Sine oculis homeobox 1 promotes proliferation and migration of human colorectal cancer cells through activation of Wnt/β-catenin signaling

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Sine oculis homeobox 1 (Six1) is a homeodomain transcription factor that is aberrantly expressed in a variety of human cancers, including colorectal cancer (CRC). Six1 has been reported to play a key role in the proliferation and migration of CRC cells but the underlying molecular mechanisms are still poorly characterized. In the present study, we found that Six1 overexpression promoted the proliferation and migration of CRC cells. Consistently, Six1 knockdown (KD) significantly inhibited proliferation and migration of CRC cells. In addition, we showed that Six1 promoted proliferation and migration of CRC cells through activation of Wnt/β-catenin signaling, as evidenced by promotion of nuclear localization of β-catenin. Silencing of β-catenin expression with siRNA or inhibiting Wnt signaling with a specific inhibitor, xav939, significantly blocked Six1-induced nuclear localization of β-catenin and mitigated Six1-promoted proliferation and migration of CRC cells. We further confirmed the involvement of β-catenin in Six1-promoted proliferation and migration of CRC cells by activation of Wnt signaling with lithium chloride (LiCl) in Six1 KD CRC cells and results showed that LiCl restores defective β-catenin nuclear localization and proliferation and migration of CRC cells. Taken together, these results suggest that Six1 homeoprotein promotes the proliferation and migration of CRC cells by activating the Wnt/β-catenin signaling pathway, and strategies targeting Six1 may be promising for the treatment of CRC.

KEYWORDS
β-catenin, colorectal cancer, migration, proliferation, Six1

1 | INTRODUCTION

The sine oculis (so) homeobox (Six) family proteins, first identified as homologs of Drosophila sine oculis (So), are homeodomain-containing transcription factors containing a conserved DNA binding Six-type HD and a protein-protein interaction Six-domain.1 Six family proteins are key regulators of organogenesis and play a crucial role in tumorigenesis.2 Six1, a member of Six family protein, has been reported to be an essential transcription factor involved in the development of many tissues and organs, including kidney and muscle tissue, as well as the
auditory system and certain sensory organs. Six1 was also found to be highly expressed and implicated in both tumor initiation and tumor progression in a variety of human cancers, including breast cancer, Hodgkin lymphoma, cervical cancer, osteosarcoma, hepatocellular carcinoma, colorectal cancer, ovarian cancer, rhabdomyosarcoma, and Wilms tumors. As a DNA-specific transcription factor, Six1 has no intrinsic transcriptional activation domain and requires EYA1 to initiate target gene expression, whereas in the absence of EYA1, SIX1 associates with corepressor DACH1 to inhibit transcription of target genes. It has been documented that Six1 induces the Warburg effect (or aerobic glycolysis) which facilitates breast tumor growth with elevated glucose uptake and lactate production in vitro and in vivo by directly upregulating the expression of many glycolytic genes depending on interaction with histone acetyltransferases HBO1 and AIB1. In addition, Six1 promotes tumor progression in mouse models of breast cancer through facilitation of lymphangiogenesis, EMT by upregulating vascular endothelial growth factor C (VEGF-C) and activating ERK and TGF-β signaling. When compared to normal mucosa, Six1 is highly expressed in 50% of CRC tissues and was associated with poor prognosis in two large cohorts (total n = 945). In CRC cells, Six1 KD with shRNA significantly reduced proliferative potential, migratory ability and invasiveness of colorectal cancer cells. Six1 also boosts EMT of CRC cells by suppressing E-cadherin expression partly through repression of miR-200-family expression and activation of ZEB1. This evidence provides strong support for the involvement of Six1 in CRC progression, but the molecular mechanisms remain largely unknown.

