**Name of journal:** ***World Journal of*** ***Gastroenterology***

**ESPS Manuscript NO: 27251**

**Manuscript Type: ORIGINAL ARTICLE**

***Basic Study***

**Special AT-rich sequence-binding protein 2 plays as a negative regulator of stemness in colorectal cancer**

Li Y *et al.* SATB2as a negative regulator of stemness in CRC

Ying Li, Yu-Hong Liu, Yu-Ying Hu, Lin Chen, Jian-Ming Li

**Ying Li, Yu-Ying Hu, Lin Chen, Jian-Ming Li,** Department of Pathology, Nanfang Hospital, Southern Medical University, Guangzhou 510515, Guangdong Province, China

**Yu-Hong Liu,** Department of Pathology, Baoan Hospital, Southern Medical University, Shenzhen 518101, Guangdong Province, China

**Jian-Ming Li,** Department of Pathology, Soochow University Medical School, Suzhou 215123, Jiangsu Province, China

**Author contributions:** Li Y and Liu YH contributed equally in this work; Li Y, Liu YH and Li JM contributed to study concept and design; Li Y, Hu YY and Chen L contributed to acquisition of data; Li Y and Li JM contributed to analysis and interpretation of data; Li Y and Li JM contributed to drafting of the manuscript for important intellectual content; Li Y, Hu YY and Li JM contributed to statistical analysis; Li JM contributed to obtaining funding; Liu YH contributed to technical or material support; and Li JM contributed to study supervision.

**Supported by** theNational Natural Science Foundation of China, No. 81525020, No. 81502033, No. 81272300 and No. 31570753.

**Institutional review board statement**: This study was approved by the Institutional Review Board of Nanfang Hospital, Southern Medical University, People’s Republic of China.

**Conflict-of-interest statement**: The authors declare that there are no conflicts of interest.

**Data sharing statement**: Technical appendix, statistical code, and dataset available from the corresponding author at lixinyue@fimmu.com. Participants gave informed consent for data sharing.

**Open-Access:** This article is an open-access article which was selected by an in-house editor and fully peer-reviewed by external reviewers. It is distributed in accordance with the Creative Commons Attribution Non Commercial (CC BY-NC 4.0) license, which permits others to distribute, remix, adapt, build upon this work non-commercially, and license their derivative works on different terms, provided the original work is properly cited and the use is non-commercial. See: http://creativecommons.org/licenses/by-nc/4.0/

**Manuscript source:** Unsolicited manuscript

**Correspondence to**: **Jian-Ming Li, MD, PhD, DSc, Professor, Chief,** Department of Pathology, Nanfang Hospital, Southern Medical University, No. 1838 Guangzhou Road North, Guangzhou 510515, Guangdong Province, China. lixinyue@fimmu.com

**Telephone**: +86-512-65882673

**Received:** May 19, 2016

**Peer-review started:** May 20, 2016

**First decision:** July 12, 2016

**Revised:** July 29, 2016

**Accepted:** August 19, 2016

**Article in press:**

**Published online:**

**Abstract**

**AIM:** To find the mechanisms of how special AT-rich sequence-binding protein 2 (SATB2) influences colorectal cancer metastasis.

**METHODS:** We used cell growth assay, colony-forming assay, cell adhesion assay and cell migration assay *in vitro* to evaluate the biological characteristics of colorectal cancer (CRC) cells with gain or loss of SATB2. Sphere formation assay was used to detect the self-renewal ability of CRC cells. The mRNA expressions of stem cell markers in CRC cells with the upregulated or downregulated SATB2 expression were detected by qRT-PCR. We used chromatin immunoprecipitation (ChIP) to verify the binding loci of SATB2 on genomic sequences of stem cell markers. The Cancer Genome Atlas (TCGA) database and our clinical samples were analyzed to find the correlation between SATB2 and some key stem cell markers.

**RESULTS:** We found that downregulation of SATB2 led to an aggressive phenotype in SW480 and DLD-1 cells characterized with increasing migration and invasion abilities. Meanwhile, overexpression of SATB2 suppressed the cells’ migration and invasion abilities in SW480 and SW620 cells. Importantly, using sequential sphere formation assay to detect the self-renewal abilities of CRC cells, we found more secondary sphere formation but not primary sphere formation in SW480 and DLD-1 cells after SATB2 expression was knocked down. Moreover, most markers for stem cells such as CD133, CD44, AXIN2, MEIS2 and NANOG were increased in cells with SATB2 knockdown and decreased in cells with SATB2 overexpression. ChIP assay showed that SATB2 bound to regulatory elements of CD133, CD44, MEIS2 and AXIN2 genes. Using TCGA database and our clinical samples, we found SATB2 was correlated with some key stem cell markers including CD44 and CD24 in clinical tissues of CRC patients.

**CONCLUSION:** SATB2 can directly bind to the regulatory elements of genetic locus of several stem cell markers and consequently inhibit the progression of CRC by negatively regulating stemness of CRC cells.

**Key words:** Special AT-rich sequence-binding protein 2; Colorectal cancer; Stemness; Metastasis

**© The Author(s) 2016.** Published by Baishideng Publishing Group Inc. All rights reserved.

**Core tip:** We found special AT-rich sequence-binding protein 2 (SATB2) had a suppressive effect on the tumor growth, adhesion and migration *in vitro*. Moreover, SATB2 could negatively regulate stemness of colorectal cancer (CRC) cells by directly binding to the regulatory elements of genetic locus of several stem cell markers. Our study provides a new mechanism for SATB2 involving in CRC progression and helps us to better understand the metastasis traits of cancer stem cells.

Li Y, Liu YH, Hu YY, Chen L, Li JM. Special AT-rich sequence-binding protein 2 plays as a negative regulator of stemness in colorectal cancer**.** *World J Gastroenterol* 2016; In press

**INTRODUCTION**

Special AT-rich sequence-binding protein 2 (SATB2) is an important [DNA-binding protein](http://en.wikipedia.org/wiki/DNA-binding_protein) involved in [transcriptional regulation](http://en.wikipedia.org/wiki/Transcriptional_regulation) and [chromatin remodeling](http://en.wikipedia.org/wiki/Chromatin_remodeling). SATB2 plays the key roles in osteoblastic differentiation, cortical neuron differentiation and skeletal development[[1-4](#_ENREF_1)]. However, the role of SATB2 in cancer initiation and progression is still not well-understood. We first found that SATB2 was a potential marker for metastasis of colorectal cancer (CRC) and low expression of SATB2 was correlated with tumor progression and poor prognosis in CRC patients[[5](#_ENREF_5)]. Interestingly, more evidences show that SATB2 is involved in progression of breast cancer, head and neck squamous cell carcinomas and osteosarcoma[[6-8](#_ENREF_6)]. Importantly, SATB2 is strongly expressed in normal colorectal and appendiceal epithelium [[9](#_ENREF_9)], demonstrating SATB2 as a diagnostic marker for CRC.

