



# The expression and prognostic value of Src homology 2 domain-containing transforming protein C3 (SHC3) and its potential role in colorectal cancer

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**Background:** To determine the prognostic value of Src homology 2 domain-containing transforming protein C3 (SHC3) in colorectal cancer (CRC).

**Methods:** The pan-cancer expression of SHC3 mRNA in TCGA was analyzed using Gene\_DE module in Tumor Immune Estimation Resource (TIMER) database. SHC3 mRNA expression in CRC was further analyzed by TCGA and OncoPrint databases. The dataset from Kaplan-Meier Plotter (<http://kmplot.com>) was used to analyze the overall survival (OS) of CRC patients in relationship of SHC3 expression. SHC3 mRNA expression in the CRC HCT116 and RKO cell lines was measured by qRT-PCR. Both cell lines were transduced with shSHC3 or shCtrl lentiviruses, and the knockdown was validated by qRT-PCR and Western blotting. The effects of SHC3 knockdown were analyzed by MTT assay, Celigo-based cell counting, colony formation assay, scratch assay and Transwell migration assay.

**Results:** SHC3 is upregulated in tumor tissues relative to normal tissues across multiple cancer types including CRC in TCGA database, and associated with poor OS (HR =3.27, 95% CI: 1.31–8.16, log-rank P=0.0072). Consistent with this, SHC3 mRNA levels were significantly high in CRC cell lines. SHC3 knockdown in the HCT116 and RKO cells markedly reduced their proliferation and migration, and promoted apoptosis.

**Conclusions:** SHC3 is upregulated in CRC tissues and cell lines, and likely functions as an oncogene in CRC.

**Keywords:** Colorectal cancer (CRC); Src homology 2 domain-containing transforming protein C3 (SHC3); proliferation; apoptosis; metastasis

Submitted Feb 17, 2021. Accepted for publication May 21, 2021.

doi: 10.21037/tcr-21-294

**View this article at:** <https://dx.doi.org/10.21037/tcr-21-294>

## Introduction

Colorectal cancer (CRC) ranks as the second most common cause of cancer-related deaths in the United States (1). An estimated 147,950 new cases of CRC were diagnosed in 2020 alone, of which 53,200 succumbed to the disease. In addition, 17,930 new cases and 3,640 deaths were recorded

for individuals aged younger than 50 years (2). So far, *RAS*, *BRAF* and microsatellite instability (MSI) represent the validated biomarkers in CRC, which have been known as the therapeutic targets or the prognostic factors. However, due to the heterogeneous, the identification of the most effective treatment for an individual patient is still a challenging issue,

**Table 1** The clinicopathological characteristics of paired colorectal cancer samples

| Clinicopathological characteristics | Subcategory | Patient (N=50) |    |
|-------------------------------------|-------------|----------------|----|
|                                     |             | N              | %  |
| Gender                              | Female      | 27             | 54 |
|                                     | Male        | 23             | 46 |
| Age                                 | <50         | 6              | 12 |
|                                     | ≥50         | 44             | 88 |
| T                                   | T1          | 2              | 4  |
|                                     | T2          | 7              | 14 |
|                                     | T3          | 35             | 70 |
|                                     | T4          | 6              | 12 |
| N                                   | N0          | 34             | 68 |
|                                     | N1          | 8              | 16 |
|                                     | N2          | 8              | 16 |
| M                                   | M0          | 35             | 70 |
|                                     | M1          | 8              | 16 |
|                                     | MX          | 6              | 12 |
|                                     | Unavailable | 1              | 2  |
| Stage                               | Stage I     | 8              | 16 |
|                                     | Stage II    | 23             | 46 |
|                                     | Stage III   | 10             | 20 |
|                                     | Stage IV    | 8              | 16 |
|                                     | Unavailable | 1              | 2  |

which needs to identify novel predictive and/or prognostic biomarkers to guide the management of CRC patients (3). The pathogenesis of colon cancer is driven by both environmental and genetic factors. Although several studies have identified mRNAs, microRNAs and long non-coding RNAs (lncRNAs) as diagnostic and prognostic biomarkers of CRC (4), the mechanisms involved in its initiation and progression have not been completely elucidated due to the highly heterogeneous nature of this malignancy. Therefore, it is crucial to identify novel molecules and signaling pathways underlying CRC pathogenesis.

The Src homology 2 domain-containing transforming protein C3 (SHC3; also known as Rai, N-Shc or ShcC) belongs to the Shc-like adaptor protein family (5). It is expressed in the mature neurons of the central nervous system (CNS), and relays neurotrophin signals from

the TrkB receptor to the Ras/mitogen-activated protein kinase (MAPK) pathway (6). Furthermore, SHC3 was recently identified as a novel susceptibility locus for nicotine dependence in African- and Caucasian-American populations (7), and is associated with schizophrenia in a northeast Chinese Han population (8). In addition, aberrantly high expression of SHC3 has been implicated in brain tumor progression (9,10), as well as increased microvascular invasiveness, advanced stages and poor prognosis in hepatocarcinoma (HCC) (11). Another study identified 15 differentially expressed immune-related genes (DEIRGs) including SHC3 as independent predictive factors of overall survival (OS) in non-small cell lung cancer (NSCLC) patients (12). Thus, SHC3 is a potential prognostic biomarker and therapeutic target for various cancers, although its role in CRC has not been investigated in detail. To this end, we analyzed the expression of SHC3 in CRC using TCGA and Oncomine databases, and assessed its function in CRC cell lines by shRNA-mediated knockdown. Our research possibly provided the evidence that SHC3 may function as an oncogene in CRC. We present the following article in accordance with the Materials Design Analysis Reporting (MDAR) reporting checklist (available at <https://dx.doi.org/10.21037/tcr-21-294>).

