Original Article

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Overexpression of DJ-1 enhances colorectal cancer cell proliferation through the cyclin-D1/MDM2-p53 signaling pathway

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SUMMARY Emerging evidence indicates that DJ-1 is highly expressed in different cancers. It modulates cancer progression, including cell proliferation, cell apoptosis, invasion, and metastasis. However, its role in colorectal cancer (CRC) remains poorly defined. The current study noted increased DJ-1 expression in CRC tumor tissue and found that its expression was closely related to clinical-pathological features. Similarly, DJ-1 increased in CRC cells (SW480, HT-29, Caco-2, LoVo, HCT116, and SW620), and especially in SW480 and HCT116 cells. Functional analyses indicated that overexpression of DJ-1 promoted CRC cell invasion, migration, and proliferation in vitro and in vivo. Mechanistic studies indicated that DJ-1 increased in CRC cell lines, activated specific protein cyclin-D1, and modulated the MDM2/p53 signaling pathway by regulating the levels of the downstream factors Bax, Caspase-3, and Bcl-2, which are related to the cell cycle and apoptosis. Conversely, knockdown of DJ-1 upregulated p53 expression by disrupting the interaction between p53 and MDM2 and inhibiting CRC cell proliferation, revealing the pro-oncogenic mechanism of DJ-1 in CRC. In conclusion, the current findings provide compelling evidence that DJ-1 might be a promoter of CRC cell invasion, proliferation, and migration via the cyclin-D1/MDM2-p53 signaling pathway. Findings also suggest its potential role as a postoperative adjuvant therapy for patients with CRC.

Keywords DJ-1, MDM2, p53, cyclin-D1, proliferation, CRC

1. Introduction

Colorectal cancer (CRC) is a malignancy that is prevalent worldwide. Recent statistics indicate that more than 1 million CRC cases are diagnosed annually. Moreover, the mortality rate of patients with CRC is as high as 33% in developed countries (*1*). In China, the incidence of CRC has risen annually given the changes in people's lifestyles and eating habits (*2*). Despite continuous advancements and improvements in comprehensive treatment of CRC over the past few years, the overall prognosis for CRC is poor, and its five-year survival rate is less than 50% (*3*). This is mainly due to the highly metastatic nature of CRC. Consequently, approximately 90% of CRC-related deaths occur as a result of metastatic disease (*4*). Numerous studies have been conducted to determine the biological (molecular) mechanism of CRC development in its early stages, and many metastasis-related oncogenes and tumor suppressor factors have been discovered (e.g. p53, K-ras, and APC). As an example, tumor invasion and growth is inhibited by targeting the gain-of-function mutant p53 in CRC *via* Stat3. Notably, patients with CRC and stable mutp53 have poorer survival rates and elevated Jak2/Stat3 activity (*5*). Peptidomimetic inhibitors of APC-Asef protein-protein interaction influence the migratory ability of CRC cells (*6*). Most of the prevailing evidence indicates that the presence of the K-ras mutation can predict the survival of patients with CRC (*7,8*). Despite these insights, the molecular pathways of CRC metastasis are diverse and are not well understood. Therefore, further studies are needed to better understand the drivers of CRC metastasis.

Originally, DJ-1 was discovered and named in a mouse NIH3T3 cell transformation study by a Japanese researcher, Nagakubo (*9*). It was described as a new mitogen-dependent oncogene and was found on the short arm of human chromosome 36 site 1 (1p36.12-1p36.33), which encodes the DJ-1 protein. This protein is highly conserved and is formed as a homodimer that is widely expressed in many human tissues. It participates in many pathological and physiological activities in cells, such as transcriptional regulation (*10*), oxidative stress (*11*), cell apoptosis, cell proliferation (*12*), and molecular

chaperoning (*13*). Early studies on DJ-1 mainly focused on its role in Parkinson's syndrome. Results from those studies suggested that a mutation in the DJ-1 gene is strongly linked to Parkinson's disease (of the human autosomal recessive early-onset type). However, DJ-1 was found to promote the transformation of normal immortalized fibroblasts with oncogenes such as H-Ras or c-MYC, so the relationship between DJ-1 and tumors has garnered considerable attention. Experiments have indicated that DJ-1 is overproduced in gastric, liver, pancreatic, and esophageal cancers (*14-17*) and is strongly correlated with the development, progression, and metastasis of and prognosis for numerous digestive system tumors. However, few studies have investigated the clinical value, function, and molecular mechanism of DJ-1 in CRC. Therefore, the current study explored whether DJ-1 participates in the development of CRC. The findings of this study should provide important scientific and clinical knowledge to prevent and treat CRC, and especially new methods and effective interventions for CRC invasion and metastasis.

2. Materials and Methods

2.1. Clinical samples

Ninety pairs of CRC specimens and adjacent nontumor tissue specimens were collected from patients (43 males and 47 females) seen at the Second Hospital Affiliated with Nanchang University from August 2016 to December 2018. Tumor-adjacent tissue was obtained at a distance of more than 5 cm from the periphery of the tumor in the aforementioned patients with CRC. No patient received radiotherapy or chemotherapy prior to surgery. The collected tissues samples were identified as CRC in a pathological examination. Each patient provided informed consent for tissue collection for research purposes (No. 2017[031]).

2.2. Cell preparation

The colorectal carcinoma cell lines SW480, HT29, HCT116, SW620, and LoVo were cultured in DMEM reconstituted with 1% penicillin-streptomycin and 10% North American FBS at 37°C in a humid atmosphere with 5% CO_2. All cells were washed with PBS every day, changed once, and then passaged for 2-3 days. All experiments were performed when the cells reached 75-85% confluence and were in the logarithmic growth phase.

2.3. Stable knockdown and overexpression of DJ-1 in CRC cells

To obtain cells overexpressing DJ-1, HCT116 and SW480 cells were transfected with the lentiviral vector carrying human DJ-1 (Shanghai China). Cells transfected with the empty vector (Mock) served as the negative control. HCT116 and SW480 cells were transfected with lentivirus-mediated control shRNA or shRNA targeting DJ-1, a designated vector, and shDJ-1. The lentivirus-mediated shRNA was obtained from Hanbio Biotechnology (Shanghai, China); specific fragments were inserted into a vector. The sequences used were as follows: vector shRNA, 5′-TTCTCCGAACGTGTCACGT-3′. shRNA-1, 5′-ACTCTGAGAATCGTGTGGAAA-3′ (DJ-1, 412 nt); shRNA-2, 5′-GCGCTTGCAATTGTTGAAGCC-3′ (DJ-1,499 nt); shRNA-3, 5′-GCAATTGTTGAAGCCCTGA AT-3′ (DJ-1, 541 nt).

The cells were subsequently harvested 72 h postinfection and cultured in a medium containing 5 μg/ml puromycin (Sigma, Missouri, USA) until all uninfected cells were killed by puromycin. The stably transfected cell lines were verified with RT-PCR and Western blotting before use in subsequent experiments.

2.4. Cell proliferation assay

SW480 and HCT116 cells in mock/OE-DJ-1 groups and vector/shDJ-1 groups were inoculated onto 6-well plates. When the cell density reached 75%-85%, 100 μL of 10 uM preheated Edu working fluid was pipetted into each well, and cells were cultured at 37°C for 2 h. After EDU labeling, 4% polyformaldehyde was used to fix the cells at room temperature for 15 min. Fifty uL of 2 mg/mL glycine solution was used to quench the reaction for 5 min, 0.3% TriX-100 PBS was incubated for 10 min, and Apollo and Hoechst staining were performed in a stepwise manner. After fluid staining, laser scanning confocal microscopy and photography were performed (Hoechst 33342 emits blue fluorescence).

