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ORIGINAL ARTICLE

Special AT-rich sequence-binding protein 1 promotes cell growth and metastasis in colorectal cancer

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Abstract

AIM: To evaluate the expression of special AT-rich sequence-binding protein 1 (*SATB1*) gene in colorectal cancer and its role in colorectal cancer cell proliferation and invasion.

METHODS: Immunohistochemistry was used to detect the protein expression of SATB1 in 30 colorectal cancer (CRC) tissue samples and pair-matched adjacent non-tumor samples. Cell growth was investigated after enhancing expression of SATB1. Wound-healing assay and Transwell assay were used to investigate the impact of SATB1 on migratory and invasive abilities of SW480 cells *in vitro*. Nude mice that received subcutaneous implantation or lateral tail vein were used to study the effects of SATB1 on tumor growth or metastasis *in vivo*.

RESULTS: SATB1 was over-expressed in CRC tissues and CRC cell lines. SATB1 promotes cell proliferation and cell cycle progression in CRC SW480 cells. SATB1 overexpression could promote cell growth *in vivo*. In addition, SATB1 could significantly raise the ability of cell migration and invasion *in vitro* and promote the ability of tumor metastasis *in vivo*. SATB1 could up-regulate matrix metalloproteases 2, 9, cyclin D1 and vimentin, meanwhile SATB1 could down-regulate E-cadherin in CRC.

CONCLUSION: SATB1 acts as a potential growth and metastasis promoter in CRC. SATB1 may be useful as a therapeutic target for CRC.

Key words: Special AT-rich sequence-binding protein 1; Colorectal cancer; Proliferation; Migration; Invasion

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INTRODUCTION

The special AT-rich sequence-binding protein 1 (SATB1),

which locates at human chromosome 3p23, is a thymocyte-specific matrix association region-binding protein that links specific DNA elements to its unique cage-like network[1]. Phosphorylation of SATB1 serves as a molecular switch in determining whether it acts as a transcriptional activator or repressor[2]. SATB1 is predominantly expressed in thymocytes and regulates the spatiotemporal expression of numerous genes that involved in T cell proliferation, development, and differentiation[3]. SATB1 has recently attracted considerable attention in cancer research and its overexpression is a frequent event in various cancers, such as breast cancer, laryngeal cancer, gastric cancer and liver cancer[4-9]. Furthermore, accumulating evidence showed that SATB1 is also associated with tumor growth and metastasis[4-6,8-10]. Han *et al*[5] found that SATB1 up-regulated the expression of matrix metalloproteases (MMP)2, MMP9 and down-regulated E-cadherin in breast cancer. On the other hand, SATB1 depletion blocks the up-regulation of E-cadherin and extracellular matrix (ECM) protein vimentin. Meng *et al*[11] showed that SATB1 plays a pivotal role in epithelial to mesenchymal transition (EMT) process and promotes liver cancer invasion. Only one study suggested that SATB1 is over-expressed in human rectal cancer and the expression of SATB1 is associated with clinicopathological parameters, including invasive depth and tumor-node-metastasis (TNM) stage in rectal cancer. Despite its importance, the roles and mechanisms of SATB1 in growth and metastasis of human colorectal cancer (CRC) remain poorly understood.

CRC is the third most common malignancy and the fourth cause of cancer mortality in the world[12-14]. Although novel molecule-based therapies including monoclonal antibodies are currently widely used in the treatment of CRC, many patients with CRC still die from disease recurrence and metastasis[15,16]. Consequently, further elucidation of the molecular mechanisms of CRC will be beneficial for developing novel therapeutic strategies to conquer this disease.

In this study, we analyzed the expression of SATB1 in CRC tissues and found that it was over-expressed in the cancerous tissue samples compared with the normal adjacent tissue samples. We also carried out *in vitro* and *in vivo* functional analysis of SATB1 by ectopical SATB1 expression in SW480 CRC cells. Further investigations focused on the regulation of SATB1 in potential downstream molecules MMPs (MMP2, MMP9), cyclin D1 (CCND1), E-cadherin and vimentin.