Recently, SATB2 and its analogue protein SATB1 are found to regulate embryonic stem cell differentiation by a direct bind with NANOG genomic locus[[10](#_ENREF_10)]. Renew and differentiation of trophoblast stem cells are also considered to be related with SATB proteins[[11](#_ENREF_11)]. In the process of cancer development and progression, very few cancer cells with stem cell-like properties have greatly enhanced tumor-initiating potential within a tumor and these cells are termed as cancer stem cells (CSCs)[[12](#_ENREF_12),[13](#_ENREF_13)]. However, the regulatory mechanisms of CSCs and relations between CSCs and cancer metastasis are still needed to be answered.

In this study, we found SATB2 had a suppressive effect on the tumor growth, adhesion, and migration *in vitro*. Moreover, SATB2 could negatively regulate stemness of CRC cells by directly binding to the regulatory elements of genetic locus of several stem cell markers. Our study provides a new mechanism for SATB2 involving in CRC progression and helps us to better understand the metastasis traits of CSCs.

**MATERIALS AND METHODS**

***Cell lines***

The CRC cell lines we used in our experiments were bought from the Cell Bank at the Chinese Academy of Sciences. Then the cells were cultured in RPMI-1640 medium in which fetal bovine serum (Hyclone, United States) was added in to a final concentration of 10%. The cells were sustained in the incubator with 5% CO2 at 37 °C.

***Plasmid and lentivirus preparation***

The information of pCAG-SATB2 vector and its control vector had been mentioned in our previous paper[5]. The pLKO.1-TRC vectors with different interference fragments targeting SATB2 were purchased from Thermo scientific company (Thermo scientific, United States, Item No. TRCN0000020684 to TRCN0000020688).

***Transfection and lentiviral transduction***

The pCAG-SATB2 was transfected into CRC cell lines to establish cells in which SATB2 expression was upregulated. The packaged virus with pLKO.1-TRC vectors were used to establish cells with stably downregulated expression of SATB2.

***Quantitative real time polymerase chain reaction***

The mRNA expression levels of SATB2 and stem cell markers in CRC cell lines were measured by quantitative real time polymerase chain reaction (qRT-PCR) using SYBR Green (Takara, China) running in 7500 real-time PCR system (ABI, United States). Expression levels of SATB2 and stem cell markers were evaluated using the △△Ct method and normalized according to the mRNA level of GAPDH. Primer sequences for qRT-PCR are listed in Table 1.

***Western blotting analysis***

Protein extracts were obtained using the lysis buffer (KeyGen Biotech). After being quantiﬁed, equivalent amounts of protein extracts were separated using SDS-PAGE and transferred to the PVDF membrane (Roche Applied Sciences). The primary antibody was added onto each membrane and incubated at 4 °C overnight. The appropriate second antibody was used on the following day. Mouse monoclonal anti-SATB2 antibody (1: 100, Abcam, United Kingdom), rabbit polyclonal anti-CD133 antibody (1: 500, Abnova, China) and mouse monoclonal anti-α-tubulin antibody (1: 1000, proteintech, United States) were used. The targeted bands were visualized and photographed by FluorChem system (Alpha Innotech).

***Cell growth assay in vitro***

Cell aliquots (100 μL) were transferred into each well of 96-well microtiter plates at a concentration of 1 × 104 cells/mL. CCK-8 (Dojindo Laboratories, Japan) was used to test the cell proliferation ability every 24 h and would last for 6 d. Each day we added 10 µL CCK-8 reagents into each well and then incubated the plates at 37 °C for 2 h. After the incubation, the absorbance of each well was measured at 450 nm using the Vmax microplate spectrophotometer (Molecular Devices, CA).

***Colony-forming assay***

1 × 102 cells were seeded into each well of 6-well culture plates and incubated at 37 °C for 14 d. Then the cells were stained with crystal violet solution and the pictures of stained cells were taken by the digital camera. Under the microscope, the colonies containing more than 50 cells were counted. The colony formation efficiency of each group was calculated as follows. The colony numbers were divided by inoculated cell number and then multiplied by 100%.

***Cell adhesion assay***

Fibronectin (Invitrogen, United States) was added into each well of 96-well plates at a concentration of 10 µg/mL and plates were incubated overnight at 4 °C. After that, 1% BSA was added and incubated at 37 °C for 1 h. Then diluted cells (1 × 105 cells/100 µL) were added to the coated wells and incubated at 37 °C for 1 h. Then the non-adherent cells were washed out. Subsequently, we added CCK-8 reagent into each well and the plates were incubated at 37 °C for 2 h. The absorbance of each well was measured at 450 nm.

***Cell migration assay in vitro***

Transwells (BD Biosciences, United States) inserted with 8 µm pores were put into wells of 24-well plate. Cells were suspended with serum free medium and 2 × 105 cells were added inside the chamber. And below the matched chamber, 600 µL RMPI-1640 medium containing 10% FBS was added. After incubation for 24 h, noninvasive cells on the membrane inside the transwell were removed. Invaded cells were fixed with methanol, stained with Giemsa or crystal violet and photographed.

***Immunoﬂuorescence analysis***

CRC cells were stained with CD133 antibody as mentioned previously. Then the goat anti-rabbit secondary antibody conjugated with Alexa Fluor 594 (ZSGB-Bio, China) was used. DAPI was used to counterstain nuclei. The fluorescence was scanned and photographed by the confocal laser scanning microscope (Olympus, Japan). And the average fluorescence intensity was calculated with ImageJ software.

***Sphere formation assay***

The low attachment plates (Corning Incorporated, United States) were used to culture cells using serum-free medium according to the previous study[[14](#_ENREF_14)]. We prepared the serum-free medium for sphere culturing by adding 10 μg EGF, 5 μg LIF and 10 μg bFGF (Invitrogen, United States) into 500 mL DMEM/F12 medium. Cells were cultured in the 24-well ULLA plates at a density of 5000 or 10000 cells/well for 1 wk. Spheres (> 50 µm) were counted using immunofluorescent microscope (Olympus, Japan).

