials and methods

Data collection. The percent spliced index (PSI) values for CCA AS events were downloaded from the TCGASpliceSeq database (http://bioinformatics.mdanderson.org/TCGASpliceSeq) (61). PSI is the ratio of normalized read counts indicating inclusion of a transcript element to the total normalized read counts for that event (i.e., both inclusion and exclusion read counts). PSI values range from 0 to 1, indicating the likelihood of the existence of an exon. Information related to seven types of AS events: exon skip (ES), mutually exclusive exons (ME), retained intron (RI), alternate promoter (AP), alternate terminator (AT), alternate donor (AD) site and alternate acceptor (AA) site events, was included in the analysis. Corresponding clinical information and gene expression levels of the samples were obtained from TCGA database. The database included data from 32 patients with CCA with complete clinical information and total survival time >90 days. Of the 32 patients with CCA, 13 were male and 19 were female. The age of the patients was 29-82, and the median age was 66.5, with 27 patients older than 50 and 5 patients younger than 50.

Association between AS events and survival. R software (R version 3.4.2) (62) was used to perform univariate Cox regression analysis to investigate the PSI prognostic values from the seven types of AS events and the differentially expressed genes in the 32 CCA cases. AS events with P<0.05 were selected for further analysis. The event-dependent survival curve for the top three AS events was plotted (collating P-values from low to high).

Systematic review. To confirm that the identified AS events were CCA-specific, all prognostic AS events from different types of cancer were extracted from published articles on PubMed (https://www.ncbi.nlm.nih.gov/), Wiley Online Library (https://onlinelibrary.wiley.com/), EBSCO (https://www.ebsco.com/), Web of Science (https://www.webofknowledge.com/), and Google Scholar (https://scholar.google.com/) on or prior to 1st May 2019 for comparison. The key words were as follows: 'cancer OR carcinoma OR adenocarcinoma OR tumour OR tumor OR malignanc* OR neoplas*' AND 'prognostic OR prognosis OR predict*' AND 'alternative splicing' OR AS'. The studies which were included needed to meet the following criteria: i) Studies describing cancer prognosis with a predicted model constructed using prognostic AS events; and ii) the prognostic AS events could be extracted from the studies. All the included studies were assessed using the bias assessment for studies of diagnostic accuracy (QUADAS) guidelines (63) and had a QUADAS score of ≥7. The following data were extracted from the included studies: First author's name, year of publication and prognostic AS events.

AS event interaction analysis and gene network construction. The interactions of the seven types of AS events were analyzed using the UpSetR package for R (64), and an UpSet plot was drawn. The network was constructed using the Reactome FI plug-in (65) for Cytoscape 3.6.0 (66), with AS events identified using univariate Cox regression analysis (P<0.01). Genes with AS events were processed using the Kyoto Encyclopedia of Genes and Genomes (KEGG) and Gene Ontology (GO) gene enrichment analyses. Due to the occurrence of a large number of significant GO pathways, only the top five pathways per group were presented using the ggplot2 package for R (67).

PI construction and prognostic value assessment. To assess the association between AS events and OS, univariate Cox regression was carried out with a significance threshold of P<0.05. The top ten most promising AS events, based on
their prognostic value, AS events selected for each splicing type. Multivariate Cox regression analysis was performed, splicing events with P<0.05 were selected as PIs. The model was established by grouping together all splicing events of the seven splicing event types. The samples were divided into two groups according to the median PSI value of the splicing events. The formula used to calculate the PI was as follows:

$$\text{Risk score} = \sum_{i}^n \text{PSI}_i \times \beta_i$$

The clinical prognostic value of the PI was assessed using Kaplan-Meier (K-M) curve analysis and time-dependent receiver operator characteristic (ROC) curve analysis. The time-dependent ROC curve was drawn using the survival ROC package for R (68).