## Methods

### *Tumor Immune Estimation Resource (TIMER) database analysis*

TIMER is a comprehensive resource for systematical analysis of immune infiltrates across diverse cancer types (13). The differential expression levels of relevant genes between tumor and adjacent normal tissues across all TCGA datasets were analyzed by the Gene DE module, and displayed using box plots. Statistical significance was calculated using the Wilcoxon test.

### *TCGA data extraction and analysis*

The data of 461 colon adenocarcinoma (COAD) and 171 rectal adenocarcinoma (READ) samples were downloaded from TCGA database. There were 458 COAD cases with RNAseq data, and 41 with paired RNAseq and clinicopathological data. RNAseq data was available for 166 READ cases, and 9 cases had paired RNAseq and clinicopathological data. The information is summarized in *Table 1*. The individual RNAseq files were separated from

the raw read files based on the barcode sequences, and the raw counts of SHC3 were compared between carcinoma and adjacent normal tissues.

### *Oncomine database analysis*

The transcriptomic data of CRC samples were analyzed using the Oncomine database ([www.oncomine.org](http://www.oncomine.org)) (14) to determine SHC3 expression levels between CRC and normal tissues, using  $P < 0.01$  and fold change (FC)  $\geq 1.5$  as the thresholds.

### *Kaplan-Meier plotter survival analysis*

The prognostic value of SHC3 expression in CRC was further assessed by analyzing the OS of patients using the Kaplan-Meier plotter (15) (<http://kmplot.com/analysis/>). Briefly, SHC3 was entered into the database, which includes 165 CRC patients with a cutoff follow-up of 49 months and Kaplan-Meier survival plots with the number-at-risk indicated below. Hazard ratio (HR), 95% confidence interval (CI) and log-rank P were obtained on the webpage. P value of  $< 0.05$  was considered as statistically significant.

### *Cell lines and culture conditions*

Human CRC cell lines HCT116 was obtained from Type Culture Collection of the Chinese Academy of Sciences (Shanghai, China) and RKO was obtained from American Type Culture Collection (USA). The cells were cultured in DMEM (Gibco-BRL, Gaithersburg, MD, USA) supplemented with 10% fetal bovine serum and 1% penicillin at 37 °C under 5% CO<sub>2</sub>.

### *RNA interference and lentiviral transduction*

The SHC3 and scrambled shRNA sequences were synthesized by Shanghai GeneChem Co. Ltd. (Shanghai, China), and cloned into the GV115 vector at the AgeI and EcoR I sites. The GV-NC-GFP-shRNA and GV-SHC3-GFP-shRNA lentiviruses were prepared by standard protocols. The shRNA sequences are as follows: 5'-TTCTCCGAACGTGTCACGT-3' for control, and 5'-TTCTTTAAGGTCAAGTTT-3' for SHC3.

### *RNA isolation and qRT-PCR*

Total RNA was extracted from cells using Trizol reagent

(Invitrogen, USA). The primer sequences for PCR were as follows: SHC3, F: 5'-TTTTGGAATGCTGTGATGGGC-3'; R: 5'-GAGCGGGAATCTTGGTAGGAC-3'; GAPDH, F: 5'-TGACTTCAACAGCGACACCCA-3'; R: 5'-CACCTGTTGCTGTAGCCAAA-3'. Each sample was analyzed in triplicate.

