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Original Article

Serine/arginine (SR)-rich-specific-protein kinase 2 promotes the epithelial-mesenchymal transition by upregulating Twist-related protein 1 expression in colon cancer cells

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Abstract: Serine/arginine (SR)-rich-specific-protein kinase 2 (SRPK2), which promotes the growth and migration of colon cancer cells, is overexpressed in colon cancer specimens. However, the role of SRPK2 in epithelial-to-mesenchymal transition (EMT) and its association with the transcription factor Twist-related protein 1 (TWIST1), are not fully understood. This study aimed to investigate the role of SRPK2, in EMT, in colon cancer and to test whether TWIST is involved in the process. The expression of SRPK2 in a normal colonic epithelial cell line and colon cancer cell lines was performed by qRT-PCR and western blot. After transfection with SRPK2 siRNA or TWIST, the cells were treated with or without TGF-β1 and the expression of SRPK2, TWIST, and epithelial marker E-cadherin and mesenchymal marker vimentin, were detected. We found the mRNA and protein expression levels of SRPK2 in colon cancer cell lines, was significantly higher than in the normal colonic epithelial cell line NCM460. After the expression of SRPK2 was inhibited, the expression of epithelial marker E-cadherin, was significantly increased, whereas the expression of vimentin, was significantly downregulated. TWIST expression was significantly inhibited, by knockdown of SRPK2. TGF-β significantly induced the expression of TWIST, SRPK2 and E-cadherin, whereas it significantly downregulated the vimentin expression. TWIST Knockdown did not significantly alter SRPK2 expression, indicating SRPK2 is upstream of TWIST. In contrast, knockdown of TWIST, significantly upregulated the TGF-β-inhibited expression of E-cadherin, whereas it significantly downregulated the TGF-β-induced expression of vimentin. Collectively, SRPK2 might promote the EMT by upregulating TWIST expression in colon cancer cells, which provides a novel strategy and early diagnosis of colorectal cancer.

Keywords: SRPK2, TWIST, EMT, colon cancer

Introduction

Colorectal cancer (CRC) is the third most commonly diagnosed cancer worldwide and a major cause of morbidity and mortality [1-3]. Although witnessed advances in CRC treatment have been made in the past decade, with the introduction of new surgical techniques, radiotherapy and chemotherapy, such as the use of anticancer drug including angiogenesis inhibitors and antibodies targeting cancer-associated proteins, the prognosis and overall survival rate of CRC patients, has not shown any promising improvement [4-6]. Metastasis plays an important role in the poor prognosis and low 5-year survival rate, of cancer patients [7, 8]. Therefore, identification of an appropriate CRC target and elucidation of the mechanism underlying its progression, are important for the development of novel treatment to suppress the progression in CRC [9-11].

Within primary tumors, the majority of carcinoma cells generally exhibit predominantly epithelial characteristics. However, to invade, circulate to other tissues and, ultimately, form metastatic colonies, neoplastic epithelial cells must transform, at least transitorily, into a more mesenchymal phenotype [12]. The epithelial-to-mesenchymal transition (EMT), is a physio-

logical process found in embryonic development, tissue remodeling and wound healing [13, 14]. EMT mediates neoplastic cell dissociation, from primary lesions to distant organs, promoting cancer cell proliferation, migration and invasion, and resulting in increased resistance to apoptosis, drug resistance, and the acquisition of stem cell-like properties. Therefore, the EMT process is an important target for therapy and diagnosis in CRC.

Serine/arginine (SR)-rich specific protein kinases (SRPKs) are a family of cell-cycle-regulated kinases that specifically phosphorylate their substrates at Ser residues located in regions rich in SR dipeptides. In the cell nucleus, SRPKs phosphorylate SR proteins found in nuclear speckles, to mediate pre-mRNA splicing [15-17]. In head and neck squamous cell carcinoma, SRPK2 is highly expressed and the inhibition of SRPK2, resulted in a significant decrease in the colony forming and invasive ability of head and neck squamous cell carcinoma cells [18]. Manipulation of the SRPK2 protein level, significantly affects the cell cycle profile and mediates cell proliferation, in human patients with myeloid hematological malignancies [19]. Overexpression of SRPK2 increases leukemia cell proliferation [20]. In a recent study, elevated expression of SRPK2 was observed in non-small cell lung cancer samples [21] and in colon cancer samples [17]. It was demonstrated that overexpression of SRPK2, promoted the growth and migration of colon cancer cells, while, knocking down the expression of SRPK2, inhibited the growth, migration and tumorigenesis of colon cancer cells [17]. However, the role of SRPK2 in EMT and its underlying mechanism remains unclear.