**Experimental validation of AS events in clinical CCA tissue samples.** In addition to the aforementioned analyses, the existence of the AS events predicted by the TCGASpliceSeq database was verified using PCR analyses with six samples of CCA tumor tissues separately collected from a 37-year-old male patient and a 38-year-old female patient during surgery at the Pathology Department of the First Affiliated Hospital of Guangxi Medical University (Nanning, China) between February 2017 and October 2017. The study was approved by the Ethics Committee of the First Affiliated Hospital of Guangxi Medical University, and written informed consent was obtained from all patients. Total RNA was extracted using the AxyPrep Multisource Total RNA Miniprep kit (Axygen; Corning, Inc.), quantified using a NanoDrop 2000 (Thermo Fisher Scientific, Inc.) and 500 ng total RNA was reverse-transcribed into cDNA using MiScript® II RT SuperMix kit (Vazyme). AS events in phytanoyl-CoA 2-hydroxylase (PHYH)_100582_ES and transferrin receptor 2 (TFR2)_80979_ES were selected for verification. The mean PSI of PHYH_100582_ES in TCGASpliceSeq database was 0.78, and ES was present in exons 7 and 8. The forward primer (primer 1) was designed based on exon 6, and the sequence was 5'-GATACTGCACTC TCCCCGAG-3'. The reverse primer (primer 2) was designed based on exon 9, and the primer sequence was 5'-GACCCAG ATCCGTTGATGAC-3'. The PCR system contained 10 µl 2X PCR Master mix (Thermo Fisher Scientific, Inc), 1 µl each of the forward and reverse primers (10 µM), 1 µl cDNA and 7 µl nuclease-free water (total volume, 20 µl). The thermocycling conditions were as follows: 95°C for 3 min, followed by 35 cycles of 95°C for 30 sec, 60°C for 30 sec and 72°C for 1 min. PCR products were subjected to electrophoresis on 2% agarose gels. The mean PSI of TFR2_80979_ES in the TCGASpliceSeq database was 1, and ES was present in exon 10. The forward primer (primer 3) was designed based on exon 9, and the primer sequence was 5'-CAGCCCATCAGTGA CACAT-3'. The reverse primer (primer 4) was designed based on exon 11, and the primer sequence was 5'-TTGTGACC ACTAGCCGCAG-3'. The PCR system and thermocycling conditions were as mentioned above.

**Correlation analysis between splicing factors and prognosis-related AS events.** A splicing factor that serves as a splicing activator when bound to an intronic enhancer element may function as a repressor when bound to its splicing element in the context of an exon (69). Therefore, the correlations between splicing factors and the prognosis-related AS events were investigated. Splicing factor information was obtained from the SpliceAid 2 database (http://193.206.120.249/splicing_tissue. html) (70). Univariate Cox regression analysis was performed on the splicing events and splicing factors, followed by Pearson's correlation analysis between the significant splicing events, splicing factors and the OS-related AS events identified by multivariate Cox analysis. The results were visualized using Cytoscape version 3.4.0 (66).

**Results**

**CCA AS events.** A total of 38,804 AS events were obtained from 9,673 genes in 32 CCA cases from the TCGA SpliceSeq dataset. The numbers of each type of AS event and the corresponding genes are presented in Fig. 1. The results demonstrated that a single gene may undergo multiple AS events simultaneously. ES was the most frequent AS event in this dataset.

**AS events associated with CCA OS rates.** Univariate Cox analysis revealed that 1,639 AS events were associated with CCA OS rates (P<0.05) and that certain genes had undergone multiple AS events simultaneously. Visualization of the intersecting sets was performed using UpSet plotting software (Fig. 2), and ≤3 AS events were identified to be associated with OS rates within the same gene. KEGG pathway enrichment analysis revealed that the genes corresponding to these AS events were primarily enriched in ‘biosynthesis of antibiotics’, ‘axon guidance’, ‘pancreatic cancer’, ‘RAP1 signaling pathway’ and ‘SNARE interactions in vesicular transport’ (Fig. 3A). GO gene enrichment analysis demonstrated that the genes with OS-related AS events corresponded to ‘protein transport’, ‘cell-cell adhesion’, ‘apoptotic process’, ‘protein localization to the nucleus’ and ‘positive regulation of transcription from RNA polymerase II promoters’ (Fig. 3B). Using a network map of these genes, MYC associated factor X (MAX), mitogen-activated protein kinase 11 (MAPK11), γ-amino butyric acid type A receptor-associated protein-like 1 (GABARAPL1), checkpoint kinase 1 (CHEK1), X-linked...
inhibitor of apoptosis (XIAP) and forkhead box A1 (FOXA1) exhibited the highest degrees of connectivity and were thus considered critical positions of the network (Fig. 4).

**OS-related AS events are CCA-specific.** Comparisons with other types of cancer demonstrated that ME-GTF2H3-306194 occurred in papillary thyroid cancer and CCA, whereas the other identified AS events were CCA-specific (Table I).