### *Western blotting*

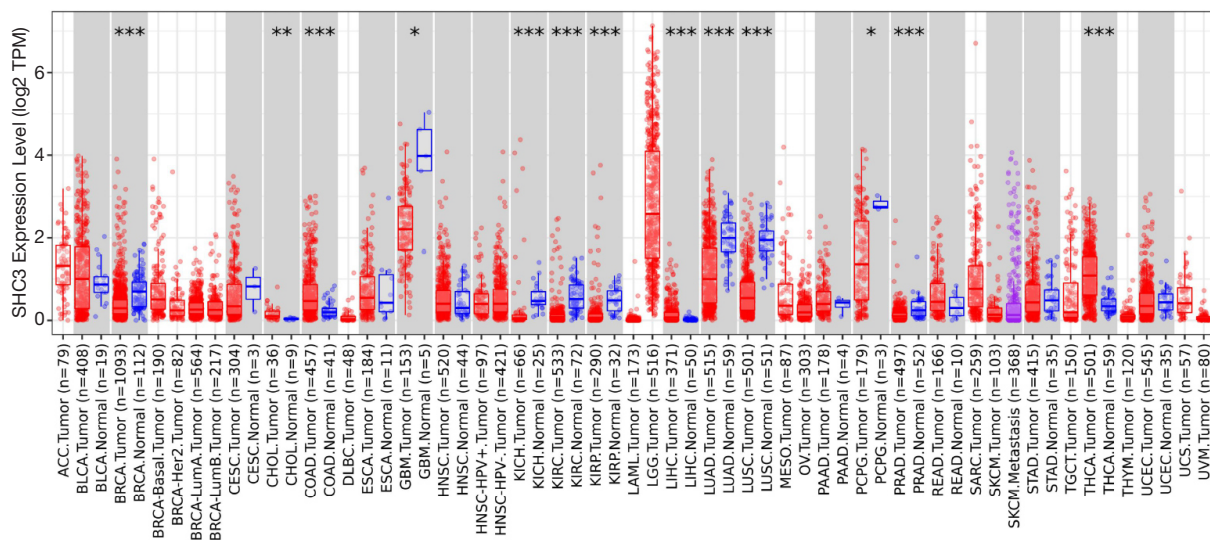
Cells were harvested 72 h after transduction, washed twice with PBS, and homogenized in lysis buffer on ice. The protein concentration in the lysates was measured using the BCA reagent. Following SDS-PAGE, nitrocellulose membrane transfer and blocking, the blots were incubated overnight at 4 °C with anti-SHC3 (1:500; Sigma, SAB1401715) and anti-GAPDH (1:10,000; Abcam, ab37168) antibodies. The blots were then incubated with the secondary antibodies at room temperature for 1 h, and the protein bands were detected by enhanced chemiluminescence (ECL) reagents (Millipore; Billerica, MA, USA) and ECL imaging system (Bio-Rad; Richmond, CA, USA) according to the manufacturer's instructions.

### *Cell proliferation assay*

CRC cells stably transduced with control or shSHC3 lentiviral constructs were seeded in 96-well plates at the density of  $(1-1.5) \times 10^3$ /well in quintuplicate. Cell proliferation was estimated on days 1-5 of culture by the fluorescence using Celigo Imaging Cytometer (Nexcelom Bioscience, Lawrence, MA, USA). In addition, for MTT assay, 20  $\mu$ L MTT solution was added to each well, and the cells were incubated for 4 h at 37 °C. The formazan crystals were dissolved by DMSO and measured at 490 nm. At least three independent experiments were performed.

### *Colony formation assay*

The transduced cells were trypsinized into a single-cell suspension, and seeded in a 6-well plate at the density of 500 cells/well. After culturing for 14 days or once the number of cells in most single colonies was more than 50, the colonies were photographed under a fluorescence microscope. The cells were washed once with PBS, fixed with 1 mL 4% paraformaldehyde for 30-60 min, rinsed again with PBS, and stained with 1 mL crystal violet for 10-20 min. After washing several times with ddH<sub>2</sub>O, the stained colonies were air-dried, photographed using a digital



**Figure 1** The pan-cancer expression of SHC3 in TCGA database. The red bars represent cancer tissues (T), and the blue bars represent normal tissues (N). \*, P<0.05; \*\*, P<0.01; \*\*\*, P<0.001 according to Wilcoxon test.

camera, and counted. Three independent experiments were performed.

### Cell apoptosis assay

The CRC cells in logarithmic growth phase were harvested 72 h after transduction, washed twice with PBS, and incubated with 10  $\mu$ L Annexin V-APC at room temperature for 10–15 min. The percentage of apoptotic cells were analyzed by flow cytometry (BD FACSCalibur). Each sample was analyzed in triplicates.

### Scratch assay

The transduced cells were seeded in 96-well plates and cultured till 90% confluent. The monolayer was gently scratched using a scraping device, and the dislodged cells were rinsed 2–3 times with PBS. Fresh low-serum (1% FBS) medium was added, and the scratched area scanned with Celigo at 0 and 24 h after scraping. The migration rate was calculated as  $(X\text{-h cell area} - 0\text{-h cell area}) / (1 - 0\text{-h cell area}) \times 100\%$ .

### Cell migration assay

The cells were seeded in 24-well Transwell inserts on day 2 post-transduction in serum free medium at the density of  $1 \times 10^6$  cells/well, and the bottom chambers were filled

with DMEM supplemented with 30% FBS. After culturing for 48 h at 37  $^{\circ}$ C, the inserts were overturned on absorbent paper to remove the medium, and the non-transferred cells were gently removed with a cotton swab. The inserts were fixed in 4% paraformaldehyde for 30 min and stained with crystal violet for 1–3 minutes. The number of migrated cells per field was counted at 200 $\times$  magnification. Each sample was analyzed in triplicates. The study was conducted in accordance with the Declaration of Helsinki (as revised in 2013).

