Knockdown of Histone Methyltransferase hSETD1A Inhibits Progression, Migration, and Invasion in Human Hepatocellular Carcinoma

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Our aim was to study the expression of human SET domain containing protein 1A (hSETD1A) in hepatocellular carcinoma patients and its relationship with human hepatocellular carcinoma cell function. A total of 30 patients with hepatocellular carcinoma were enrolled in this study. The expression of hSETD1A was detected by real-time polymerase chain reaction (PCR) and Western blotting. The immortalized normal human liver cell line including SMMC-7721 was subjected to real-time PCR for hSETD1A mRNA. Furthermore, hSETD1A-small hairpin RNA (shRNA) was used to knock down hSETD1A expression in SMMC-7721 cells. Cell proliferation, cell apoptosis, and cell migration were determined by CCK8, flow cytometry, and Transwell assays. The positive expression rate level of hSETD1A mRNA and protein in liver carcinoma tissues was 73.33%. hSETD1A knockdown using a specific hSETD1A-shRNA inhibited cell proliferation and promoted cell apoptosis in SMMC-7721 cells. It was also found that downregulation of hSETD1A inhibited cell migration ability but did not affect cell invasion. In conclusion, the expression of hSETD1A occurs at a high rate in hepatocellular carcinoma patients. The expression state of hSETD1A may be a prognostic factor in hepatocellular carcinoma.

Key words: Hepatocellular carcinoma (HCC); hSETD1A; Clinical samples; SMMC-7721; Cell function

INTRODUCTION

Hepatocellular carcinoma (HCC), which is frequently caused by hepatitis virus B and C infections, is the fifth most common malignancy and the second leading cause of cancer-related deaths worldwide (1,2). Moreover, its incidence grows continually. The mechanisms of initiation and development of liver neoplasms, which is related to the immunological status of body and gene abnormality, are still unclear (3). In the past few years, patients with HCC have not received unsurpassed clinical efficacy even with improvements in surgical and medical treatments, mainly because of frequent postsurgical recurrence and metastasis (4). The risk of recurrence may be defined by cancer classification based on biomarkers (5,6). A better prognosis with a significant biomarker will help choose an appropriate treatment in advance. However, current methods for HCC diagnosis are classified into the following main categories: imaging [abdominal ultrasonography, magnetic resonance imaging (MRI), contrast-enhanced computed tomography (CT)] and laboratory biomarker analysis [serum alpha-fetoprotein (AFP) levels] (7,8). However, the diagnostic performance of imaging technologies is unsatisfactory, particularly for the diagnosis of small lesions and early stage HCC, which gives us a clue that we may need a useful molecular biomarker (9–11). Unfortunately, some molecular biomarkers related to the risk of cancer recurrence have not been explored completely in HCC patients, and the underlying mechanisms responsible for metastasis remain largely unknown (11–14). Therefore, reliable biomarkers predicting HCC relapse need to be developed, and the mechanisms underlying cancer metastasis need to be explored.

Previous research shows that some proteins, such as programmed death-ligand 1 (PD-L1) (15), heat shock protein 90 (HSP90) (16,17), and SIRT3 (17), are important regulatory molecules in HCC. Their alterations are found in a variety of tumor tissues including breast, gastric, esophageal, lung, bladder, and renal cell cancers (18–23), which means that they may not be the best specific biomarkers for HCC. Furthermore, all previous studies are focused on data in vitro, which suggests that we still know little about these important protein levels in HCC patients.

The hSETD1A gene belongs to the trithorax (TrxG) family of histone methyltransferases (HMT) (24). As we know, alterations in histone modification can drive important cancerous processes such as proliferation, invasion, angiogenesis, and differentiation by perturbing normal genes (25). hSETD1A has been shown to play a role in cell differentiation and tissue development, but very little is known about the role of hSETD1A in cancer, particularly in solid tumors (24,26). Recent studies show that hSETD1A may play an oncogenic role in human colorectal cancer through targeting the Wnt signaling pathway and controlling tumor growth, and in human breast cancer with the link among hSETD1A, MMPs, and metastasis (24).