**AS events that may be used as CCA prognostic indicators.** The three most statistically significant AS events were selected, and their gene expression levels were used to calculate their prognostic values in CCA (Fig. 5). K-M analysis revealed that these prognostic signatures significantly separated patients with distinct prognoses. To assess the ability of these splicing events to predict CCA prognosis, a time-dependent ROC curve was established. The results of the ROC curve suggested that a single indicator was not enough to predict patient prognosis, as the area under the curve (AUC) values were low for the AS events and gene expression values (Fig. 5). Therefore, ten AS events with the most significant prognostic values in each splicing type were selected to construct the PIs (Table II). The results suggested that these PIs had higher predictive values compared with single indicators (Fig. 6). The AUCs for each of the seven subtypes of AS were as follows: AA, 0.901; AD, 0.858; AP, 0.986; AT, 0.839; ES, 0.939; ME, 0.779; and RI, 0.859. In addition, the ten most significant AS events from all types combined were selected to construct a final prognostic model; the AUC was 0.984 (Fig. 6). It is worth noting that the predictive efficacy of the prognostic model with seven combined splicing events (0.984) was slightly lower than that obtained with AP alone (0.986).

**Confirmation of PHYH_100582_ES and TFR2_80979_ES by clinical samples.** Towards PHYH_100582_ES, the PCR products were predicted to comprise two bands; the band without ES was predicted to be 414 bp in length, whereas the band size following ES was predicted to be 144 bp in length (Fig. 7A). If the TFR2_80979_ES event did not occur, the PCR product was predicted to be 238 bp; following the ES event, the PCR product size was predicted to be 140 bp (Fig. 7B). Since the PSI value of this ES event was 1, ES events occurred in all TFR2 mRNAs; if the TCGASpliceSeq database was accurate, the PCR product was predicted to be a single band. The results of PHYH_100582_ES and TFR2_80979_ES were consistent with those predicted by the TCGASpliceSeq database. The PCR products of PHYH_100582_ES were 414 and 144 bp (Fig. 7C), suggesting that, in CCA tissues, a proportion of PHYH mRNA had undergone an ES event. The PSI of TFR2_80979_ES in the TCGASpliceSeq database was 1, indicating that ES events occurred in all TFR2 mRNAs. The electrophoresis result demonstrated that the product of TFR2_80979_ES was a single band of 140 bp; no band of 238 bp was observed (Fig. 7D), suggesting that ES events occurred in all TFR2 mRNA. These results were consistent with those suggested by the RNA sequencing data from TCGASpliceSeq.

**Correlation between splicing factors and prognosis-related AS events.** A total of 11 AS events and corresponding splicing...
factors significantly related to CCA OS rates (P<0.05) were identified. Multivariate Cox analysis identified 23 prognosis-related AS events (Table III; P<0.001); 17 of these exhibited positive correlations with OS, whereas 6 exhibited negative correlations with OS. Of these 23 AS events, 15 were correlated with a group of splicing factors, the expression of which was directly associated with AS events (|r|>0.3; P<0.05). AS events are primarily regulated by splicing factors, which often bind to pre-mRNAs and regulate RNA splicing by influencing exon selection and splicing site. Therefore, the associations between survival-related AS events and splicing factors were determined (Fig. 8).

Discussion

Using data mined from TCGASpliceSeq and in silico approaches, the present study identified that a number of AS events are closely associated with survival in CCA. The present study is the first to report this type of result. The results of the present study also demonstrated that AS events may be used to construct a PI model that effectively determines CCA prognosis.

The incidence of CCA has increased in recent years. The prognosis of advanced CCA is poor, with an extremely low 5-year survival rate (7). The prognosis depends on the synergistic effects of various factors; however, no clear and effective molecular markers have been identified for CCA diagnosis and treatment. Carcinoembryonic antigens (CEAs) and carbohydrate antigen (CA) 199, CA 125, CA 50 and CA 242 are currently used as CCA tumor markers, but these biomarkers have disadvantages in clinical application. For example, the majority of studies that examined CA 199 as a biomarker for the detection of CCA have reported its suboptimal accuracy, with wide variation in reported sensitivity (38-93%) and specificity (67-98%) (71). CA 125 is upregulated in 65% of patients with CCA and is of value in predicting survival (72);
Figure 4. A network diagram of overall survival-related alternative splicing events in cholangiocarcinoma. Genes corresponding to alternative splicing events were identified and a network map was constructed using Cytoscape software. The genes with the highest degree of connectivity are marked in red.