MATERIALS AND METHODS

Cell lines and plasmids

Human CRC cell lines SW480, SW620, RKO, HT29, HCT116 and Lovo were obtained from Shanghai Institute of Cell Biology (Shanghai, China) and were cultured in RPMI 1640 medium (Invitrogen, Carlsbad, CA, United States), supplemented with 10% fetal bovine serum. The pcDNA3.1 (Invitrogen, Carlsbad, CA, United States) was used to construct a SATB1 over-expressing plasmid. DNA fragment with mature SATB1 or a negative control sequence was inserted to this vector. Stable transfection of the plasmids was carried out using Lipofectamine2000 (Invitrogen, Carlsbad, CA, United States) according to the manufacturer's instruction.

Immunohistochemistry and immunoblot analysis

Paraffin-embedded tumors and paired normal tissue samples were obtained from 30 CRC patients with the approval from the Ethics Committee of the Second Hospital of Zhejiang University Medical College. Immunohistochemical (IHC) analyses were performed on 3-μm, formalin-fixed and paraffin-embedded sections. Primary antibodies for SATB1 were diluted at 1:250 (BD Biosciences, California, United States) for IHC[17,18]. For immunoblot analysis, 20 g total cellular protein was loaded per lane, separated by 4%-12% SDS-polyacrylamide gel electrophoresis, and then transferred to nitrocellulose (Invitrogen, Carlsbad, CA, United States) by electroblotting. The membranes were incubated with either SATB1 antibody (diluted 1:1000; BD Biosciences, California, United States) or α-tubulin antibody (diluted 1:200; Santa Cruz Biotechnology) at 4 ℃ overnight[19].

Cell proliferation assay and colony formation assay

Cell proliferation assay was determined by standard 3-(4,5-cimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide (MTT) assays. Briefly, the cells were seeded at a density of 2×10^3 cells per well in 96-well culture plates (Costar). Cell proliferation was assessed 24, 48 and 72 h later. One-tenth volume of 5 mg/mL MTT was added to each well, and the plate was further incubated at 37 ℃ for another 4 h; thereafter, the medium was replaced and the formazan crystals formed were dissolved in 150 μL dimethyl suophoxide with oscillation for 10 min. The optical density was determined with a multiwell spectrophotometer (BioTek, VT, United States) at 570 nm. Absorbance values were presented as percentages relative to untreated controls. The MTT assays were repeated at least three times[20]. For colony formation assay, cells were trypsinized and counted. One hundred cells were seeded in six-well plates. After 2 wk of growth, colonies with a diameter greater than 4 mm were counted. Experiments were performed in quadruplicate[21].

Scratch wound healing assay

Scratch wound healing assay was performed as previously described. Briefly, transfected cells in 6-well plates were cultured until cells reached confluence and starved overnight. Cell layers were wounded using a 200 μL pipette tip and cultured for another 48 h. Photographs were taken at time 0, 48 and 72 h[22].

Cell migration and invasion assay

A transwell cell migration and Matrigel invasion assay was used to investigate the impact of SATB1 on migratory and invasive ability of SW480 cells. For migration detec-

tion, transfected cells were placed in transwell Chamber at 2×10^4 cells/well. The lower transwell chamber contained 10% fetal bovine serum for use as a chemoattractant. For invasion assay, the bottom of the culture inserts (8-mm pores) were coated with 30 μL of the mixture containing serum-free RPMI-1640 and Matrigel (1:8; BD Biosciences, Bedford, MA, United States). The Matrigel was allowed to solidify at 37 ℃ overnight. After solidification, cells (2×10^4 cells/well) were reseeded onto the upper chamber. Twenty-four hours later, the cells that had migrated or invaded through the membrane were fixed with 95% alcohol and stained with crystal violet. The number of migrated cells or invaded cells was quantified by counting 5 independent symmetrical visual fields under microscope[23].

Xenograft studies

Cells of 2×10^4 were harvested, washed and resuspended in 200 mL phosphate-buffered saline, and was subcutaneously injected into the flanks of 5-wk-old female nude mice. Animal experimental procedures were performed strictly in accordance with the related ethics regulations of our university. Tumor sizes were measured in two dimensions with calipers every week. Tumor volumes (mm^3) were calculated using the following formula: V = (length × width^2)/2[24]. For *in vivo* metastasis assays, SW480-SATB1 cells or SW480-negtive control (SW480-NC) cells were transplanted into nude mice (5-wk-old BALB/c-nu/nu, ten per group, 1×10^6 cells for each mice) through the lateral tail vein. Mice were killed after 10 wk. The lungs were dissected and subjected to hematoxylin and eosin staining. The numbers of metastases in the lungs were examined histologically.