An anchorage-independent colony formation assay was performed using soft agar growth of 3×10^3 cells for 10-14 days. Cell colonies were counted.

2.5. Analysis of apoptosis

Cells were grouped into: Control groups and a test group with CRC cells treated with shDJ-1-1# and shDJ-1-2# for 48 h followed by staining with Annexin V-FITC/PI (BD Pharmingen, USA). Apoptosis was determined as Annexin V +/PI + (late apoptotic) cells and Annexin V +/PI− (early apoptotic).

2.6. Cell cycle analysis

CRC cells were incubated with shDJ-1 for different time periods. Thereafter, they were fixed with 70% ice-cold ethanol at -20°C for 24 h. Cold PBS was used to wash the cells, which were then cultured with 300 uL of the staining solution (5 U/mL RNaseA and 5 ug/mL PI, BD Pharmingen, USA) for 30 min at 4°C. The results were examined with BD FASCanto II flow cytometry

and assessed with the software ModFitLT 3.2.

2.7. Cell invasion and migration assay

Transwell chambers were pre-coated with Matrigel. Matrigel was pre-cooled at 4°C and then added to cover the bottom of the Transwell chamber. Subsequently, the Transwell chamber was placed in an incubator until the Matrigel solidified, after which it was removed. After digestion and re-suspension, SW480 and HCT116 cells (control group and shDJ-1/OE-DJ-1 group) were inoculated onto the upper layer of the serum-free chamber at a density of 2×10^5/mL, and the lower chamber was filled with 20% FBS medium. After incubation for 24 h, unmigrated cells in the upper chamber were wiped away using a sterile cotton swab. Cells that migrated were examined using crystal violet (0.1%) staining and counted under a microscope (Migration experiment without Matrigel).

2.8. RNA isolation and qRT-PCR

TRIzol reagent (Invitrogen) was added to the samples to isolate total RNA, which was then used to synthesize cDNA with the PrimeScript reverse transcriptase reagent kit (TaKaRa, Shiga, Japan). qRT-PCR was performed to analyze the level of DJ-1, cyclin-D1, and p-MDM2 in patient samples and cell lines (all gene primer sequences are shown in Table 1). mRNA expression was calculated using the $2^{-\Delta\Delta CT}$ method, and the level of expression of target genes was normalized to that of GAPDH expression. All experiments were repeated at least thrice.

2.9. Western blotting

Following successful transfection of cells, cells were obtained at the indicated time-points. They were lysed to obtain total cell proteins. The quantity of protein was assessed with the BCA assay kit (Beyotime Biotechnology, China). Next, 30 μg of protein was separated using SDS-PAGE (concentration of 8% or 10%). Then, the protein was transferred to PVDF membranes. The membranes were treated with primary antibodies: GAPDH and Tubulin served as internal standards (8884s/2128s, CST, 1:1000), DJ-1 (ab18257, Abcam, 1:1000), p53(ab1101, Abcam, 1:1000), p-MDM2 (ab170880, Abcam, 1:5000), MDM2 (ab38618, Abcam, 1:1000), cyclin-D1 (55506T, CST, 1:1000), Bcl-2 (ab32124, Abcam, 1:1000), Bax (ab32503, Abcam, 1:1000), and cleaved caspase-3 (ab2302, Abcam, 1:1000).

2.10. HE and immunohistochemistry

The protein expression in the cancer specimens was determined immunohistochemically (IHC). The samples were from subjects with confirmed CRC who were seen at the Second Hospital Affiliated with Nanchang University from 2016 to 2018. The samples were cultured with p-MDM2 (ab170880, Abcam, 1:50), cyclin-D1 (55506T, CST, 1:500), and DJ-1 (ab18257, Abcam, 1:200) antibodies. The staining intensity of the cancer samples was scored as follows: 3 (strong staining, brown), 2 (moderate staining, yellowish-brown), 1 (weak staining, light yellow), and 0 (no staining). Intensity scores < 2 indicated low expression while intensity scores ≥ 2 indicated overexpression. Stained slides were independently examined by two researchers blinded to the clinical outcomes and patient allocation. Metastatic cancer nodules were confirmed with H&E staining.

Table 1. The sequences for RT-qPCR

GAPDH	
Forward Primer	5'-TGTGGGCATCAATGGATTTGG-3'
Reverse Primer	5'-ACACCATGTATTCCGGGTCAAT-3'
DJ-1	
Forward Primer	5'-AACGCTGAAGCGTCCAGAAT-3'
Reverse Primer	5'-TGACCACATCACGGCTACAC-3'
cyclin-D1	
Forward Primer	5'-CTGATTGGACAGGCATGGGT-3'
Reverse Primer	5'-GTGCCTGGAAGTCAACGGTA-3'
p-MDM2	
Forward Primer	5'-CTGTGTTGAGCCCTCTTCCAT-3'
Reverse Primer	5'-TTCTCCGTCAGGGACACTTC-3'

2.11. *In vivo* experiments

All animal experiments were approved by the Institutional Animal Care and Use Committee of the Second Hospital Affiliated with Nanchang University (No. 2017[028]). SW480 cells transfected with the shRNA/Vector were reconstituted in 100 μL of PBS. The cells were subcutaneously transplanted in 5-week-old female nude mice. Every 7 days, the tumor size was measured with Vernier calipers. The tumor volume was determined as: shortest diameter 2× longest diameter/2 at 7-day intervals post-transplantation. One month after cell implantation, mice were sacrificed and the xenografts specimens were used in Western blotting.

2.12. Statistics and data analysis

Groups were compared using the log-rank test; the Kaplan-Meier method was used to plot patient survival curves. Pearson's correlation test or an unpaired *t*-test was used to compare quantitative variables. Multivariate analysis was performed using the Cox regression model. Linear regression was calculated with the Spearman rank correlation. $P < 0.05$ was regarded as statistically significant.

3. Results

3.1. DJ-1 is upregulated in human CRC cells

To examine the effect of DJ-1 on CRC tissues, its level of expression was compared between 90 pairs of CRC samples and adjacent nontumor tissue samples. In both sets of samples, DJ-1 was largely localized in the cytoplasm, and a higher percentage of cells highly expressed DJ-1 in CRC tissues (62.2%, 56 of 90) compared to the level in adjacent normal tissues (13.3%, 12 of 90) (Figure 1A-B and Table 2). In addition, the level of DJ-1 protein expression was markedly higher in 39 CRC specimens compared to that in normal tissues (Figure 1C-D, $p < 0.01$). RT-qPCR revealed a consistently similar pattern of expression in 39 pairs of CRC samples (66.7%, 26 of 39) and matching normal colon samples (15.4%, 6 of 39), suggesting that DJ-1 mRNA was highly expressed in tumors (Figure 1E-F). In conclusion, these findings imply that levels of DJ-1 protein and mRNA expression were markedly higher in human CRC samples than those in adjacent noncancerous colon samples.