Figure 5. Kaplan-Meier survival and ROC curves of alternative splicing events and gene expression of the top three factors identified using univariate Cox analysis. Blue, low-risk group; red, high-risk group. (A-C) Kaplan-Meier curves of alternative splicing events. (A) FRMD8_16850_ES (HR, 0.736; 95% CI, 0.627-0.824; P<0.001). (B) MBLAC2_72765_AT (HR, 1.186; 95% CI, 1.084-1.298; P<0.001). (C) MBLAC2_72766_AT (HR, 0.843; 95% CI, 0.771-0.923; P<0.001). (D-F) Kaplan-Meier curves of gene expression levels. (D) CDADC1 (HR, 1060.208; 95% CI, 15.259-73662.548; P=0.001). (E) ABCA4 (HR, 13.302; 95% CI, 2.596-68.156; P=0.002). (F) C19orf12 (HR, 0.0004; 95%CI, 0.000001-0.081; P=0.004). (G) ROC curves of alternative splicing events and gene expression levels: FRMD8_16850_ES, MBLAC2_72765_AT, MBLAC2_72766_AT, CDADC1, ABCA4 and C19orf12. AA, alternate acceptor; AD, alternate donor; AP, alternate promoter; AT, alternate terminator; ROC, receiver operating characteristic; AUC, area under the curve; ES, exon skip; ME, mutually exclusive exon; RI, retained intron; HR, Hazard ratio; CI, confidence interval; FRMD, FERM domain-containing 8; MBLAC2, metallo-β-lactamase domain-containing 2; CDADC1, cytidine and DCMP deaminase domain-containing 1; ABCA4, ATP-binding cassette subfamily A member 4; C19orf12, chromosome 19 open reading frame 12.
however, there are no further studies on CA 125 in CCA. CA 50 exhibits cross-antigenicity with CA 199, and elevated serum CA 50 levels are commonly used to diagnose or prognose pancreatic and colorectal cancers (73). However, Shan et al. (74) demonstrated that elevated serum CA 50 levels are not always associated with the expression of CA 50 in cancer tissues. Despite the clinical utility of CA 242, it is not sufficiently effective in the early detection of cancer, since elevated classical tumor biomarker levels indicate the presence of a significant number of cancer cells (75).