Real-time polymerase chain reaction analysis

Total RNA was extracted from cells expressing SATB1 and negative control cells with Trizol (Invitrogen, Carlsbad, CA, United States). The expression of CCND1, E-cadherin, vimentin, MMP2 and MMP9 was detected by quantitative real-time polymerase chain reaction (PCR). The primers are as follows: CCND1, the forward primer 5'-TATTGCGCTGCTACCGTTGA-3' and the reverse primer 5'-CCAATAGCAGCAAACAATGTGAAA-3'; MMP2, the forward primer TCTTCAAGGACCGGTTCATTTG and the reverse primer GATGCTTCCAAACTTCACGCTC; MMP9, the forward primer CACTGTCCACCCCTCAGAGC and the reverse primer GCCACTTGTCGGCGATAAGG; E-cadherin, the forward primer 5'-TGCCCAGAAAATGAAAAAGG-3' and the reverse primer 5'-GTGTATGTGGCAATGCGTTC-3'; Vimentin, the forward primer 5'-TGGCCGACGCCATCAACACC-3' and the reverse primer 5'-CACCTCGACGCGGGCTTTGT-3'; β-actin was used as an internal control. The primers for β-actin were 5'-TGACGGGGTCACCCACACTGTGCCCATCT-3' and 5'-GAAGTAGTAAGTGGGAACCGTGT-3'. Real-time PCR was performed using the SYBR® Green (Invitrogen) dye detection method on ABI PRISM 7900 HT Sequence Detection System under default conditions: 95 ℃ for 10 min, and 35 cycles of 95 ℃ for 15 s and 55 ℃ for 1 min. Comparative C_t method was used for quantification of the transcripts[25].

Statistical analysis

Each experiment was repeated at least 3 times. All results were expressed as mean ± SD. The difference between means was analyzed with Student's *t* test or the χ^2 test. All statistical analysis were performed using SPSS 16.0 software (Chicago, IL, United States). Differences were considered significant when $P < 0.05$[26].

RESULTS

Expression of SATB1 increased in CRC tissue samples

To assess the role of SATB1 in CRC, we examined the protein expression of SATB1 in 30 human CRC tissue samples and pair-matched adjacent non-tumor tissue samples by IHC. We observed positive immunoreactivities in CRC in 53% (16 of 30) of cancer tissue samples, compared with only 10% (3 of 30) in the adjacent mucosa tissue cells. The representative examples of IHC staining results are shown in Figure 1A. Statistical analysis using Pearson χ^2 (df = 1, two-sided) indicates that the difference in SATB1 expression between cancer and adjacent tissues was significant ($P < 0.01$) (Table 1). Western blot analysis of SATB1 in established CRC cell lines showed that the expression level of SATB1 in SW620 was higher in the SW480 (Figure 1B). SW480 and SW620 were a matched pair of primary and metastatic population of cells from the same patient[27]. SW620 cells were derived from the metastasis lymph node of Dukes' type C colorectal adenocarcinoma.

SATB1 expression promotes CRC cell proliferation in vitro

In order to investigate the role of SATB1 in CRC carcinogenesis, we tested the effect of SATB1 on the proliferation of SW480 cells. We established stable SATB1 expressing CRC cells. As shown in Figure 2A, SATB1 levels were higher in cells stably expressing SATB1 than the negative control cells. MTT assay showed that introduction of SATB1 caused a remarkable promotion of cell proliferation in SW480 cells ($P < 0.05$; Figure 2B). Furthermore, expression of SATB1 in SW480 cells significantly enhanced the numbers of colony formation. As shown in Figure 2C, the colony number for the negative control cells was 55.3 ± 5.0, while that for the SATB1 overexpression was 23.7 ± 3.2 ($P = 0.018$). To clarify the mechanisms underlying growth promotion by SATB1 in CRC cell lines, we performed cell-cycle analysis using flow cytometry on the cells stained with propidium iodide. SW480-SATB1 cells showed a higher proportion of cells in S phase (17.59%), compared with the control cells (13.02% for SW480-NC cells) (Figure 2D).