In the current study, we found that hSETD1A expression was further increased in carcinoma tissue from HCC patients compared with adjacent normal tissue in these patients. It is implied that hSETD1A was associated with the multiple malignant characteristics of HCC. Using real-time polymerase chain reaction (PCR), we explored the mRNA level of hSETD1A in the SMMC-7721 cell line. Furthermore, we synthesized and transfected the special small hairpin RNA (shRNA), which silenced hSETD1A mRNA expression in SMMC-7721. Multiple analyses revealed that the downregulation of hSETD1A was an independent risk factor for cancer cell proliferation, cell apoptosis, and cell migration. The results suggested that the expression of hSETD1A and its relationship with the clinical pathology and prognosis of patients with HCC might provide a basis for the treatment of liver cancer.

MATERIALS AND METHODS

Patient Characteristics, Clinical Features, and Tissue Harvest

As shown in Table 1, primary HCC tissue was obtained from surgical specimens of 30 hospitalized patients in Nanfang Hospital (Guangzhou, China) between October 2014 and May 2015. A diagnosis of HCC was confirmed by pathology. Liver carcinoma tissue and adjacent normal mucosa from these 30 patients were used to examine the expression of hSETD1A.

Cell Lines Culture and Treatment

Human HCC cell line SMMC-7721 [American Type Culture Collection (ATCC), Manassas, VA, USA] was maintained in RPMI-1640 with 5% heat-inactivated fetal bovine serum (FBS) and 1% antibiotic–antimycotic solution (Invitrogen, Carlsbad, CA, USA) at 37°C in a humidified atmosphere of 95% air and 5% CO_2. SMMC-7721 cells were transfected with hSETD1A-shRNA (siRNA sequence: cagaagaatggtacaaatccaag) (50 μM) through Lipofectamine 2000 (Invitrogen) for 24, 48, 72, and 96 h. NC-shRNA (50 μM) was used as a negative control.

Table 1. Demographic, Clinical, and Biological Data of Hepatocellular Carcinoma Patients in This Study

Items	Cases ($N = 30$)
Sex	
Male	23 (76.67%)
Female	7 (23.33%)
Age	
≤50	14 (46.67%)
≥50	16 (53.33%)
Different degree	
High differentiation	4 (13.33%)
Medium, low differentiation	26 (86.67%)
Tumor size	
≤5 cm	7 (23.33%)
≥5 cm	23 (76.67%)
Clinical stages	
Stage I–II	8 (26.67%)
Stage III–IV	22 (73.33%)
HBV infection	
Negative	3 (10.00%)
Positive	27 (90.00%)
Portal vein thrombosis	
No	19 (63.33%)
Yes	11 (36.67%)
Level of serum AFP	
≤400 μg/L	14 (46.67%)
≥400 μg/L	16 (53.33%)

Total RNA Isolation, Reverse Transcription, and Real-Time PCR

Total RNA was isolated by using a UNIQ-10 column and TRIzol Total RNA Isolation Kit (Sangon, Shanghai, China). One microgram of total RNA was used for reverse transcription in a reaction volume of 20 μl using Cloned AMV Reverse Transcriptase (Invitrogen). Two microliters of cDNA was used for real-time PCR using TaKaRa Ex Taq RT-PCR Version 2.1 kit (TaKaRa, Shiga, Japan). Gene-specific PCR primers for hSETD1A and *GAPDH* are listed in Table 2, and PCR signals were detected with a DNA Engine Opticon 2 Continuous Fluorescence Detection System (Bio-Rad, Hercules, CA, USA). PCR was monitored for 45 cycles using an annealing temperature of 60°C. At the end of the PCR cycles, melt curve analysis and 2% agar electrophoresis were performed to assess the purity of the PCR products. Negative control reactions (no template) were routinely included to monitor potential contamination of reagents. Relative amounts of hSETD1A mRNA were normalized to that of GAPDH mRNA.

Protein Isolation and Western Blotting Analysis

The concentration of protein extracts from mouse hepatic microsomes was determined using a BCA kit (Pierce, Rockford, IL, USA), and 20 μg of protein lysates was separated on 10% SDS-polyacrylamide gel

Table 2. Sequence of the Primers Used for Detection in Hepatocellular Carcinoma Patients

Gene	Primer Sequence (5′–3′)
hSETD1A	
Forward	AAACCTCCACCTTACCTCCG
Reverse	GAACTTCTCACTCAGGGCCT
GAPDH	
Forward	CGGAGTCAACGGATTTGGTCGTAT
Reverse	AGCCTTCTCCATGGTGGTGAAGAC

electrophoresis (SDS-PAGE) gels followed by transfer to nitrocellulose membranes. Western blotting analysis was performed as previously described (27), and the signal was detected using an ECL system (Millipore, Billerica, MA, USA). Antibodies used in this study included anti-human SETD1A (1:2,000; Santa Cruz Biotechnology).

