

Research Paper

Activation of the PI3K/Akt/mTOR/p70S6K Pathway is Involved in S100A4-induced Viability and Migration in Colorectal Cancer Cells

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Abstract

The S100 protein family member S100A4 regulates various cellular functions. Previous studies have shown that elevated expression of S100A4 is associated with progression and metastasis of colorectal cancer (CRC). However, little is known about whether and how S100A4 contributes to CRC development. In our present study, the elevated expression of S100A4 in CRC tissues compared to matched adjacent normal tissues was confirmed by immunohistochemistry, semi-quantitative RT-PCR and Western blot. Adenovirus-mediated S100A4 overexpression obviously enhanced viability and migration of CRC cells, which was detected by MTT assay and transwell assay, respectively. Additionally, S100A4 overexpression increased the phosphorylation levels of Akt, mTOR and p70S6K. These effects of S100A4 were abolished by treatment with either the specific PI3K/Akt inhibitor LY294002, or the specific mTOR/p70S6K inhibitor rapamycin. Furthermore, overexpression of S100A4 resulted in upregulation of VEGF and downregulation of E-cadherin, which were strongly reversed by either LY294002 or rapamycin. Altogether, our results demonstrate that activation of the PI3K/Akt/mTOR/p70S6K signaling pathway is involved in S100A4-induced viability, migration, upregulation of VEGF and downregulation of E-cadherin in CRC cells.

Key words: colorectal cancer; S100A4; PI3K/Akt/mTOR/p70S6K; viability; migration.

Introduction

Colorectal cancer (CRC) is the most predominant malignant digestive tumor worldwide, in which distant metastasis accounts for the leading cause of mortality in CRC patients [1]. In the United States, the 5-year survival rate is about 90% in patients with local tumor, 69% in patients with regional lymph node metastasis, and 12% in patients with distant metastasis [2]. Therefore, there is an urgent need to investigate the pathological mechanism in CRC for devel-

oping therapy and prevention strategies against this malignancy.

S100A4, also known as metastasin (Mts1), p9Ka, CAPL, calvasculin, fibroblast-specific protein (FSP1), pEL-98, 18A2, and 42A [3], is a member of the low-molecular calcium binding S100 protein family, which consists of at least 21 members that possess a various biological functions including cell survival, motility, adhesion and migration [4, 5, 6]. Aberrant

expression of S100A4 was found in different types of tumors including CRC [7-11], gastric cancer [12], breast cancer [13], pancreatic carcinoma [14] and lung squamous cell carcinoma [15]. Overexpression of S100A4 has been implicated in tumor growth, angiogenesis, epithelial-mesenchymal transition (EMT), extracellular matrix remodeling and metastasis [16, 17]. Thus, S100A4 is assumed to be a marker for poor prognosis and high risk of distant metastasis [3, 7, 8]. However, the precise role and potential molecular mechanism of S100A4 in CRC tumorigenesis still remain to be fully elucidated.

The phosphatidylinositol-3-kinase/protein kinase B (PI3K/Akt) signaling pathway is associated with multiple cellular functions such as cell proliferation, differentiation and intracellular trafficking, all of which are involved in cancer development. Mammalian target of rapamycin (mTOR), an important downstream target of PI3K/Akt, positively regulates the serine/threonine kinase p70 S6 kinase (p70S6K) and eukaryotic translation initiation factor 4E binding protein 1 (4E-BP1), which constitutes a main pathway for cell proliferation, survival, differentiation and angiogenesis [18, 19]. Previous study has demonstrated that Akt, mTOR and p70S6K are significantly activated in CRC tumor tissues [20-23].

In this study, we investigated the effects of S100A4 on viability and migration in CRC cells and the potential role of the PI3K/Akt/mTOR/p70S6K signaling pathway in these functions of S100A4.

Materials and Methods

Human colorectal cancer tissue samples

Tumor tissues and matched adjacent normal mucosa tissues from five randomly clinical-diagnosed CRC patients were provided by the First Affiliated Hospital of Chongqing Medical University, Chongqing, China. No patient underwent any therapy before surgery. This study was approved by the Ethics Committee of Chongqing Medical University (protocol number 2012-19). All tumor tissue samples were acquired at the time of surgery, immediately frozen in liquid nitrogen and kept at -80°C.

Cell culture

Human CRC cell lines SW480, LoVo and human embryonic kidney cell line HEK293 were purchased from American Type Culture Collection (Manassas, VA, USA). All cells were cultured in high-glucose Dulbecco's Modified Eagle Medium (Invitrogen, CA, USA) supplemented with 10% fetal bovine serum (Sigma-Aldrich, Saint Louis, MO, USA), 100 U/ml penicillin and 100 µg/ml streptomycin (Sigma-Aldrich, Saint Louis, MO, USA) at 37°C in a humidified atmosphere of 5% CO₂.

