SRPK1/2 and PP1α exert opposite functions by modulating SRSF1-guided MKNK2 alternative splicing in colon adenocarcinoma

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Abstract

Background: The Mnk2 kinase, encoded by MKNK2 gene, plays critical roles in MAPK signaling and was involved in oncogenesis. Human MKNK2 pre-mRNA can be alternatively spliced into two splicing isoforms, the MKNK2a and MKNK2b, thus yielding Mnk2a and Mnk2b proteins with different domains. The involvement of Mnk2 alternative splicing in colon cancer has been implicated based on RNA-sequencing data from TCGA database. This study aimed at investigating the upstream modulators and clinical relevance of Mnk2 alternative splicing in colon adenocarcinoma (CAC).

Methods: PCR, western blotting and immunohistochemistry (IHC) were performed to assess the expression of Mnk2 and upstream proteins in CAC. The function of Mnk2 and its regulators were demonstrated in different CAC cell lines as well as in xenograft models. Two independent cohorts of CAC patients were used to reveal the clinical significance of MKNK2 alternative splicing.

Results: Comparing with adjacent nontumorous tissue, CAC specimen showed a decreased MKNK2a level and an increased MKNK2b level, which were correlated with KRAS mutation and tumor size. The SRSF1 (serine/arginine-rich splicing factor 1) was further confirmed to be the major splicing factor targeting MKNK2 in CAC cells. Higher expression of SRPK1/2 or decreased activity of PP1α were responsible for enhancing SRSF1 phosphorylation and nucleus translocation, subsequently resulted in a switch of MKNK2 alternative splicing.

Conclusions: Our data showed that phosphorylation and subcellular localization of SRSF1 were balanced by SRPK1/2 and PP1α in CAC cells. High nucleus SRSF1 promoted MKNK2 splicing into MKNK2b instead of MKNK2a, consequently enhanced tumor proliferation.

Keywords: Alternative splicing, Colon adenocarcinoma, MKNK2, SRSF1, SRPK1/2

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Background
Colorectal cancer ranks the third on both malignant morbidity and cancer-related deaths worldwide [1]. Approximately 20% of colorectal cancer patients were diagnosed at an extensive-stage with distant distribution, and the 5-year survival rate is less than 15% [2]. As the most common histological subtype, colon adenocarcinoma (CAC) can be treated with surgical resection, adjuvant chemotherapy, and radiation treatment. Unfortunately, molecular understanding of CAC progression remains limited although its incidence and mortality rates have been declined during the past decades [3].

Numerous well-known kinase networks are considered to play roles in CAC pathogenesis, such as EGFR, MAPKs, c-Src, et al. The MAP kinase-interacting serine/threonine-protein kinases (Mnks), downstream of MAPKs, are protein kinases that can phosphorylate eIF4E and enhance oncogenic mRNA translation [4]. Mnk2a and Mnk2b are two protein isoforms that derive from the predominant splicing factor that promotes synthesis of MKNK2a (SR-specific protein kinase) and protein phosphatase α (PP1α) on directly modulating SRSF1 phosphorylation and subsequent nucleus transportation. Interestingly, SRSF1-MAPK switch is dependent on its А180Su17, Shanghai Outdo Biotech, Shanghai, China). The siRNAs and shRNAs were listed in Supplementary Table S6.

DNA constructs, siRNAs, shRNAs
pCDNA3.1-vector was obtained from Invitrogen. Full-length of KRAS-G12V cDNA was cut from pBabe K-Ras 12 V vector (Cat. #12544, Addgene) and cloned into pcDNA3.1-vector (Invitrogen) via BamH1 and Xba1 restriction sites, thus generating the pCDNA3.1-RAS-G12V construct. Other DNA constructs including pCDNA3.1-Mnk2a, pCDNA3.1-Mnk2b, pCDNA3.1-SRPK1-WT, pCDNA3.1-SRPK1-K109A, pCDNA3.1-SRPK2-WT, pCDNA3.1-SRPK2-K110A, pCDNA3.1-PP1α-WT, pCDNA3.1-PP1α-T320A, and pCDNA3.1-PP1α-H125A were generated by GenePharma (Shanghai, China). The siRNAs and shRNAs were listed in Supplementary Table S6.

Transient transfections were conducted by using Lipofectamine3000 Transfection Reagent (Cat. # L3000015, Thermo Fisher Scientific, Pittsburgh, PA, USA) according to the manufacturer’s instructions. Puromycin was used to select and maintain stable overexpression cells. Lentivirus infection of SRPK1 and SPRK2 shRNAs were conducted as previously described [11].

Patients and tissue samples
Two independent cohorts of patients were retrospectively enrolled in this study. The primary cohort enrolled 32 CAC patients who underwent surgical treatment in Qilu Hospital of Shandong University (Jinan, China) from 2016 to 2017. The fresh-resected carcinoma tissues were frozen in liquid-nitrogen until experimental test. The validation cohort comprised of 100 CAC cases that were formalin-fixed paraffin-embedded (#HCo-180Su17, Shanghai Outdo Biotech, Shanghai, China). The patients in validation cohort were followed up until July 2015 (ranging 2–97 months). All the patients in primary cohort and validation cohort underwent R0
resection and classified with TNM stage I-IIIC at the time of surgical intervention according to the 7th AJCC/UICC TNM classification system. None of the patients in the two cohorts received any adjunctive therapy before tissue sample collection. The clinicopathological characteristics of the patients in two cohorts were summarized in Supplemental Table S1 and Supplemental Table S3, respectively. Informed consents were obtained from all patients or immediate relatives. All experiments were approved and supervised by the Ethics Committee of Qilu Hospital of Shandong University.