SATB1 promotes tumorigenesis potential in vivo

In order to assess the role of SATB1 on CRC tumorigenesis *in vivo* , equal numbers of SW480-SATB1 cells and

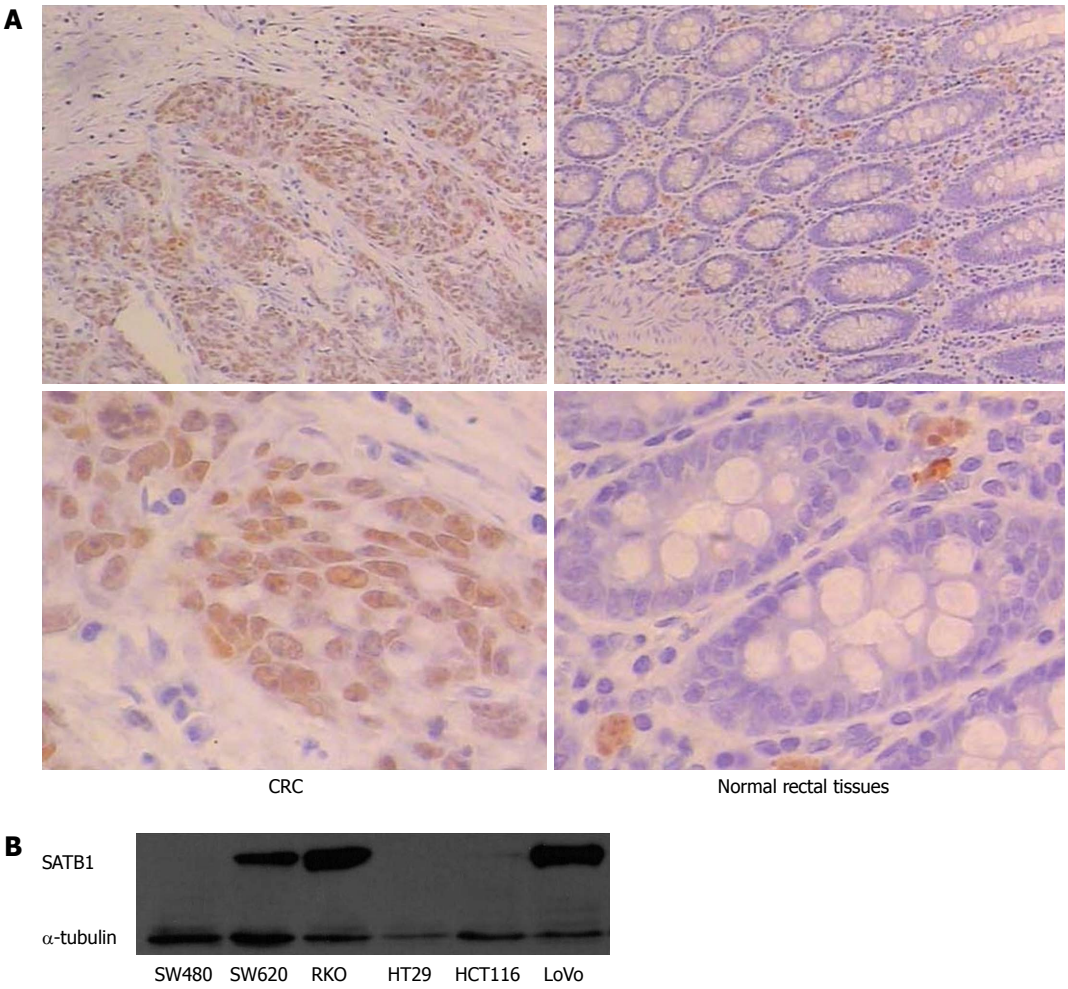

Figure 1 Upregulation of special AT-rich sequence-binding protein 1 in human colorectal cancer. A: Immunostaining for SATB1 protein in tissue of carcinomas and adjacent normal tissue mucosa. Top: Representative pictures of carcinomas (left) and normal tissue (right); B: Western blot detection of SATB1 protein in different colorectal cancer cell lines. SATB1: Special AT-rich sequence-binding protein 1; CRC: Colorectal cancer.

Table 1 Summary of the immunohistochemistry findings

	Cancer tissues	Normal tissues
Total number of samples	30	30
Samples with SATB1 expression in nucleus	16	3

SATB1: Special AT-rich sequence-binding protein 1.

SW480-NC cells were implanted onto flanks of 5-wk-old female nude mice, and the growth of the implanted tumors was measured at weeks 1-4. The results indicated that SW480 cells with enhanced SATB1 expression could promote the growth of subcutaneous tumors (Figure 2E) ($P < 0.01$).