Reagents

Adenovirus expressing S100A4 and green fluorescent protein (Ad-S100A4), and adenovirus expressing green fluorescent protein (Ad-GFP) were kindly provided by Dr. Tongchuan He (Medical Center, Chicago University, Chicago, USA). The PI3K/Akt inhibitor LY294002 and the mTOR/p70S6K inhibitor rapamycin were purchased from Sigma-Aldrich (Saint Louis, MO, USA). All antibodies used were: goat anti-S100A4 antibody (Cat#19948, Santa Cruz, CA, USA), mouse anti-β-actin antibody (Cat#47778, Santa Cruz, CA, USA), mouse anti-E-cadherin antibody (Cat#8426, Santa Cruz, CA, USA), rabbit anti-Akt antibody (Cat#4691, Cell Signaling, MA, USA), rabbit anti-p-Akt (Ser473) (Cat#4060, Cell Signaling, MA, USA), rabbit anti-mTOR antibody (Cat#2983, Cell Signaling, MA, USA), rabbit anti-p-mTOR (Ser2448) (Cat#2971, Cell Signaling, MA, USA), rabbit anti-p70S6K antibody (Cat#2708, Cell Signaling, MA, USA), rabbit anti-p-p70S6K (Thr421/Ser424) (Cat#9204, Cell Signaling, MA, USA), rabbit anti-goat IgG (Cat#2306, Zhongshan Golden Bridge, Beijing, China), goat anti-mouse IgG (Cat#2305, Zhongshan Golden Bridge, Beijing, China), goat anti-rabbit IgG (Cat#2301, Zhongshan Golden Bridge, Beijing, China).

Immunohistochemistry

The expression of S100A4 in human CRC tissues and matching adjacent normal mucosa tissues was examined by immunohistochemistry. After fixed with formalin and embedded with paraffin, the sections were deparaffinized in xylene and dehydrated in a gradually decreasing concentrations of ethanol series, then incubated in 10 mM sodium citrate buffer in a microwave oven (500 W) for 20 min. After inactivation of the endogenous peroxidase activity with 3% hydrogen peroxide at room temperature for 10 min, the sections were blocked with 10% normal goat serum for 1 h, followed by incubated with primary S100A4 antibody (1:100) overnight at 4°C. Subsequently, the sections were washed with phosphate buffered saline (PBS) three times, incubated with biotinylated secondary antibody at 37°C for 30 min, and visualized with 0.05% 3,3'-diaminobenzidine hydrochloride (DAB). Finally, the sections were counterstained with 10% hematoxylin, and photographs were taken using a microscope with a digital camera.

Semi-quantitative RT-PCR

Total RNA from human CRC tissues was isolated using Trizol Reagent (Invitrogen, CA, USA). RT-PCR was performed by using the TaqMan reverse transcription kit according to the manufacturer's instructions (TaKaRa Biotechnology, Dalian, China) and

the T100 Thermal Cycler (Bio-Rad, CA, USA). PCR products were identified by electrophoresis with 1.5% agarose gels and recorded using the Gel Doc 1000 imaging system (Bio-Rad, CA, USA). GAPDH RNA was served as an input control. The following primers were used: S100A4, forward primer: 5'-TCAGAACTAAAGGAGCTGCTGACC-3', reverse primer: 5'-TTTCTTCCTGGGCTGCTTATCTGG-3'; GAPDH, forward primer: 5'-CAGCGACACCCACTCCTC-3', reverse primer: 5'-TGAGGTCCACCACCTGT-3'.

Western blot

Human CRC tissues and CRC cell lines SW480 and LoVo were collected, and lysed in ice-cold RIPA lysis buffer (Beyotime, Shanghai, China) containing protease inhibitor and phosphatase inhibitor (Roche, Mannheim, Germany). Protein extracts were separated by 6%-15% sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) and transferred onto polyvinylidene difluoride membranes (Millipore Corporation, MA, USA). The membranes were blocked in TBST containing 5% skimmed milk for 2 h, followed by incubation with primary antibodies (1:1000) overnight at 4°C. Subsequently, the membranes were washed with TBST and incubated with horseradish peroxidase-conjugated secondary antibodies (1:5000) for 1 h. After washed with TBST, the interest proteins were detected using enhanced chemiluminescence (Millipore Corporation, MA, USA), followed by exposure on the Gel Doc 1000 Electrophoresis Documentation (Bio-Rad, CA, USA).

Amplification and infection of recombinant adenoviruses

The recombinant adenoviruses Ad-S100A4 and Ad-GFP were amplified in HEK293 cells. Ad-S100A4 was used to increase S100A4 expression in CRC cells while Ad-GFP was used as a negative control.