3.2. Increased DJ-1 expression in CRC is related to a poor prognosis and the TNM stage

Further experiments were conducted to explore the relationship between the level of DJ-1 protein and the pathological features of CRC. The results are shown in Tables 3 and 4. The level of DJ-1 expression was lower in samples from patients with early stage cancer than in samples from those with advanced disease (Figure 2A-B). The level of expression was strongly related to the tumor TNM stage ($p = 0.0001$) and lymphatic metastasis ($p = 0.0003$). That said, the level of expression did not differ significantly in terms of tumor histological grade and location (Table 4). Kaplan–Meier survival curves indicated that high levels DJ-1 were inversely correlated with the survival rate (Figure 2C, $p < 0.001$). Thus, the current results indicate that DJ-1 contributes to the

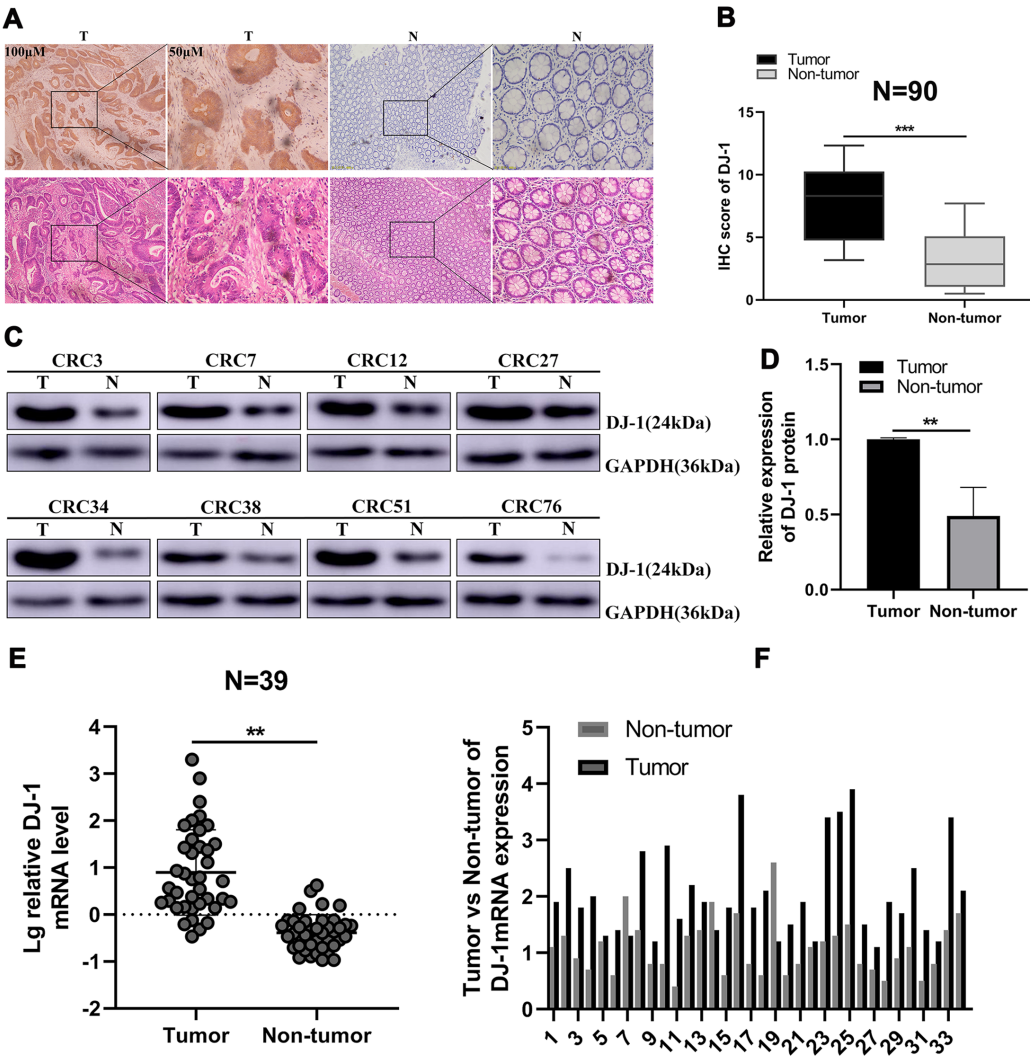

Figure 1. DJ-1 is overexpressed in human CRC tissues. (**A**), Typical IHC and H&E staining images of DJ-1 in normal colon tissue and CRC samples (N: normal colon tissue; T: tumor; scale bar = 100 μM, 50 μM); (**B**), IHC scores for 90 pairs of normal and CRC samples; (**C**), Typical Western blot indicating DJ-1 expression (N: normal colon tissue; T: tumor); (**D**), DJ-1 protein levels in CRC tumor tissues and matching adjacent normal tissues were determined with Western blotting; (**E**), Level of DJ-1 mRNA in39 paired CRC and adjacent normal specimens; (**F**), Ratio of expression (DJ-1/GAPDH) for each the 39 paired CRC and normal specimens. ($^{**}p < 0.01$, $^{***}p < 0.001$).

progression of CRC and is closely related to a poor prognosis.

3.3. Knockdown and overexpression of DJ-1 in CRC cell lines

The baseline level of DJ-1 was measured with Western blotting and RT-qPCR in 6 CRC cell lines: SW480, HT-29, Caco-2, LoVo, HCT116, and SW620. DJ-1 increased particularly in the HCT116 and SW480 cell lines (Figure 3A-B). Therefore, HCT116 and SW480 cells were selected to construct stably transfected cell lines with lentivirus-mediated shRNA targeting DJ-1 to knockdown DJ-1 expression. Transfection efficiency was verified with Western blotting. Silencing efficiency was 82.64% in SW480 cells and 85.26% in HCT116 cells (Figure 3C-D). Analysis was performed with ImageJ. mRNA levels consistently indicated a similar level of silencing efficiency; the mRNA level was reduced by 58.13% in SW480 cells and by 61.92% in HCT116 cells (Figure 3E).

3.4. DJ-1 enhances the invasion, proliferation, and migration of CRC cells

To assess the impact of DJ-1 on the progression of CRC, DJ-1 was knocked down or overexpressed in SW480 and HCT116 cells (Figure 3C-E). Results indicated that SW480 and HCT116 cells with knocked down DJ-1 invaded and migrated into the lower chamber at a slower rate than the vector groups (Figure 4C-F, 24h, $p < 0.05$; 48, $p < 0.01$). After transfection with OE-DJ-1, there were more migrating and invading CRC cells compared to the number in mock cells (Figure 4C-F, $p < 0.001$). Similar findings were noted in HCT116 cells. The role of DJ-1 in the development of colon cancer was evaluated. Results revealed that DJ-1 inhibition decreased the growth of colon cancer cells while DJ-1 overexpression significantly facilitated their growth and proliferation (Figure 4A-B, $p < 0.05$). In addition, DJ-1 regulation of

Table 2. DJ-1 levels in CRC and adjacent nontumor tissues

	DJ-1 (High)	DJ-1 (Low)	*p* value
Tumor	62	28	$p < 0.001$
Adjacent nontumor tissue	12	78	

Table 3. The clinical characteristics of the CRC samples used in this study

Feature	WHO grade		
	I ($n = 6$)	II ($n = 43$)	III ($n = 41$)
Gender			
Male	2	22	19
Female	4	21	22
Age at diagnosis			
< 65	1	2	7
≥ 65	5	41	34
Depth of invasion			
Submucosa	1	2	1
Muscular layer	1	3	9
Serous layer	2	31	20
Every layer	2	7	11
Location			
Transverse colon	2	3	7
Ascending colon	0	1	5
Descending colon	1	17	14
Sigmoid colon	3	12	16

Table 4. The correlation of DJ-1 expression and clinical-pathological characteristics of CRC

Group	Expression of DJ-1 protein		
	DJ-1 (High, $n = 62$)	DJ-1 (Low, $n = 28$)	*p* value
Age at diagnosis			0.733
< 65	13 (21.0%)	5 (17.9%)	
≥ 65	49 (79.0%)	23 (82.1%)	
Gender			0.626
Male	30 (48.4%)	12 (42.9%)	
Female	32 (51.6%)	16 (57.1%)	
Location			0.929
Transverse colon	12 (19.4%)	6 (21.4%)	
Ascending colon	10 (16.1%)	5 (17.9%)	
Descending colon	15 (24.2%)	5 (17.9%)	
Sigmoid colon	25 (40.3%)	12 (42.8%)	
Lymph node status			0.0003*
Positive	41 (66.1%)	7 (25.0%)	
Negative	21 (33.9%)	21 (75.0%)	
Histological grade			0.55
Highly/Moderately differentiated	29 (8.3%)	15 (53.6%)	
Poorly differentiated	33 (66.7%)	13 (46.4%)	
TNM stage			0.0001**
I + II	19 (30.7%)	22 (80%)	
III + IV	43 (69.3%)	6 (20%)	

*$p < 0.001$; **$p < 0.0001$.