Real-time PCR (RT-qPCR) and real-time quantitative PCR (RT-qPCR)
Paired tumor and nontumorous specimens from primary cohort were subjected for RNA isolation and RT-qPCR using GAPDH as internal control. Briefly, total RNA was extracted from tissues and cells using TRIzol reagent. RNA was reversely transcribed into cDNA by PrimeScript RT Master Mix (TaKaRa, Kyoto, Japan). Quantitative polymerase chain reaction (qPCR) was conducted with SYBR Premix Ex Taq II kits (TaKaRa, Kyoto, Japan). Quantitative polymerase chain reaction (qPCR) was performed using the one step RT-PCR kit (QIAGEN) following the manufacturer's protocol. The primers used for RT-PCR and RT-qPCR were listed in Supplemental Table S6.

Cell proliferation and colony formation
The proliferation assay colony formation assay were conducted as previously described [13, 14]. Proliferation capacity was assessed by 3-(4,5-dimethylthiazol-2-yl)-2, 5-diphenyl-2H-tetrazolium bromide (MTT) assays at designated time points (6 h, 24 h, 48 h, 72 h, 96 h). Colonies were tested after incubation for 10 days in DMEM.

RNA-binding protein immunoprecipitation (RIP)
The RIP assay was conducted by using an RNA-Binding Protein Immunoprecipitation Kit (Millipore) according to the manufacturer’s procedure. The binding between the SRSF1 protein and MKNK2a/2b-mRNA was determined by RT-PCR and Western blotting of immunoprecipitated mixture.

Sample lysis and immunoblotting
Tissue samples or cultured cells were lysed using RIPA lysis buffer (Cat. #P0013, Beyotime, Beijing, China) containing protease and phosphatase inhibitors for total protein test. For nucleus isolation, cells were processed using the Nucleus and Cytosol Protein Extraction Kit (Cat. #P0027, Beyotime) according to the manufacturer’s instruction. The immunoblotting was conducted as described previously [14]. The antibodies used in our study were listed in Supplementary Table S6 at 1:1000 dilution.

Immunohistochemical (IHC) staining
The IHC staining was performed as described before [14] using anti-SRSF1, anti-SRPK1, anti-SRPK2, anti-PP1α, anti-PP1α-p-Thr320 antibodies (Table S6) at 1:300 dilution. The staining results were semi-quantified by multiplying the positive percentage score (0, no positive stained cells; 1, 0–25% positive; 2, 26–50% positive; 3, 51–75% positive; 4, 76–100% positive) and staining intensity score (0, negative staining; 1, slightly yellow; 2, dark yellow; 3, yellow brown), ranging 0–12. As for the quantification of SRSF1 staining, the nucleus staining and cytosol staining were scored separately. Either the nucleus staining or the cytosol staining were scored as 0–12 as described above. The cut-off of IHC scores were obtained by using receiver operating characteristic (ROC) curves to distinguish high- or low-expression groups.

Immunoprecipitation
Transfected cells were harvested and lysed with RIPA buffer. After protein quantification using the BCA kit (Thermo Fisher Scientific), equal amount of lysates were incubated with HA agarose beads overnight an 4 °C. The immunoprecipitated mixture were subjected to Western blotting after washing out the nonspecific bindings with RIPA buffer.

GST-pull down
The purified GST protein was obtained from Prof. Jinping Sun in Shandong University (Jinan, China). The purified GST-tagged SRPK1 and GST-tagged SRPK2 proteins were purchased from Novus Biologicals (CO, USA). The His-SRSF1 protein was purchased from Creative Biomart (NY, USA). The GST-pull down assay was conducted as described before using GST agarose beads [15].

In vitro phosphorylation test
Purified SRPK1 or SRPK2 proteins were incubated with SRSF1 proteins in kinase reaction buffer (25 mM Tris, pH 7.5, with 10 mM MgCl2, 2 mM DTT, 5 mM β-Glycerophosphate, 0.1 mM Na3VO4, and 2 mM EGTA) containing 20 μM ATP for 30 min at 37 °C water bath. Reactions were then quenched by adding SDS loading buffer and subjected to western blot assay.

Mice model and xenograft
Transfected SW480 cells (4 × 10⁶) were subcutaneously injected into six-week male Balb/c nude mice. Each group contained 5 mice. Tumor size was measured every 5 days using the following formula: V = (length×width²)/2. The mice were sacrificed on the 21st day after injection, and the xenograft tumors were removed and weighed as described before [13]. The Animal Welfare
Committee of Shandong University approved all procedures involving animals.