At the genetic level, a number of genes are abnormally expressed in CCA. For example, the abnormal expression of genes such as transforming growth factor β1, SMAD4, c-MET, matrix metallopeptidase 7, vascular endothelial growth factor (VEGF)-A, VEGF-B, VEGF-C and VEGF-D are associated with poor prognosis (76-79). Mutations in genes such as human epidermal growth factor receptor 2, TP53, KRAS, cytosolic NADP-dependent isocitrate dehydrogenase and mitochondrial NADP-dependent isocitrate dehydrogenase can also affect the prognosis of patients with CCA, but the prognostic value of
| Author, year | Type | PMID | Alternative splicing events | (Refs.) |
|-------------|------|------|-----------------------------|--------|
| Lin et al., 2019 | Papillary thyroid cancer | 30986203 | AA-SHP1-8302, AA-CASK-88861, AD-FBXL19-36205, AD-SAT2-39030, AD-TRO-89255, AD-CSTF2-89611, AP-ZC3H11A-9456, AP-STK32C-13483, AP-GRB2-43439, AP-CRTC1-48500, AP-ERCC1-50440, AP-ESR1-78161, AT-MAGI3-4271, AT-TPM1-30982, AT-ATP8B3-46544, AT-MAST1-47878, AT-SPAG16-57327, AT-CBWD5-86498, AT-OLFM1-88103, ME-NSMF-193275, ME-GTF2H3-306194, RI-C11orf49-15609, RI-ZNF276-38138, RI-USP36-43917, RI-NUDT18-82937, RI-NAPRT1-85430 | (95) |
| Gao et al., 2019 | Uteri corpus endometrial carcinoma | 30640723 | AP-BDNF-14763, AP-DDX58-86057, AP-FYTTD1-28538, AP-GNAL-44643, AP-GPATCH2L-28538, AP-HUS1-79610, AP-MAP4-64545, AT-IPO11-72190, AT-ZFAND4-11368, ES-CKMT2-72660, ES-CMC2-37735, ES-FBXL12-47421, ES-NDUFB1-28987, ES-PSMD12-43112, ES-ZNF528-51457, RI-AP3M2-83565, RI-DNASE1L3-65424, RI-GABARAP-38871 | (34) |
| Huang et al., 2018 | Prostate adenocarcinoma | 30221674 | ES-TCEB2-33303, AD-ABHD17A-46558, AP-FKBP2-16602, AD-YPEL3-36074, ES-STXBP2-47124, AT-PTGDS-88235, AT-HMGA2-22879, ES-NHLRC3-25701 | (36) |
| He et al., 2018 | Bladder urothelial carcinoma | 30048970 | AA-B4GALT2-1228, ES-TMTC2-9217, ES-TIMM9-11224, ES-APOBEC3D-26508, AP-TPD52-35921, ES-MICU1-4164, ES-DDX11-8115, ES-SMC6-22132 | (94) |
| Zhang et al., 2019 | Breast carcinoma | 30984247 | AA-CARM1-47598, AA-ZBTR25-27884, AA-GPBP1-72126, AA-ZNRF1-37578, AA-DDX41-74796, AA-CTDSPL1-57478, AD-OS9-22701, AD-HN1-43371, AD-THTPA-26757, AD-NTM1-87866, AD-MGME1-58753, AD-SEC31A-69735, AP-SEC22A-66462, AP-ALG3-67851, AP-PACS2-29630, AP-ECE2-67857, AP-HSP90AB1-76378, AT-MAGT1-89535, AT-RNASEH2B-25898, AT-SIN3B-48214, AT-SARNP-22252, AT-ZN5F6-48822, AT-STOX2-71298, AT-NIPAL3-1110, ES-NDUFA12-23737, ES-UBR4-880, ES-COPS3-94686, ES-ACE1-70753, ES-CCNL-69628, ES-RPAP1-30095, ES-RPAP1-30095, ES-RPAP1-30095, RI-RBM48-80441, RI-RBM6-64936, RI-RPAP1-30095, RI-METTL17-26746, RI-POMGNT1-2787, RI-TRABD-62792, RI-WDR6-64794, RI-FASK-82335, RI-NAA38-81579 | (96) |
| Lin et al., 2018 | Esophageal adenocarcinoma | 30131306 | AA-U2AF1L4-49280, AA-TICRR-32428, AA-RSRC2-24968, AA-PREPL-53439, AA-PPL2-61247, AA-FAM135A-76637, AA-CDV3-66839, AA-ABC57-89517, AA-ZNF384-19927, AD-RPP14-65344, AD-PQBP1-89028, AD-MFSD11-43690, AD-COX6A5-84682, AP-ZNF623-85469, AP-KIAA0513-37876, AP-FAM19A5-62732, AP-ALDH6A1-28367, AT-TRIM4-80864, AT-RNASEH2B-25927, AT-RNASEH2B-25926, AT-MCPH1-82574, AT-MAPK1-65732, AT-AHI1-77886, ES-TNC-87345, ES-FML-31651, ES-NFATC5-91080, ES-MYLF6-22384, ES-MRPL43-12878, ES-IREF-117161, ME-SDR39U1-27012, ME-CLC1L2-70138, ME-CMC2-37707, RI-ZNF131-71926, RI-SLC52A3-58464, RI-PPARGC1B-74501, RI-PCGFI-68404, RI-MDK-15570, RI-MAF-37687, RI-FAM1C-88504 | (60) |
| Lin et al., 2018 | Stomach adenocarcinoma | 30131306 | AA-RPLP0-24277, AA-NAT6-64990, AA-MRV11-14373, AA-LM07-26065, AA-BDKRB2-29192, AD-YIPF2-47605, AD-SPHK2-20793, AD-SENP1-21411, AD-PGAP2-14004, AD-NFATC1-46241, AD-CCDC51-64635, AP-RCAN1-60494, AP-PLC1D1-64009, AP-LTP1B1-53719, AP-FAM56B-75537, AP-ABL2-9010, AT-ZFNY8-6094, AT-ZFYVE28-68559, AT-STEAP4-80362, AT-STEAP4-80361, AT-KIF1B-602, AT-KIF1B-601, AT-CXCL12-11344, AT-CLDN11-6767, AT-ABC58-78909, ES-UBXN1-11263, ES-TMEM230-58637, ES-SRSF3-75985, ES-SORBS1-12641, ES-P4HA2-73263, ES-CREM-11245, ME-NAB2-12559, ME-KDM6A-98323, ME-FYN-77273, ME-CCDC53-106010, RI-TREX1-64682, RI-SRFF7-53276, RI-RPS15-46490, RI-LDHA-14642, RI-BICD2-86883, RI-AL52C-64462 | (60) |
these genes remains controversial and has not been applied clinically (72). Due to the complexity of cancer, diagnosis or prognosis based on a single molecule is limited. The advent of high-throughput sequencing technologies allows us to address this issue by identifying more genomic abnormalities related to CCA.