SATB1 promotes CRC cell migration and invasion in vitro and in vivo

Wound-healing assay was performed to examine the effect of SATB1 expression on cell migration. We found that SW480-SATB1 cells healed the scratch wound earlier than negative controls cells (Figure 3A). We also estimated the effects of SATB1 on the migration and invasion of SW480 cells using a Transwell cell migration and Matrigel invasion assay. The data demonstrated that the overexpression of SATB1 markedly promoted the migration and invasion of SW480 cells. The number of SW480-SATB1 cells (307 ± 20, $P < 0.0001$) that had migrated through the membrane without Matrigel was significantly higher than that of SW480-NC cells (104 ± 20) (Figure 3B). A similar result was found with the invaded cells; the number of SW480-SATB1 cells (237 ± 19, $P < 0.0001$) passing through the Matrigel was significantly higher than that of SW480-NC cells (82 ± 10) (Figure 3B). To further explore the effects of SATB1 on tumor metastasis *in vivo*, SW480-SATB1 cells or SW480-NC cells were transplanted into nude mice through the lateral tail vein. Histological analysis of the lung of mice confirmed that SATB1 could promote lung metastasis formation. Lung metastasis of SW480 cells was apparent in mice injected with SW480-SATB1 cells (Figure 3C). In contrast, few metastatic tumors were detected in mice injected with SW480-NC cells (Figure 3C). Our results indicate that SATB1 could promote CRC cell metastasis *in vivo*.