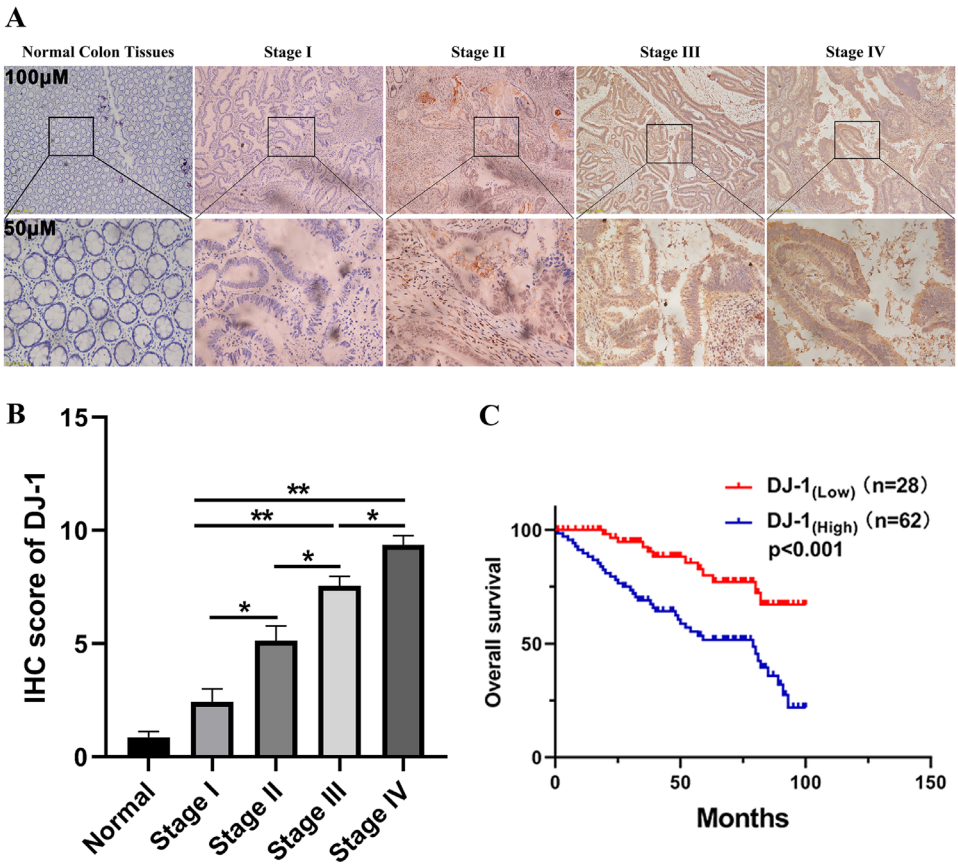

Figure 2. The prognostic value of DJ-1 expression in CRC. (**A**), Results of DJ-1 staining of CRC tumors and normal colorectal mucosa samples obtained from patients with cancer in various TNM stages (scale bar = 100 μM, 50 μM); (**B**), Results of an IHC assay of DJ-1 expression for all stages of cancer. Tumors with a low TNM stage had lower DJ-1 expression than those with a high TNM stage; (**C**), Kaplan-Meier survival curves revealed that increased DJ-1 expression ($n = 62$) in the 90 CRC samples was related to a shorter overall survival compared to samples with low DJ-1 levels ($n = 28$) ($p < 0.001$) ($^{*}p < 0.05$, $^{**}p < 0.01$).

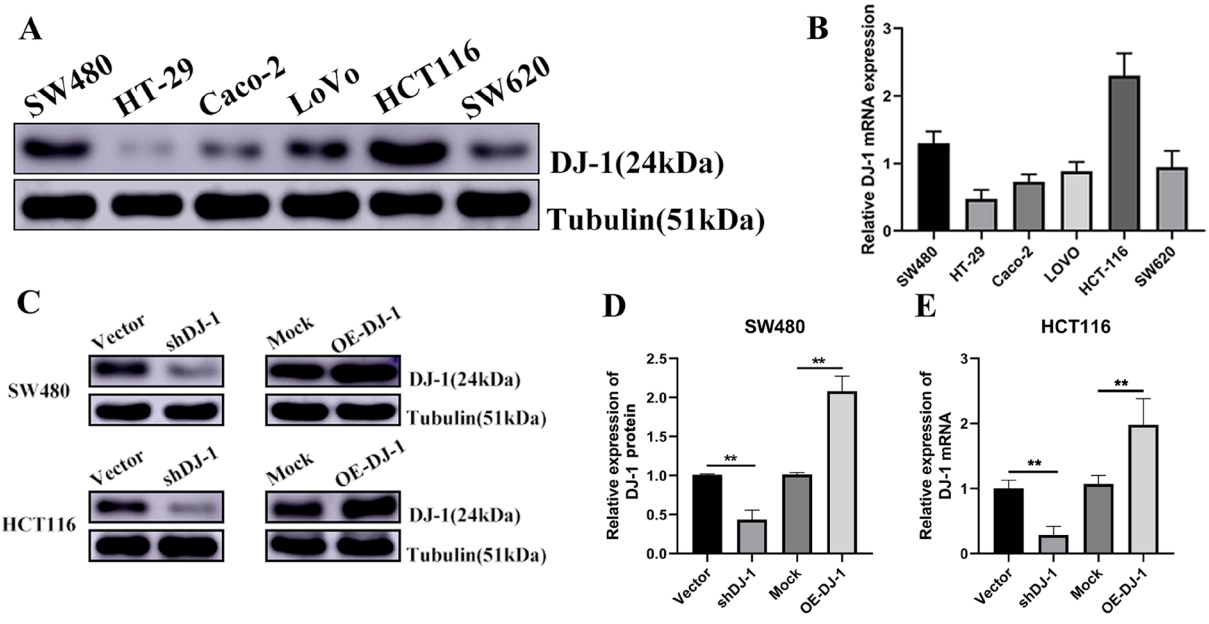

Figure 3. DJ-1 knockdown and overexpression in CRC cell lines. HCT116 and SW480 cells were transfected with lentiviral vectors to inhibit or overexpress DJ-1; An empty vector and scrambled shRNA served as negative controls. (**A-B**), The level of expression of DJ-1 protein and mRNA in 6 CRC cell lines; (**C-E**), The level of expression of DJ-1 protein and mRNA was verified with Western blotting ($p < 0.01$) and RT-qPCR. (OE-DJ-1: Overexpressed DJ-1, $^{**}p < 0.01$).