Twist-related protein 1 (TWIST1) is elevated in many primary tumors including colon, melanoma, prostate, breast, and gastric carcinomas [22-25]. In agreement with its role in embryonic cell migration, TWIST1 overexpression has been linked to increased tumor cell migration, invasion, and metastasis [26, 27]. Although the action of TWIST1 has been correlated with the changes in classical EMT markers, such as epithelial cadherin (E-cadherin) and neural cadherin (N-cadherin), in gastric cancer cells, hepatocellular carcinoma, and melanoma [26-29]. However, the association of TWIST1 with SRPKs is not fully understood. This study aimed to investigate the role of SRPK2, in EMT, in colon cancer and to test whether TWIST, is involved in the process. The findings are expected, to indicate a promising therapeutic target, for colon cancer.

Materials and methods

Cell culture

Human colon cancer cell lines HCT-15, HT-39, SW620 and normal human colon epithelial cell line NCM460 were purchased from the Institute of Biochemistry and Cell Biology (Chinese Academy of Sciences, China) and maintained in a Roswell Park Memorial Institute 1640 medium (Thermo Fisher Scientific, USA) supplemented with 10% (v/v) fetal bovine serum (FBS, Gibco, USA), 100 IU/mL penicillin and 100 mg/mL streptomycin (Beyotime, China), at 37°C in a humidified atmosphere containing 5% CO_2.

Cell transfection

Cells were transfected with SRPK2 siRNA or TWIST siRNA in accordance with the manufacturer's instructions (Invitrogen, USA). Cells were harvested at 48 h. The SRPK2 siRNA sequence was 5'-UCCCAGUACAUUGGACCUAAA-3', and the TWIST siRNA sequence was 5'-CACCGGATCAAACTGGCCTGCAATTCAAGAGATTGCAGGCCAG TTTGATCC-3'. The NC siRNA, used as a control, was 5'-UCGGCUCUUACGCAUUCAA-3'. After transfection, the levels of target genes were detected by quantitative reverse transcription polymerase chain reaction (qRT-PCR), and the cells were treated with 5 ng/ml TGF-β1 (PeproTech, USA) for 24 h.

qRT-PCR

Total RNA was extracted from the cells, using TRIzol reagent (Invitrogen, USA). Using the reverse transcription kit (Promega, USA), the RNA was synthesized to cDNA. The qRT–PCR, was performed using a Stratagene MX3005P apparatus (Agilent, USA). The specific primers used for mRNA amplification were as follows: SRPK2 forward: 5'-GTATCATGTTATTAGAAAGC-3'; SRPK2 reverse: 5'-GATACTCTTCACACAACGTA-3'; TWIST forward: 5'-AGTCTTACGAGGAGCTGCAGACG-3'; TWIST reverse: 5'-AGGAAGTCGATGTACCTGGCCG-3'; glyceraldehyde-3-phosphate dehydrogenase (GAPDH) forward: 5'-CGAGATCCCTCCAAAATCAA-3'; GAPDH reverse: 5'-ATCCACAGTCTTCTGGGTGG-3'. Gene expression was normal-