Statistics
Data are presented as the mean ± standard deviation (SD). Statistical analyses were conducted using SPSS 20.0 Software. Correlations between mRNAs were analyzed using Spearman correlation test. Correlations between proteins and patients’ characteristics were analyzed using the Kaplan-Meier method and significance was analyzed using the log-rank test. Multivariate cox regression analyses were carried out to identify independent prognostic factors. For the cellular and xenograft data, the difference between two groups was tested using Student’s t-test, while One-way ANOVA analysis was used to compare data among more than two groups. A value of \( P < 0.05 \) indicated a statistically significant result.

Results
**MKNK2 alternative splicing is altered in CAC tissues and affected by KRAS mutation**
MKNK2 can be alternatively spliced to MKNK2a and MKNK2b mRNAs and consequently translated into two protein isoforms: Mnk2a and Mnk2b (Fig. 1a). By analyzing the mRNAs level of MKNK2 isoforms in 32 paired tissues (Table S1), we found that MKNK2a was downregulated in CAC tumor tissues while MKNK2b was upregulated (Fig. 1b). Mutations of KRAS and TP53 are the most well-characterized onco-driver of CAC, and we next investigated whether they have effects on MKNK2 alternative splicing (Fig. 2c). Although TP53 seemed to have no impact on MKNK2 mRNA levels, MKNK2a splicing was inhibited while MKNK2b was remarkably upregulated in KRAS-mutated CAC samples. Therefore, we enrolled both KRAS-WT cells (Caco-2 and HT-29) and KRAS-mutated cells (HCT-116 and SW480) to conduct cellular validation. Comparing with Caco-2 and HT-29 cells, HCT-116 and SW480 cells showed lower MKNK2a but higher MKNK2b mRNA levels (Fig. 1d). Furthermore, transfecting Caco-2/HT-29 cells with KRAS-G12V plasmids significantly altered the predominant MKNK2 isofrom from MKNK2a to MKNK2b (Fig. 1e), indicating that KRAS can affect MKNK2 alternative splicing. We next aimed to seek whether the KRAS-G12V-induced MKNK2b was resulted from increased RNA synthesis or decreased RNA degradation using NCM460 cells. Actinomycin D is a potent inhibitor that can inhibit poly(A)RNA synthesis in a dose-dependent manner [16]. According to our data, actinomycin D treatment significantly attenuated the KRAS-G12V-induced MKNK2b expression at 20 nM or higher concentrations (Fig. 1f), implying this MKNK2b alteration was correlated with RNA synthesis instead of RNA degradation. Interestingly, MKNK2a and MKNK2b showed opposite effects on KRAS-G12V-induced colony formation of NCM460 cells (Fig. 1g), indicating their distinct roles in modulating tumor development.

**MKNK2 alternative splicing is correlated with tumor growth instead of LN metastasis**
Additionally, MKNK2a was relatively lower in tumors with larger size while MKNK2b was higher in larger CAC tissues (Fig. 1h). Spearman correlation test showed that tumor diameter was negatively correlated to MKNK2a level and positively correlated to MKNK2b level (Fig. S1A, S1B). Apparently, the MKNK2a/MKNK2b ratio was decreased in tumor specimens with larger diameter (Fig. S1C). In contrast, we didn’t identify any significant correlation between MKNK2 mRNA levels with lymph node (LN) metastasis (Figure S1D), indicating MKNK2 may have little effect on modulating tumor invasion. Since we showed that MKNK2 alternative splicing is closely correlated with KRAS mutation, we chose HCT-116 and SW480 cell for following cellular tests in this study. Although transfection of Mnk2a showed no obvious effect on CAC cell proliferation (Fig. 1i) or colony formation (Fig. 1j), overexpressing Mnk2b significantly enhanced cell proliferation capacity. Both our clinical and cellular findings suggested a novel tumor-promoting role of Mnk2b on CAC progression.

**Nucleus SRSF1 is closely correlated with MKNK2 alternative splicing in tumor tissues**
Since KRAS is not a splicing factor and is less possible for directly regulating MKNK2 alternative splicing, we next aimed to figure out the upstream splicing factors targeting MKNK2. Serine/arginine-rich splicing factors (SRSFs) are the major splicing factors that regulate alternative splicing process in mammalian cells [17]. By testing the mRNA levels of SRSF family members, we found that SRSF2 showed a positive relationship with KRAS-G12V level. In contrast, SRSF3, SRSF4 and SRSF9 showed negative correlations with MKNK2a level (Figure S2, Table S2). However, only SRSF1 was negatively correlated with MKNK2a and positively correlated with MKNK2b simultaneously, indicating its predominant role on modulating MKNK2a-MKNK2b switch in CAC tissues (Fig. 2a, b). Moreover, RNA-immunoprecipitation results confirmed the interaction between SRSF1 protein and MKNK2a/2b-mRNA in SW480 cells (Fig. 2c).

