**Original Article**

ANKHD1 silencing suppresses the proliferation, migration and invasion of CRC cells by inhibiting YAP1-induced activation of EMT

Ping'an Yao1*, Yecheng Li1*, Wenqi Shen1, Xiaohui Xu1, Wei Zhu2,3, Xiaodong Yang1, Jianping Cao2,3, Chungen Xing1

1Department of General Surgery, The Second Affiliated Hospital of Soochow University, No.1055 Sanxiang Road, Suzhou 215004, China; 2School of Radiation Medicine and Protection, Medical College of Soochow University, Suzhou 215123, China; 3State Key Lab of Radiation Medicine and Protection, Collaborative Innovation Center of Radiation Medicine of Jiangsu Higher Education Institutions, Soochow University, Suzhou 215123, China. *Equal contributors.

Received August 15, 2018; Accepted October 11, 2018; Epub November 1, 2018; Published November 15, 2018

**Abstract:** Ankyrin repeat and KH domain containing 1 (ANKHD1) is a protein with multiple ankyrin repeat domains and a single KH domain, and it is encoded by the ANKHD1 gene in humans. ANKHD1 has been reported to be highly expressed in various cancer tissues, and it is involved in cancer progression, including proliferation and invasion. However, its functional roles in colorectal cancer (CRC) remain unclear. In our study, we first found that high expression of ANKHD1 in CRC tumor tissue was associated with tumor infiltration depth (P=0.03). ANKHD1 was highly expressed in HCT116 and SW480 cells. Downregulation of ANKHD1 inhibited CRC cell proliferation, migration and invasion both in vitro and in vivo. ANKHD1 silencing inhibited the expression of MMP2, MMP9, the mesenchymal marker vimentin, and the epithelial-to-mesenchymal transition (EMT) transcription factors Snail and ZEB1, while increasing the expression of the epithelial marker E-cadherin. As a cofactor of YAP1 in the Hippo signaling pathway, ANKHD1 silencing reduced the expression and increased the phosphorylation of YAP1. Moreover, the phosphorylation of AKT was inhibited when ANKHD1 was knocked down. The mechanism study revealed that the effect of ANKHD1 might be associated with the expression of YAP1 and that AKT signaling and EMT played crucial roles in this process. Overexpression of YAP1 reversed the effect of ANKHD1 silencing on CRC cell proliferation, migration and invasion. In conclusion, these findings suggest that ANKHD1 might act as a novel regulator that promotes CRC cell proliferation, migration and invasion by activating EMT via YAP1.

**Keywords:** ANKHD1, YAP1, EMT, proliferation, migration, invasion, CRC

**Introduction**

Colorectal cancer (CRC) is one of the most common malignant tumors in the gastrointestinal tract, the third most commonly diagnosed cancer and the third leading cause of cancer-related death in both males and females in the United States [1]. Surgery and adjuvant therapy, including chemotherapy and radiotherapy, are currently the main treatments for CRC [2]. Despite the advances in treatments, the prognosis of CRC patients is not optimistic, with a 65% five-year relative survival rate for patients diagnosed in 2013 [1]. Metastasis of CRC is one of the main reasons for its high mortality and poor prognosis. Approximately 90% of CRC-related deaths occur as a result of metastatic disease [3]. Epithelial-to-mesenchymal transition (EMT) has been shown to play a vital role in cell metastasis and invasion [4]. Aberrant activation of EMT-inducing transcription factors is an important part of metastasis [5]. Once this conversion occurs, immobile epithelial cells lose their epithelial characteristics and acquire mesenchymal properties, including the ability to migrate and invade [6]. The occurrence of EMT also promotes the progression of malignant tumors [7]. Despite these insights, the molecular mechanisms of EMT are varied, and mechanisms by which it will be possible to influence the metastasis of CRC still need to be explored.
Ankyrin repeat and KH domain containing 1 (ANKHD1) is a protein with multiple ankyrin repeat domains and a single KH domain that is encoded by the ANKHD1 gene. ANKHD1 is a human orthologous protein of MASK and was first identified in Drosophila melanogaster [8]. The presence of multiple ankyrin repeats suggests that it mediates protein-protein interactions and participates in biological processes, including the cell cycle, transcription, differentiation, apoptosis, cell survival, and even the development of cancer [8, 9]. ANKHD1 was found to be overexpressed in both malignant cells from patients diagnosed with acute leukemia and multiple myeloma (MM) and several cancer cell lines, including K562 (leukemia), MM1R, MM1S, RPMI 8226 and U266 (MM) and LNCaP (prostate) [10-12]. In leukemia cells, ANKHD1 silencing significantly inhibited cell proliferation and migration [13]. Studies have found that ANKHD1 is a novel component of the Hippo signaling pathway that interacts with YAP1 and promotes cancer progression [12]. However, the expression of ANKHD1 in CRC and its effect on the progression of CRC has not been reported thus far.