Using the TCGASpliceSeq CCA data, 38,804 AS events were identified in 9,673 CCA genes, some of which exhibited multiple AS events occurring simultaneously. AS events are widespread in CCA and may be related to its occurrence and development. For instance, the first AS event reported to be associated with CCA prognosis was exon 2 skipping of the trefoil factor 2 (\textit{TFF2}) gene (80). \textit{TFF2} is highly expressed in a variety of tumors, including CCA. Exon 2 skipping AS events lead to the loss of exon 2, resulting in a decrease in wild-type \textit{TFF2} proteins. A high proportion of this AS event in \textit{TFF2} is associated with improved prognosis. Another example is \textit{P53}, as alterations in the N-terminus of this protein by AS has been demonstrated to worsen the prognosis in patients with CCA (81).

The present study identified a large number of CCA AS events, 1,639 of which were correlated with patient prognosis (\(P<0.05\)). Bioinformatics analyses of all genes with significant prognostic values identified the following genes to be located at the center of the gene network: \textit{MAX}, \textit{MAPK11}, \textit{GABARAPL1}, \textit{CHEK1}, \textit{XIAP} and \textit{FOXA1}. These genes have been broadly reported to be involved in cell cycle regulation, autophagy, proliferation, apoptosis and have been previously demonstrated to be associated with cancer (82-87). Tumor-specific AS events have been intensively studied by investigating the differentially alternatively spliced genes between tumor and adjacent normal tissues (88) in certain cancer types, such as breast (89), lung (56) and ovarian (57) cancer. Yosudjai \textit{et al} (90) also demonstrated that aberrant AS of anterior gradient protein 2 homolog promoted cell proliferation, migration, invasion and adhesive potential in CCA; however, there has been a lack of research on its association with patient prognoses. Among the genes identified above, \textit{MAX} forms a heterodimer with the proto-oncogene \textit{MYC}, which binds to DNA to regulate the transcription of multiple genes, thus regulating cell
proliferation, differentiation and apoptosis (82). The MAPKII gene encodes a serine/threonine kinase that is widely involved in various cellular signal transduction pathways, such as p38 the MAPK signaling pathway by phosphorylating multiple target proteins (87). CHEK1 is a cell cycle monitoring-related protein, abnormalities of which result in DNA damage and the bypassing of the cell cycle checkpoints (91). FOXA1 is a transcription factor, the aberrant expression of which is closely associated with hepatocellular carcinoma (84); however, its association with CCA has not been previously reported.

The current study attempted to predict the prognosis of patients with CCA using the three most significant AS events and genes. The results were not satisfactory (the maximum AUC was 0.747; data not shown), likely due to individual differences between patients. Similarly, no single factor was able to accurately predict patient prognosis. Therefore, ten AS events with the most significant prognostic values, as calculated by multivariate Cox regression analysis for each type of AS event, were selected to construct a PI. The prognostic models based on the combined seven types of splicing events demonstrated comparable predictive efficacy to the model that used only the AP splicing events. However, the combined model was more successful at predicting patient prognosis compared with models based on any other single type of splicing event.