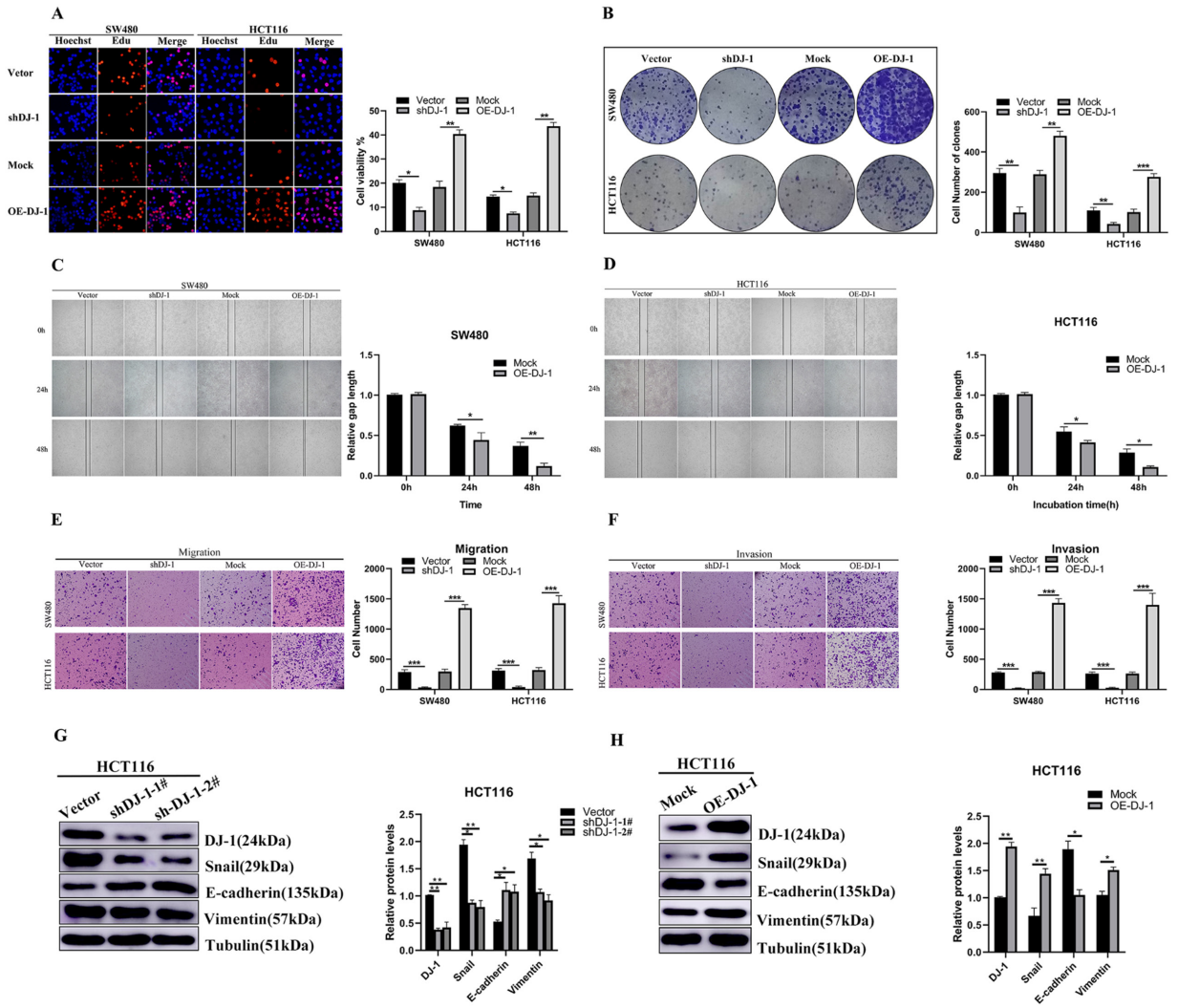

Figure 4. DJ-1 enhances the invasion, proliferation, and migration of CRC cell lines. (**A**), The Edu kit cell proliferation assay was performed in different groups; (**B**), A colony formation assay of the Vector/shDJ-1 and Mock/OE-DJ-1 groups was performed after 14 days of culturing; (**C-D**), Wound-healing migration at the indicated times (Scale bar, 100μm); (**E-F**), A cell Matrigel assay and migration & invasion assay were performed as quantitative assays. The migratory process was assessed at 24 h, and invasion at 48 h (Scale bar, 50μm); (**G-H**), Western blotting was performed to evaluate the effect of DJ-1 on levels of Snail, E-cadherin, and Vimentin expression in HCT116 cells. Data are expressed as the mean ± SD of 3 replicates. (OE-DJ-1: Overexpressed DJ-1, $^{*}p < 0.05$, $^{**}p < 0.01$, $^{***}p < 0.001$).

the expression of proteins involved in tumor metastasis was determined in CRC cell lines. Results indicated that the expression of Snail and Vimentin, two important proteins related to tumour metastasis, decreased markedly ($p < 0.05$) whereas that of E-cadherin increased significantly in shDJ-1 CRC cells (Figure 4G, $p < 0.05$), suggesting that changes in levels of these proteins may play a role in shDJ-1-mediated malignant progression. When DJ-1 was overexpressed in CRC cells, the opposite results were noted (Figure 4H, $p < 0.05$).

3.5. DJ-1 regulates the proliferation and apoptosis of CRC cells

To explore the potential function of DJ-1 in CRC cell apoptosis and proliferation, the impact of DJ-1 knockdown was examined in CRC cell lines. Results indicated that DJ-1 knockdown slightly increased the quantity of cells in the G1 phase and decreased the proportion of cells in the S phase (Figure 5A, $p < 0.05$). In other words, cell viability markedly decreased as a result of inhibition with DJ-1 compared to that in the shRNA groups. That said, SW480 and HCT116 cells transfected with shDJ-1 had an increased rate of apoptosis (Figure 5B, $p < 0.05$).

3.6. DJ-1 promotes CRC cell proliferation *via* the cyclin-D1/MDM2-p53 signaling pathway

DJ-1 may antagonize p53 by inhibiting the expression of the p53 and eventually promoting the proliferation of cancerous cells (*18*). Unfortunately, a co-immunoprecipitation assay revealed that DJ-1 does not directly interact with p53 in CRC (data not shown). Several studies have reported that an oncogene signaling pathway contributes to apoptosis by targeting

MDM2 and indirectly activating the p53 pathway (*19,20*). Thus, the state of the MDM2/p53 pathway was assessed to determine whether it underlies the oncogenic functions of DJ-1 in CRC. Western blotting was performed to evaluate the mechanism responsible for the suppressive effects of DJ-1 on proliferation *in vitro*. Results indicated that the expression of p-MDM2 (MDM2, no significant change) decreased in CRC cells treated with shDJ-1 ($p < 0.05$). However, the expression of p53 increased for some unknown reason ($p < 0.05$). DJ-1 knockdown reduced the level of the apoptotic protein Bcl-2 and enhanced the level of the proteins Bax and Caspase-3 ($p < 0.05$). In contrast, DJ-1 overexpression produced similar results (Figure 6C-D). Thus, DJ-1 is involved in regulating the process of cell apoptosis. In addition, the level of cyclin-D1 protein increased after DJ-1 overexpression compared to that in cells transfected with the control DJ-1-free vector ($p < 0.05$). DJ-1 influenced the amount of cyclin-D1 and promoted expression of Bax and cleaved caspase-3 but inhibited the level of Bcl-2 protein (Figure 6A-B, $p < 0.05$). Similarly, IHC revealed that DJ-1 inhibition decreased the expression of cyclin-D1 and p-MDM2 in the same human CRC tissues (Figure 7A-B, $p < 0.05$). The level of DJ-1 protein was related to that of cyclin-D1 and p-MDM2 in the same CRC tissue samples (Figure 7C-D, and Table 5, $r = 0.2137$, $p = 0.0068$; $r = 0.2056$, $p = 0.0037$).