Hence, our study aimed to study the effect of ANKHD1 on CRC cell proliferation and metastasis both in vitro and in vivo. Inhibition of ANKHD1 reduced YAP1 expression and inhibited the occurrence of EMT and the phosphorylation of AKT. Then, we investigated the role played by YAP1 in the effect of ANKHD1 on CRC progression.

Materials and methods

Cell culture

The human colorectal carcinoma cell lines HCT8, HCT116, SW480, SW620, LOVO and RKO were purchased from the Shanghai Chinese Academy of Sciences (Shanghai, China) and cultured in Dulbecco’s modified Eagle’s medium (DMEM) (HyClone, Logan, USA) supplemented with 10% fetal bovine serum (FBS) and 1% penicillin-streptomycin at 37 °C in a humidified atmosphere with 5% CO₂. The experiments were performed when the cells reached 70-80% confluence.

Transduction and transient transfection

HCT116 and SW480 cells were transduced with lentivirus-mediated control shRNA or lentivirus-mediated shRNA targeting ANKHD1, designated shNC and shANKHD1, respectively. The lentivirus-mediated shRNA was obtained from Hanbio Biotechnology (Shanghai, China); specific fragments were inserted into shNC (top strand: GATCCGTCTCCGACGCAGTCACGATTACGAGTACGTCGAGAATTTCGCTACGAGTTAGCTCTGTGACACGTTCGGAGAACG) and shANKHD1 (top strand: GATCCGTCTCCGACGCAGTCACGATTACGAGTACGTCGAGAATTTCGCTACGAGTTAGCTCTGTGACACGTTCGGAGAACG) and shANKHD1 (top strand: GATCCGTCTCCGACGCAGTCACGATTACGAGTACGTCGAGAATTTCGCTACGAGTTAGCTCTGTGACACGTTCGGAGAACG) and shANKHD1 (top strand: GATCCGTCTCCGACGCAGTCACGATTACGAGTACGTCGAGAATTTCGCTACGAGTTAGCTCTGTGACACGTTCGGAGAACG) and shANKHD1 (top strand: GATCCGTCTCCGACGCAGTCACGATTACGAGTACGTCGAGAATTTCGCTACGAGTTAGCTCTGTGACACGTTCGGAGAACG). The cells were subsequently harvested at 72 h postinfection and selected in medium containing 8 μg/ml puromycin (Sigma-Aldrich, St. Louis, Missouri, USA) until all uninfected cells were killed by the puromycin. The stably transfected cell lines were validated by Western blotting and real-time PCR and then used for the subsequent experiments. The YAP1 lentiviral vector (YAP1 cDNA) was purchased from Applied Biological Materials (Richmond, BC, Canada), and transfection was performed using the Lipofectamine 2000 reagent (Invitrogen, Carlsbad, CA, USA). The subsequent experiments were performed 48 h after transfection. HCT116-shANKHD1 cells were transfected with the YAP1 lentiviral vector and designated HCT116shANKHD1+YAP1.