Therefore, we further investigated whether SRSF1 participate in CAC progression via MKNK2a-MKNK2b switch. RT-qPCR and immunoblotting data showed that SRSF1 was upregulated in tumor tissues compared with adjacent nontumorous tissues on both RNA and protein levels (Fig. 2d, e). Interestingly, IHC data identified a
diffused staining of SRSF1 protein in both nucleus and cytoplasm (Fig. 2f). To better evaluate the alteration of SRSF1 in CAC tissues, we separately assessed its expression in nucleus or cytoplasm. No statistical difference was observed between tumor tissues and adjacent nontumorous tissues regarding to cytoplasm-SRSF1. Nevertheless,
CAC samples showed higher total SRSF1 and nucleus-SRSF1 than nontumorous tissues (Fig. 2g). In addition, both total SRSF1 and nucleus-SRSF1 were positively correlated to MKNK2b-mRNA level, while negatively correlated to MKNK2a and MKNK2a/MKNK2b (Figure S3A, S3B). In contrast, cytoplasm-SRSF1 showed no significant correlation with MKNK2 alternative splicing (Figure S3C). Using ROC method (Figure S3D), we next divided the primary cohort into subgroups based on SRSF1 levels in different subcellular locations. Consistent with Spearman correlation test, Chi-square results also revealed a significant difference of MKNK2 alternative splicing between low nucleus-SRSF1 and high nucleus-SRSF1 groups (Fig. 2h). Similar results were obtained based on total SRSF1 expression but not cytoplasm-SRSF1 (Fig. 2h).

**TNPO3-dependent nucleus transportation of SRSF1 determines MKNK2 alternative splicing and cell proliferation**

We set out to explore the possible effects of SRSF1 on MKNK2 alternative splicing in CAC cells. Knockdown of SRSF1 can significantly inhibit MKNK2a-MKNK2b switch according to RT-qPCR (Fig. 3a) and RT-qPCR data (Fig. 3b), respectively. Of note, cell proliferation and colony formation capacities were also attenuated by silencing SRSF1 (Fig. 3c, d). On the other hand, overexpressing SRSF1 resulted in MKNK2a-MKNK2b switch and enhanced cell proliferation (Fig. 3e-h). Taking into consideration that only nucleus-SRSF1, instead of cytoplasm-SRSF1, showed a significant correlation with MKNK2 alternative splicing in CAC samples, we
hypothesize that nucleus transportation is critical for SRSF1-guided MKNK2a/MKNK2b switch. Previous studies had revealed that transportin 3 (TNPO3) can assist nucleus trafficking of SRSF members [18], thus we blocked the nucleus transportation of SRSF1 by silencing TNPO3 (Figure S3E). Accordingly, TNPO3-siRNA abolished the role of SRSF1 on promoting MKNK2a-MKNK2b switch (Fig. 3e, f) and resulted in attenuated cell proliferation (Fig. 3g, h).

SRPK1 and SRPK2 are positively correlated with MKNK2a-MKNK2b switch and SRSF1 nucleus translocation

Since phosphorylation plays vital roles on intracellular trafficking of SRSF proteins [19], we were interested to identify the upstream kinases responsible for SRSF1 nucleus transportation. CDC2-like kinase 1 (CLK1) and SR-specific protein kinases (SRPKs) had been proved to phosphorylate the RS domain of SRSF1 through in vitro kinase assays [20]. Therefore, we tested the statistical relevance between MKNK2a-MKNK2b switch and CLK1, SRPK1, SRPK2, respectively. Correlation test revealed that CLK1 has no significant correlation with MKNK2 alternative splicing (Fig. S4A). In contrast, the level of SRPK1 mRNA is positively correlated with MKNK2b level, while negatively correlated with MKNK2a level or MKNK2a/MKNK2b ratio (Figure S4B). As for SRPK2, although no significance was
observed towards MKNK2a level, it showed a significant correlation with MKNK2b (Fig. S4C). Besides, both SRPK1 and SRPK2 mRNA levels were upregulated in tumor tissues comparing with adjacent tissues in our primary cohort (Fig. 4a). TCGA database also revealed upregulated transcriptions of SRSF1 and SRPKs (Figure S5). Moreover, SRPK1 as well as SRPK2 showed positive correlations with nucleus-SRSF1 protein level (Fig 4b), indicating that SRPKs may promote nucleus translocation of SRSF1. We next evaluated protein expression level of SRPKs in primary cohort by immunoblotting. Consistent with mRNA data, both SRPK1 and SRPK2 proteins were elevated in tumor tissues (Fig. 4c).

**Prognostic effects of SRSF1, SRPK1, and SRPK2**

The findings in primary cohort and TCGA database promoted us to further investigate the prognostic significance of SRSF1 and SRPKs by introducing another independent validation cohort (n = 100). IHC experiments were conducted to explore the protein expression and localization. SRPK1 and SRPK2 were predominantly localized in cytoplasm, while SRSF1 was stained in both nucleus and cytoplasm (Fig. 4d). In accordance with primary cohort, CAC samples in validation cohort showed higher nucleo localization of SRSF1 and higher expression of SRPK1/2 (Fig. 4e). Besides, patients with higher SRPK1 or SRPK2 were characterized with higher nucleus-SRSF1 level (Fig. 4f). Furthermore, higher SRPK1 was observed in patients with larger tumor size and advanced tumor stages (Table S3).

Kaplan-Meier survival analyses revealed that patients with higher nucleus-SRSF1 had a poorer overall survival (Fig. 4g). Higher SRPK1 or higher SRPK2 also indicated unfavorable prognosis, respectively (Fig. 4h, i). Of note, patients with both high SRPK1 and high SRPK2 showed a lowest overall survival time comparing with other subgroups (Fig. 4j). Prognostic effects of other clinicopathological factors were also summarized, which demonstrated the clinical significance of lymph node metastasis and TNM stage (Fig. 4k, Table S4). Additionally, multivariate analysis was performed to explore independent prognostic variables (Fig. 4l, Table S5). Higher nucleus-SRSF1, higher SRPK1/2, and advanced TNM stage were all identified as independent risk factors of CAC overall survival.