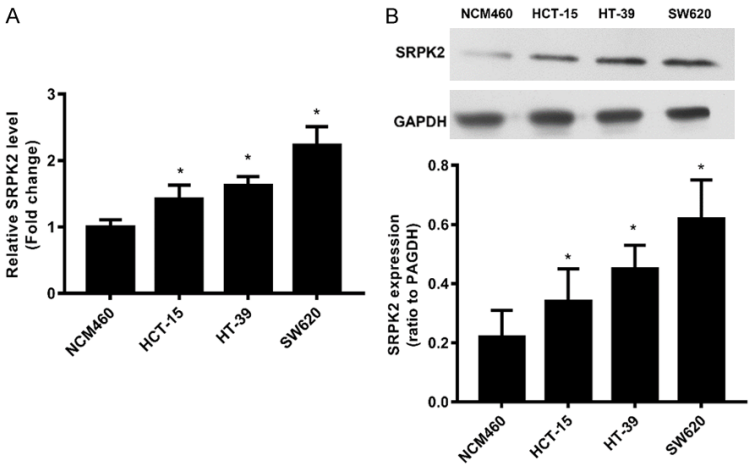

Figure 1. Expression levels of SRPK2 in normal colonic epithelial cell line NCM460 and colon cancer cell lines HCT-15, HT-39 and SW620. A. The mRNA expression levels of SRPK2 in NCM460 and colon cancer cell lines HCT-15, HT-39 and SW620 were detected by qRT-PCR. B. The level of SRPK2 protein was detected by western blot. The grayscale values were normalized to GAPDH, and presented as percentage of GAPDH. *$p < 0.05$.

ized to the level of GAPDH within each sample, using the 2-ΔΔCT methods [30].

Western blot

Following siRNA treatment, cells were pelleted and lysed in radioimmunoprecipitation (RIPA) buffer. Protein concentration was examined by the bicinchoninic (BCA) assay (Beyotime, China). Protein was separated by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) and transferred to a polyvinylidene difluoride (PVDF) membrane. Then, blots were blocked in non-fat milk for 1 h, and incubated with primary antibody overnight at 4°C. The antibodies TWIST 2c1a, SRPK2, E-cadherin and vimentin (Santa Cruz, USA), were used at 1:500 dilutions, GAPDH (Sigma-Aldrich, USA) at 1:2500 dilution; and horseradish peroxidase (HRP) conjugated anti-mouse secondary antibodies. GAPDH was used as internal control. The chemiluminescent substrate ECL Plus (Thermo Fisher), was used for protein detection. Data quantification was performed, using ImageJ software. The grayscale values were normalized to GAPDH, and presented as percentage of GAPDH.

Statistical analysis

All experiments were repeated at least three times and the statistical analysis were performed by Microsoft Excel (Microsoft, USA). The data were presented as mean ± SD. Two-tailed Student's *t* test was used for the analysis. A *p*-value < 0.05, was considered statistically significant.

Results

SRPK2 overexpressed in colon cancer cells

In order to detect the role of SRPK2 in colon cancer cell lines, the expression of SRPK2 in the normal colonic epithelial cell line NCM460 and colon cancer cell lines HCT-15, HT-39 and SW620, was monitored by qRT-PCR and western blot (**Figure 1**). Results revealed that the expression of SRPK2 in the colon cancer cell lines HCT-15, HT-39 and SW620, was significantly higher than that NCM460, at both, the mRNA and protein levels. SW620 expressed the highest SRPK2, hence, these cells were used in subsequent experiments, to investigate the role of SRPK2 in EMT.

Knockdown of SRPK2 inhibited EMT in colon cancer cells

SRPK2 was knocked out in SW620 cells, to identify its role in EMT (**Figure 2**). After transfection with SRPK2 siRNA for 48 h, the expression of SRPK2, was significantly inhibited in SW620 cells as shown in both qRT-PCR and western blot assays (**Figure 2A** and **2B**).

By inhibiting the expression of SRPK2, the expression of E-cadherin was significantly increased, whereas the expression of vimentin, was significantly downregulated (**Figure 2B**), suggesting the knockdown of SRPK2, inhibited EMT in colon cancer cells. Furthermore, knockdown of SRPK2 significantly inhibited TWIST expression, indicating the SRPK2 promotes EMT, by upregulating TWIST expression in colon cancer cells (**Figure 2B**).