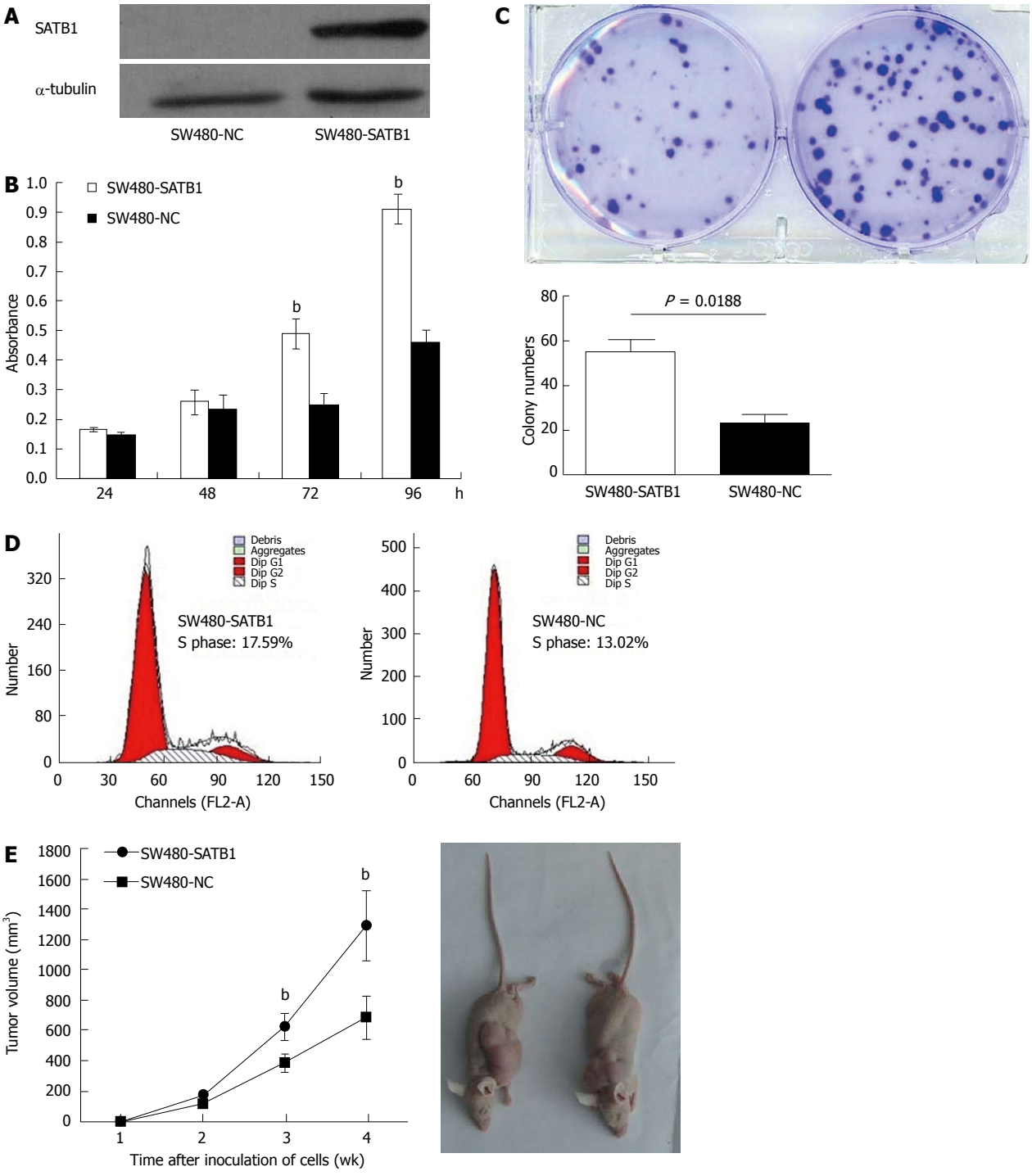

Figure 2 Special AT-rich sequence-binding protein 1 promotes cell growth and *in vivo* tumorigenesis potential in SW480 cells. A: Western blot analysis of special AT-rich sequence-binding protein 1 (SATB1) protein expression in colorectal cancer (CRC) cells that stably expressing SATB1 and SW480 negative control (NC) cells; B: Effect of SATB1 overexpression on cell proliferation by 3-(4,5-cimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide assay. $^{b}P < 0.01$ *vs* NC cells; C: Colony formation assays for SW480-SATB1 cells and NC cells. Data are representatives of three independent experiments; D: Growth rates of SW480-SATB1 cells and negative control cells in an *in vivo* mouse model. Volumes of tumors were monitored every week. Bottom: Representative pictures of tumor samples. $^{b}P < 0.01$ *vs* NC cells.

SATB1 induces proliferation and metastasis related gene expression change in SW480 cells

We detected the potential downstream molecules regulated by SATB1 *via* real-time PCR analysis to probe into the possible mechanism that SATB1 promotes CRC cell proliferation and metastasis. The results showed that the expression of CCND1 was up-regulated in SW480 cells with enhanced SATB1 expression. We also found that MMP2 and MMP9, the major MMPs that have a key role in the proteolytic cascade-leading ECM cleavage during metastasis in colon carcinoma, were up-regulated in SW480-SATB1 cells (Figure 3D). In addition, the expression of