Cell Proliferation Detection

SMMC-7721 cells were seeded at a density of 1.0×10^5 cells/ml in six-well plates to achieve ~50% confluence the next day and were then transfected with hSETD1A-shRNA (50 µM) using Lipofectamine 2000 (Invitrogen). Thereafter, 100 µl of CCK8 (Dojindo, Japan) solution was added into each well and cells were incubated for 1 h. The absorbance was measured at 450 nm using a microplate reader.

Apoptosis Assay

After shRNA transfection for 72 h, the apoptotic cells were quantified using the Annexin-V/Propidium Iodide (PI) apoptosis kit (Multiscience, Hangzhou, China). SMMC-7721 cells were collected, washed with phosphate-buffered saline (PBS), and resuspended in 200 µl of binding buffer containing 5 µl of annexin V (10 µg/ml) for 10 min in the dark. The cells were then incubated with 10 µl of PI (20 µg/ml), and the samples were immediately analyzed using flow cytometry (Epics XL; Beckman Coulter, Brea, CA, USA). Data acquisition and analysis were performed using CellQuest software.

Transwell Assay

SMMC-7721 (untransfected), SMMC-7721/NC-shRNA, and SMMC-7721/hSETD1A-shRNA cells were plated at 1.0×10^5 cells/well in 0.5 ml of serum-free medium in 24-well Matrigel-coated Transwell units with polycarbonate filters (8-µm pore size; CoStar Inc., Milpitas, CA, USA). The outer chambers were filled with 0.5 ml of medium supplemented with 10% FBS. After 24 h, the cells were fixed in methanol and stained with crystal violet. The top surface of the membrane was gently scrubbed with a cotton bud, and the cells that had invaded through the membrane filters were counted. The invasion inhibition rate (%) was calculated as $[(A - B)/A] \times 100$], where A is the invading cells' percentage

for the NC-shRNA group, and B is the invading cells' percentage for the hSETD1A-shRNA group.

Statistical Analysis

The differences between each group were expressed as the mean ± SD. Statistical significance was assessed by Student's t-test and one-way analysis of variance (ANOVA) followed by a Tukey post hoc test. Differences were considered statistically significant with a value of $p < 0.05$.

RESULTS

*hSETD1A Expressed Differentially in HCC Patients and Human HCC*

mRNA level and protein level detection of hSETD1A was conducted using real-time PCR and Western blotting. As shown in Figure 1A and B, both the mRNA level (Fig. 1A) and the protein level (Fig. 1B) of hSETD1A are highly expressed in liver carcinoma tissues compared to the adjacent normal tissues from the same patient.

Validation of hSETD1A Expression in Human HCC by Real-Time PCR

In order to confirm the results obtained from clinical samples, the expression of hSETD1A mRNA was analyzed by real-time PCR in SMMC-7721 compared to HepG2, Hep3B, huh7, and LM3. The results showed that hSETD1A mRNA is relatively highly expressed in SMMC-7721 (Fig. 2). Therefore, we chose SMMC-7721 to complete the following experiment.