The results of the present study were compared with those reported in previous studies. All AS events identified in the present study were uniquely associated with CCA, with the exception of ME-GTF2H3-306194, which has been previously identified to have prognostic value in papillary thyroid cancer.
Table III. Predictive factors identified for cholangiocarcinoma using multivariate logistic regression.

| Splicing type | Gene symbol | AS ID |
|---------------|-------------|-------|
| AA            | ST3GAL4     | 19399 |
| AA            | TECR        | 47998 |
| AA            | TGIF1       | 44506 |
| AD            | SYNGR1      | 62301 |
| AD            | ZHX3        | 59398 |
| AP            | C12orf65    | 25058 |
| AP            | CHMP3       | 54439 |
| AP            | SH3KBP1     | 88640 |
| AT            | MBLAC2      | 72765 |
| AT            | TGF8B       | 28531 |
| ES            | PLEKHG2     | 49826 |
| ES            | ACAD9       | 66674 |
| ES            | TP53I11     | 15489 |
| ES            | FRMD8       | 16850 |
| ES            | UBE2F       | 58170 |
| ME            | FGFR3       | 68513 |
| ME            | RNF146      | 114496|
| ME            | GRB10       | 79717 |
| ME            | SORBS2      | 71377 |
| ME            | GTF2H3      | 306194|
| RI            | C11orf88    | 18667 |
| RI            | DET1        | 32385 |
| RI            | GAREML      | 52884 |

AA, alternate acceptor; AD, alternate donor; AP, alternate promoter; AT, alternate terminator; ES, exon skip; ME, mutually exclusive exon; RI, retained intron; AS ID, alternative splicing event ID.

and CCA, indicating that ME-GTF2H3-306194 may serve similar functions in CCA and papillary thyroid cancer and suggesting a new treatment possibility for CCA and papillary thyroid cancer by targeting ME-GTF2H3-306194.

In addition, the existence of two AS events predicted by the TCGASpliceSeq database was verified using PCR analysis of six CCA tumor tissue samples. Metastasis was common in cholangiocarcinoma (7,92). The direct infiltration of cholangiocarcinoma cells along the bile duct wall is one of the main features of cholangiocarcinoma metastasis (7). Cancer cells diffuse invasively in the bile duct wall and coexist with the bile duct and surrounding connective tissue, making the infiltration range of cholangiocarcinoma difficult to identify, and thus non-cancerous bile duct tissues cannot be easily obtained during surgery (93). Therefore, only two AS events were verified, and the difference of the splicing events between tumor and non-tumor tissues was not determined.

To investigate the causes of AS events in CCA, the relationship between AS events and splicing factors was analyzed. Splicing factors are a class of RNA-binding proteins that affect the selection of cleavage sites by recognizing the cis-acting elements of mRNA precursors (27). Previous studies on lung (56), ovarian (57) and bladder (94) cancer have demonstrated that the expression of splicing factors was correlated with prognosis-related AS events; however, this phenomenon was not observed in the current study. Pearson's correlation analysis between a group of splicing factors, the expression of which was directly related to AS events, and general OS-related splicing events identified 15 AS events that were mutually associated. Therefore, in CCA, splicing factors may affect the structure of their own proteins by creating their own AS event and then subsequently affecting the AS events of other genes.

In summary, TCGASpliceSeq data were mined to identify the characteristics of AS events associated with CCA to establish a model that can predict CCA prognosis using PIs from AS events. The prognostic effect was estimated using ROC curves and was favorable, with AUCs >0.9. However, due to the small sample size, the validity of this model should be verified with additional clinical samples and in different populations. Several additional limitations of the present study also need to be addressed. The data in the current study were obtained from online databases and thus remain at the bioinformatics level. Additionally, among the AS events selected to construct the PI model, one event was not CCA-specific, which reduced the specificity of the PI model. In addition, although splicing factors have been demonstrated to contribute to changes in the splicing patterns of target genes and tumorigenesis, and certain cancer-specific splicing factors are associated with OS rates, there has been a lack of research on CCA-related splicing factors, and whether the splicing factors identified in the present study are truly CCA-specific requires additional research. Finally, although an accurate PI model was constructed by screening the significant prognostic AS events identified by univariate and multivariate Cox regression, the results are based on a single cohort and should be validated in additional cohorts; this is intended to be performed in our subsequent study.

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Availability of data and materials

The datasets generated and analyzed during the current study are available from the TCGA data portal (portal.gdc.cancer.gov/).

Authors' contributions

HYW analyzed and interpreted data, and drafted the manuscript. YW, LML, ZBC, QPH and SLP obtained information from the database, supervised the data mining, and revised
the manuscript. All authors read and approved the final manuscript.

Ethics approval and consent to participate

The study was approved by the Ethics Committee of the First Affiliated Hospital of Guangxi Medical University (ethics approval no. 20170303-2). All the patients provided written informed consent to participate in this study.

Patient consent for publication

Not applicable.

Competing interests

The authors declare that they have no competing interests.

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