***Chromatin immunoprecipitation***

SW480 cells were cultured and harvested. Following [procedure](javascript:void(0);)s were provided by CHIP-IT Express Enzymatic and Enzymatic shearing Kit (Active Motif). Mouse monoclonal anti-SATB2 antibody (Abcam, Cambridge, United Kingdom) was used. The positive and negative control antibodies were provided in CHIP-IT control (Active Motif). The immunoprecipitated DNA was amplified by PCR. And the primer sequences for PCR are listed in Table 2.

***Correlation analysis using The Cancer Genome Atlas database and clinical samples of CRC patients***

RNA-Seq expression data (combining level 3 data from IlluminaGA\_RNASeqV2 platforms) from CRC patients were downloaded from The Cancer Genome Atlas (TCGA), which had been analyzed in Cancer Browser (<https://genome-cancer.ucsc.edu/>). Correlations between SATB2 and stem cell markers were analyzed according to data from these clinical samples.

We collected 68 fresh samples from colorectal cancer patients operated during March to April in 2010 in Nanfang hospital. Among them, there were 45 cases of male and 23 cases of female. The average age was 63.77 ± 16.22 years old. We collected the tumor tissue and its adjacent normal tissue. Then the tissues were saved in liquid nitrogen and the RNA was extracted and analyzed subsequently.

***Statistical analysis***

SPSS V.13.0 statistical software package was used to perform all statistical analyses. The Student’s *t*-test was used to compare two groups of independent samples. The one-way ANOVA was used to analyze differences among multiple groups and differences between groups were analyzed by LSD pairwise comparison. And we used Pearson correlation analysis to calculate the correlation between SATB2 and stem cell markers. *P* < 0.05 was considered to be statistically significant for all the analyses.

**RESULTS**

***Overexpression of SATB2 inhibited the proliferation and migration of CRC cells in vitro***

SATB2 was successfully overexpressed in SW480 and SW620 both in mRNA (SW480, *P* < 0.001; SW620, *P* < 0.001; Figure 1A) and protein (SW480, *P* < 0.05; SW620, *P* < 0.01; Figure 1B) levels. CCK-8 cell proliferation assay showed that overexpression of SATB2 inhibited cell proliferation in SW480 (*P* < 0.001) and SW620 (*P* < 0.001) cells (Figure 1C). Moreover, the plate colony formation assay indicated that cells with SATB2 overexpression had a deceased formation of clones compared with control cells (SW480, *P* < 0.001; SW620, *P* < 0.01; Figure 1D). A significant decrease in cell migration was showed in CRC cells after the exogenous expression of SATB2 (SW480, *P* < 0.001; SW620, *P* < 0.001; Figure 1E).

***Knockdown of SATB2 promoted adhesion, colony-formation and migration of CRC cells in vitro***

To further confirm the function of SATB2 on the biological properties of CRC cells, we used the pLKO.1-TRC system with shRNA interferences targeting SATB2 to produce virus to knock down SATB2 expression in CRC cells. The lentiviruses with different shRNA interferences targeting SATB2 were tested in SW480, SW620 and DLD-1 cells for optimal selection. The lentivirus with shRNA#1 targeting SATB2 had the optimal efficiency to knock down SATB2 expression in three tested CRC cell lines (SW480, *P* < 0.001; SW620, *P* < 0.01; DLD-1, *P* < 0.01) and was then used to establish the cell lines with SATB2 stable knockdown (Figure 2A). Single cells were isolated from the cells infected by the lentivirus with shRNA#1 targeting SATB2 and cultured for 2 weeks to establish clones with SATB2 stable knockdown (Figure 2B). SW480/clone7 (*P* < 0.001) and DLD-1/clone5 (*P* < 0.01) were used in our next experiments. Contrast to our previous results, enhanced adhesion ability (SW480, *P* < 0.001; DLD-1, *P* < 0.001; Figure 2C), colony-forming capacity (SW480, *P* < 0.05; DLD-1, *P* < 0.01; Figure 2D) and migration ability (SW480/shRNA#1, *P* < 0.05; DLD-1/shRNA#1, *P* < 0.001; SW480/clone7, *P* < 0.001; DLD-1/clone5, *P* < 0.001; Figure 2E and F) were found in SW480 and DLD-1 cells after SATB2 was down-regulated.

***SATB2 knockdown enhanced secondary sphere formation of CRC cells in vitro***

In our previous studies, we found SATB2 expression was closely correlated with tumor invasion, lymph node metastasis, distant metastasis and Dukes’ classification in CRC patients[[5](#_ENREF_5)]. Further, we found that SATB2 overexpression inhibited the proliferation and migration of CRC cells while knockdown of SATB2 promoted adhesion, colony-formation and migration of CRC cells *in vitro*. There is a subpopulation of CSCs that contributes to the biological traits of high-grade malignancy[[15-17](#_ENREF_15)]. The CSCs were possibly the main cause of new tumor formation and tumor metastasis. We checked whether SATB2 could influence phenotype of stemness of CRC cells. As we know, self-renew is one of the basic characteristics of stemness of CRC cells. So we observed the self-renew of CRC cells using sequential sphere formation assay. Nevertheless, SATB2 knockdown had no effects on primary sphere formation in CRC cells (Figure 3A). Interestingly, more secondary sphere formation was found in SW480 and DLD-1 cells after SATB2 expression was knocked down (SW480/shRNA#1, *P* < 0.05; DLD-1/shRNA#1, *P* > 0.05; SW480/clone7, *P* < 0.05; DLD-1/clone5, *P* < 0.05; Figure 3B), indicating that SATB2 repressed the self-renewal ability of CRC cells.