Effects of hSETD1A-shRNA on Cell Proliferation

The result in Figure 3A suggests that the hSETD1A mRNA level was effectively silenced by the specific
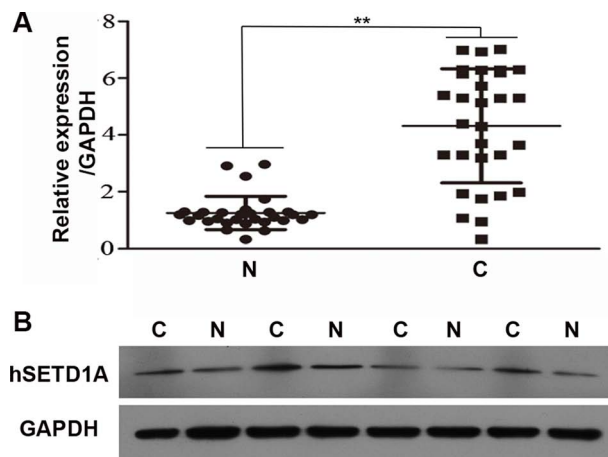
Figure 1. Expression of human SET domain containing protein 1A (hSETD1A) in liver carcinoma tissue (C), adjacent normal tissue (N), respectively, of HCC patients as detected by real-time polymerase chain reaction (PCR) (A) and Western blotting (B). **$p < 0.01$.
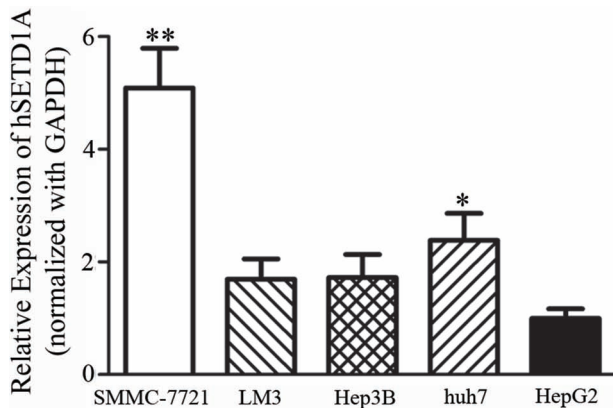
Figure 2. Expression of hSETD1A in SMCC-7721, LM3, Hep3B, huh7, and HepG2 as detected by real-time PCR. *$p < 0.05$; **$p < 0.01$.

shRNA. Furthermore, we examined the effects of hSET-D1A-shRNA on SMMC-7721 cell proliferation. As shown in Figure 3B, cellular population was reduced time independently at median inhibitory concentration (IC_{50}) levels in the hSETD1A-shRNA group compared to the NC-shRNA group.

Effects of hSETD1A-shRNA on Cell Apoptosis

Cell apoptosis was analyzed using flow cytometry after hSETD1A-shRNA transfection for 72 h based on CCK8 results. Compared to the NC-shRNA group, SMMC-7721 cells exposed to hSETD1A-shRNA presented typical promotion from apoptotic morphology with cell shrinkage, nuclear fragmentation, and cellular rupture into debris. The occurrence of apoptosis was significantly higher in cells treated with NC-shRNA as a negative control with respect to the hSETD1A-shRNA group (Fig. 4A and B).

Effects of hSETD1A-shRNA on Cell Migration

As shown in Figure 5, the invasive potential of hSETD1A-shRNA cells was not significantly different from that of the NC-shRNA cells (79.14 vs. 77.63%, $p > 0.05$, data not shown). The invasion inhibition rates of the SMMC-7721 cells and the NC-shRNA cells were not significantly different. In addition, the migration potential of the hSETD1A-shRNA cells was significantly lower than that of the NC-shRNA cells (52.26 vs. 80.23%, $p < 0.01$). The migration inhibition rate in the hSETD1A-shRNA cells reached up to 34.86%. The migration inhibition rates of the SMMC-7721 cells and the NC-shRNA cells were not significantly different.

DISCUSSION

Through exploration of the hSETD1A expression in primary HCC, this study found that the expression of the hSETD1A protein in HCC patients was significantly
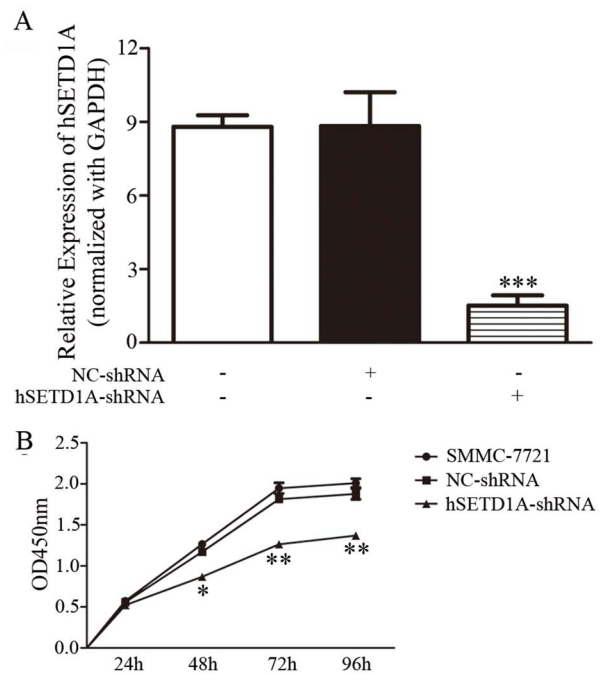