β-Catenin is a pivotal component of the Wnt pathway. During activation of Wnt signaling, β-catenin was released from a large protein complex mainly composed of Axin, APC, CK1, and glycogen synthase kinase 3 beta (GSK-3β) and accumulated in the cytoplasm. The accumulated β-catenin translocated into the nucleus to activate gene transcription by converting the TCF/LEF repressor complex into a transcriptional activator complex. Wnt/β-catenin signaling regulates many fundamental biological processes, including cell fate determination, cell proliferation, and stem cell maintenance. Deregulation of Wnt signaling is present in about 90% of sporadic colon cancers. For example, APC mutation is a very early event in colorectal tumorigenesis and restoration of APC expression induces apoptosis in CRC cells with deficiency in endogenous APC expression. Frameshift mutation of AXIN2, a substitute of AXIN1 in the β-catenin destruction complex under certain circumstances, has been reported to be closely associated with CRC with defective DNA MMR. Given that Six1 and Wnt/β-catenin play important roles in CRC development, it would be
very interesting to determine whether there is any crosstalk between Six1 and the Wnt/β-catenin pathways and the contribution of this interaction in the progression of CRC.

In the present study, we showed that overexpression of Six1 can promote the proliferation and migration of human CRC cells and Six1 silencing reduced their growth and migration. Importantly, we found that Six1 exerts its effects in CRC cells by activating the Wnt/β-catenin signaling pathway. Our data show an important and novel correlation between Six1 and Wnt signaling that may have important implications for therapeutic treatment of CRC patients.

2 MATERIALS AND METHODS

2.1 Cell culture

Human colorectal cancer cell lines LoVo and HCT116 were obtained from the ATCC (Manassas, VA, USA), and were cultured in RPMI-1640 (Hyclone, Logan, UT, USA) supplemented with 10% (v/v) FBS (Hyclone) and 1% penicillin/streptomycin (Invitrogen, Carlsbad, CA, USA) at 37°C in a humidified incubator containing 5% CO₂. All cells were tested and found to be free of Mycoplasma contamination.

2.2 Plasmids and siRNA knockdown

Full-length cDNA of the Six1 gene was synthesized by Sangon (Shanghai, China), and subcloned into the pXJ40-Myc expression vector containing a Myc-tag between the BamHI and XhoI restriction enzyme sites with specific primers, generating pXJ40-Six1 expression constructs. This recombinant Six1 plasmid was then transfected into HCT116 cells using Lipofectamine 2000 (Invitrogen), while cells transfected with vector pXJ40-Myc served as the control. All transfections were carried out in accordance with the manufacturer’s protocol.
Three sequences specific to human Six1 were selected to generate siRNA, target 1: GCA CAA GAA CGA GAG CGU ATT; target 2: CCA ACU CUC UCC UGU GGA ATT; target 3: GGA GCU CAA ACU AUU CUC UTT. For the human CTNNB1 gene, the following siRNA target sequences were selected: target 1: GCU UUA UUC UCC CAU UTT; target 2: GGA CAC AGC AAU UUG UTT; target 3: GGA UGU GGA UAC CUC CCA ATT. All siRNA constructs were synthesized by GenePharma (Shanghai, China). Three scrambled sequences with no homology to any human genes were used as negative controls for RNAi experiments. For siRNA transfection, LoVo cells were subcultured in six-well plates. Once they grew to 40% confluence, cells were transfected with 10 nmol/L siRNA for 48 hours prior to further treatment.