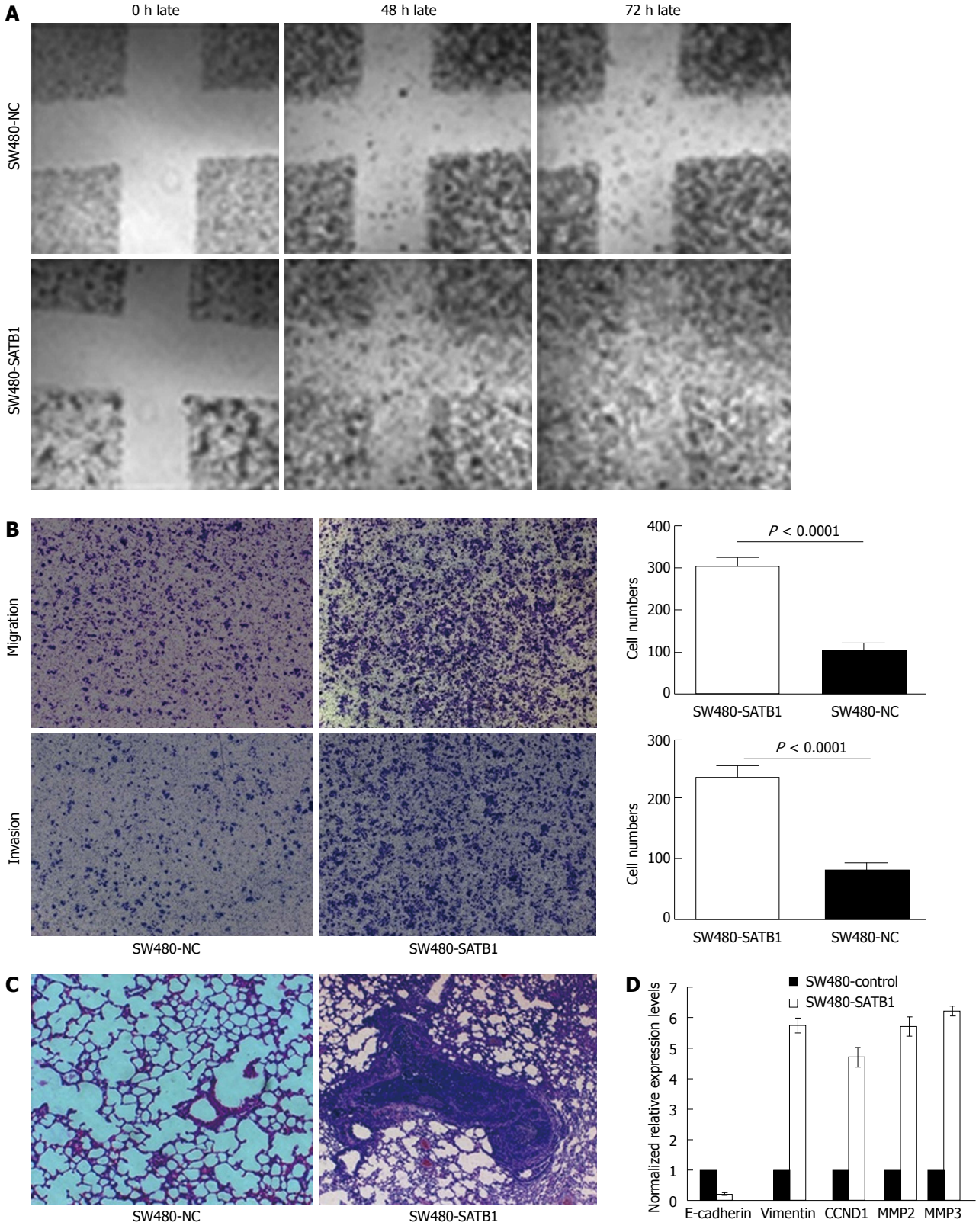

Figure 3 Special AT-rich sequence-binding protein 1 promotes migratory property of SW480 cell *in vitro* and *in vivo* metastasis potential. A: A wound-healing assay of SW480-special AT-rich sequence-binding protein 1 (SATB1) cells and negative control (NC) cells. Photographs were taken at the time of 0, 48 and 72 h. Representative photos from one of three replicate experiments are shown (40× original magnification); B: Representative photo-micrographs of Transwell results for SW480-SATB1 cells and NC cells were taken (40× original magnification). The number of SW480-SATB1 cells passing through the membrane with or without Matrigel was significantly lower than that of NCs; C: Representative hematoxylin and eosin stained sections of the lung tissues isolated from mice implanted with SW480-SATB1 cells and NC cells through the lateral tail vein. The data shown are the number of lung metastases from each group; D: A bar chart showing downstream molecules regulated by SATB1 using real-time polymerase chain reaction in SW480-SATB1 cells and NC cells. MMP: Matrix metalloproteases; CCND1: Cyclin D1.

EMT related gene vimentin was increased and E-cadherin was decreased in SW480-SATB1 cells (Figure 3D).

DISCUSSION

Previous studies have suggested the important role of SATB1 in tumor growth and metastasis. But there have been few researches on the relationship between SATB1 and CRC. More recently, Meng *et al*[11] reported that high level of SATB1 expression was closely correlated with invasive depth and TNM stage in 93 paired samples of human rectal cancer. However, the effects of SATB1 on CRC remain poorly understood. In this study, we showed that overexpression of SATB1 in CRC tissue samples and cell lines could promote cell growth *in vitro* and *in vivo*. In addition, SATB1 could significantly increase the ability of cell migration and invasion *in vitro* and promote the ability of tumor metastasis *in vivo*. We further showed that SATB1 could up-regulate MMPs 2, 9 and vimentin, meanwhile SATB1 could down-regulate E-cadherin in CRC. The data from the current study suggested that SATB1 acts as a potential growth and metastasis promoter in CRC.

Immunohistochemical results showed that the SATB1 protein was overexpressed in CRC tissues and was localized in the nuclei of cancer cells. We also found that SATB1 was overexpressed in cell lines derived from CRC. Our finding is consistent with a recent report showing that the expression of SATB1 was increased in rectal cancer and cell lines[11]. These data prompted us to analyze the functional effects of SATB1 in CRC cells. We found that SATB1 promotes cell proliferation and cell cycle progression in CRC SW480 cells. In addition, SATB1 expression could promote cell growth *in vivo*. These results suggested that SATB1 may play a tumor promoter role in CRC carcinogenesis.