Figure 3. Downregulation of hSETD1A promotes growth of SMCC-7721 cells in vitro. (A) Expression of hSETD1A in SMCC-7721 after downregulation of hSETD1A. (B) Cell numbers were counted in the following time points: 24, 48, 72, and 96 h. *$p < 0.05$; **$p < 0.01$; ***$p < 0.001$.

high. Among the enrolled patients in this study, we also found that we had more patients with medium/low differentiation, bigger tumor size (≥ 5 cm), later clinical stage (III–IV), and positive hepatitis B virus (HBV), which implied that patients with these categories may have a high expression of hSETD1A (data now shown). The underlying connection needs further research. Results in vitro showed that expression of the SETD1A protein promoted liver cancer cell proliferation and migration and protected liver cancer cells from apoptosis. These results demonstrated that there may be some connection between hSETD1A and HCC, but there has been no study to make the mechanism known yet. According to previous research, the hSETD1A protein had not been found to be the prognostic factor in liver cancers. This study was the first to report that the expression level of hSETD1A in primary HCC may affect the prognosis and have a negative correlation to cancer cell function.

As we know, the hSETD1A gene belongs to HMT that methylates lysine 4 at histone H3 tails (H3K4), which plays an important role in cancerous processes (24). It is reported that ablation of hSETD1A exhibits some dramatic effects on the trimethylation of H3K4 (H3K4me3) enrichment and expression, which controls gene transcription. However, little is known about the target genes affected by the altered H3K4me3 expression
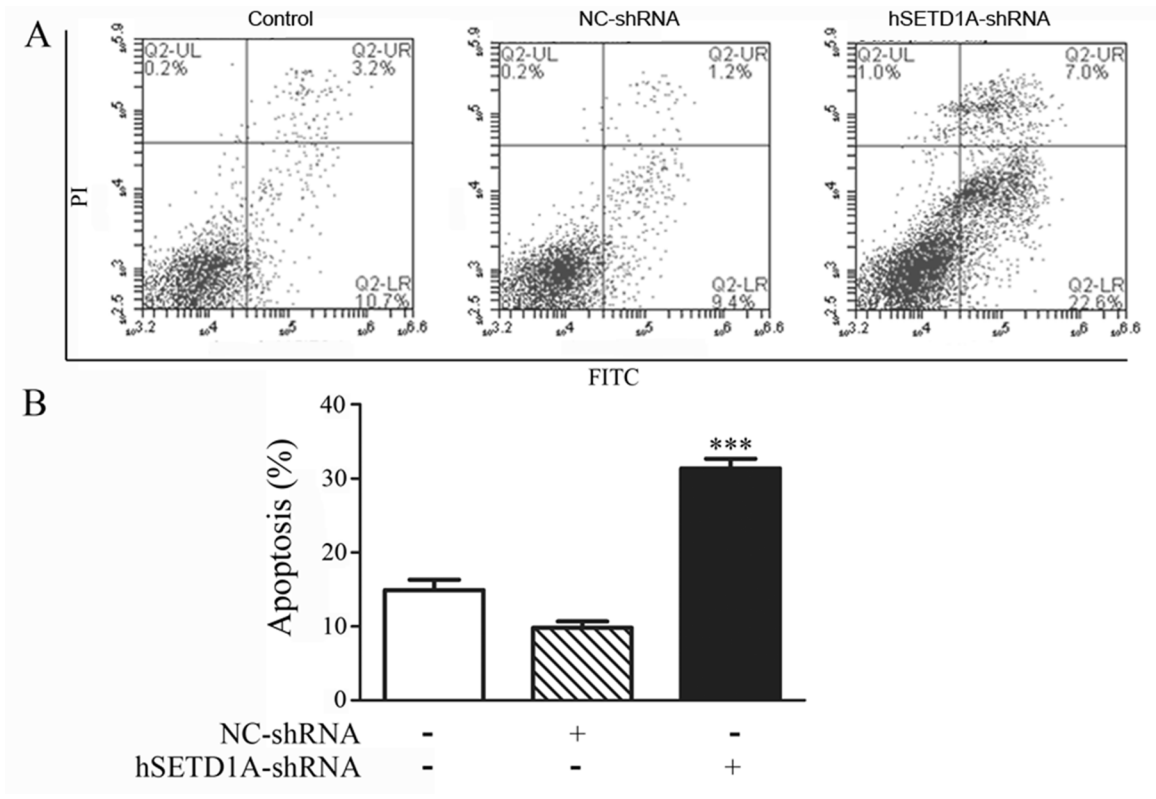
Figure 4. Induction of apoptosis change. (A) SMMC-7721 cells were transfected with small hairpin RNAs (shRNAs) for 72 h before being harvested for apoptosis test. (B) Percentage of SMMC-7721 cell apoptosis in the 72-h time point. ***$p < 0.001$.