Cell proliferation assay

Cell proliferation was measured by the Cell Counting Kit 8 (Beyotime Biotechnology, China) according to the manufacturer’s instructions. The stably transfected cell lines HCT116-shANKHD1 and SW480-shANKHD1 were seeded in 96-well plates. Then, 100 μl of DMEM containing 10% CCK8 reagent was added to each well at 24 h, 48 h, 72 h and 96 h post-cell seeding and cultured for 2 h. The absorbance was measured at a wavelength of 450 nm by a microplate reader (Bio-Rad, Hercules, CA, USA). Three independent experiments were performed.

Cell invasion and migration assay

Cell invasion and migration assays were performed using 8-μm pore size transwell chambers (Corning, New York, USA). For the invasion assay, the upper chamber of the transwell was precoated with Matrigel and incubated at 37 °C for 2 h to form the Matrigel layer in the chamber. The stably transfected cells were starved in serum-free DMEM; then, 4 × 10⁴ cells with
0.5% BSA in DMEM were seeded in the upper chamber of the transwell, while complete DMEM with 10% FBS was added to the lower chamber. At 24 h after inoculation, the cells remaining in the upper chamber were disposed of, and the invaded cells were stained by crystal violet. For the cell migration assay, 4 × 10^4 starved cells with 0.5% BSA in DMEM were seeded in the upper chamber of the transwell, and the lower chamber was filled with complete DMEM with 10% FBS. At 24 h after inoculation, the cells in the upper transwell chamber were disposed of, and the migrated cells were stained by crystal violet. Each experiment was repeated three times independently.

**RNA extraction and quantitative real-time PCR**

Total RNA was extracted using TRIzol reagent (Invitrogen, Carlsbad, CA, USA), and 1 μg of total RNA from the stably transfected cells was reverse transcribed to cDNA using the Prime Script PRT Reagent Kit (Takara, Kyoto, Japan) according to the manufacturer’s instructions. Quantitative real-time PCR was performed using SYBR-Green Mix and detected by the ABI PRISM 7500 Sequence Detection System (Applied Biosystems, Foster City, CA, USA). The mRNA level of ANKHD1, YAP1, E-cadherin and Vimentin was detected by quantitative real-time PCR, and the amplified transcript level was normalized to that of GAPDH. The primer pairs were as follows: ANKHD1 (forward primer: 5'-CCTGCTTGGAACCCTATGATAAA-3'; reverse primer: 5'-CGTGCCAGGCCAAATCTG-3'), GAPDH (forward primer: 5'-CATGAGAAGTATGACCTTAGA-3'; reverse primer: 5'-AGTCCCTTCACGATCCAAATG-3'), YAP1 (forward primer: 5'-CGCTTCTCAACGCGCTCA-3'; reverse primer: 5'-AGTACTGCTGTGCAGGGAGT-3'), E-cadherin (forward primer: 5'-ATTCTGATTCTGCTGCTCTTG-3'; reverse primer: 5'-AGTAGTCATAGTCCTGGTCTTT-3') and Vimentin (forward primer: 5'-GGACAGCTAACCAACAGACA-3'; reverse primer: 5'-AAGGTCAGACGGTGCAGAG-3').

**Western blot assay**

The stably transfected cells and transiently transfected cells were collected at specific times and then lysed by cell lysis buffer for Western blotting. The total protein concentration was detected using a BCA Protein Assay Kit (Beyotime Biotechnology, China). After that, 30 μg of proteins were separated by 8% or 10% SDS-PAGE and transferred to PVDF membranes. The following primary antibodies were used for the Western blots: ANKHD1, YAP1, p-YAP1, ZEB1, Snail, E-cadherin, vimentin, MMP2, MMP9, AKT, p-AKT, Bcl-2 and Bax (1:1000, Abcam, Cambridge, MA, USA), Tubulin and GAPDH (Beyotime Biotechnology, China) were used as internal controls.