Upregulation of SRPK2-induced by TGF-β-promoted EMT, was inhibited by TWIST siRNA

In order to further elucidate the role of TWIST in EMT of colon cancer cells, TWIST siRNA, was

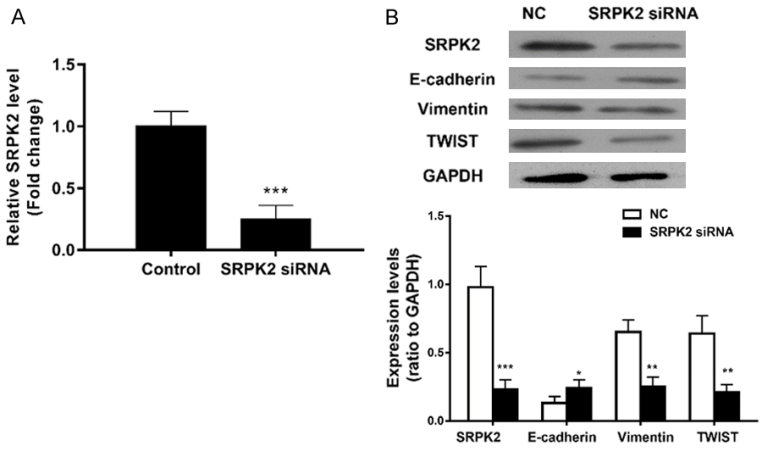

Figure 2. siSRPK2 inhibited EMT of colon cancer cells. After transfected with siSRPK2 in SW620 cells, the EMT marks including E-cadherin and Vimentin were detected by western blot, and the potential signaling pathway TWIST was also detected by western blot. A. The inhibition of SRPK2 by siSRPK2 was detected by qRT-PCR. B. The expression of SRPK2, EMT marks including E-cadherin and Vimentin, and TWIST were detected by western blot and data was quantified. The grayscale values were normalized to GAPDH, and presented as percentage of GAPDH. $*p < 0.05$, $***p < 0.001$ vs. NC.

transfected into the SW620 cells. After treatment with TGF-β for 24 h, the expression of SRPK2, E-cadherin and vimentin, were detected (**Figure 3**). Results revealed that TGF-β significantly induced the expression of TWIST, SRPK2 and E-cadherin, whereas it significantly downregulated the expression of vimentin, indicating TGF-β induced EMT in colon cancer cells, which might involve the upregulation of SRPK2 and TWIST.

A previous study showed that the overexpression of SRPK2, induces the migration and invasion of colon cancer cells [17]. In the current study, transfection of TWIST siRNA, significantly inhibited the expression of TWIST and TGF-β-induced TWIST. Knockdown of TWIST, did not significantly alter the expression of SRPK2, indicating SRPK2 is upstream of TWIST. Furthermore, knockdown of TWIST, significantly upregulated the TGF-β-inhibited expression of E-cadherin, whereas it significantly downregulated the TGF-β-induced expression of vimentin. In summary, SRPK2 might promote EMT, by upregulating TWIST expression in colon cancer cells.

Discussion

SRPK2 is elevated in many types of primary cancer including head and neck squamous cell carcinoma, hepatocellular carcinoma, and colon cancer [17, 18, 31]. SRPK2 plays an important role in cancer progression. It has been previously demonstrated that SRPK2 binds and phosphorylates acinus, an SR protein essential for mRNA splicing, and redistributes it from the nuclear speckles to the nucleoplasm, resulting in upregulation of cyclin A1, but not A2, in leukemia cells [20], thus, contributes to leukemia tumorigenesis. Pharmacological inhibition of SRPK2, can trigger early and late events of apoptosis [32]. Moreover, regulation of alternative splicing by SRPK2, could be a potential therapeutic strategy in angiogenic pathologies [21]. Inhibition of SRPK2, significantly inhibits the colony forming and invasive ability of various head and neck squamous cell carcinoma cell lines [18]. In the current study, the role of SRPK2 and, in addition, the involvement of TWIST, in the EMT of colon cancer cells were investigated.

We found that the expression of SRPK2 in colon cancer cell lines HCT-15, HT-39 and SW620, was significantly higher than that in the normal colonic epithelial cell line NCM460, at both, the mRNA and protein levels. The SW620 cells expressed the highest SRPK2, hence, these cells were used in subsequent experiments, to examine the role of SRPK2 in the EMT of colon cancer cells. After transfection with SRPK2 siRNA, for 48 h, the expression of SRPK2 was significantly inhibited in SW620 cells, and the expression of epithelial marker E-cadherin, was significantly increased, whereas the expression of mesenchymal marker vimentin, was significantly downregulated, suggesting the knockdown of SRPK2, inhibited EMT in colon cancer cells. TWIST expression, was significantly inhibited by the SRPK2 knockdown, indicating SRPK2 promotes EMT, by upregulating TWIST expression, in colon cancer cells.