Cell viability assay

The CRC cells were seeded at a density of 1.5×10^3 cells per well in 96-well plates and incubated at 37°C. At 24 h, 48 h, 72 h after infection with adenoviruses, and with or without chemical inhibitors in DMEM containing 1% FBS, 20 μ l of 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium-bromide (MTT, Promega, WI, USA) was added to each well. After incubation for 4 h, the culture medium supernatant was removed and the formazan was dissolved with dimethyl sulfoxide for 10 min at room temperature. The spectrophotometric absorbance was measured at 492 nm using a microtiter plate reader (Tecan Sunrise, DE, USA).

Cell migration assay

The cell migration assay was performed with 24-well Millipore transwell chambers (Millipore Corporation, MA, USA). CRC cells (4×10^4 cells per well) at 24 h after Ad-S100A4 infection, with or without inhibitors, were trypsinized, washed, and suspended in 400 μ l of serum-free DMEM and seeded in the upper chamber. The lower chamber was filled with 600 μ l of DMEM containing 20% FBS as a chemoattractant. After incubation at 37°C for 24 h, the migrated cells on the lower surface of membrane were washed with PBS, fixed in 4% paraformaldehyde for 20 min, stained with crystal violet solution (Sigma-Aldrich, Saint Louis, MO, USA). The migrated cells were photographed and counted.

Statistical analysis

All experiments were repeated at least thrice, and the values were expressed as mean \pm standard deviation (mean \pm SD). All data were analyzed by using SPSS version 15.0 software (SPSS Inc, IL, USA). Statistical significance was evaluated using the independent sample t-test, and a value of $P < 0.05$ was considered to be statistically significant.

Results

Increased S100A4 expression in human CRC tissues

To validate that S100A4 expression is elevated in CRC, we examined the mRNA and protein levels of S100A4 in human CRC and matched adjacent normal mucosa tissues. As expected, elevated mRNA and protein levels of S100A4 were detected in CRC tissues from five randomly clinical-diagnosed CRC patients by semi-quantitative RT-PCR and Western blot, respectively (Fig. 1A, 1B). Elevated S100A4 protein expression was further confirmed by immunohistochemistry, in which S100A4 was mainly detected in the cytoplasm and cell membrane in tumor cells (Fig. 1C). In contrast, S100A4 was undetectable in matched adjacent normal tissues (Fig. 1A, 1B, 1C).

S100A4 overexpression promotes viability and migration in CRC cells

To explore the effects of S100A4 on viability and migration in CRC cells, we first infected two CRC cell lines SW480 and LoVo with Ad-S100A4 for S100A4 overexpression (Fig. 2A). While compared with the Ad-GFP group, S100A4 overexpression strongly increased the viability of SW480 and LoVo cells at 48 h and 72 h (Fig. 2B). Twenty-four hour after viral infection, the numbers of migrated SW480 or LoVo cells in S100A4 overexpression groups were much more than that in the GFP groups (Fig. 2C).

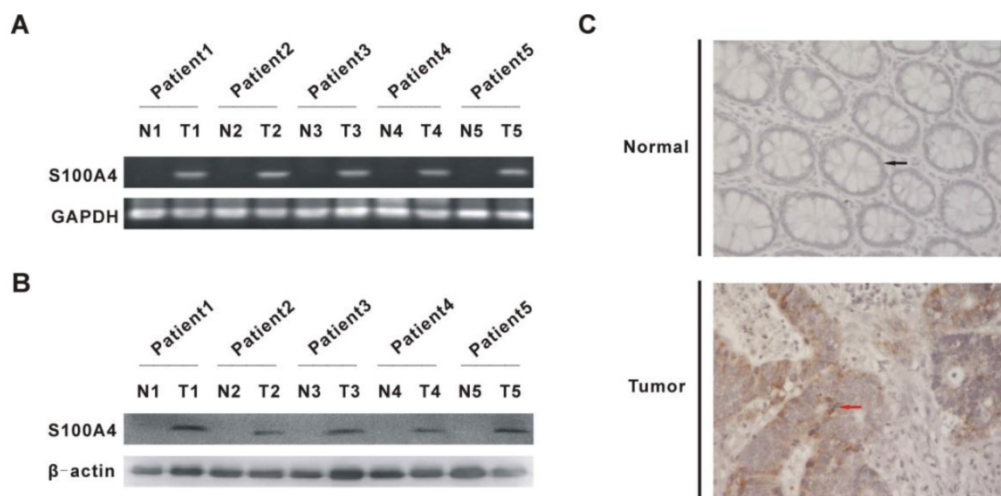


Figure 1. S100A4 expression is increased in human CRC tissues. (A) S100A4 mRNA in human CRC tissues and matched adjacent normal mucosa tissues from five randomly clinical-diagnosed colorectal cancer patients was detected by semi-quantitative RT-PCR. N, matched adjacent normal mucosa tissues; T, tumor tissues. (B) S100A4 Protein was detected by Western blot. N, matched adjacent normal mucosa tissues; T, tumor tissues. (C) S100A4 expression was detected by immunohistochemistry. Representative images are shown. The black arrow showed normal glandular epithelial cells (upper) and the red arrow showed tumor glandular epithelial cells (bottom), $\times 400$.