**In vivo experiments**

Male BALB/c nude mice (5 weeks old) were purchased from the SLAC Laboratory Animal Company (Shanghai, China). Then, 4 × 10^6 HCT116-shANKHD1 cells or HCT116-shNC cells in 0.1 mL of PBS were injected subcutaneously into the right flanks of the nude mice (five mice per group). Tumors were measured every two days by caliper, and the longest diameter (A) and the shortest diameter (B) of every tumor were recorded separately to calculate the tumor volumes according to the following formula: π/6 × A × B^2 [14]. For the metastasis assay, 1 × 10^6 HCT116-shANKHD1 cells or HCT116-shNC cells in 0.1 ml PBS were injected into the nude mice via the tail vein. After 40 days, the lungs and livers were dissected and photographed and then stained with hematoxylin and eosin (H&E). All nude mouse experiments were approved by the ethics committee of Soochow University.

**Immunohistochemistry and HE analysis**

Immunohistochemistry (IHC) was performed to investigate protein expression levels in cancer tissue. All surgically resected specimens were obtained from patients diagnosed with CRC at the Second Affiliated Hospital of Soochow University from 2009 to 2014. The sections of tissue for IHC were incubated with antibodies against ANKHD1. The intensity of staining of the cancer tissues was scored as follows: 0 (no staining), 1 (weak staining, light yellow), 2 (moderate staining, yellowish brown), and 3 (strong staining, brown). An intensity score ≥ 2 was considered overexpression, whereas intensity scores < 2 were considered indicators of low expression. All slides were evaluated independently by two investigators blinded to the patient identities and clinical outcomes. H&E staining was performed to verify the presence of metastatic cancer nodules.

**Statistical analysis**

The X^2 test was performed to detect the correlation between ANKHD1 expression and clinical
Effect of ANKHD1 on proliferation, invasion and metastasis of colorectal cancer

Table 1. Correlation between tumor ANKHD1 expression and the pathologic features of CRC patients

<table>
<thead>
<tr>
<th>Variables</th>
<th>All cases</th>
<th>ANKHD1 expression</th>
<th>P</th>
</tr>
</thead>
<tbody>
<tr>
<td>Colorectal cancer</td>
<td>136</td>
<td>Negative (%)</td>
<td>Positive (%)</td>
</tr>
<tr>
<td>Age</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>&lt; 60</td>
<td>35</td>
<td>4 (2.9)</td>
<td>31 (22.8)</td>
</tr>
<tr>
<td>≥ 60</td>
<td>101</td>
<td>8 (5.9)</td>
<td>93 (68.4)</td>
</tr>
<tr>
<td>Gender</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Male</td>
<td>82</td>
<td>7 (5.1)</td>
<td>75 (55.1)</td>
</tr>
<tr>
<td>Female</td>
<td>54</td>
<td>5 (3.7)</td>
<td>49 (36.1)</td>
</tr>
<tr>
<td>Differentiation</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>High/intermediate</td>
<td>101</td>
<td>8 (5.9)</td>
<td>93 (68.4)</td>
</tr>
<tr>
<td>Low/null</td>
<td>35</td>
<td>4 (2.9)</td>
<td>31 (22.8)</td>
</tr>
<tr>
<td>Invasion</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Infiltration of shallow muscularis</td>
<td>6</td>
<td>2 (1.5)</td>
<td>4 (2.9)</td>
</tr>
<tr>
<td>Infiltration of deep muscularis</td>
<td>21</td>
<td>3 (2.2)</td>
<td>15 (11.1)</td>
</tr>
<tr>
<td>Infiltration of seroso</td>
<td>109</td>
<td>7 (5.1)</td>
<td>105 (77.2)</td>
</tr>
<tr>
<td>Lymph node metastasis</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Yes</td>
<td>56</td>
<td>3 (2.2)</td>
<td>53 (39.0)</td>
</tr>
<tr>
<td>No</td>
<td>80</td>
<td>9 (6.6)</td>
<td>71 (52.2)</td>
</tr>
<tr>
<td>Nerve invasion</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Yes</td>
<td>49</td>
<td>4 (2.9)</td>
<td>45 (33.1)</td>
</tr>
<tr>
<td>No</td>
<td>87</td>
<td>8 (5.9)</td>
<td>79 (58.1)</td>
</tr>
<tr>
<td>Vascular invasion</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Yes</td>
<td>20</td>
<td>1 (0.7)</td>
<td>19 (14.0)</td>
</tr>
<tr>
<td>No</td>
<td>116</td>
<td>11 (8.1)</td>
<td>105 (77.2)</td>
</tr>
<tr>
<td>Duck’s A</td>
<td>20</td>
<td>4 (2.9)</td>
<td>16 (11.8)</td>
</tr>
<tr>
<td>B</td>
<td>60</td>
<td>5 (3.7)</td>
<td>55 (40.4)</td>
</tr>
<tr>
<td>C</td>
<td>56</td>
<td>3 (2.2)</td>
<td>53 (39.0)</td>
</tr>
</tbody>
</table>