***SATB2 knockdown increased expression of several markers for cancer stem cells in CRC cells in vitro***

We found that SATB2 knockdown enhanced secondary sphere formation of CRC cells *in vitro*. It is logically supposed that SATB2 may affect expressions of markers of cancer stem cells as a key transcriptional factor which controls gene expressions. Therefore, we detected mRNA expressions of several key markers of cancer stem cell, such as CD133, CD44, AXIN2, MEIS2 and NANOG, by qRT-PCR in CRC cells with gain or loss of SATB2 expression. Accordingly, most markers for stem cells were increased in cells with SATB2 knockdown and decreased in cells with SATB2 overexpression (Figure 4A and B), especially CD133 (SW480/pCAG-SATB2, *P* < 0.05; DLD-1/pCAG-SATB2, *P* < 0.001; SW480/clone7, *P* < 0.001; DLD-1/shRNA#1, *P* < 0.05; DLD-1/clone5, *P* < 0.001), CD44 (SW480/pCAG-SATB2, *P* < 0.05; HCT-116/pCAG-SATB2, *P* < 0.05; DLD-1/pCAG-SATB2, *P* < 0.05; SW480/clone7, *P* < 0.01; DLD-1/clone5, *P* < 0.001) and PRL1 (HCT-116/pCAG-SATB2, *P* < 0.01; SW480/shRNA#1, *P* < 0.01; SW480/clone7, *P* < 0.01; DLD-1/shRNA#1, p < 0.01; DLD-1/clone5, *P* < 0.01). Specifically, CD133 expression was further analyzed by western blotting (SW480, *P* < 0.05; SW620, *P* < 0.001; DLD-1, *P* < 0.05) and immunofluorescent staining (SW480, *P* < 0.001; SW620, *P* < 0.001; DLD-1, *P* < 0.001). And CD133 expression was increased in CRC cells after SATB2 was knocked down (Figure 4C and D).

***SATB2 binds to regulatory elements of CD133, CD44, MEIS2 and AXIN2 genes***

As a transcriptional factor, SATB2 may affect gene expressions of stem cell markers by directly binding to regulatory elements of those genes. We used the Genomatix online software to find the possible SATB2 binding loci of those stem cell marker genes. We found that SATB2 may bind to regulatory elements of CD133 (Figure 5A), CD44 (Figure 5B), MEIS2 (Figure 5C) and AXIN2 (Figure 5D), at single or multiple sites. Then, we employed ChIP, followed by PCR, to test whether SATB2 could bind to regulatory elements of these genes. Chromatin fragments were prepared from SW480 cells. Mouse monoclonal anti-SATB2 antibody was used to precipitate the needed chromatin. Anti-RNA pol II and anti-IgG antibodies were used as positive and negative control separately. Our results indicated that regulatory elements of CD133 (Figure 5A), CD44 (Figure 5B), MEIS2 (Figure 5C) and AXIN2 (Figure 5D) contained SATB2-binding sequences.

***SATB2 is correlated with some key stem cell markers including CD44 and CD24 in clinical tissues of CRC patients***

To further analyze the correlation between SATB2 and some key stem cell markers, RNA-Seq expression data in clinical samples of CRC from TCGA (The Cancer Genome Atlas, http://cancergenome.nih.gov/) were used. Using data from TCGA, we found that SATB2 was negatively correlated with expressions of CD44 (*P* < 0.001), CD26 (*P* < 0.001), CD166 (*P* < 0.01), CD29 (*P* < 0.001) and KRT19 (*P* < 0.01) and positively correlated with expressions of CD24 (*P* < 0.001) and LGR5 (*P* < 0.01) (Figure 6A). Significantly, in our clinical CRC tissues, we further confirmed that SATB2 was positively correlated with CD24 (*P* < 0.001) expression (Figure 6B). However, the correlation between SATB2 and CD133 was marginal for significance analysis both in TCGA data (*P* = 0.072, Figure 6A) and our own clinical samples of CRC (*P* = 0.052, Figure 6B), suggesting that limited samples were included in both studies.

**DISCUSSIONS**

In our previous studies, SATB2 has been found to be a potential novel prognostic factor for CRC because of its strong correlation with local invasion, lymph node metastasis and distant metastasis in CRC[[5](#_ENREF_5)]. After then, more evidences have confirmed SATB2 as a useful marker for CRC metastasis [[18-21](#_ENREF_18)]. Even so, the mechanisms by which SATB2 is involved in CRC metastasis are still largely unclear.

Here, we found SATB2 was a tumor suppressor in CRC. Gain-of-function studies showed that overexpression of SATB2 inhibited the proliferation and migration of CRC cells *in vitro*. Meanwhile, loss-of-function studies indicated that knockdown of SATB2 promoted adhesion, colony-formation and migration of CRC cells *in vitro*. These results were consistent with our clinical data, supporting the importance of SATB2 on tumor metastasis in CRC.

We also discovered SATB2 was a negative regulator of stemness in CRC. At present, many solid tumors, including brain, colon, lung, breast, liver, prostate and bladder cancers, have been identified to have CSCs[[13](#_ENREF_13),[22-26](#_ENREF_22)]. CSCs, commonly identified by their cell-surface-marker expression, have self-renewal and tumor-initiating ability and account for cancer relapse and metastasis[[27](#_ENREF_27)]. Self-renewal ability of CSCs, also called as stemness, is one of the basic characteristic of CSCs. Using primary and secondary sphere formation assay to detect self-renew of CRC cells, we found that SATB2 knockdown enhanced secondary sphere formation of CRC cells *in vitro*. Consistently, expressions of CD133, NANOG and CD44, the key markers for CRC, were significantly increased when SATB2 was stably knocked down in CRC cells. Meanwhile, CD133 and CD44 were downregulated when SATB2 was overexpressed in CRC cells. As a transcriptional factor, SATB2 may regulate gene expressions by directly binding to the regulatory elements of these stemness genes. NANOG was found to be directly regulated by SATB2 because of the binding to its promoter region[[10](#_ENREF_10)]. In our study, Genomatix, an online web-based bioinformatic system, was first used to predict the potential genetic locus which might be recognized and bound by SATB2. We found that SATB2 could bind to one or more regulatory elements of CD133, CD44, MEIS2 and AXIN2. Importantly, ChIP assay confirmed that SATB2 could bind directly to the regulatory elements of CD133, CD44, MEIS2 and AXIN2. Interestingly, in TCGA clinical database and our clinical data of CRC, SATB2 was correlated with expressions of several stem cell markers such as CD44 and CD24. Much more evidences are needed to explore to know the precision mechanisms by which SATB2 regulates stemness of cancer cells.

In general, our studies found that SATB2 could directly bind to the regulatory elements of genetic locus of several stem cell markers and consequently inhibit the progression of CRC by negatively regulating stemness of CRC cells.