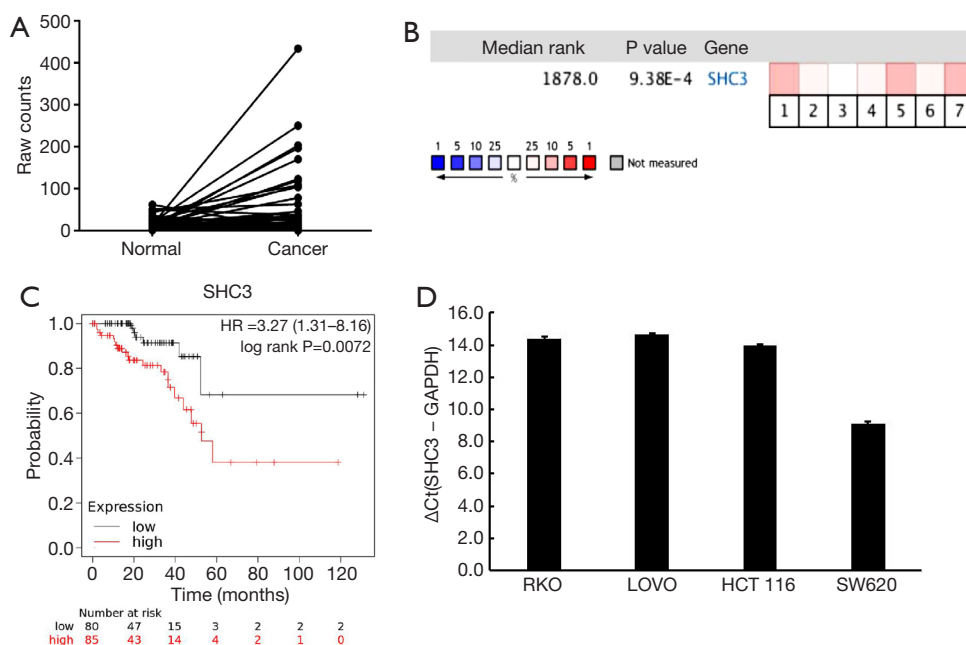
### Statistical analysis

Statistical analysis was performed using the SPSS 19.0 software. Data were confirmed to be normally distributed by Kolmogorov-Smirnov test and presented as the mean  $\pm$  standard deviation (SD). The differences between shSHC3 group and shCtrl group were compared by Student's *t*-test unless otherwise stated. P<0.05 in a two-sided test was considered statistically significant.

## Results

### The pan-cancer expression of SHC3 in TCGA database

The pan-cancer expression profile of SHC3 was determined using the Gene\_DE module of TIMER database. As shown in Figure 1, SHC3 was differentially expressed between the tumor and adjacent normal tissues



**Figure 2** SHC3 is upregulated in CRC and associated with poor prognosis. (A) The expression of SHC3 in 50 paired CRC and normal colorectal tissues in TCGA database. (B) Meta-analysis of SHC3 gene expression from three OncoPrint datasets. Colored squares indicate the median rank for SHC3 (vs. normal tissue) across 3 analyses. Gaedcke Colorectal [1]; Graudens Colon [2]; Kaiser Colon [3-6]; Skrzypczak Colorectal 2 [7]. The rank of a gene is the median rank for that gene across each of the analyses. The P value of a gene is that for the median-ranked analysis. (C) Kaplan-Meier curves showing overall survival (OS) of SHC3<sup>high</sup> vs. SHC3<sup>low</sup> CRC patients. (D) SHC3 mRNA expression in different CRC cell lines. CRC, colorectal cancer.

for all TCGA tumor datasets.