**Restoration of Mnk2b reverses the anti-proliferation effects of SRPK knockdown**

The effect of SRPKs on CAC progression were validated by cellular experiments. The nonspecific SRPK inhibitor, SRPIN340, can significantly inhibit cell proliferation and colony formation of CAC cells (Fig. 5a, b). After testing the transfection efficiency of SRPK1-siRNAs and SRPK2-siRNAs (Figure S6A, S6B), we knocked down the two kinases in CAC cells and found that silencing either SRPK1 or SRPK2 showed no statistically significant effect on cell proliferation. However, simultaneously knockdown of both SRPK1 and SRPK2 remarkably attenuated cell proliferation and colony formation (Fig. 5c, d). Interestingly, overexpressing Mnk2b restored the cell proliferation capacity of SRPK1/2-silenced cells (Figure S6C-E). In contrast, SRPK1 and SRPK2 overexpression enhanced cell proliferation while TNPO3-siRNA almost abolished this effect (Figure S6F-H).

**SRPK1 and SRPK2 regulate MKNK2 alternative splicing by directly phosphorylating SRSF1 and promoting SRSF1 nucleus transportation**

We also compared the effects of SRPK1/2 knockdown or SRPK1/2 overexpression on SRSF1 phosphorylation, nucleus shuttling, and MKNK2 alternative splicing in CAC cell lines (Fig. 5e, f; Figure S7A-D). As expected, either SRPK1-siRNA or SRPK2-siRNA significantly inhibited SRSF1 phosphorylation and nucleus translocation. In contrast, SRPK1 or SRPK2 overexpression upregulated the phosphorylation and nucleus accumulation of SRSF1. Besides, overexpressing SRPK1/2 led to an enhanced MKNK2a-MKNK2b switch, while silencing SRPK1/2 showed opposite effects according to RT-PCR and RT-qPCR results. To further investigate whether kinase activity of SRPK1/2 were critical for SRSF1-guided MKNK2 alternative splicing, we generated the kinase-dead mutants SRPK1-K109A and SRPK2-K110A. Either kinase-dead mutant or inhibitor SRPIN340 significantly attenuated, if not abolished, the role of SRPK1/2 on modulating SRSF1 phosphorylation, nucleus transportation as well as downstream MKNK2 alternative splicing (Fig. 5g, h; Figure S7E-H). Moreover, immunoprecipitation data identified the intracellular interaction between SRSF1 with SRPK1/2 (Fig. 5i). Extracellular GST pull down assay was then conducted and confirmed their direct binding activity (Fig. 5j). We next investigate whether the interaction between SRPK1/2 and SRSF1 is a kinase-substrate process. In vitro phosphorylation test showed that both SRPK1 and SRPK2 are capable of phosphorylating SRSF1, which can be inhibited by SRPIN340 (Fig. 5k). Therefore, SRPK1 and SRPK2 may directly phosphorylate SRSF1 and promote it nucleus translocation, subsequently modulate MKNK2 alternative splicing.

**PP1α activity is downregulated in colon adenocarcinoma tissues**

It has been reported that PP1α is the major phosphatase that dephosphorylates SRSF1 [21], thus we explored whether PP1α is responsible for antagonizing SRPK1/2 during CAC development. To our surprise, no significant difference of PPP1CA mRNA level was observed
Fig. 4 (See legend on next page.)
between tumor tissues and adjacent tissues (Fig. 6a). Taking into consideration that the phosphatase activity of PP1α is as important as its expression level, we next tested whether the activity of PP1α was altered in CAC tissues by testing its phosphorylation on T320 site, which inhibits its catalyzation capacity. IHC data showed cytoplasmic localization of both PP1α and pT320-PP1α (Fig. 6b). Consistent with RT-qPCR data, PP1α protein level showed no difference between tumor and adjacent tissues. However, the pT320-PP1α level was upregulated in tumor tissues, indicating a decreased PP1α activity (Fig. 6c).

PP1α negatively modulates SRSF1 nucleus transportation, MKNK2 alternative splicing, and tumor proliferation

Furthermore, patients with higher nucleus-SRSF1 showed higher pT320-PP1α level but not total PP1α level (Fig. 6d; Figure S8A). Spearman correlation test also revealed a positive correlation between pT320-PP1α and nucleus-SRSF1 (Fig. 6e) while no correlation was observed regarding total PP1α (Figure S8B). Similarly, although no association was identified between total PP1α and MKNK2 alternative splicing (Figure S8C), pT320-PP1α was positively correlated to MKNK2a-MKNK2b switch (Fig. 6f).