Parameters [15]. Student’s t-test was performed to determine differences in the data from the *in vitro* experiments, and the results are expressed as the mean ± standard deviation (SD) of three independent experiments. All statistical analyses were performed using GraphPad Prism 6.0 software (GraphPad Software Inc., San Diego, USA). P < 0.05 was considered statistically significant.

Results

ANKHD1 expression in colorectal cancer tissue sample

To determine the expression of ANKHD1 in CRC, tumor tissues of 136 colorectal cancer patients were used for immunohistochemical staining. Our results showed that ANKHD1 was expressed in 91.2% of the samples (Table 1) and widely expressed in both normal colorectal tissues and colorectal cancer tissues (Figure 1A-C). The expression of ANKHD1 in colorectal cancer was highly correlated with the tumor infiltration depth (P=0.03; Table 1), while its expression was not significantly associated with age, sex, degree of differentiation and lymph node metastasis.

Construction of cell lines

The baseline expression level of ANKHD1 was detected by Western blot assay in 6 CRC cell lines, including HCT8, SW480, SW620, HCT116, LOVO and RKO. ANKHD1 was highly expressed in SW480 and HCT116 cell lines (Figure 1D). HCT116 and SW480 cells were selected to construct stably transfected cell lines with lentivirus-mediated shRNA targeting ANKHD1 to knockdown ANKHD1 expression. The infection efficiency was verified by Western blotting. Silencing efficiency was 85.26% and 82.64% in HCT116 and SW480 cells, respectively. Analysis was conducted with ImageJ. The mRNA levels exhibited similar silencing efficiency and were reduced by 61.92% and 56.00%, respectively (Figure 1E, 1F).

ANKHD1 silencing inhibits colorectal cancer cell proliferation in vitro

To investigate whether ANKHD1 affects the proliferation of colorectal cancer cells, a CCK8 assay was performed after HCT116 and SW480 cells were stably infected with shANKHD1 for 24, 48, 72 and 96 hours. Our results showed that the silencing of ANKHD1 by shRNA suppressed the proliferation of colorectal cancer cells (Figure 2A, 2B). The relative proliferation...
rate was significantly reduced by 22.5%, 31.9% and 36.7% in HCT116-shANKHD1 cells at 48 h, 72 h and 96 h compared with HCT116-shNC and HCT116-mock cells (Figure 2A, P < 0.001). This marked inhibition of proliferation was also observed in SW480-shANKHD1 cells, and the relative proliferation rate was significantly reduced by 19.1%, 28.9% and 32.4% at 48 h, 72 h and 96 h compared with SW480-shNC and SW480-mock cells (Figure 2B, P < 0.01), respectively.

To determine whether ANKHD1 silencing affects apoptosis of colorectal cancer cells, a flow cytometry-based apoptosis assay was performed. Our results showed that ANKHD1 silencing did not significantly affect apoptosis of colorectal cancer cells compared with control group (Figure 2D). Consistently, ANKHD1 silencing did not significantly affect the expression of Bcl-2 and Bax in colorectal cancer cells (Figure 2C).