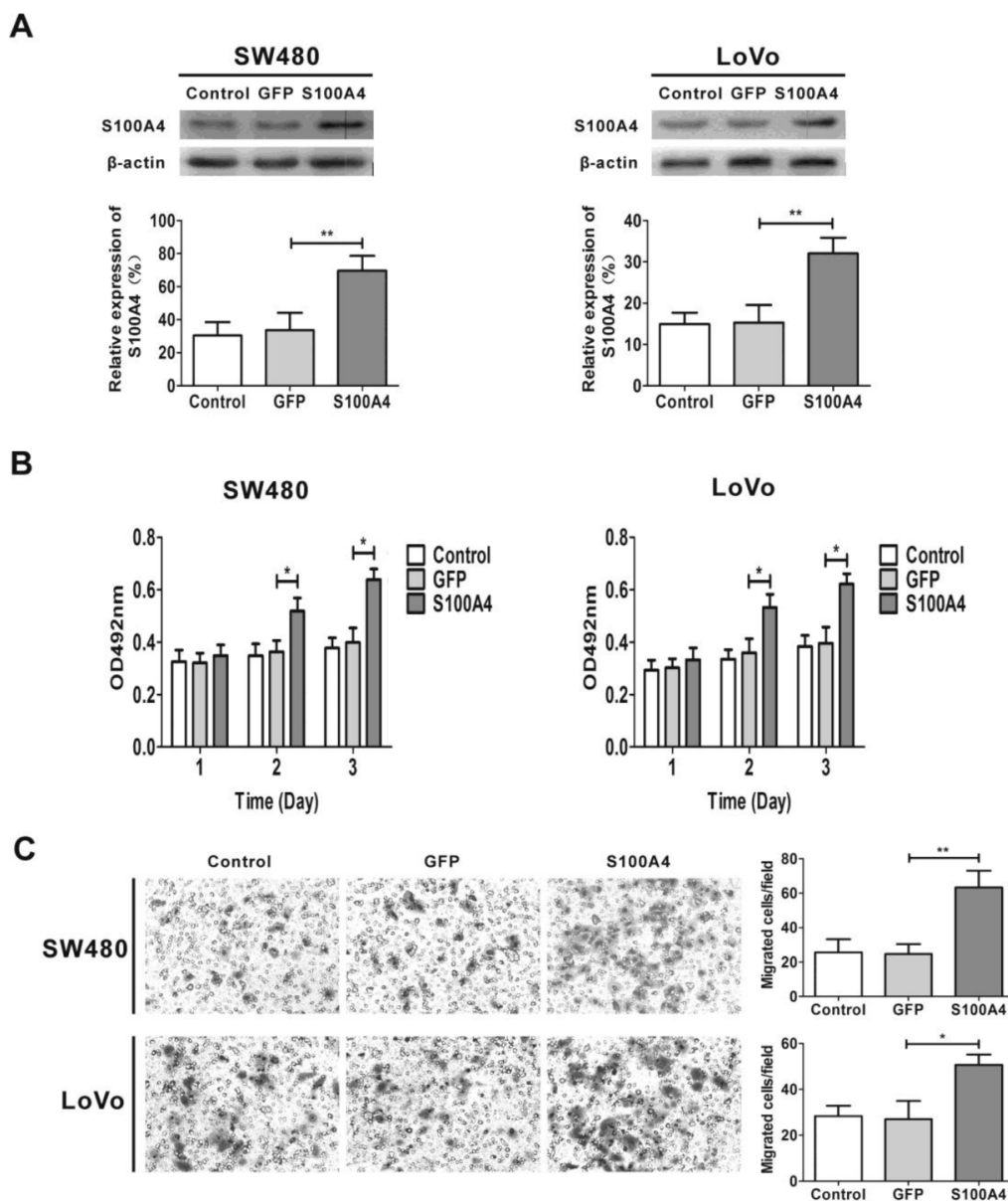


Figure 2. S100A4 promotes viability and migration in CRC cells. (A) Increased protein levels of S100A4 in SW480 and LoVo cells after infection with Ad-S100A4 were detected by Western blot. $**P < 0.01$ vs GFP group. (B) Effects of S100A4 overexpression on viability of SW480 and LoVo cells by MTT assay. $*P < 0.05$ vs GFP group. (C) Effects of S100A4 overexpression on migration of SW480 and LoVo cells by transwell migration assay, $\times 200$. $*P < 0.05$, $**P < 0.01$ vs GFP group.