3.7. The DJ-1-cyclin-D1/p53-MDM2 pathway enhances CRC progression *in vivo*

Additional tests were performed to examine the impact of DJ-1 suppression on the oncogenic activity of SW480

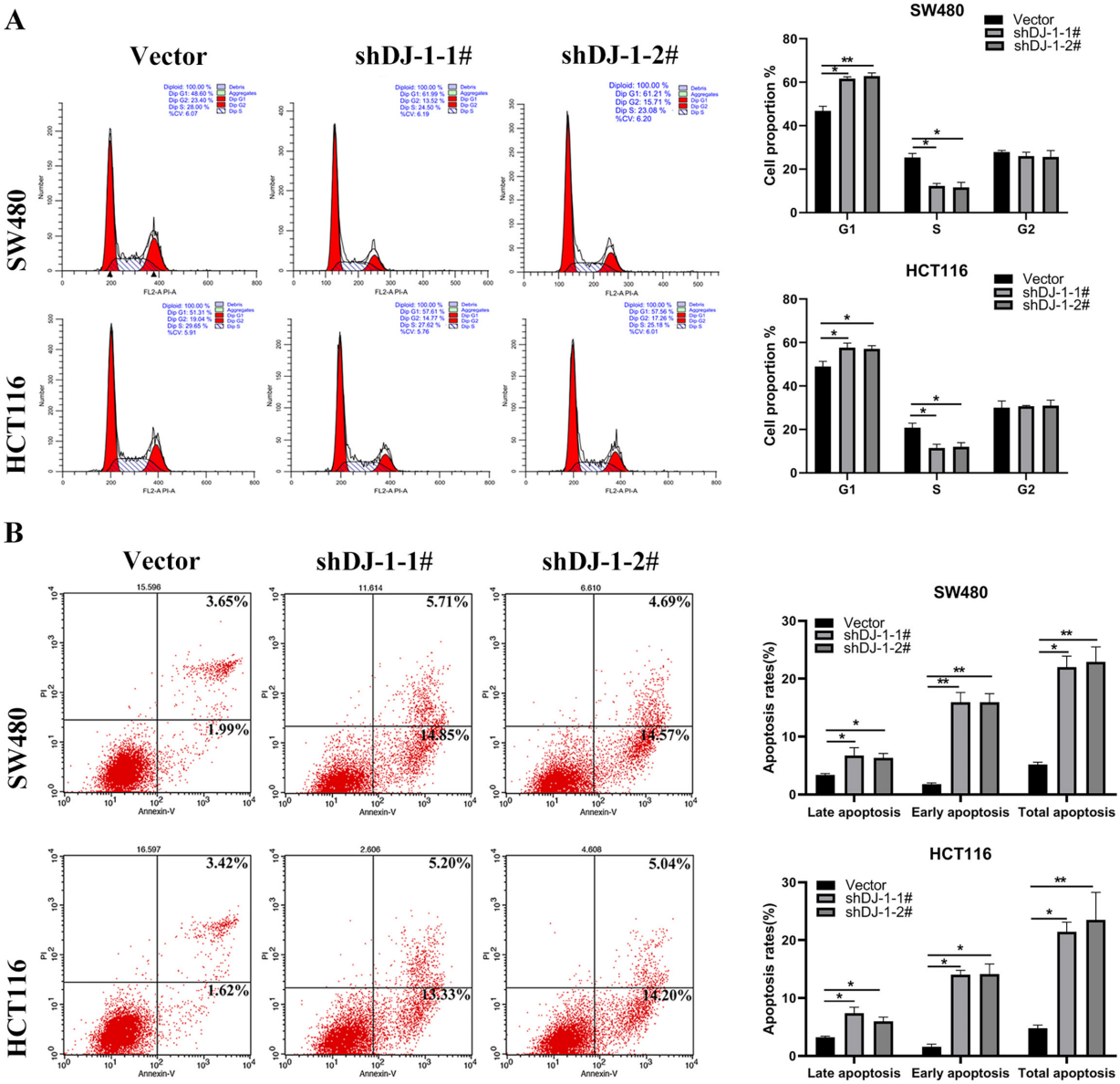

Figure 5. DJ-1 promotes cell cycle progression and decreases cell apoptosis. (**A**), Cell cycle analyses were performed with HCT116 and SW480 cells transfected with shDJ-1-1# and shDJ-1-2#; (**B**), Flow cytometry indicated that DJ-1 knockdown increased cell apoptosis in CRC cells ($^{*}p < 0.05$, $^{**}p < 0.01$).

cells, and a subcutaneous xenograft model was created in nude mice. Implantation of DJ-1-knockdown cells produced xenograft tumors with a smaller average weight compared to those formed by control cells (Figure 8A, B and D, $p < 0.01$), and those tumors displayed slower growth (Figure 8E, $p < 0.01$). Furthermore, H&E staining indicated successful subcutaneous tumor formation in nude mice (Figure 8C). In addition, p53 expression increased while p-MDM2 and cyclin-D1 decreased in tumors obtained from DJ-1-knockdown cells compared to those obtained from control cells (Figure 8F-H, $p < 0.05$). This suggested that the DJ-1/cyclin-D1/MDM2-p53 pathway participated in the development of colon cancer cells *in vivo*.

4. Discussion

At present, tumor metastasis and invasion are the leading factors that decrease the efficacy of tumor therapy, leading to death in patients with CRC (*21*). Thus, more detailed molecular studies need to be conducted to reveal the mechanisms involved in CRC metastasis to promote the development of an early intervention for high-risk cases involving metastasis. Numerous studies have indicated that DJ-1 is a key modulator of metastasis in various types of cancer (*22,23*). The current results revealed that DJ-1 expression increased in CRC, and levels were closely related to the pathological features of the