**COMMENTS**

***Background***

Special AT-rich sequence-binding protein 2 (SATB2) is a key factor for [transcriptional regulation](http://en.wikipedia.org/wiki/Transcriptional_regulation) and [chromatin remodeling](http://en.wikipedia.org/wiki/Chromatin_remodeling). Previously, we found that decreased expression of SATB2 was correlated with metastasis in colorectal cancer (CRC). Unfortunately, how SATB2 influences CRC metastasis is still unclear.

***Research frontiers***

SATB2 and its analogue protein SATB1 are found to regulate embryonic stem cell differentiation by a direct bind with NANOG genomic locus. And cancer stem cells (CSCs) with stem cell-like properties have been reported to enhance greatly tumor-initiating potential within a tumor. This implies the possible relationship between SATB2 and CSCs.

***Innovations and breakthroughs***

This is the first study to report the relationship between SATB2 and stemness of CRC cells. And our study provides a new mechanism for SATB2 involving in CRC progression.

***Applications***

SATB2 inhibits the progression of CRC by negatively regulating stemness of CRC cells which provides a new possible therapy for CRC patients.

***Terminology***

SATB2 is a protein that binds AT sequence on the targeted genes to regulate their transcriptions. Low expression of SATB2 has been reported in CRC tissues. And SATB2 expression was closely correlated with tumor invasion, lymph node metastasis, distant metastasis and Dukes’ classification in CRC patients. This predicts a possible role of SATB2 on regulating tumor metastasis.

***Peer-review***

This is a very interesting and maybe an useful future technique. CRC is a leading cancerous disease, so many people affected therefore every method that could predict influence factors on metastatisation is very important to choose the correct treatment or even follow up schedule.

**REFERENCES**

1 **Dobreva G**, Chahrour M, Dautzenberg M, Chirivella L, Kanzler B, Fariñas I, Karsenty G, Grosschedl R. SATB2 is a multifunctional determinant of craniofacial patterning and osteoblast differentiation. *Cell* 2006; **125**: 971-986 [PMID: 16751105 DOI: 10.1016/j.cell.2006.05.012]

2 **Britanova O**, Depew MJ, Schwark M, Thomas BL, Miletich I, Sharpe P, Tarabykin V. Satb2 haploinsufficiency phenocopies 2q32-q33 deletions, whereas loss suggests a fundamental role in the coordination of jaw development. *Am J Hum Genet* 2006; **79**: 668-678 [PMID: 16960803 DOI: 10.1086/508214]

3 **Britanova O**, de Juan Romero C, Cheung A, Kwan KY, Schwark M, Gyorgy A, Vogel T, Akopov S, Mitkovski M, Agoston D, Sestan N, Molnár Z, Tarabykin V. Satb2 is a postmitotic determinant for upper-layer neuron specification in the neocortex. *Neuron* 2008; **57**: 378-392 [PMID: 18255031 DOI: 10.1016/j.neuron.2007.12.028]

4 **Alcamo EA**, Chirivella L, Dautzenberg M, Dobreva G, Fariñas I, Grosschedl R, McConnell SK. Satb2 regulates callosal projection neuron identity in the developing cerebral cortex. *Neuron* 2008; **57**: 364-377 [PMID: 18255030 DOI: 10.1016/j.neuron.2007.12.012]

5 **Wang S**, Zhou J, Wang XY, Hao JM, Chen JZ, Zhang XM, Jin H, Liu L, Zhang YF, Liu J, Ding YQ, Li JM. Down-regulated expression of SATB2 is associated with metastasis and poor prognosis in colorectal cancer. *J Pathol* 2009; **219**: 114-122 [PMID: 19557828 DOI: 10.1002/path.2575]

6 **Patani N**, Jiang W, Mansel R, Newbold R, Mokbel K. The mRNA expression of SATB1 and SATB2 in human breast cancer. *Cancer Cell Int* 2009; **9**: 18 [PMID: 19642980 DOI: 10.1186/1475-2867-9-18]

7 **Chung J**, Lau J, Cheng LS, Grant RI, Robinson F, Ketela T, Reis PP, Roche O, Kamel-Reid S, Moffat J, Ohh M, Perez-Ordonez B, Kaplan DR, Irwin MS. SATB2 augments ΔNp63α in head and neck squamous cell carcinoma. *EMBO Rep* 2010; **11**: 777-783 [PMID: 20829881 DOI: 10.1038/embor.2010.125]

8 **Seong BK**, Lau J, Adderley T, Kee L, Chaukos D, Pienkowska M, Malkin D, Thorner P, Irwin MS. SATB2 enhances migration and invasion in osteosarcoma by regulating genes involved in cytoskeletal organization. *Oncogene* 2015; **34**: 3582-3592 [PMID: 25220418 DOI: 10.1038/onc.2014.289]

9 **Zhao X**, Qu Z, Tickner J, Xu J, Dai K, Zhang X. The role of SATB2 in skeletogenesis and human disease. *Cytokine Growth Factor Rev* 2014; **25**: 35-44 [PMID: 24411565 DOI: 10.1016/j.cytogfr.2013.12.010]

10 **Savarese F**, Dávila A, Nechanitzky R, De La Rosa-Velazquez I, Pereira CF, Engelke R, Takahashi K, Jenuwein T, Kohwi-Shigematsu T, Fisher AG, Grosschedl R. Satb1 and Satb2 regulate embryonic stem cell differentiation and Nanog expression. *Genes Dev* 2009; **23**: 2625-2638 [PMID: 19933152 DOI: 10.1101/gad.1815709]

11 **Asanoma K**, Kubota K, Chakraborty D, Renaud SJ, Wake N, Fukushima K, Soares MJ, Rumi MA. SATB homeobox proteins regulate trophoblast stem cell renewal and differentiation. *J Biol Chem* 2012; **287**: 2257-2268 [PMID: 22123820 DOI: 10.1074/jbc.M111.287128]

12 **Al-Hajj M**, Wicha MS, Benito-Hernandez A, Morrison SJ, Clarke MF. Prospective identification of tumorigenic breast cancer cells. *Proc Natl Acad Sci U S A* 2003; **100**: 3983-3988 [PMID: 12629218 DOI: 10.1073/pnas.0530291100]

13 **Singh SK**, Clarke ID, Terasaki M, Bonn VE, Hawkins C, Squire J, Dirks PB. Identification of a cancer stem cell in human brain tumors. *Cancer Res* 2003; **63**: 5821-5828 [PMID: 14522905]