S100A4 activates the PI3K/Akt/mTOR/p70S6K signaling pathway in CRC cells

To explore the signaling pathways involved in S100A4-induced viability and migration in CRC cells, we focused on the PI3K/Akt signaling pathway that plays a critical role in cancer cell viability, migration and cancer progression. Interestingly, S100A4 over-expression enhanced phosphorylation levels of Akt, mTOR and p70S6K in SW480 and LoVo cells. However, S100A4 had no significant effect on total protein levels of Akt, mTOR and p70S6K (Fig. 3A). Furthermore, the S100A4-induced phosphorylation levels of Akt, mTOR and p70S6K were suppressed by LY294002 (10 μ M), while rapamycin (100nM) only reduced the phosphorylation levels of mTOR and p70S6K (Fig. 3B).

S100A4 induces cell viability and migration via the PI3K/Akt/mTOR/p70S6K signaling pathway

Because S100A4 induced viability and migration of CRC cells and activated the PI3K/Akt/mTOR/p70S6K signaling pathway, we proceeded to examine whether the activation of the PI3K/Akt/

mTOR/p70S6K signaling pathway is involved in S100A4-induced cell viability and migration in CRC cells. Ad-S100A4-infected SW480 and LoVo cells were treated with the LY294002 or rapamycin, and then cell viability and migration were measured. We found that both LY294002 and rapamycin significantly suppressed S100A4-induced cell viability and migration (Fig. 4A, 4B).

S100A4 regulates expression of VEGF and E-cadherin via the PI3K/Akt/mTOR/p70S6K signaling pathway

Previous studies showed that the expression of VEGF and E-cadherin was regulated by the PI3K/Akt/mTOR signaling pathway, and VEGF and E-cadherin are involved in viability and migration of various tumor cells [44, 45, 47]. We next examined the effect of S100A4 on the expression of VEGF and E-cadherin. The results showed that S100A4 overexpression obviously increased VEGF expression and decreased E-cadherin expression in SW480 and LoVo cells (Fig. 5A), which were abolished by treatment with either LY294002 or rapamycin (Fig. 5B).