ANKHD1 silencing inhibits the growth of xenograft tumors

To further confirm the inhibition of proliferation observed in vitro, we injected HCT116-shANKHD1 and HCT116-shNC cells into the right flanks of nude mice. Consistent with our
observation in vitro, the volumes of tumors formed by HCT116-shANKHD1 cells were significantly smaller than those formed by HCT116-shNC cells (Figure 2E, 2F, P < 0.05). The results demonstrated that ANKHD1 promoted tumorigenesis in vivo.

ANKHD1 silencing reduced CRC cell migration and invasion

Our migration assay revealed that the down-regulation of ANKHD1 markedly suppressed HCT116 and SW480 cell migration (Figure...
Effect of ANKHD1 on proliferation, invasion and metastasis of colorectal cancer

Figure 3. ANKHD1 promotes colorectal cancer cell migration and invasion in vitro and in vivo. Representative images of the migration and invasion of HCT116 cells (A) and SW480 cells (B) infected with shANKHD1 or shNC or none. ANKHD1 silencing significantly repressed cell migration and invasion (C, D). ***P < 0.001. The cell counts are for the invasion assay with at least five random microscope fields (invasion: × 200 magnification). Cell migration is expressed as the percentage of migrating cells in at least five random microscope fields of view (Analysis conducted with ImageJ software, migration: × 200 magnification). In total, 1 × 10^6 HCT116-shNC and HCT116-shANKHD1 cells were injected into nude mice through the tail vein, and the mice were reared for 40 days before being sacrificed to evaluate the lung metastases. Representative figures for lung metastatic tumors (E) show that mice injected with HCT116-shANKHD1 cells had fewer metastatic nodules than those injected with HCT116-shNC cells (**P < 0.05). (F) H&E staining of mouse lung metastases and normal lung tissue (× 200 magnification).

3A-D shows cell migration reduced by 39.6% and 29.7%, respectively, both P < 0.001. Similarly, an invasion assay also indicated that ANKHD1 silencing significantly reduced cell invasion by 45.4% and 57.5% compared to control HCT116 and SW480 cells, respectively (Figure 3A-D, both P < 0.001). These results suggested that ANKHD1 silencing clearly inhibited CRC cell migration and invasion.

ANKHD1 silencing inhibits HCT116 cell lung metastasis in nude mice

To further validate the effect of ANKHD1 silencing on CRC cell migration and invasion, a pulmonary metastasis model was generated by tail vein injections of HCT116-shANKHD1 and HCT116-shNC cells in nude mice. The nude mice were sacrificed after 40 days, and the
vital organs were removed and observed. The formation of pulmonary nodules was used to assess the capacity for metastasis. Compared with the HCT116-shANKHD1 group, more and larger migrated nodules were observed in the lungs in the HCT116-shNC group (Figure 3E, \( P < 0.001 \)). Then, the lung tissue was analyzed by H&E staining to further evaluate the metastases (Figure 3F). These observations further confirmed that ANKHD1 plays an important role in the metastasis and invasion of colorectal cancer.

**ANKHD1 silencing inhibited EMT and AKT phosphorylation**

Matrix metalloproteinases (MMPs) play a role in ECM degradation and promote tumor progression, leading to metastasis. Furthermore, MMPs activate TGF-β and promote EMT [16]. Consistently, we found that ANKHD1 silencing clearly reduced MMP2 and MMP9 expression by Western blotting (Figure 4A). As indicated by the Western blot assay, the expression of the epithelial marker protein E-cadherin was increased, while the expression of the mesenchymal marker protein vimentin was decreased in the shANKHD1 group compared with the shNC group and the mock group in both HCT116 and SW480 cells (Figure 4B). These changes also occurred at mRNA level (Figure 4E). The present study showed alterations in EMT-associated transcription factors, including ZEB1 and Snail; Figure 4B shows that ZEB1 and Snail were markedly decreased in the shANKHD1 groups. These results confirmed that ANKHD1 played an important role in the progression of EMT.