Invasion and metastasis are the most influential factors for clinical outcome of CRC. Recent studies have shown that SATB1 contributes to tumor metastasis in many types of tumors, such as breast cancer, gastric cancer, and liver cancer[5,7-9]. Up to date, there was only one report about SATB1 expression and clinical feature in rectal cancer which found that high levels of SATB1 expression were closely correlated with invasive depth and TNM stage in human rectal cancer samples[11]. This report suggested that SATB1 may facilitate CRC metastasis. In this study, we found that ectopical SATB1 expression endows the non-aggressive SW480 cells with a capability of migration and invasion *in vitro* and metastasis *in vivo*. So we consider that SATB1 may play a crucial role in promoting cancer invasion and metastasis in CRC.

MMP2 and 9, which degrade ECM and promote tumor invasion[28,29], were up-regulated in the SW480-SATB1 cells that ectopically expressed SATB1. We also found up-regulation of vimentin and down-regulation of E-cadherin in mRNA level in the SW480-SATB1 cells. As a genome organizer, SATB1 recruits chromatin remodeling factors and regulates the spatiotemporal expression of numerous genes involving tumor growth and metastasis. SATB1 has been found to promote breast tumor metastasis and reprograms the genome to change the expression profiles consistent with invasive tumors[5]. MMP2 and MMP9 are gelatinases that belong to multigene family of proteolytic enzymes[30]. MMP2 and MMP9 are capable of degrading essentially all the ECM components and the basal membrane, both of which play an crucial role in preventing the migration of cancer cells[31]. In this sense, MMP2 and MMP9 play an important role in the proteolytic cascade-leading ECM degradation during metastasis in colon carcinoma[32,33]. E-cadherin is an adherent junction protein and tumor suppressor. Low E-cadherin and high vimentin are traditional markers currently accustomed to discern cells that have undergone a EMT process[34]. EMT is a process that epithelial cells lose polarity, cell-to-cell contacts, and cytoskeletal integrity contributing to the dissemination of carcinoma cells from epithelial tumors[35,36]. EMT is thought to be responsible for seeding distant dissemination, eventually leading to cancer-related mortality[34]. Han *et al*[5] firstly reported that SATB1 regulated EMT related gene such as E-cadherin, vimentin, fibronectin, N-cadherin, SNAIL and SIP1, and SATB1 depletion restores cell polarity and reduces aggressive phenotypes of breast cancer MDA-MB-231 cells *in vitro*. Another link between SATB1 and EMT was emphasized by Tu *et al*[9], suggesting that SATB1 mainly induces EMT concomitant with increased expression of Snail1, Slug, Twist and vimentin and decreased expression of E-cadherin, tight junction protein ZO-1 and desmoplakin in liver cancer cell lines. The data from the current study suggest that SATB1 can promote CRC metastasis by degrading ECM and inducing EMT in part.

In conclusion, this study demonstrated that SATB1 is over-expressed in CRC and can promote the growth and metastasis of CRC cells *in vitro* and *in vivo*. We thus have found a new potential promoting factor for the development and progression of CRC.

COMMENTS

Background

Elucidation of the molecular mechanisms of colorectal cancer (CRC) is beneficial for developing novel therapeutic strategies to conquer this disease and in this study the authors analyzed the role of special AT-rich sequence-binding protein 1 (SATB1) in CRC carcinogenesis.

Research frontiers

Only one study suggested that the expression of SATB1 is associated with clinicopathological parameters in CRC. But the roles and mechanisms of SATB1 in growth and metastasis of human CRC remain poorly understood.

Innovations and breakthroughs

SATB1 promotes CRC cell growth, migration and invasion *in vitro* and the ability of tumor metastasis *in vivo*.

Applications

SATB1 acts as a potential growth and metastasis promoter in CRC. SATB1 may be useful as a therapeutic target for CRC.

Terminology

SATB1 gene locates at human chromosome 3p23, and is a thymocyte-specific matrix association region-binding protein. SATB1 is predominantly expressed in thymocytes and could regulate the spatiotemporal expression of numerous genes that involved in T cell proliferation, development, and differentiation.

SATB1 has recently attracted considerable attention in cancer research and its overexpression is a frequent event in various cancers. Furthermore, accumulating evidence showed that SATB1 is also associated with tumor growth and metastasis.

Peer review

The manuscript is well written and the findings are important in its field of investigation.

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