### SHC3 is upregulated in CRC and correlates with poor prognosis

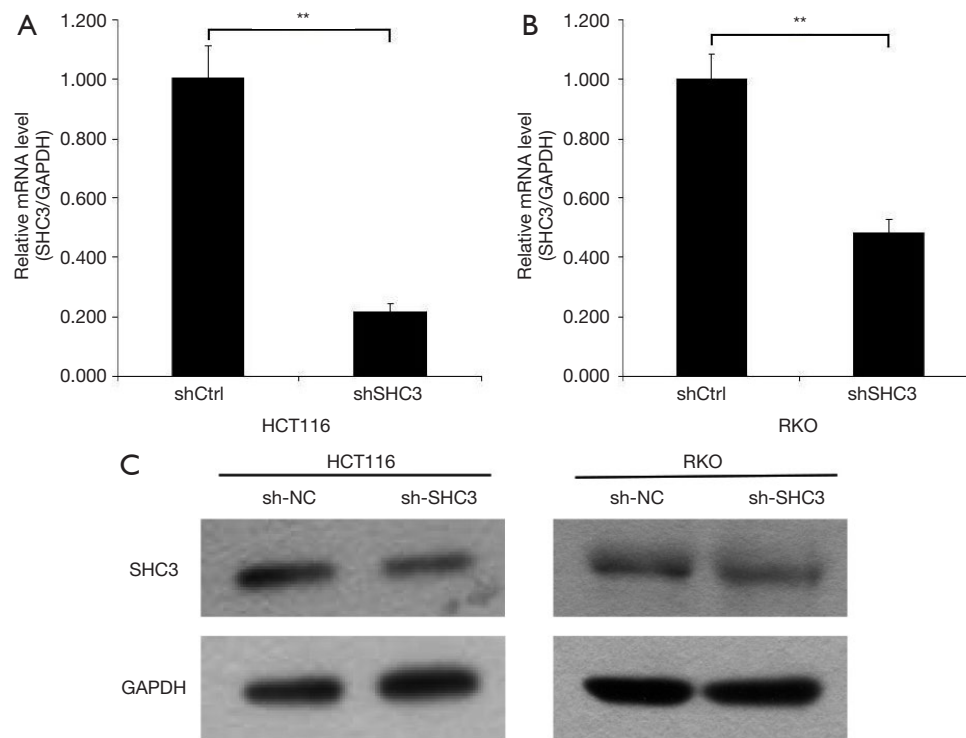
In addition, SHC3 was significantly upregulated in the CRC tissues compared to normal colon tissues across 50 paired samples in TCGA database (Figure 2A). Further analysis of the OncoPrint database revealed that SHC3 expression was markedly higher in the CRC tissues in 7 analyses across 4 independent datasets (16-19). Based on a meta-analysis using OncoPrint algorithms (205 samples,  $P=9.38E-4$ , Figure 2B), the median expression level of SHC3 in CRC was 1,878. Furthermore, higher expression of SHC3 correlated with shorter OS and poor prognosis in CRC (HR = 3.27, 95% CI: 1.31-8.16, log-rank  $P=0.0072$ , Figure 2C). The upper quartile survival of low SHC3 expression cohort was 52.2 months, while high SHC3 expression cohort was 36.53 months. We also measured SHC3 expression in different CRC cell lines, and detected high levels in the RKO, LOVO and HCT116 cell lines (Figure 2D).

### SHC3 knockdown in CRC cells

The functional role of SHC3 in CRC was further assessed by shRNA-mediated silencing in the HCT116 and RKO cells, and the knockdown efficiency by Lv-shSHC3 was 78.1% and 51.5% respectively ( $P<0.01$ ,  $P<0.01$ ; Figure 3A). In addition, Lv-shSHC3 also suppressed SHC3 expression at the protein level (Figure 3B).

### Knockdown of SHC3 inhibited cancer cell proliferation and promoted cell apoptosis

As shown in Figure 4A, cell proliferation rates were significantly reduced in the Lv-shSHC3 versus the control group for both HCT116 and RKO cells. Likewise, Celigo-based cell counting also indicated slower growth in the Lv-shSHC3 group (Figure 4B). Furthermore, SHC3 knockdown in HCT-116 and RKO cells significantly decreased the number of colonies (Figure 4C) and increased the percentage of apoptotic cells (Figure 4D). These results indicated that knockdown of SHC3 could inhibit cell



**Figure 3** The knock-down efficiency of SHC3 in CRC cells. (A) SHC3 mRNA level in HCT116 and RKO cells transduced with shCtrl and shSHC3. GAPDH was the internal standard. (B) Immunoblot showing SHC3 protein levels in the indicated groups. \*\*,  $P < 0.01$ . CRC, colorectal cancer.

proliferation and growth and promote cell apoptosis.