Our results revealed that AKT phosphorylation was significantly inhibited in the shANKHD1 group compared with the shNC group and the mock group but the expression of AKT remained unchanged (Figure 4C). These results suggest that ANKHD1 silencing inhibits AKT activation.

**YAP1 is required for ANKHD1 silencing-induced inhibition of proliferation and migration via AKT signaling and EMT**

According to reports, ANKHD1 is a novel member of the Hippo signaling pathway and is essential for the full activity of YAP1 [12, 17, 18]. Is YAP1 involved in the effects of ANKHD1 on CRC cell proliferation, migration and invasion? Western blot analysis showed that YAP1 was downregulated when ANKHD1 was knocked down (Figure 4D), real-time PCR assay revealed that this inhibition was occurred in transcriptional level (Figure 4E). Due to phosphorylated YAP1 was able to be degraded by ubiquitination, we tested p-YAP1 expression under ANKHD1 was knocked down, and found that p-YAP1 was significantly increased (Figure 4D). The increase of p-YAP1 may enhanced the degradation of YAP1. YAP1 is an important downstream EMT-inducing molecule [14]. Then, HCT116-shANKHD1 cells were transfected with the YAP1 overexpression vector to explore the role of YAP1 in ANKHD1 silencing-mediated inhibition of CRC cell proliferation, migration and invasion. Transfection efficiency was verified by Western blotting, with a 1.64-fold overexpression efficiency in HCT116-shANKHD1 cells (Figure 5A). A CCK8 assay revealed that the upregulation of YAP1 counteracted ANKHD1 silencing-induced inhibition of cell proliferation. The relative proliferation rates were increased by 22.7%, 14.7% and 17.8% in the shANKHD1+YAP1 group compared with the shANKHD1 group at 48 h, 72 h and 96 h, respectively, in HCT116 cells (Figure 5B, \( P < 0.01 \)). The migration assay indicated that HCT116-shANKHD1 cell migration was increased by 26.7% when the expression of YAP1 was upregulated (Figure 5C, \( P < 0.001 \)). Similarly, the invasion assay also showed a significant 46.0% increase in invasion (Figure 5C, \( P < 0.001 \)). These results revealed that ANKHD1 promotes CRC cell proliferation, migration and invasion via YAP1.

Western blot analysis showed that upregulation of YAP1 increased the protein expression of vimentin and the EMT transcription factors Snail and ZEB1, while the expression of E-cadherin was decreased (Figure 5D, 5E). Furthermore, we found that the phosphorylation of AKT in HCT116-shANKHD1+YAP1 cells was activated compared with HCT116-shANKHD1 cells (Figure 5F). These results indicate that ANKHD1 activates EMT and AKT signaling via YAP1. These results suggested that YAP1 is essential for ANKHD1 silencing-induced inhibition of cell proliferation, migration and invasion through the inhibition of AKT signaling and EMT.

**Discussion**

ANKHD1 is a multiple ankyrin repeat-containing protein that functions as a scaffolding pro-
Published studies have revealed that ANKHD1 is highly expressed in cancer cells, including acute leukemia, MM and prostate cancer cells, and that it plays an important role in cancer progression, including in proliferation, migration and invasion [10, 11, 19]. Nevertheless, the mechanisms by which ANKHD1 affects CRC development remain unclear. The present study retrospectively analyzed ANKHD1 expression in 136 CRC cases. Our research showed that ANKHD1 was expressed in both cancerous and paracancerous nontumor tis-

Figure 4. ANKHD1 silencing inhibits EMT and AKT signaling. Western blot analyses of the relative expression levels of MMP2, MMP9 (A), EMT-associated markers E-cadherin and vimentin and EMT transcription factors Snail and ZEB1 (B), AKT (C), and YAP1 (D). GAPDH and Tubulin were detected as internal controls. Real-time PCR detected the expression of ANKHD1, YAP1, E-cadherin and Vimentin at mRNA level (E, *P < 0.05, **P < 0.01).
Effect of ANKHD1 on proliferation, invasion and metastasis of colorectal cancer