TGF-β1 plays an important role in the EMT induction. It has been shown that EMT in A549 and hepG2 cells is regulated by TGF-β1 autocrine, contributing to pulmonary fibrosis or hepatocellular carcinoma metastasis [7, 33]. In

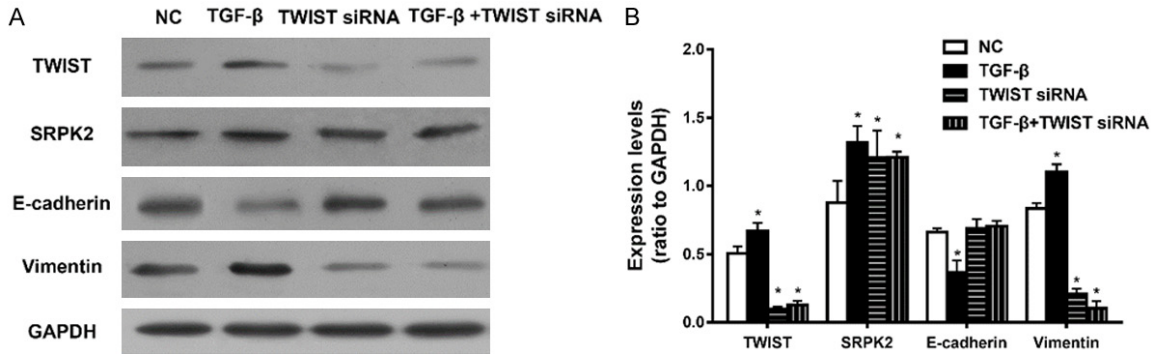

Figure 3. TGF-β induced expression of SRPK2, and its mediated EMT were suppressed by siTWIST. SW620 cells were transfected with siTWIST, and treated with TGF-β for 24 h, then, the expression of SRPK2 and the EMT marks including E-cadherin and Vimentin were detected by western blot. The grayscale values were normalized to GAPDH, and presented as percentage of GAPDH. $*p < 0.05$.

this study, TGF-β1 induced the transition from an epithelial phenotype to a mesenchymal phenotype, thereby, the epithelial cells become more migratory and less adhesive. Moreover, TGF-β1 downregulated E-cadherin, a marker for the epithelial phenotype and upregulated vimentin, a marker for the mesenchymal phenotype, in colon cancer cells.

The transcription factor TWIST1, is elevated in various types of primary tumors including colon, melanoma, prostate, breast, and gastric carcinomas [22-25]. Consistent with its role in embryonic cell migration, TWIST1 overexpression has been linked to increased tumor cell migration, invasion, and metastasis [26, 27]. The action of TWIST1, has been associated with the changes in classical EMT markers, such as E-cadherin and N-cadherin in gastric cancer cells, hepatocellular carcinoma, and melanoma [26-29]. In colon cancer cells, after treatment with TGF-β for 24 h, the expression of TWIST, SRPK2 and E-cadherin, was significantly increased, whereas the expression of vimentin was significantly downregulated, indicating that TGF-β-induced EMT in colon cancer cells, which might involve the upregulation of SRPK2 and TWIST. This scenario concurs with the previous description that overexpression of SRPK2, induces the migration and invasion of colon cancer cells [17]. Transfection of TWIST siRNA, significantly inhibited the expression of TWIST and TGF-β-induced TWIST. Knockdown of TWIST, did not significantly alter the expression of SRPK2, indicating SRPK2 is upstream of TWIST. However, TWIST knockdown, significantly upregulated the TGF-β-inhibited expression of E-cadherin, whereas it significantly downregulated the TGF-β-induced expression of vimentin. Collectively, our results demonstrated, for the first time that SRPK2 might promote the EMT, by upregulating TWIST expression in colon cancer cells, which provides novel insights into the oncogenic mechanism of SRPK2, in colon cancer and provides a novel strategy and early diagnosis of colon cancer.

Disclosure of conflict of interest

None.

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