2.3 Western blotting

Cells (HCT116 or LoVo) were washed twice with cold PBS (0.5 mmol/L KH$_2$PO$_4$, 1.3 mmol/L KCl, 3.2 mmol/L Na$_2$HPO$_4$, 140 mmol/L NaCl, pH 7.4) and proteins were extracted using cold lysis buffer (20 mmol/L Tris pH 7.4, 150 mmol/L NaCl, 1 mmol/L EDTA, 1 mmol/L EGTA, 5 mmol/L NaF, 0.5% Triton X-100, 2.5 mmol/L sodium pyrophosphate, 1 mmol/L β-glycerolphosphate, 1 mmol/L Na$_3$VO$_4$, 1mg/L leupeptin, and 0.5% Na-deoxycholate) at 4°C overnight. Samples were then centrifuged at 15 000 g for 10 minutes at 4°C, and supernatants were collected. Supernatant protein concentrations were measured using the Bradford assay (Bio-Rad, Hercules, CA, USA). Total protein (10-20 μg) was separated on an 8%-10% SDS-PAGE gel, and transferred to a PVDF membrane (Millipore, Billerica, CA, USA). After blocking with TBST (0.1% Tween-20 in TBS) containing 5% (w/v) non-fat milk at room temperature for 1 hour, membrane was probed with appropriate primary antibodies diluted to working concentrations in 3% bovine serum albumin (BSA) (Genview, Pompano Beach, FL, USA) at 4°C overnight. The membrane was then washed with TBST for 3 times (5-10min each) and incubated with a horseradish peroxidase-conjugated secondary antibody (Vazyme Biotech, Nanjing, China) at room temperature for over 1 h, and developed using Pierce’s West Pico Chemiluminescence substrate. Immune-reactive bands were visualized using a luminescent image analyzer (Amersham Imager 600; GE Healthcare, Butler, PA, USA). Primary antibodies were purchased from Cell Signaling Technology (Danvers, MA, USA; Six1, LaminB1, p-JNK, JNK, p-p53, p53, cyclinD1, p-AKT, AKT, p-AMPKα, AMPKα, p-p38, p38 and β-catenin) and Sigma Chemical Co. St Louis, MO, USA (β-actin). All results were from three independent experiments.

2.4 Fractionation of nuclear and cytoplasmic proteins

Cytoplasmic proteins and the nuclear proteins were extracted using a nuclear and cytoplasmic protein extraction kit (Beyotime Institute of Biotechnology, Haimen, China) according to the manufacturer’s instructions. Briefly, after collecting and washing cells with PBS, cell pellets were resuspended in ice-cold cytoplasmic protein extraction buffer A containing PMSF on ice. Samples were vigorously vortexed for 15 seconds and then incubated on ice for 15 minutes. After adding cytoplasmic protein extraction buffer B containing PMSF, lysate was vortexed and centrifuged at 16 000 g for 10 minutes at 4°C, and supernatant was transferred (cytoplasmic extract) to a clean prechilled tube. The pellet was resolved with nuclear protein extraction buffer and incubated on ice for 30 minutes. After centrifuge at 16 000 g for 10 minutes at 4°C, supernatant was collected as nuclear protein.

![FIGURE 3](https://example.com/figure3.png) Sine oculis homeobox 1 (Six1) activated Wnt/β-catenin signaling by promoting β-catenin nuclear localization. A, Western blot shows that β-catenin nuclear localization was elevated in HCT116 cells transfected with pXJ-40-Six1. B, β-catenin nuclear localization was perturbed in LoVo cells transfected with Six1 siRNA. β-Actin was used as a cytoplasmic loading control and LaminB1 was used as a nuclear loading control.
2.5 | Cell proliferation assay

MTT assay was used to determine cell proliferative ability with Cell Proliferation Kit I (Roche Applied Science, Penzberg, Germany) according to the manufacturer’s instructions. In brief, 2000 cells were plated in 96-well plates and, at specified time points (12, 24, 48, 72, and 96 hours) cell proliferation was quantified using the MTT assay in fresh medium for 4 hours and then with DMSO overnight. Absorbance was measured at a wavelength of 490 nm using a SpectraMax Plus microplate reader (Thermo Fisher Scientific, Waltham, MA, USA).

2.6 | Wound-healing assay

LoVo cells or HCT116 cells treated appropriately were seeded into 60-mm dishes and cultured at 37°C until 90% confluent. Then, cells were cultured in serum-free medium for 24 hours and scratched with a 200-μL sterile pipette tip to create a wound that was run along the dish bottom. Wounded monolayer cells were washed twice with fresh normal medium to remove cell debris. Changes in wound size were photographed under the microscope at different time points, and the rate of wound healing was calculated according to the percentage of cells filling in the scratched area over time.

2.7 | In vitro migration assays

Human CRC cell migration was evaluated using a Transwell assay system. Briefly, LoVo and HCT116 cells suspended in 100 μL serum-free media were seeded into the upper compartment of each chamber of a 24-well plate (with an 8-μm pore size insert; BD Biosciences, San Jose, CA, USA). RPMI-1640 (600 μL) containing 10% FBS was added into the lower chamber to serve as a chemoattractant. After incubation for 24 hours, cells on the upper surface of the membrane were removed with a cotton swab and cells migrating to the lower compartment of the insert were fixed and stained using a 0.5% crystal violet solution for 45 minutes and were then counted under a microscope. This experiment was conducted in triplicate.