In addition, the constitutively active mutant of PP1α, PP1α-T320A, significantly inhibited proliferation of CAC cells (Fig. 6g-i). Cellular assays were then performed to test crosstalk between PP1α and SRSF1 by overexpressing constitutively active mutant (T320A) or inactive mutant (H125A) of PP1α, respectively. We found that either PP1α-WT or PP1α-T320A can decrease SRSF1 phosphorylation and nucleus translocation, while PP1α-H125A showed opposite effects (Fig. 6j; Figure S8D). Consistently, PP1α-T320A inhibited MKNK2a-MKNK2b switch while PP1α-H125A promoted it (Fig. 6j; Figure S8D). The PP1α specific inhibitor, tautomycin, can efficiently block the effects of PP1α-WT or PP1α-T320A on altering SRSF1 phosphorylation, nucleus translocation and MKNK2 alternative splicing (Fig. 6k; Figure S8E). Furthermore, we observed similar findings on SW620 cells, the metastatic cell line of SW480 (Figure S9). Therefore, we concluded that PP1α can suppress CAC progression at least partially by inhibiting SRSF1-guided MKNK2a-MKNK2b switch, which is dependent on SRSF1 phosphorylation and nucleus shuttling.

SRPKs and PP1α have opposite effects on modulating MKNK2 alternative splicing and tumor growth in mice model

Xenograft models were utilized to verify tumor-related roles of SRPK1/2 and PP1α in vivo. SW480 cells that stably overexpressed SRPK1&2 or PP1α, or stably silenced SRPK1/2 with shRNAs were seeded subcutaneously in Balb/c nude mice. There was no statistical difference among the groups on the aspects of body weight nor food consumption (Fig. 7a, b). Among the four groups, SRPK1&2-overexpressing group showed the highest proliferation capacity and largest tumor size. In contrast, PP1α-T320A group and sh-SRPK1/2 group both showed impaired growth process comparing to control group (Fig. 7b-e).

The mRNA levels of MKNK2a and MKNK2b in isolated xenografts were further tested by RT-qPCR, which revealed consistent results with cellular assays. On one hand, SRPK1&2 downregulated MKNK2a splicing and upregulated MKNK2b level. On the other hand, PP1α-T320A or sh-SRPK1/2 attenuated MKNK2a-MKNK2b switch (Fig. 7f). Proliferation index of xenografts was also evaluated by calculating Ki-67 positive staining (Fig. 7g). As a result, SRPK1&2 group showed the highest Ki-67 staining percentage, while PP1α-T320A group and sh-SRPK1/2 group showed decreased Ki-67 level, which is consistent with tumor growth curve (Fig. 7h). Taken together, our data revealed an oncogenic role of SRPK1/2 signaling in colon adenocarcinoma cells (Fig. 7i). In nontumorous cell, MKNK2 pre-mRNA is predominantly spliced into MKNK2a and translated to Mnk2a protein, which exerts anti-tumor effects. In colon...
Fig. 5 (See legend on next page.)
adenocarcinoma cells, SRSF1 is hyperphosphorylated due to elevated SRPK1/2 and decreased PP1α activity, resulting in SRSF1 nucleus shuttling. Nucleus SRSF1 accumulation finally leads to alternative splicing of MKNK2 pre-mRNA to MKNK2b and upregulated the Mnk2b oncoprotein level (Fig. 7i).

Discussion

Alternative splicing expands the proteomic complexity by generating splicing variants on mRNA level and encoded distinct protein isoforms including kinases. In the present study, we focused on MKNK2a and MKNK2b alternative splicing, encoding Mnk2a and Mnk2b, respectively. Our data revealed a downregulation of MKNK2a and an upregulation of MKNK2b in colon adenocarcinomas, which is closely correlated with KRAS mutation. The hypothesis of MKNK2b is observed in samples with larger tumor size, implying its role on promoting tumor proliferation. Maimon et al. had recently reported that Mnk2b is deficient on activating p38-induced cell death pathway, while maintaining its capacity of promoting eIF4E-induced oncoprotein translation. In contrast, Mnk2a can induce both oncogenesis as well as p38-MAPK stress apoptosis pathway. Therefore, they elucidated downstream mechanism of how Mnk2a and Mnk2b exert opposite effects on tumor progression in Ras-activated cells [10].

Consistently, our data also revealed a significant correlation between MKNK2 splicing shift with KRAS mutation in colon adenocarcinomas, while no obvious association with TPS53 was identified. Additionally, cell lines possessing KRAS mutation showed a prevalence of MKNK2a-MKNK2b switch in comparison with KRAS-WT cell lines. Strikingly, KRAS-G12V transfection enhanced MKNK2b mRNA splicing even in nontumorous colon epithelial cells, indicating an oncogenesis correlation between KRAS mutation and MKNK2 alternative splicing. According to the data reported by Maimon et al. [10], oncogenic transformation caused by activated Ras can induce MKNK2a-MKNK2b switch in breast cancer cells, pancreatic cancer cells and in lung cancer cells. Furthermore, coexpressing Mnk2a inhibited the oncogenic phenotype of Ras-transformed MCF-10A cells, indicating that Mnk2a can block Ras-induced transformation [10]. Therefore, it is high likely that oncogenic Ras induces tumor transformation at least partially through affecting MKNK2a-MKNK2b switch. Another study by Maimon et al. artificially induced a strong switch from MKNK2b to MKNK2a by using designated oligonucleotides, resulting in inhibited glioblastoma development and re-sensitization to chemotherapy [22]. Their study provides evidence on the therapeutic potential of manipulating MKNK2 alternative splicing as a novel approach to treat glioma. Our present results regarding MKNK2a-MKNK2b switch in colon adenocarcinoma implicated that similar intervention may be a novel direction for CAC treatment.