14 **Zappone MV**, Galli R, Catena R, Meani N, De Biasi S, Mattei E, Tiveron C, Vescovi AL, Lovell-Badge R, Ottolenghi S, Nicolis SK. Sox2 regulatory sequences direct expression of a (beta)-geo transgene to telencephalic neural stem cells and precursors of the mouse embryo, revealing regionalization of gene expression in CNS stem cells. *Development* 2000; **127**: 2367-2382 [PMID: 10804179]

15 **Charafe-Jauffret E**, Ginestier C, Iovino F, Wicinski J, Cervera N, Finetti P, Hur MH, Diebel ME, Monville F, Dutcher J, Brown M, Viens P, Xerri L, Bertucci F, Stassi G, Dontu G, Birnbaum D, Wicha MS. Breast cancer cell lines contain functional cancer stem cells with metastatic capacity and a distinct molecular signature. *Cancer Res* 2009; **69**: 1302-1313 [PMID: 19190339 DOI: 10.1158/0008-5472.can-08-2741]

16 **Pang R**, Law WL, Chu AC, Poon JT, Lam CS, Chow AK, Ng L, Cheung LW, Lan XR, Lan HY, Tan VP, Yau TC, Poon RT, Wong BC. A subpopulation of CD26+ cancer stem cells with metastatic capacity in human colorectal cancer. *Cell Stem Cell* 2010; **6**: 603-615 [PMID: 20569697 DOI: 10.1016/j.stem.2010.04.001]

17 **Marcato P**, Dean CA, Pan D, Araslanova R, Gillis M, Joshi M, Helyer L, Pan L, Leidal A, Gujar S, Giacomantonio CA, Lee PW. Aldehyde dehydrogenase activity of breast cancer stem cells is primarily due to isoform ALDH1A3 and its expression is predictive of metastasis. *Stem Cells* 2011; **29**: 32-45 [PMID: 21280157 DOI: 10.1002/stem.563]

18 **Eberhard J**, Gaber A, Wangefjord S, Nodin B, Uhlén M, Ericson Lindquist K, Jirström K. A cohort study of the prognostic and treatment predictive value of SATB2 expression in colorectal cancer. *Br J Cancer* 2012; **106**: 931-938 [PMID: 22333599 DOI: 10.1038/bjc.2012.34]

19 **Yang MH**, Yu J, Chen N, Wang XY, Liu XY, Wang S, Ding YQ. Elevated microRNA-31 expression regulates colorectal cancer progression by repressing its target gene SATB2. *PLoS One* 2013; **8:** e85353 [PMID: 24386467 DOI: 10.1371/journal.pone.0085353]

20 **Yang MH**, Yu J, Jiang DM, Li WL, Wang S, Ding YQ. microRNA-182 targets special AT-rich sequence-binding protein 2 to promote colorectal cancer proliferation and metastasis. *J Transl Med* 2014; **12**: 109 [PMID: 24884732 DOI: 10.1186/1479-5876-12-109]

21 **Dragomir A**, de Wit M, Johansson C, Uhlen M, Pontén F. The role of SATB2 as a diagnostic marker for tumors of colorectal origin: Results of a pathology-based clinical prospective study. *Am J Clin Pathol* 2014; **141**: 630-638 [PMID: 24713733 DOI: 10.1309/AJCPWW2URZ9JKQJU]

22 **Haraguchi N**, Ishii H, Mimori K, Tanaka F, Ohkuma M, Kim HM, Akita H, Takiuchi D, Hatano H, Nagano H, Barnard GF, Doki Y, Mori M. CD13 is a therapeutic target in human liver cancer stem cells. *J Clin Invest* 2010; **120**: 3326-3339 [PMID: 20697159 DOI: 10.1172/JCI42550]

23 **Eramo A**, Lotti F, Sette G, Pilozzi E, Biffoni M, Di Virgilio A, Conticello C, Ruco L, Peschle C, De Maria R. Identification and expansion of the tumorigenic lung cancer stem cell population. *Cell Death Differ* 2008; **15**: 504-514 [PMID: 18049477 DOI: 10.1038/sj.cdd.4402283]

24 **Wright MH**, Calcagno AM, Salcido CD, Carlson MD, Ambudkar SV, Varticovski L. Brca1 breast tumors contain distinct CD44 /CD24-and CD133 cells with cancer stem cell characteristics. *Breast Cancer Res* 2008; **10**: R10 [PMID: 18241344 DOI: 10.1186/bcr1855]

25 **O'Brien CA**, Pollett A, Gallinger S, Dick JE. A human colon cancer cell capable of initiating tumour growth in immunodeficient mice. *Nature* 2007; **445**: 106-110 [PMID: 17122772 DOI: 10.1038/nature05372]

26 **Collins AT**, Berry PA, Hyde C, Stower MJ, Maitland NJ. Prospective identification of tumorigenic prostate cancer stem cells. *Cancer Res* 2005; **65**: 10946-10951 [PMID: 16322242 DOI: 10.1158/0008-5472.CAN-05-2018]

27 **Gupta PB**, Chaffer CL, Weinberg RA. Cancer stem cells: mirage or reality? *Nat Med* 2009; **15**: 1010-1012 [PMID: 19734877 DOI: 10.1038/nm0909-1010]