Figure 5. Overexpression of YAP1 reversed the ANKHD1 silencing-induced inhibition of CRC cell proliferation, migration and invasion. (A) Transfection efficiency was verified by Western blotting. (B) CCK8 assays were performed at 24, 48, 72 and 96 h after HCT116-shANKHD1 cells were transfected with the YAP1 vector. The relative proliferation rate was significantly increased in the shANKHD1+YAP1 group compared with the shANKHD1 group (***P < 0.001). (C) Representative images of the migration and invasion of HCT116-shANKHD1 cells infected with the YAP1 vector or not. Cell counts are for the invasion assay with at least five random microscope fields (invasion: ×200 magnification). Cell migration is expressed as the percentage of migrating cells in at least five random microscope fields of view (migration: ×200 magnification). Overexpression of YAP1 increased HCT116-shANKHD1 cell migration and invasion (***P < 0.001). Western blot analyses of the relative expression levels of the EMT-associated markers E-cadherin and vimentin (D), the EMT transcription factors Snail and ZEB1 (E) and AKT (F). The data are presented as the means ± SD of three independent experiments.
Effect of ANKHD1 on proliferation, invasion and metastasis of colorectal cancer

The molecular mechanisms by which YAP1 modulates cell proliferation and EMT are varied. In the present study, we found that ANKHD1 silencing markedly inhibited the phosphorylation of AKT. AKT signaling plays a significant role in cell proliferation, invasion and migration. The downregulation of p-AKT inhibits proliferation and invasion in colon [36], pancreas [37] and lung [38] cancers. Moreover, mounting evidence has revealed that AKT activation pro-
Effect of ANKHD1 on proliferation, invasion and metastasis of colorectal cancer

motivates EMT via upregulated Snail expression and downregulated expression of the epithelial marker E-cadherin [39]. In our study, the inhibition of AKT signaling induced by ANKHD1 silencing was reversed when YAP1 was overexpressed. Taken together, we hypothesized that ANKHD1 silencing inhibits YAP1 action and then suppresses proliferation and EMT via AKT signaling. This molecular mechanism still needs more in-depth research.

In conclusion, we have proposed the concept that the ANKHD1-YAP1-EMT axis might regulate CRC cell proliferation, migration and invasion. The axis conferred decreased proliferative and metastatic capacities on CRC cells when ANKHD1 was knocked down. The knockdown of ANKHD1 attenuated the viability of YAP1, thereby reducing the activity of downstream AKT signaling and inhibiting the occurrence of EMT. Our study elucidates the effect of ANKHD1 on the development of CRC cells and might provide a novel target for anti-colorectal cancer therapy.

Acknowledgements

This work was supported by the National Natural Science Foundation of China (No 81672970 and 81673100), the Suzhou Key Medical Center (No LCZX201505), the project of Suzhou Science and Technology Development Plan (No SJS201618 and SS201753), the Health and Family Planning Commission Fund Project of Jiangsu Province (No LCZX201505), the Second Affiliated Hospital of Soochow University Preponderant Clinic Discipline Group Project Funding, Graduate Student Scientific Research Innovation Projects of Jiangsu Province (No KYC18_2534), and Key Scientific Development Program of China (No 2016YFC0904702).

Disclosure of conflict of interest

None.

Address correspondence to: Chungen Xing and Xiaodong Yang, Department of General Surgery, The Second Affiliated Hospital of Soochow University, No. 1055 Sanxiang Road, Suzhou 214004, China. Tel: +86-512-67784106; E-mail: xingcg@suda.edu.cn (CGX); Tel: 13812740990; E-mail: wjyxd@163.com (XDY); Jianping Cao, School of Radiation Medicine and Protection, Medical College of Soochow University, No. 199 Ren’ai Road, Suzhou 215123, China. Tel: +86-512-65880037; Fax: +86-512-65880037; E-mail: jpciao@suda.edu.cn

References

Effect of ANKHD1 on proliferation, invasion and metastasis of colorectal cancer