In addition, our RT-qPCR screening assay indicated SRSF1 as a promising modulator targeting MKNK2 alternative splicing, which was confirmed by correlation test, RNA immunoprecipitation, and knockdown assays. The findings on that nuclear SRSF1, instead...
of cytoplasmic SRSF1, was correlated with patients’ clinical outcomes promoted us to further explore the upstream regulation mechanisms of SRSF1 subcellular trafficking. Consistent with electron microscopy data [23], we verified the critical role of TNPO3 on importing SRSF1 into nucleus of colon adenocarcinoma cells. Further study showed that the tumor-promoting role of SRSF1 can be significantly attenuated by silencing TNPO3, confirming that only nucleus SRSF1 exerts pro-oncogenic effects.

Post-translational modification has remained the focus of protein function investigation during the past decades, and phosphorylation is one of the most important modifications which we focused on [24, 25]. Abnormal expression of kinases or phosphatases can result in dysregulated phosphorylation events, leading to various malignancies as we previously reported [14, 26]. It has been reported that multisite phosphorylation of SRSF1 is a prerequisite for its nucleus transportation [27, 28], which is the key mechanism for its functional alternative
splicing. Therefore, we next aimed to investigate the up-steam kinase and phosphatase which controls SRSF1 phosphorylation. Three major kinases had been reported to phosphorylate SRSF1 including SRPK1, SRPK2, and CLK1 [19, 20]. Considering that enzyme-substrate relationship can be difference in various cell types, we initially explored whether those kinases could affect SRSF1 nucleus translocation in colon adenocarcinoma cells. Although we didn’t observe any statistical correlation between CLK1 and nucleus SRSF1 level, both SRPK1 and SRPK2 indeed correlated with SRSF1 nucleus translocation. Additionally, SRPK1 and SRPK2 showed upregulated expression in CAC tissues compared with adjacent tissues, which is consistent with their expression patterns in acute myeloid leukemia, breast cancer, and pancreatic cancer [29–31].

In accordance with their underlying crosstalk, SRPK1/2 and SRSF1 are all identified as independent risk factors for the overall survival of CAC patients. Following cellular results again confirmed that silencing SRPK1 and SRPK2 can lead to decreased SRSF1 phosphorylation, nucleus transportation, \( \text{MKNK2a-MKNK2b} \) switch, and cell proliferation. Similar results were obtained by introducing kinase-dead mutants or using their inhibitor SRPIN340. Of note, we systematically verified that both SRPK1 and SRPK2 can directly interact and phosphorylate SRSF1 by immunoprecipitation, GST pull-down, and in vitro phosphorylation test. The anti-tumor effects of SRPK inhibition had been recently reported in other malignancies [31, 32], our data enlarged the therapeutic potential of developing SRPK inhibitor for CAC treatment.

Interestingly, a previous study by Wang et al. demonstrated that SRPK1 can act as both oncogene and tumor suppressor, either higher or lower level of SRPK1 induces cell transformation [33]. The underlying mechanism is determined by the interaction between SRPK1 and PHLPP, thus aberrant SRPK1 expression in either direction could result in hyperactivation of Akt by blocking its phosphatase PHLPP. Considering that specific effect of kinase is largely depend on its substrate, identification of other SRPK1 substrates will be invaluable for further illustrating its tumor-related role. Anyway, here our data confirmed the pro-oncogenic axis of SRPK1/2-SRSF1-MKNK2 in CAC progression.