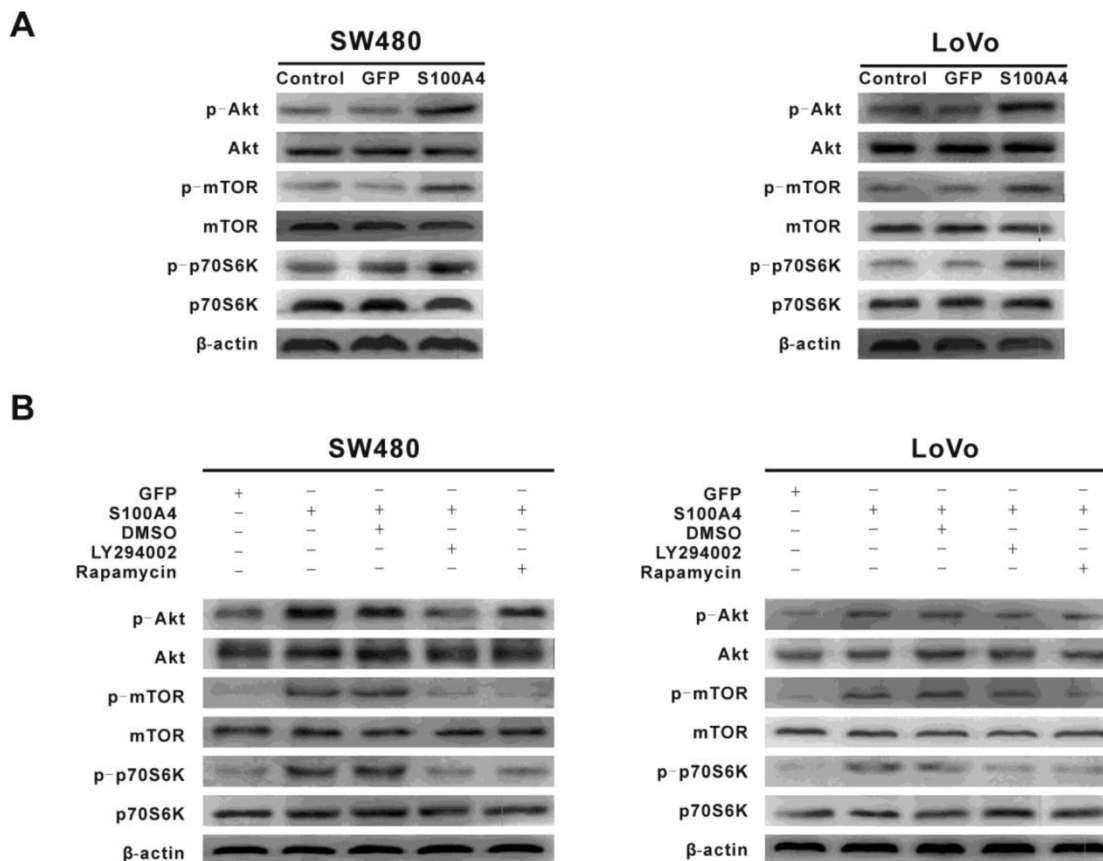


Figure 3. S100A4 activates the PI3K/Akt/mTOR/p70S6K pathway in CRC cells. (A) Increased phosphorylation levels of Akt, mTOR, p70S6K in Ad-S100A4-infected SW480 and LoVo cells were detected by Western blot. (B) Effects of LY294002 and rapamycin on S100A4-induced activation of Akt, mTOR, p70S6K in SW480 and LoVo cells.