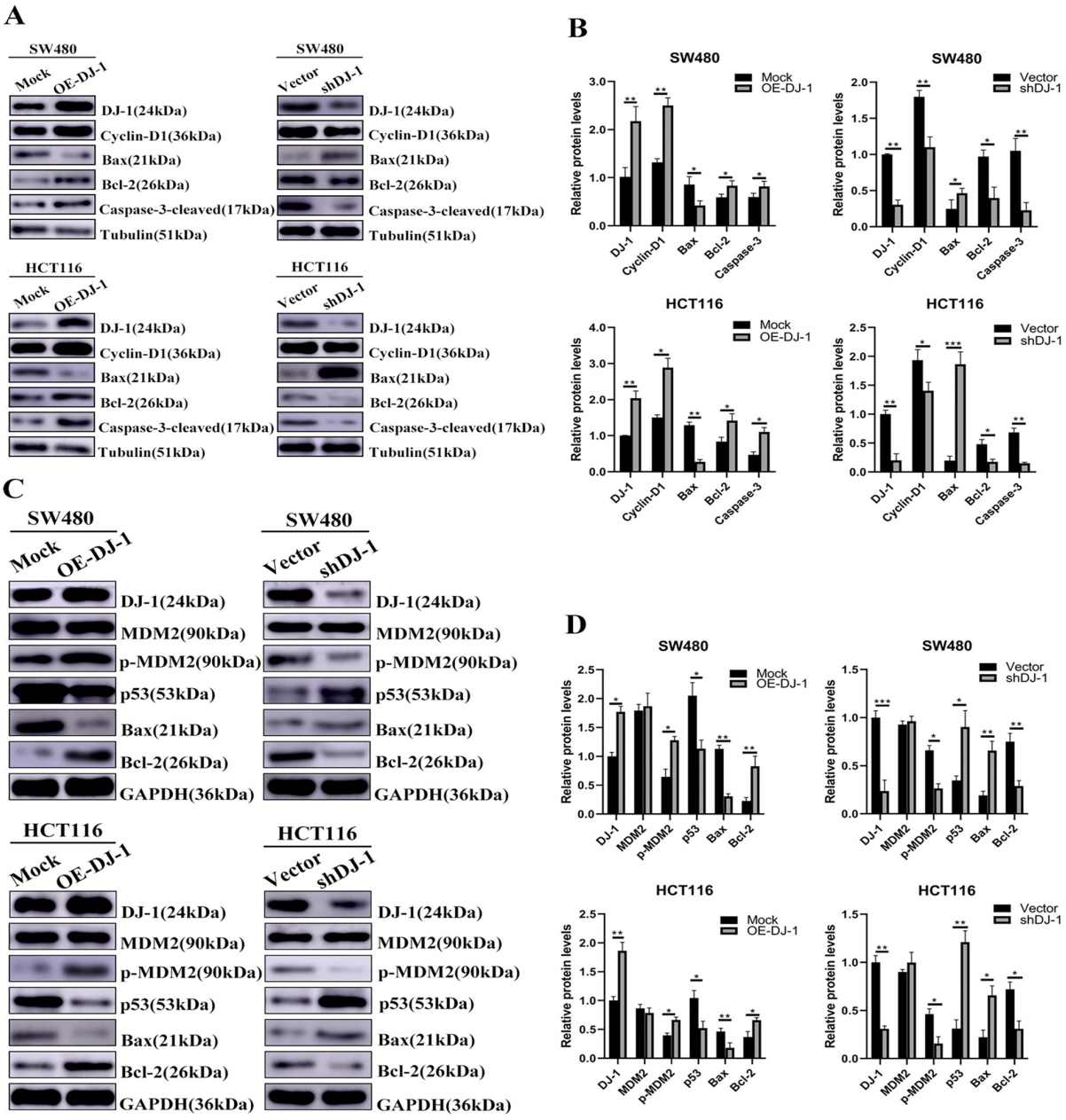

Figure 6. DJ-1 promotes CRC cell proliferation *via* the cyclin-D1/MDM2-p53 signaling pathway. (**A-D**), The effect of DJ-1 on cyclin-D1, Bax, Bcl-2, Caspase-3, p53, p-MDM2, and MDM2 protein levels was evaluated with Western blotting in SW480 and HCT116 cells. Data are expressed as the mean ± SD of 3 replicates. (OE-DJ-1: Overexpressed DJ-1, $^{*}p < 0.05$, $^{**}p < 0.01$).

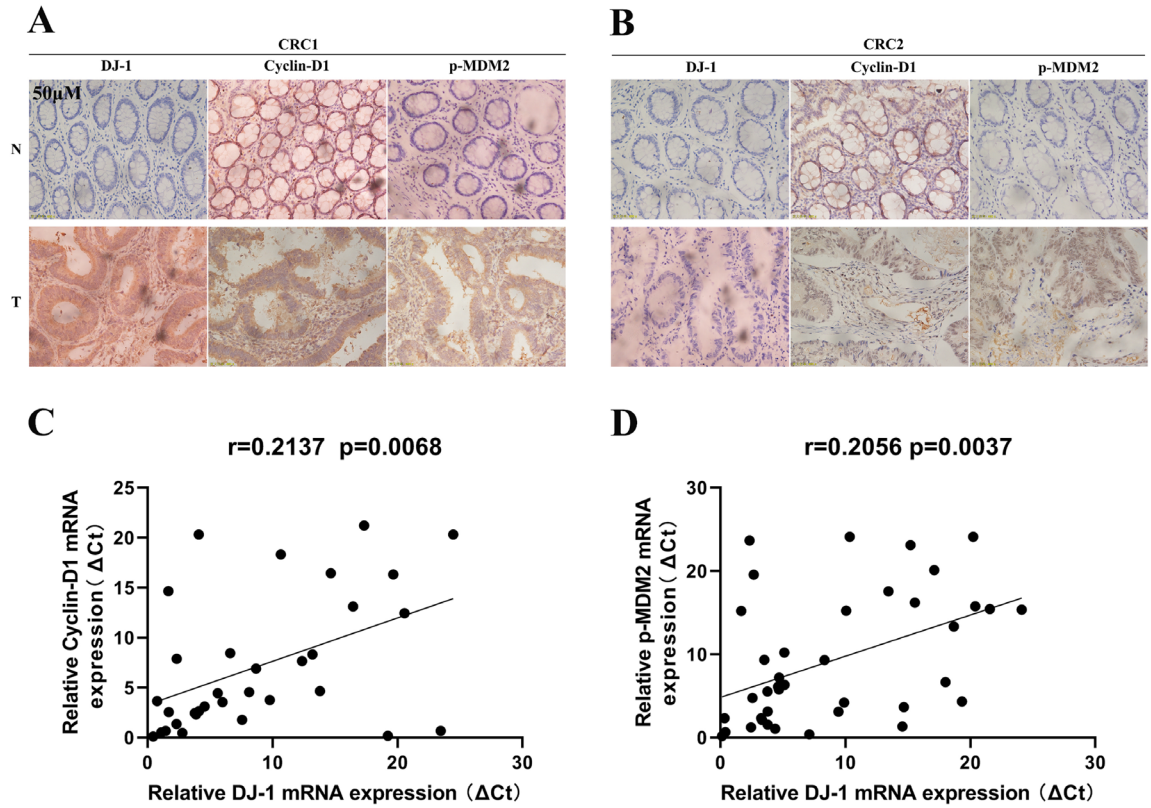

Figure 7. DJ-1 and cyclin-D1 and DJ-1 and p-MDM2 are correlated in CRC. (**A-B**), Representative immunohistochemistry images of DJ-1, cyclin-D1, and p-MDM2 protein levels in tumor specimens from two patients with CRC are shown. In Patient 1, DJ-1 overexpression upregulated cyclin-D1 and p-MDM2, while the opposite occurred in Patient 2 (N: normal colon tissue; T: tumor; scale bar = 50 μM); (**C**), The linear correlation between levels of DJ-1 and cyclin-D1 mRNA in CRC tissues (r = 0.2137) (p = 0.0068); (**D**), The linear correlation between levels of DJ-1 and p-MDM2 mRNA in CRC tissues (r = 0.2056) (p = 0.0037).