### ***Inhibition of SHC3 suppressed migration in human colonic cancer cells***

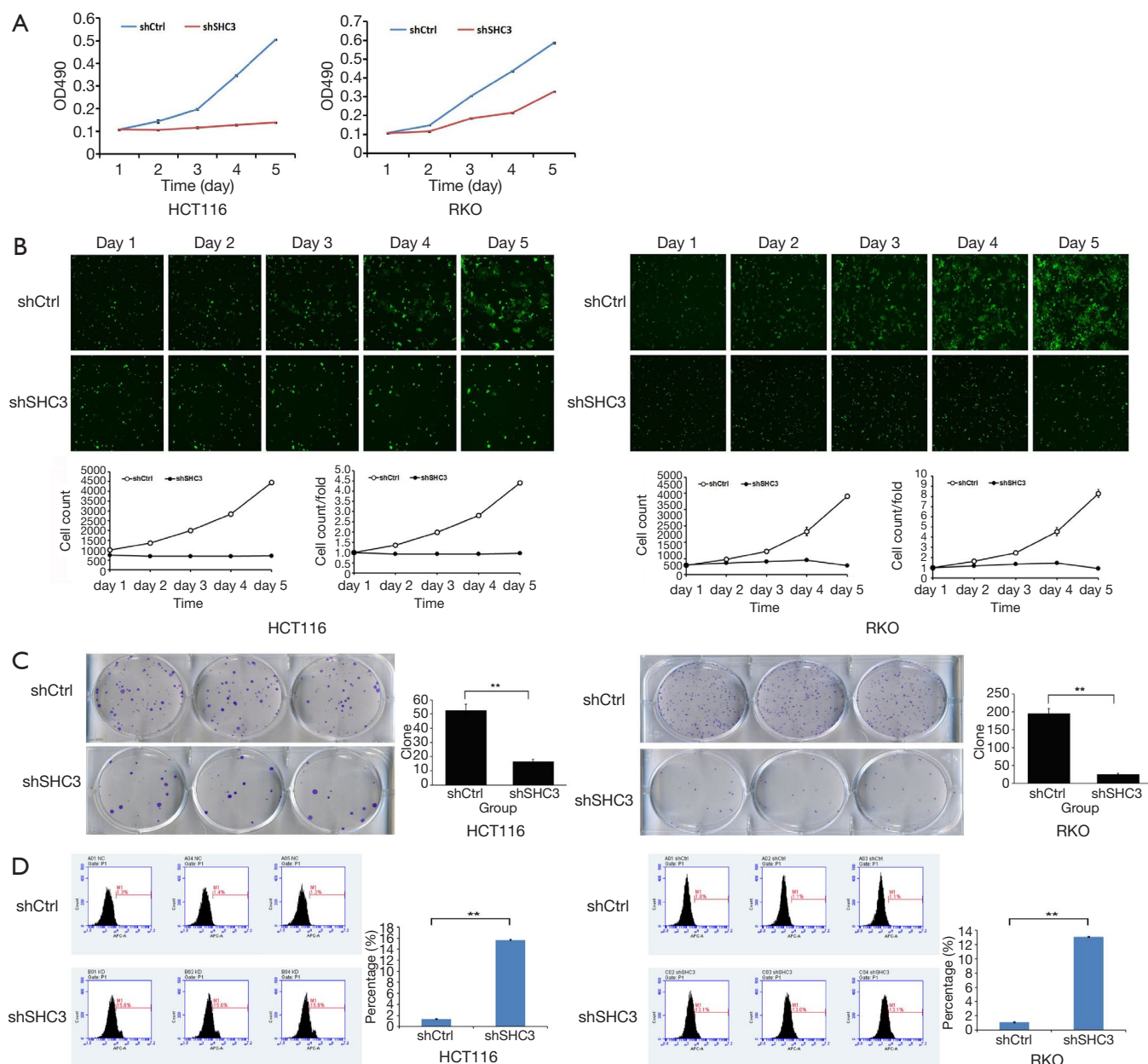
Finally, inhibition of SHC3 also reduced *in vitro* migration of HCT116 and RKO cells as indicated by the wound scratch and Transwell assays (*Figure 5A,B*). Taken together, SHC3 knockdown in human CRC cells inhibited proliferation and migration and promoted apoptosis, which indicates an oncogenic role in CRC.

### **Discussion**

CRC is a genetically heterogeneous disease (20) associated with high mortality rates due to frequent metastasis (21). Early detection and radical surgical excision of colorectal tumors increase the 5-year survival rate to 90–95%, which underscores the need for identifying novel early diagnostic markers (22). The advent of bioinformatics and machine learning has helped define and validate novel “omics-

based” markers with important diagnostic and prognostic implications (23). Integrated analysis of CRC RNAseq and microarray data of TCGA and Oncomine databases showed that SHC3 mRNA was significantly upregulated in the CRC tissues and associated with poor patient survival and outcome. These results indicated that SHC3 likely functions as an oncogene in CRC progression. Consistent with bioinformatics data, SHC3 mRNA expression was moderate to high in CRC cell lines. So far, bioinformatic approach has become an alternative to find out novel biomarkers for cancer research, provide predictions and guidance for our study. However, the bioinformatics tool has its restrictions, which need to be identified by experiments in further.