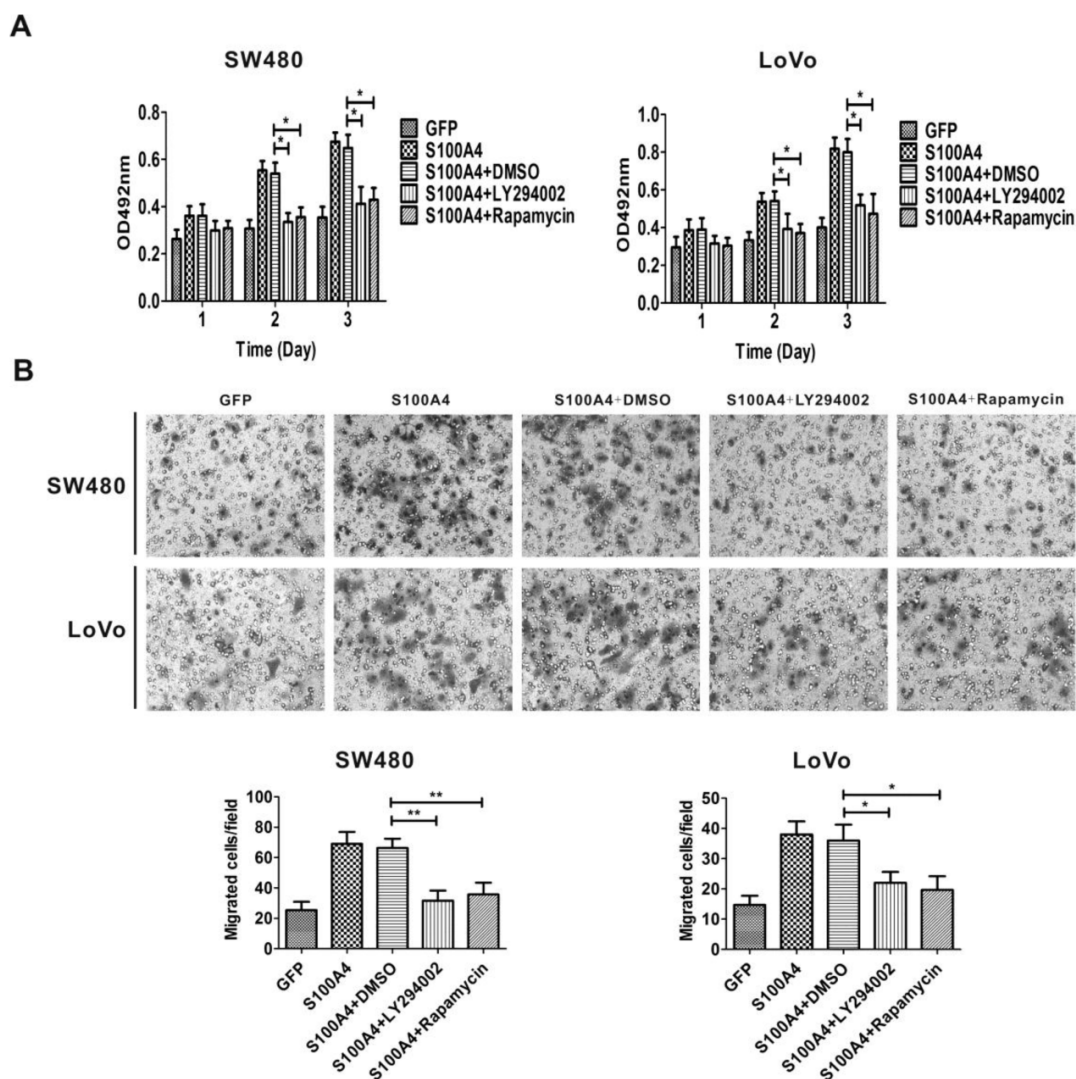


Figure 4. LY294002 and rapamycin suppress S100A4-induced viability and migration of CRC cells. (A) Effects of LY294002 and rapamycin on S100A4-induced viability of SW480 and LoVo cells were detected by MTT assay. * $P < 0.05$ vs S100A4 group. (B) Effects of LY294002 and rapamycin on S100A4-induced migration of SW480 and LoVo cells were detected by transwell assay, $\times 200$. * $P < 0.05$, ** $P < 0.01$ vs S100A4 group.

Discussion

In this study, we confirmed that S100A4 expression is upregulated in CRC tissues and that S100A4 overexpression promotes viability and migration of CRC cells. In addition, S100A4 induced upregulation of VEGF expression and downregulation of E-cadherin expression. We also provided evidence showing that activation of the PI3K/Akt/mTOR/p70S6K signaling pathway is involved in S100A4-induced cell viability, migration, and VEGF upregulation and E-cadherin downregulation in CRC cells. These results suggest that activation of the PI3K/Akt/mTOR/p70S6K signaling pathway is involved in the oncogenic property of S100A4.

Over the past decades, molecular genetic studies have revealed gene alterations including a number of well-known oncogenes and tumor suppressors underlying the pathogenesis mechanism of CRC [24, 25]. Substantial clinical evidences have implied S100A4 as

an important gene associated with CRC progression, metastasis, and survival [7, 8, 10]. Consistent with previous reports, mRNA and protein expression of S100A4 in human CRC tissues were obviously increased. Accumulating evidences suggest that S100A4 modulates a wide range of cellular processes ranging from angiogenesis to cell motility, adhesion, migration, and invasion [9]. For instance, S100A4 regulates the interaction between myosin and actin to impact rearrangement of the cytoskeleton [26]. Several studies have reported that S100A4 contributes to cell invasion and angiogenesis through upregulation of matrix metalloproteinases (MMPs) [27-29]. Additionally, downregulation of S100A4 expression in colorectal cancer cells suppressed cell growth, invasion and metastasis formation [30, 31]. In agreement with these studies, our data showed that CRC cells SW480 and LoVo with S100A4 overexpression had significantly increased viability and migration.