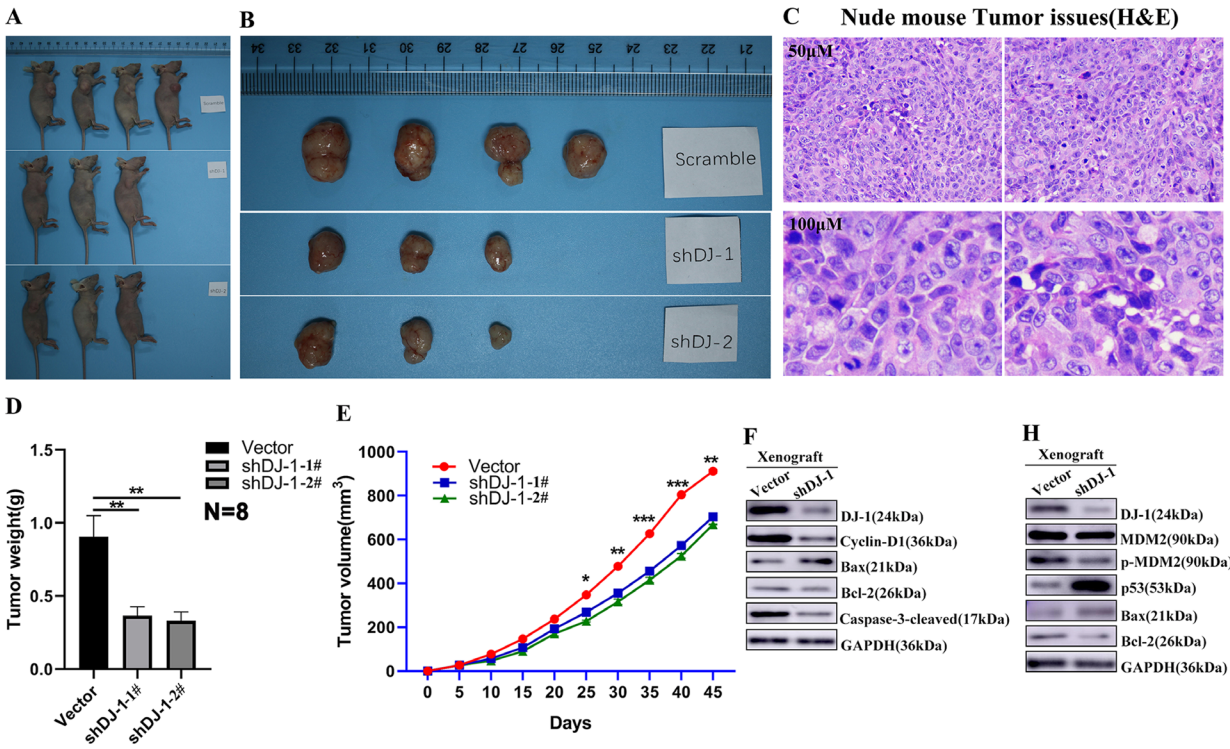

Figure 8. Knockdown of DJ-1 inhibits CRC growth *in vivo*. (**A**), Typical images of nude mice transfected with SW480 cells with shDJ-1-1#, shDJ-1-2#; (**B**), Subcutaneous implantation of SW480 cells; (**C**), Representative images of HE staining of a tumor; (**D**), Tumor weight, n = 4; (**E**), Growth curves for tumors; (**F-H**), Expression of DJ-1, cyclin-D1, p53, p-MDM2, Bax, and Bcl-2 in xenograft tumors obtained from control cells and DJ-1-knockdown. ($^{*}p < 0.05$, $^{**}p < 0.01$, $^{***}p < 0.001$).

Table 5. Association between cyclin-D1 and MDM2 expression and DJ-1 expression in CRC

Gene	*n* (%)	DJ-1 expression		
		High	Low	*r/p-value*
cyclin-D1 expression				
Low	31 (34.4%)	21	18	$r = 0.2137$
High	59 (65.6%)	41	10	$p = 0.0068^*$
p-MDM2 expression				
Low	37 (41.1%)	26	19	$r = 0.2056$
High	53 (58.9%)	36	9	$p = 0.0037^*$

$^*p < 0.001$

disease. This implies that DJ-1 is not only involved in the tumorigenesis of CRC but can also predict the prognosis for this cancer. Moreover, DJ-1 was found to enhance the metastasis and growth of CRC tumors both *in vivo* and *in vitro*. In conclusion, these findings indicate that DJ-1 plays a crucial role in the progression and development of CRC.

Similarly, many previous studies have found that DJ-1 participates in cancer initiation, progression, and multidrug resistance through multiple mechanisms in a context-dependent manner. For instance, the role of DJ-1 in mitochondrial metabolic function is modulated by 14-3-3β. DJ-1 modulation of this novel molecular mechanism of mitochondrial metabolic efficiency is potentially involved in promoting the proliferation of cancer cells (*24*).DJ-1 enhances tumor metastasis and invasion *via* activation of the PLAGL2/Wnt/BMP4 axis in CRC (*22*). In addition, DJ-1 activates the pKB/Akt signaling pathway by inhibiting PTEN, thereby promoting the progression of ovarian, lung, and breast cancer (*25-27*).

Malignant proliferation, migration, and invasion by cancer cells through the basal membrane into the lymphatic vessels, adjacent tissues, and blood is a basic characteristic of cancers and a contributor to local tumor metastasis. The current study explored the molecular mechanism of DJ-1 in CRC cell proliferation, invasion, and migration. Results showed that DJ-1 activated cyclin-D1 and MDM2-p53 signaling, which modulate CRC pathogenesis and progression. A previous study reported that DJ-1 induced G1 arrest by decreasing cyclin-E1 in HCT8 cells (*28*). The results indicated that DJ-1 triggered G1-S phase arrest in HCT116 and SW480 cells (Figure 5, A-H). During G1-S phase arrest, cyclin-D1 is a cardinal factor that orchestrates the transition from the G1 to the S phase in many cancers (*29,30*). cyclin-D1 is highly expressed during the initiation of many cancers such as CRC, indicating that downregulating cyclin-D1 may be a novel target for treatment of CRC.

Western blotting revealed that the level of cyclin-D1 protein increased following overexpression of DJ-1 in SW480 cells compared to that in cells transfected with the control DJ-1-free vector. Equivalent findings were obtained in HCT116 cells. Moreover, DJ-1 overexpression decreased the expression of the apoptosis-related proteins cleaved Caspase-3 and Bax and increased the level of Bcl-2 expression, thereby enhancing the malignant proliferation of CRC cells.

Given that the exact mechanism of DJ-1 signaling in CRC cells is not clearly known, the current study explored the involvement of p53 signaling in DJ-1-enhanced CRC aggressiveness. Mutations in p53, APC, or β-catenin, which are classic tumor suppressor genes, account for over 50% of CRC cases. These mutations often increase gene expression (c-Myc and CCND1), promote nuclear β-catenin accumulation, and increase TCF/LEF transcription activity, leading to uncontrolled proliferation (*31-33*). Moreover, prior studies have revealed that stimulation of p53 and Snail/β-catenin signaling enhances CRC metastasis (*34,35*). Decreased PDLIM claudin-3 or 1PTEN expression has been found to trigger metastasis and/or CRC EMT by promoting Wnt/β-catenin signaling, while surface-expressed FZD8 GPCR48, and FGFR4 respond to paracrine or autocrine cytokines or growth factors in a tumor microenvironment to activate p53 signaling, thereby promoting cancer aggressiveness.

Therefore, the current explored whether DJ-1 regulates p53 and promotes the malignant proliferation and metastasis of CRC cells. Interestingly, a co-immunoprecipitation assay revealed that DJ-1 does not directly interact with p53 in CRC. An oncogene signaling pathway has been found to contribute to apoptosis by targeting MDM2 and indirectly activating the p53 pathway (*36-37*). Therefore, the current study explored whether activation of the MDM2/p53 pathway mediated the oncogenic effects of DJ-1 in CRC. Western blotting was performed to assess how DJ-1 regulates anti-proliferative action *in vitro*. Several p53 targets were positively affected by DJ-1. Bax was the most markedly altered protein. Moreover, p53 knockdown ameliorated DJ-1-induced CRC cell proliferation but not invasion and migration.

Taken together, the current findings indicate that DJ-1 indirectly reduced p53 expression by upregulating p-MDM2 and then activated apoptosis signaling by decreasing expression of Bax and cleaved Caspase-3, all of which enhanced malignant proliferation of CRC cells. Interestingly, results indicated that DJ-1 activated EMT signaling and regulated Snail signaling-targeted gene expression and CRC cell proliferation (Figure 4 M-P). These findings add to the knowledge of the underlying mechanism of the pro-proliferative effects of DJ-1 and reveal new avenues for CRC therapy.

5. Conclusion

This study has provided compelling evidence that as an oncogene DJ-1 might be a promoter of CRC cell invasion, proliferation, and migration *via* the cyclin-D1/MDM2-p53 signaling pathway, and this study has

described its potential role as a postoperative adjuvant therapy for patients with CRC.

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