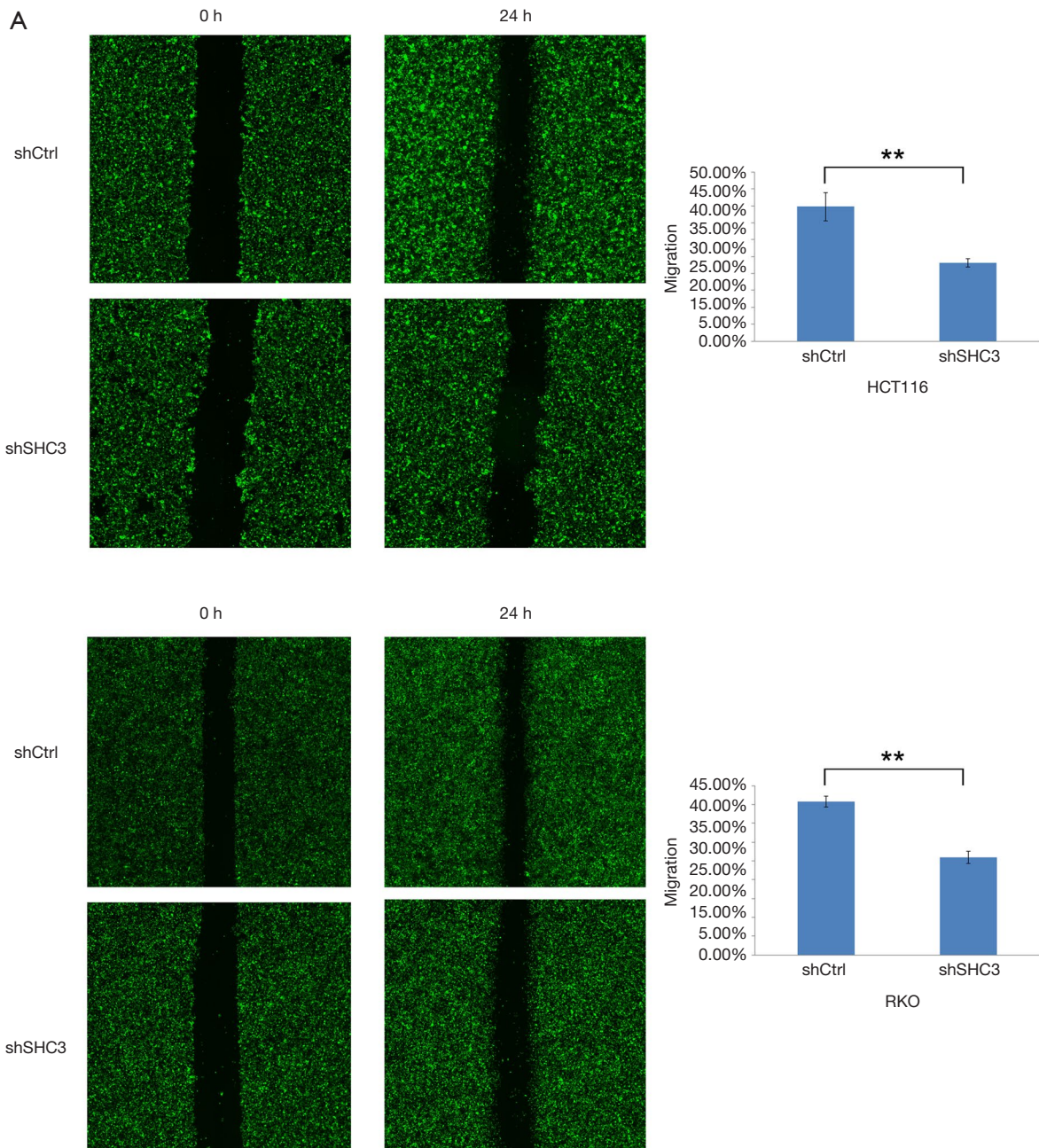
The role of SHC3 in the nervous system has been extensively documented but little is known regarding its specific functions in malignancies. Aberrant SHC3 expression has been reported in neuroblastoma, glioblastoma, ependymomas, thyroid tumors and hepatocellular carcinoma (HCC), and associated with tumor progression (10,11,24–26). In addition, GO and KEGG analyses indicate SHC3 involvement in glioma, chronic



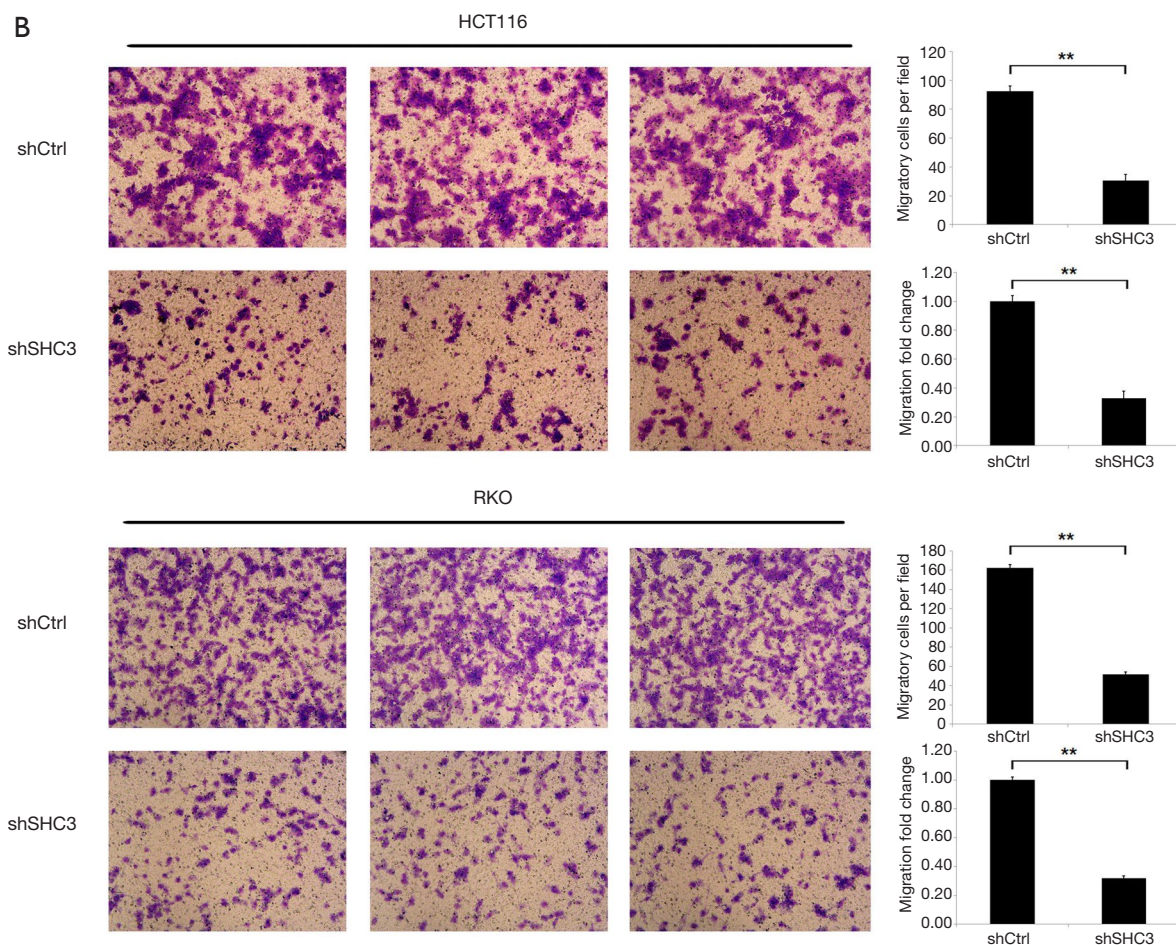
**Figure 4** SHC3 knockdown inhibited CRC cell proliferation and promoted apoptosis. (A) Proliferation index of shSHC3 and shCtrl HCT116 and RKO cells. (B) Celigo-based cell counting detection. Number of viable HCT116 and RKO cells transduced with shCtrl or shSHC3 ( $\times 100$ ). The fluorescence was measured to access cell counts using Celigo Imaging Cytometer. (C) Number of colonies formed by shCtrl and shSHC3 HCT116 and RKO cells. Colonies were stained with crystal violet. (D) Percentage of apoptotic HCT116 and RKO cells transduced with shCtrl or shSHC3. Values are mean  $\pm$  standard deviation of three experiments. \*\*,  $P < 0.01$ . CRC, colorectal cancer.