2.8 | Statistical analysis

All data are expressed as mean ± SD. Experimental results were analyzed with GraphPad Prism Software Version 5.0 (GraphPad Software Inc., La Jolla, CA, USA). P < .05 was considered to be statistically significant.
3 | RESULTS

3.1 | Sine oculis homeobox 1 overexpression promotes proliferation and migration of HCT116 cells

To determine the role of Six1 in the progression of CRC, we first compared the expression of Six1 in different CRC cells, including HCT8, HCT116, and LoVo cells. Six1 protein was highly expressed in LoVo cells but was almost undetectable in HCT116 cells (Figure S1). For this reason, these two cell lines were selected for subsequent Six1 loss of function and gain of function experiments, respectively.

To assess the effect of Six1 overexpression in the progression of CRC, Six1 was cloned into pXJ-40 vector which was then transfected into HCT116 cells. After transfection for 48 hours, western blotting was carried out to confirm transfection efficiency. We found that protein levels of Six1 were significantly increased in cells transfected with the Six1 vector (pXJ-40-Six1) as compared to the control vector (Figure 1A). We then sought to assess the effect of this overexpression on the growth and migration of CRC cells in vitro using MTT, wound healing, and Transwell assay. We found that proliferation of HCT116 cells was significantly enhanced by Six1 overexpression as measured by the MTT assay (Figure 1B). Similarly, the migratory potential of these CRC cells was enhanced in a wound-healing assay, as the Six1-overexpressing cells showed a narrower wound area 24 hours after wounding relative to control cells (Figure 1C,D). The enhanced migratory potential of these Six1-overexpressing cells was further confirmed by Transwell migration assay (Figure 1E,F). In summary, these results indicated that overexpression of Six1 promotes CRC-associated migration and proliferation in vitro.

3.2 | Sine oculis homeobox 1 knockdown decreases proliferation and migration of LoVo cells

To further confirm the role of Six1 in the proliferation and migration of CRC cells, we knocked down Six1 expression in LoVo cells with siRNA targeting human Six1 (si-Six1). Efficiency of Six1 knockdown was confirmed by western blot. As shown in Figure 2A, Six1 expression in cells transfected with siRNA (si-Six1) was significantly reduced as compared to that in cells transfected with scrambled siRNA (si-Ctrl). We then
assessed cell proliferation and migration using the same MTT, wound healing, and Transwell assay. Six1 knockdown significantly reduced the growth of colorectal cancer cells, especially at 2, 3, and 4 days after transfection (Figure 2B). LoVo cells in which Six1 had been knocked down also showed a wider wound area 24 hours after scratching in comparison with control cells (Figure 2C, D). This was further validated by Transwell migration assays (Figure 2E, F). These results suggested that siRNA knockdown of Six1 expression suppresses tumor cell growth, consistent with an oncogenic role for Six1 in CRC cells.

3.3 | Sine oculis homeobox 1 induces activation of Wnt/β-catenin signaling

To determine the signaling pathways involved in Six1-mediated progression of CRC, we screened multiple potential signaling pathways that had been reported to regulate the progression of CRC. We found that the Wnt/β-catenin pathway was activated in response to Six1 levels. However, no obvious difference could be observed for many other signaling pathways, such as JNK, p53, AKT, P38 and AMPK pathways. Specifically, we found that Six1 overexpression in HCT116 cells led to a redistribution of β-catenin from the cytoplasm to the nucleus, driving an increase in the nuclear accumulation of β-catenin (Figure 3 and Figure S2A). Consistent with this finding, Six1 knockdown by siRNA reduced Wnt activation (Figure 3 and Figure S2B). Additionally, cyclin D1, a crucial downstream effector in the Wnt/β-catenin signaling pathway, was also upregulated or downregulated as a response to the activation or inhibition of Wnt pathway, as shown in Figure 3. This suggests that Six1 facilitates activation of the Wnt/β-catenin signaling pathway in colorectal cancer cells. Importantly, total β-catenin levels were almost unchanged in HCT116 and LoVo cells in all conditions, indicating that nuclear localization rather than altered expression mediates these observed effects.