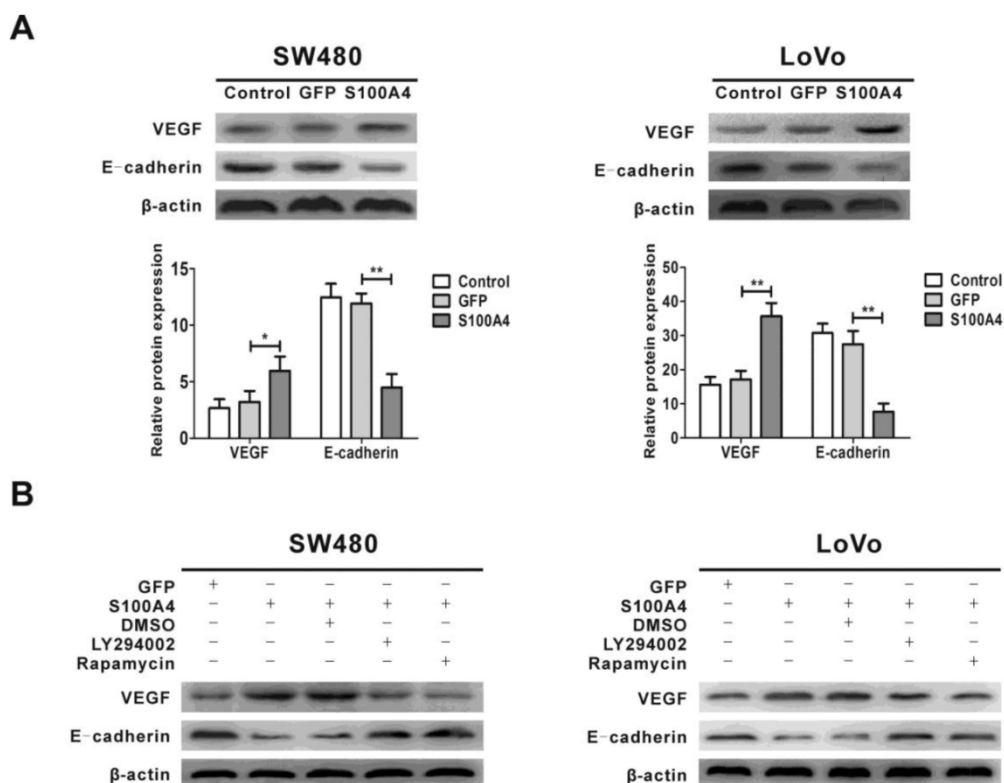


Figure 5. Regulation of VEGF and E-cadherin expression by S100A4 involves the PI3K/Akt/mTOR/p70S6K pathway in CRC cells. (A) Effects of S100A4 on protein levels of VEGF and E-cadherin in SW480 and LoVo cells were detected by Western blot. * $P < 0.05$, ** $P < 0.01$ vs GFP group. (B) Effects of LY294002 and rapamycin on S100A4-induced protein levels of VEGF and E-cadherin in SW480 and LoVo cells were detected by Western blot.

It is well known that vascular endothelial growth factor (VEGF) is one of the most potent endothelial cell mitogens [32]. It not only increases formation of vascular capillary lumens, but also promotes cancer cell proliferation, invasion, and subsequent metastasis [33]. Previous studies have shown the overexpression of VEGF in human CRC, and VEGF could be a prominent angiogenic factor for induction of angiogenesis during CRC development [34, 35]. In transgenic mice, S100A4 has been shown to be a potent stimulator of angiogenesis [36]. In gastric carcinoma, positive correlation between expression of S100A4 and VEGF was found, which was associated with lymph node metastasis and prognosis [37]. S100A4 silencing suppressed proliferation, angiogenesis and invasion in thyroid cancer cells, which was associated with downregulation of VEGF [38]. High expression of S100A4 and VEGF were concurrently detected in colorectal cancer [39]. Our data showed that VEGF was upregulated by S100A4 overexpression in CRC cells through activation of the PI3K/Akt/mTOR/p70S6K pathway. On the other hand, VEGF is recognized as a potent PI3K/Akt activating factor [40]. While how S100A4 activates the PI3K/Akt/mTOR/p70S6K pathway is currently unclear, it is interesting to explore the possibility in future study that a VEGF autocrine establishes a positive feedback loop for S100A4-induced activation of this pathway and CRC

cell viability and migration.

A possible mechanism by which S100A4 induces migration of cancer cells is to inhibit cell adhesion [41]. E-cadherin plays an important role in cell adhesion [42]. It has been reported that S100A4-mediated esophageal squamous cell invasion and metastasis involving E-cadherin downregulation [43]. Increased S100A4 expression combined with decreased E-cadherin expression was associated with tumor progression, distant metastasis and survival in CRC [44]. In the present study, we found that S100A4 reduced E-cadherin expression in CRC cells SW480 and LoVo cells. Downregulation of E-cadherin expression induced by S100A4 may inhibit intercellular adhesion, thus contributing to tumor cells' migration phenotype [45].

The PI3K/Akt signaling pathway plays a pivotal role in cell proliferation, apoptosis, survival, migration, invasion and metastasis [46]. A number of studies suggest that aberrant activation of the PI3K/Akt signaling pathway is associated with human CRC [47, 48]. The mTOR kinase lies downstream of the PI3K/Akt signaling pathway and facilitates phosphorylation of p70S6K and 4E-BP1 [49]. Previous findings demonstrated frequent changes of the PI3K/Akt/mTOR pathway in human CRC [50, 51]. The PI3K/Akt/mTOR pathway regulates VEGF expression in different tumors for angiogenesis [52, 53].

In human esophageal squamous cells S100A4 regulates E-cadherin expression through Akt [54]. In this report, we found that the phosphorylation levels of Akt, mTOR and p70S6K were activated by S100A4 overexpression and suppressed by the PI3K/Akt inhibitor LY294002 in SW480 and LoVo cells. The mTOR/p70S6K inhibitor rapamycin only inhibited the phosphorylation levels of mTOR and p70S6K. Moreover, inhibitor of PI3K or mTOR activity blocked S100A4-induced viability, migration, VEGF upregulation and E-cadherin downregulation of CRC cells. These results establish that S100A4 induces viability and migration in CRC cells through the PI3K/Akt/mTOR/p70S6K signaling pathway.

Altogether, our results suggest that activation of the PI3K/Akt/mTOR/p70S6K pathway contributes to S100A4-induced viability and migration in CRC cells. Therefore, this S100A4-mediated colorectal carcinogenesis signaling pathway could be a therapeutic and preventive target against CRC.

Acknowledgments

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Competing Interests

The authors have declared that no competing interest exists.

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