myeloid leukemia and prolactin signaling pathways (27). In HCC for instance, SHC3 activates the MVP/MEK/ERK signaling pathway and stimulates downstream tumorigenic responses such as proliferation, migration, invasion and epithelial-mesenchymal transition (EMT). SHC3

upregulation is induced by promoter demethylation (11). Hypomethylation and subsequent upregulation of SHC3 has also been demonstrated in cryptogenic HCC. A recent study showed that SHC3 is overexpressed in CRC tissues, and is the downstream target of thyroid hormone receptor







**Figure 5** Inhibition of SHC3 suppressed migration of CRC cells. (A) Scratch assay showing motility of shCtrl and shSHC3 HCT116 and RKO cells. The fluorescence was measured using Celigo Imaging Cytometer ( $\times 100$ ). (B) Percentage of migrating shCtrl and shSHC3 HCT116 and RKO cells. Migrating cells were stained with Giemsa (Sigma, USA),  $\times 100$ . Values are mean  $\pm$  standard deviation of three experiments. \*\*,  $P < 0.01$ . CRC, colorectal cancer.

interactor 13 (TRIP13) (28), which provides valuable mechanistic insights into the role of SHC3 in CRC. To further elucidate the functional role of SHC3 in CRC, we knocked down its expression in specific cell lines using shRNA. Our results showed that knocking down SHC3 reduced proliferation and migration of the CRC cell, and significantly increased the apoptosis rates. These findings strongly indicate that SHC3 is an oncogene in CRC, and this hypothesis will have to be investigated further.

## Conclusions

SHC3 is upregulated in CRC tissues and cells, and functions as an oncogene. It is a promising new diagnostic

marker and therapeutic target for CRC that warrants further investigation.

## Acknowledgments

We acknowledge the native English speaker from CureEdit for improving the grammar and readability.

*Funding:* This work was supported by Institutional Foundation of the First Affiliated Hospital of Xi'an Jiaotong University (YK201522).

## Footnote

*Reporting Checklist:* The authors have completed the MDAR

reporting checklist. Available at <https://dx.doi.org/10.21037/tcr-21-294>

*Data Sharing Statement:* Available at <https://dx.doi.org/10.21037/tcr-21-294>

*Conflicts of Interest:* All authors have completed the ICMJE uniform disclosure form (available at <https://dx.doi.org/10.21037/tcr-21-294>). The authors report this work was supported by institutional foundation of the first affiliated hospital of Xi'an Jiaotong University (YK201522). The authors have no other conflicts of interest to declare.

*Ethical Statement:* The authors are accountable for all aspects of the work in ensuring that questions related to the accuracy or integrity of any part of the work are appropriately investigated and resolved. The study was conducted in accordance with the Declaration of Helsinki (as revised in 2013). Institutional ethical approval and informed consent were waived.

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**Cite this article as:** Zhao C, Zhang J, Ma J, Zhang N, Chen W. The expression and prognostic value of Src homology 2 domain-containing transforming protein C3 (SHC3) and its potential role in colorectal cancer. *Transl Cancer Res* 2021;10(7):3418-3428. doi: 10.21037/tcr-21-294