3.4 | Wnt/β-catenin signaling is involved in Six1-mediated CRC progression

We next silenced β-catenin expression in Six1-overexpressing cells with a commercial β-catenin siRNA (Figure 4A). Interestingly, this
**DISCUSSION**

Colorectal cancer is the third most frequently diagnosed malignancy and the fourth most frequent cause of cancer death worldwide. Colorectal cancer is a heterogeneous disease at the molecular level. Extensive heterogeneity of CRC confers its resistance to targeted treatments. CRC incidence has been increasing in developing countries, especially in Western Asia and Eastern Europe. Thus, further study to determine the molecular mechanism underlying CRC progression is urgently required. In our study, we found that Six1 is involved in the proliferation and migration of human CRC cells through activating the Wnt/β-catenin signaling pathway. Our results may give rise to a new target for intervention in the treatment of CRC and may improve the future treatment of CRC.

Six1, a member of the sine oculis homeobox family proteins, is a transcription factor and has been reported to be involved in tumorigenesis. For example, Six1 overexpression can promote tumor growth and metastasis in breast cancer by amplifying TGF-β signaling. In addition, SIX1 enhanced cell proliferative and migratory potential and suppressed cell apoptosis by activating the PI3K/AKT signaling pathway through reducing phosphatase and tensin homolog (PTEN) expression in osteosarcoma cells. In CRC, Six1 facilitated angiogenesis by inducing the expression of VEGF and promoting EMT through repression of miR-200 expression and activating ZEB1. Six1 also increases metastatic potential by promoting EMT in HCT116 cells, whereas Six1 gene knockdown by lentivirus-mediated shRNA efficiently suppressed proliferation, migration and invasion in LoVo cells. Consistent with these reports, our results showed that Six1 overexpression significantly enhanced the proliferation and migration of HCT116 cells, and silencing of Six1 expression was found to reduce the proliferation and metastasis of LoVo cells. These results indicate that Six1 functions as a potential oncogene in the progression of CRC.

The underlying molecular mechanism for Six1-regulated CRC progression is identified to be related to the Wnt/β-catenin signaling pathway. β-Catenin is a transcription factor and plays a key role in the canonical Wnt pathway by translocating from the cytoplasm into the nucleus. Once in the nucleus, β-catenin binds to TCF proteins and serves as a coactivator of TCF to stimulate transcription of Wnt target genes. Aberrant activation of the canonical Wnt/β-catenin pathway occurs in almost all colorectal cancers and contributes to their growth, invasion and survival. It has been reported that Wnt/β-catenin promotes skeletal myogenesis in P19 cells by upregulating Six1 expression, suggesting that Wnt/β-catenin signaling factors lie genetically upstream of Six1. However, our present study showed strong evidences that Six1 promotes growth and migration of CRC cells by facilitating β-catenin nuclear translocation. Involvement of Wnt/β-catenin in Six1-mediated growth and migration of CRC cells was supported by two solid pieces of evidence. First, inhibiting Wnt/β-catenin signaling by gene KD or Wnt inhibitor xav939 significantly blocked Six1-promoted proliferation and migration of CRC cells. Second, activating Wnt/β-catenin signaling with Wnt activator LiCl could restore impaired proliferation and migration of CRC cells with Six-1 KD. These data may suggest that interaction between Six1 and Wnt signaling is either cell type specific or there is negative feedback between them.

In summary, we first reported a novel mechanism for Six1 in the proliferation and migration of CRC cells. Our results indicated that Six1 potentiates the progression of CRC cells through upregulating the Wnt signaling pathway. As a result of the important roles of Six1 and Wnt/β-catenin signaling in the progression of CRC, we will investigate the exact mechanism underlying Six1 regulating β-catenin nuclear localization which might serve as an attractive target for the molecular therapy of CRC.

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**CONFLICTS OF INTEREST**

Authors declare no conflicts of interest for this article.

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SUPPORTING INFORMATION

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