**Fig. 7** SRPKs and PP1α have opposite effects on MKNK2 alternative splicing and tumor growth in mice model. SW480 cells transduced with shSRPK1/2, or overexpressing SRPK1&2, or overexpressing PP1α-T320A were subcutaneously injected into six-week male Balb/c nude mice. The mice condition was monitored from the date of observing macroscopical xenograft. The body weight (a) or food consumption (b) of mice in each group showed no statistical difference. c Xenograft growth curves were plotted as described in the Method section. The mice were sacrificed after monitoring for 20 days, then the xenografts were isolated, weighted (d), and photographed (e). f Levels of MKNK2α and MKNK2b splicing variants were tested via RT-qPCR from isolated xenografts. g Representative H&E staining and Ki-67 immunostaining results of isolated xenografts. Scale bar: 100 μm. h The proliferation index was determined using the percentage of Ki-67 positive cells in isolated xenografts. i A schematic model shows the effects of SRPK1/2 and PP1α on regulating MKNK2α/MKNK2b switch. MKNK2 pre-mRNA can be alternatively spliced into two MKNK2 mRNA isoforms and further translated into two protein isoforms, namely Mnk2a and Mnk2b. Mnk2a perhaps play an anti-tumor role while Mnk2b exerts remarkable oncogenic effects according to our data. In nontumorous cells, Mnk2a is the predominantly protein isoform compared to Mnk2b. In colon adenocarcinoma cells, SRPK1 and SRPK2 are upregulated while PP1α is inactivated, resulting into a hyperphosphorylation status of SRSF1. Phosphorylation of SRSF1 promotes its nucleus transportation in a TNPO3-dependent manner. The nucleus SRSF1 can directly bind to MKNK2 pre-mRNA and significantly generate more MKNK2b isoform, while the MKNK2α proportion is decreased.
Besides kinases, we next focused on SRSF1 upstream phosphatase to impartially evaluate the importance of its phosphorylation in CAC. Adams JA et al. and her colleagues reported that protein phosphatase 1α (PP1α) directly dephosphorylates the RS domain of SRSF1 through binding to its RNA recognition motif 1 (RRM1) through an allosteric mechanism [34]. Of note, disrupting the interaction between PP1α and SRSF1 results in significant shifts of alternative splicing such as EIF5 and TIMM8B genes [35]. On the other hand, PP1α has been demonstrated to inhibit the metastasis of SW620 colon cancer cells via deactivating Src protein [36]. Therefore, we were prompted to assess whether PP1α was dysregulated in colon adenocarcinoma. Although we didn't find any significant difference of PP1α expression between CAC tissues and adjacent tissues, its catalytic inactivate type, namely the Tyr320-phosphorylated PP1α, was elevated in tumor tissues, indicating that PP1α activity was downregulated in colon adenocarcinoma. Here we also validated the role of PP1α on regulating MKNK2 alternative splicing by introducing its constitutively active mutant and inactive mutant, respectively. Besides SW480 and HCT-116 cells, PP1α and its mutations showed similar effects on the metastatic SW620 cell line, further highlighting the prognostic value of PP1α-SRSF1-MNK2 axis in CAC progression. However, we have to keep in mind that phosphorylation status of SR proteins can not only modulate their subcellular localization but also determines the splicing site selection [37], thus the more detailed effects of SRPKs and PP1α on alternative splicing need further validation. Together with the findings from xenografts, we concluded that PP1α can attenuates colon cancer progression at least partially via antagonizing SRPK1/2-induced SRSF1 phosphorylation and downstream MKNK2a-MKNK2b splicing shift.

Conclusions
Our study reveals a significant role of SRSF1-guided MKNK2a-MKNK2b splicing switch in the proliferation of colon adenocarcinoma, which is determined by SRSF1 phosphorylation and subcellular translocation. The alternative splicing of MKNK2 is balanced by SRPK1/2 and PP1α, thus providing opportunities of therapeutic intervention, such as SRPK inhibitors or PP1α allosteric activators, in treating malignancies. Additionally, the subcellular location of SRSF1, expression levels of SRPK1/2, as well as phosphorylation status of PP1α can all serve as independent prognostic predictors for the overall survival of colon adenocarcinoma.

Supplementary Information
The online version contains supplementary material available at https://doi.org/10.1186/s13046-021-01877-y.

Additional file 1: Table S1. Patients information of primary cohort. Table S2. Correlations between MKNK2 alternative splicing and splicing factors on mRNA levels. Table S3. Correlation between protein expression level and clinical features of colon adenocarcinoma patients in validation cohort. Table S4. Kaplan-Meier survival analyses for colon adenocarcinoma patients. Table S5. Cox-regression analysis for overall survival of colon adenocarcinoma patients. Table S6. Antibodies, chemicals, siRNAs, shRNAs, and primers in this study. Figure S1. Correlations between MKNK2 alternative splicing with patients’ characteristics in primary cohort. Figure S2. Correlations between splicing factors and MKNK2 alternative splicing in CAC tissues. Figure S3. Correlations between SRSF1 protein expression and MKNK2 alternative splicing in CAC tissues. Figure S4. Correlations between upstream kinases and MKNK2 alternative splicing in CAC tissues. Figure S5. SRSF1, SRPK1, and SRPK2 are upregulated in CAC tissues. Figure S6. Effects of SRPKs on cell proliferation can be altered by Mnk2b and TNPO3. Figure S7. SRPKs modulate SRSF1 phosphorylation, nucleus transportation, and MKNK2 alternative splicing. Figure S8. Functions of PP1α on modulating SRSF1-dependent MKNK2 splicing. Figure S9. Functions of PP1α on modulating proliferation and SRSF1-dependent MKNK2 splicing in metastatic SW620 cells.

Abbreviations
CAC: Colon adenocarcinoma; CLK1: CDC2-like kinase 1; eIF4E: Eukaryotic translation initiation factor 4E; Mnk: MAP kinase-interacting serine/threonine-protein kinase; PP1α: Protein phosphatase 1α; RNA-IP: RNA immunoprecipitation; SRSF: Serine and arginine rich splicing factor; SRPK: SR-specific protein kinase; TNPO3: Transportin 3

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Authors’ contributions
HL designed this project and analyzed the data. ZG help conduct xenograft experiments. KL conducted RT-PCR and RT-qPCR analyses. QZ performed cell proliferation tests and WB tests. ZX and YX were major contributors in interpreting the data and writing the manuscript. All authors read and approved the final manuscript.

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Availability of data and materials
The data regarding the current study are available on reasonable request.

Ethics approval and consent to participate
All experiments were approved and supervised by the Ethics Committee of Qilu Hospital of Shandong University. Informed consent was obtained from all individual participants included in the study. Animal studies in nude mice were approved by the Ethical Committee of Shandong University and carried out in accordance with the UK Animals (Scientific Procedures) Act, 1986.

Consent for publication
Not applicable.

Competing interests
None.
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