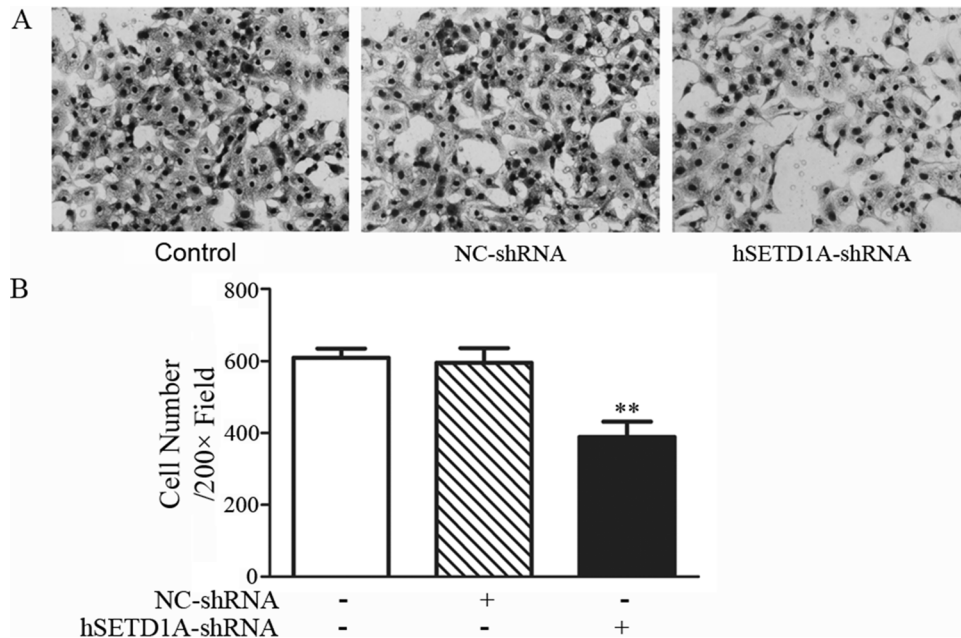
Figure 5. Suppressive effect of shRNA-mediated hSETD1A silencing on the migration potential of SMMC-7721 cells. (A) Migration tests. (B) Percentage of SMMC-7721 cell migration in the 24-h time point. **$p < 0.01$.

during carcinogenesis and whether hSETD1A is correlated to the increase of H3K4me3 levels. Previous research revealed that hSET works with mixed lineage leukemia (MLL) as a complex that is required for enzymatic activity (28,29). To understand the target gene specificity of hSET1/MLL complexes and how it works in specific types of cancers such as liver cancer will not only provide new insight into the pathogenesis of liver cancer but also lead to new strategies for cancer diagnosis and therapeutic approaches.

Recently, it was shown that histone can interact with β-catenin (30), a master regulator of the canonical Wnt signaling pathway, in human embryonic kidney 293 (HEK293) cells (31) and colorectal cancer cells (32,33). On the basis of these studies, they depicted a hypothesis which is that β-catenin and hSETD1A are upregulated and interact in the nucleus; β-catenin then recruits hSETD1A to putative Wnt promoters to confer H3K4me3, assemble preinitiation complex, activate Wnt target genes, and subsequently promote cellular growth. Identifying and targeting the β-catenin partners or cofactors, such as hSETD1A, could be an effective therapeutic approach for inhibiting the Wnt signaling pathway in colorectal cancer (24). Future studies validating this hypothesis and investigating whether hSETD1A works similarly in various types of cancers, such as HCC, may significantly contribute to our understanding of the role of hSETD1A in cancer diseases.

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