**P-Reviewer:** Furka A, Lakatos PT, Morris DLL, Perini MV

**S-Editor:** Qi Y

**L-Editor: E-Editor:**

**Specialty type:** Gastroenterology and hepatology

**Country of origin:** China

**Peer-review report classification**

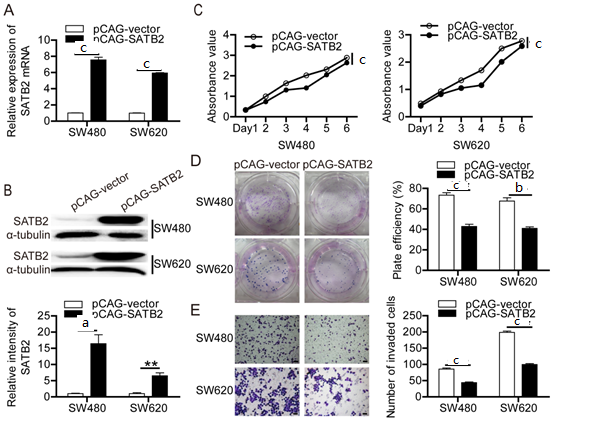
Grade A (Excellent): A

Grade B (Very good): B, B

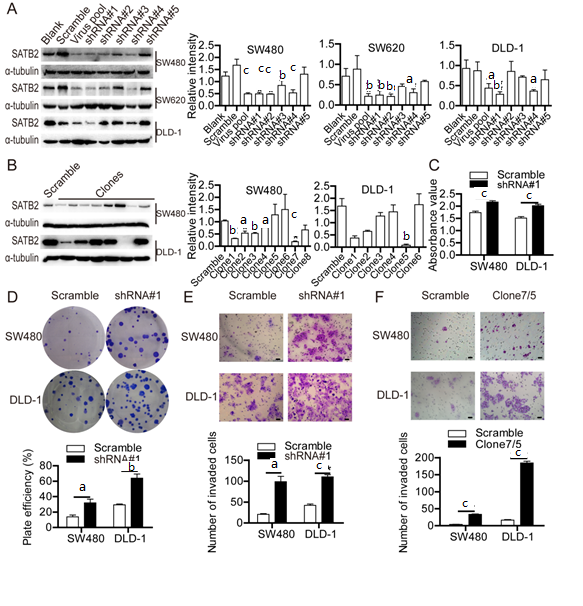
Grade C (Good): 0

Grade D (Fair): 0

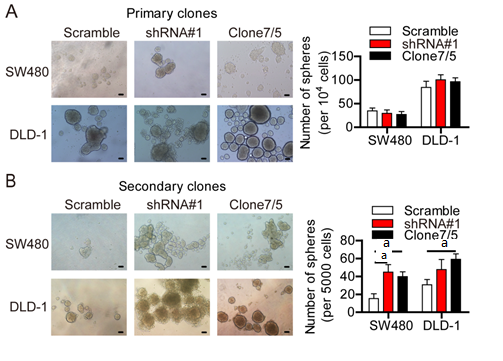
Grade E (Poor): 0



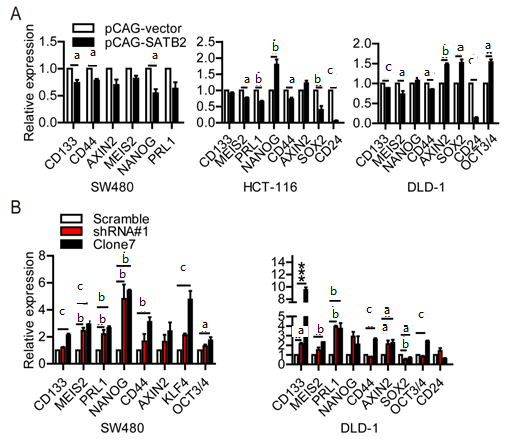
**Figure 1 Overexpression of sequence-binding protein 2** **inhibited the proliferation and migration of colorectal cancer cells *in vitro*.** A, B: Expression levels of SATB2 in SW480 and SW620 cells transfected with pCAG-SATB2 were increased whenever detected by qRT-PCR (A) or Western Blot (B); C: The proliferation abilities of cells with SATB2 overexpression were detected to have a decrease in CCK-8 cell proliferation assay. The P values of time effect, group effect and their interaction effect are all below 0.001 in SW480 and DLD-1 cells; D: Colony formation assay was used to analyze ability of clone formation in SATB2 overexpression cells. And cells with SATB2 overexpression formed less clones; E: Cell migration capacities of control cells and SATB2 overexpressed cells were compared by detecting the invaded cell numbers in transwell chambers. Less invaded cells were found in SATB2 overexpressed cells. Scale bar is 50 μm. Data shown are represented as mean ± SEM. a*P* < 0.05, b*P* < 0.01, c*P* < 0.001 *vs* control.

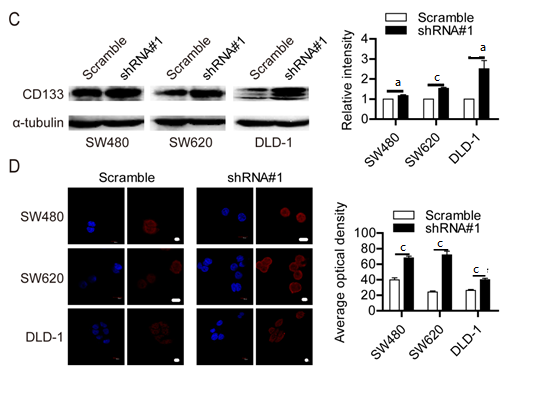


**Figure 2** **Knockdown of sequence-binding protein 2 promoted adhesion, colony-formation and migration of colorectal cancer cells *in vitro*.** A: SATB2 expression levels in cells infected by virus with different shRNA interferences targeting SATB2 were detected by western blot. And shRNA interference named shRNA#1 had the best effect in silencing SATB2 expression in SW480, SW620 and DLD-1 cells; B: Cells in which SATB2 was stably knocked down were isolated into single cells and single cells were cultured for 2 weeks to form clones. Then we detected SATB2 expression in different clones by western blot. And we chose SW480/clone7 and DLD-1/clone5 cells to be used in the following assays; C: Adhesion capabilities of cells infected by shRNA#1 virus and its control virus were compared by detecting the cells’ absorbance values to reflect numbers of adhered cells. Cells with low SATB2 expression had increased adhesion capabilities; D: Abilities of clone formation of CRC cells with stably reduced expression of SATB2 by an infection of shRNA#1 virus were detected to have an increase in colony formation assay; E and F: Transwell chambers were used to detect migration ability of cells with stably reduced expression of SATB2 by an infection of shRNA#1 virus (E) or by culturing the single cells isolated from shRNA#1 virus infected cells to clones (F) and both groups with low SATB2 expression had increased cell migration abilities. Scale bar is 50 μm. Data shown are represented as mean ± SEM. a*P* < 0.05, b*P* < 0.01, c*P* < 0.001 *vs* control.



**Figure 3** **Sequence-binding protein 2 knockdown enhanced secondary sphere formation of colorectal cancer cells *in vitro*.** A: Self-renewal abilities of CRC cells in which SATB2 was stably knocked down were evaluated by sequential sphere formation assay; B: Spheres formed primarily were isolated into single cells and then equal amounts of cells were cultured to form secondary spheres. More secondary spheres were formed in SATB2 downregulated cells in SW480 and DLD-1. Scale bar is 50 μm. Data shown are represented as mean ± SEM. a*P* < 0.05 *vs* control.





**Figure 4 Sequence-binding protein 2 knockdown increased expression of several markers for cancer stem cells in colorectal cancer cells *in vitro*.** A-B: The mRNA expressions of a series of stem cell markers were detected in SATB2 overexpression cells (A) and SATB2 knockdown cells (B) by qRT-PCR; C-D: CD133 expression level in cells with stable SATB2 knockdown was increased when detected by western blot (C) and immunofluorescence (D). Scale bar is 10 μm. Data shown are represented as mean ± SEM. a*P* < 0.05, b*P* < 0.01, c*P* < 0.001, *vs* control.

F:\papers more\satb2 crc\final version\figure5.tif

**Figure 5 Sequence-binding protein 2 binds to regulatory elements of CD133, CD44, MEIS2 and *AXIN2* genes.** A-D: ChIP was carried out with anti-SATB2 antibody, anti-RNA pol II antibody and anti-IgG antibody. Input DNA and immunoprecipitated DNA by anti-RNA pol II antibody were used as positive controls. Immunoprecipitated DNA by anti-IgG antibody and H2O were used as negative controls. PCR was used to find out the genomic binding sites for each gene. Red squares under the genomic sequence mean the predicted Satb2-binding loci. Green boxes mean the exons for each genomic sequence. And black arrows mean the translation initiation sites. As expected, SATB2 bound CD133 (A), CD44 (B), MEIS2 (C) and AXIN2 (D) at their regulatory elements.

F:\papers more\satb2 crc\final version\figure6.tif

**Figure 6** **Sequence-binding protein 2 is correlated with some key stem cell markers including CD44 and CD24 in clinical tissues of colorectal cancer patients.** A: The correlations between SATB2 and stem cell markers were analyzed by Pearson correlation analysis according to RNA-Seq expression data of 434 primary colorectal tumors from TCGA. The matched scatter grams and the statistical *P* values for different genes showed separately; B: The correlations between SATB2 and stem cell markers were analyzed by Pearson correlation analysis according to the mRNA expression level of genes in 68 cases of colorectal cancer (CRC) tissues collected from NanFang hospital. The matched scatter grams and the statistical *P* values for different genes showed separately.

**Table 1 Primer sequences used in** **quantitative real time polymerase chain reaction for detection of stem cell markers mRNA expression**

|  |  |
| --- | --- |
| **Genes** | **Sequences** |
| *CD133-F* | 5′ TTTGTCTTCTATTCTTGGCTTC 3′ |
| *CD133-R* | 5′ ACCTTGTCATAATCAATTTTGG 3′ |
| *CD44-F* | 5′ GGTTCATAGAAGGGCACGT 3′ |
| *CD44-R* | 5′ TGTCTTCGTCTGGGATGG 3′ |
| *CD24-F* | 5′ TCAAGTATTTGGGAAGTG 3′ |
| *CD24-R* | 5′ GTGTTCTAAATGTGGCTAT 3′ |
| *OCT3/4-F* | 5′ CGACCATCTGCCGCTTTGAG 3′ |
| *OCT3/4-R* | 5′ CCCCCTGTCCCCCATTCCTA 3′ |
| *KLF4-F* | 5′ TGGGTCTTGAGGAAGTGCTG 3′ |
| *KLF4-R* | 5′ TGTTTACGGTAGTGCCTGGTC 3′ |
| *SOX2-F* | 5' CACCTACAGCATGTCCTACTC 3' |
| *SOX2-R* | 5' CATGCTGTTTCTTACTCTCCTC 3' |
| *PRL-1-F* | 5′ GGCAAACTTCGAGTCTCCT 3′ |
| *PRL-1-R* | 5′ CCGGTTGATGAATGGCTAA 3′ |
| *MEIS2-F* | 5′ GTCCACGAACTGTGCGATAA 3′ |
| *MEIS2-R* | 5′ TTCGGAAGGGTACGGATG 3′ |
| *AXIN2-F* | 5′ AGCATTTTAATCAACAGCATCTA 3′ |
| *AXIN2-R* | 5' TAACTAAGAATGTGATCCAAGAA 3' |
| *NANOG-F* | 5’ CAACTGGCCGAAGAATAGCA 3 |
| *NANOG-R* | 5’ GCAGGAGAATTTGGCTGGAA 3’ |
| *GAPDH-F* | 5' GGAGCGAGATCCCTCCAAAAT 3' |
| *GAPDH-R* | 5' GGCTGTTGTCATACTTCTCATGG 3' |

**Table 2 Primer sequences used in polymerase chain reaction for detecting genomic binding sites for stem cell markers**

|  |  |
| --- | --- |
| **Genes** | **Sequences** |
| *CD133-F* | 5′ TTTGTCTTCTATTCTTGGCTTC 3′ |
| *CD133-R* | 5′ ACCTTGTCATAATCAATTTTGG 3′ |
| *CD44(1)-F* | 5′ CTCATGGCTCAGTCGCCCAATCA 3′ |
| *CD44(1)-R* | 5′ TTTGCTCCTGAGCTGTTGCGTGG 3′ |
| *CD44(2)-F* | 5′ AGATTAAGGAGCTAGGACTC 3′ |
| *CD44(2)-R* | 5′ AAGATCACTTGGCAAGAAAG 3′ |
| *CD44(3)-F* | 5′ GGCACGTGTGAAACCTTTCCATTC 3′ |
| *CD44(3)-R* | 5′ GCTGAGCTGGACGCCAAGCA 3′ |
| *CD44(4)-F* | 5′ GCCTTTCATCCCTCGGGTGTGC 3′ |
| *CD44(4)-R* | 5′ TTCCTCCCAGGGACCAGGCC 3′ |
| *MEIS2(1)-F* | 5′ GGATTCCTGGCCAAAGGACGC 3′ |
| *MEIS2(1)-R* | 5′ CTCCCCCTAAGAGCGGCTCCA 3′ |
| *MEIS2(2)-F* | 5′ ACTGCCCGCAAGGATTCCACAA 3′ |
| *MEIS2(2)-R* | 5′ GGACTGTGGACCAAATCCAGCACAG 3′ |
| *AXIN2-F* | 5′ TATTCAAGGCATCTTTTACTGGAC 3′ |
| *AXIN2-R* | 5' AGCAAAGAACTAGCCAATAAGGAG 3' |