

UCHL1 acts as a colorectal cancer oncogene via activation of the β -catenin/TCF pathway through its deubiquitinating activity

JIALING ZHONG¹, MEI ZHAO¹, YIMING MA¹, QING LUO¹, JIAN LIU¹, JIA WANG¹,
XINGHUA YUAN², JIANLI SANG³ and CHANGZHI HUANG¹

¹Department of Etiology and Carcinogenesis and State Key Laboratory of Molecular Oncology, and ²Department of Abdomen Surgery, Cancer Institute and Hospital, Chinese Academy of Medical Sciences and Peking Union Medical College, Beijing 100021; ³Institute of Cell Biology, College of Life Sciences, Beijing Normal University, Beijing 100875, P.R. China

Received March 6, 2012; Accepted May 3, 2012

DOI: 10.3892/ijmm.2012.1012

Abstract. Ubiquitin C-terminal hydrolase-L1 (UCHL1) belongs to the family of deubiquitinating enzymes (DUBs), which is involved in the ubiquitin-dependent proteolytic system. Previously, we have reported that the upregulation of UCHL1 is related to lymph node metastasis in colorectal cancer (CRC). However, its molecular mechanisms remain elusive. In this study, we transfected pcDNA3.1/UCHL1 and the pcDNA3.1/UCHL1-C90S mutant into HCT8 cells. The changes in biological features in these stable transfectants were examined both *in vitro* and *in vivo*. Western blot analysis was used to analyze the changes in the β -catenin/T cell factor (TCF) pathway. We demonstrated that UCHL1 re-expression promoted the proliferation, migration and metastasis potential of HCT8 cells both *in vitro* and *in vivo*. We also found that UCHL1 could decelerate β -catenin degradation depending on its deubiquitinating activity. The accumulated β -catenin consequently activated the β -catenin/TCF pathway and induced the expression of cyclin D1 and uPA. These observations imply that UCHL1 may contribute to CRC progression by activating the β -catenin/TCF pathway through its deubiquitinating activity.

Introduction

Colorectal cancer (CRC) is the third most common cancer, and the fourth leading cause of cancer death worldwide (1). In recent years, improved treatment has resulted in a 5-year survival rate of about 50% (2). However, the survival rate of

advanced stage patients, especially of those with lymph node metastasis, is still low. A series of genes are involved in the pathogenesis of CRC. Investigation of these genes is important to clarify the tumorigenic pathway and is helpful for developing new biomarkers for early detection and accurate diagnosis.

Ubiquitin C-terminal hydrolase-L1 (UCHL1) is a member of the deubiquitinating enzymes (DUBs), widely expressed in neuronal tissues, and functions to maintain ubiquitin balance by associating and releasing ubiquitin (3). Previous studies showed that UCHL1 played an important role in neurodegenerative diseases including Parkinson's disease and Alzheimer's disease (4). Recently, growing evidence indicates that UCHL1 also plays a role in tumorigenesis and tumor progression. Interestingly, the roles of UCHL1 are conflicting in different cancer types. UCHL1 acts as a tumor promoter in pancreatic cancer, prostate cancer, and lung cancer (5-7). However, it acts as a tumor suppressor in ovarian cancer, hepatocellular cancer, and nasopharyngeal carcinoma (8-10). In esophageal cancer, breast cancer and CRC, its function is ambiguous (10,11-15). The role of UCHL1 in cancer cells is still largely unexplored.

In our previous study, we found that the expression of UCHL1 was increased in the lymph node metastasis (LNM) CRC group compared with the non-LNM CRC group. The re-expression of UCHL1 through gene transfection increased the invasive ability of HCT8 cells (16). In this study, we further demonstrated that UCHL1 re-expression promoted the proliferation, migration, and metastatic capabilities of HCT8 cells both *in vitro* and *in vivo*. We also found that UCHL1 could decelerate β -catenin degradation depending on its deubiquitinating activity. Accumulated β -catenin consequently activated the β -catenin/T cell factor (TCF) pathway and induced the expression of cyclin D1 and uPA.

Materials and methods

Cell culture and transfection. HCT8 cells were maintained in Roswell Park Memorial Institute (RPMI)-1640 (Invitrogen, USA) supplemented with 10% heat-inactivated fetal bovine serum (FBS) at 37°C in a humidified 5% CO₂ atmosphere. DNA transfection was performed using Lipofectamine (Invitrogen) according to the manufacturer's protocol. For stable transfection, cells were selected using full growth medium containing

Correspondence to: Dr Changzhi Huang, Department of Etiology and Carcinogenesis and State Key Laboratory of Molecular Oncology, Cancer Institute and Hospital, Chinese Academy of Medical Sciences and Peking Union Medical College, 17 Panjiayuan Nanli, Chaoyang, Beijing 100021, P.R. China
E-mail: huangpumc@163.com

Key words: ubiquitin C-terminal hydrolase-L1, colorectal cancer, β -catenin/T cell factor pathway

G418 (600 $\mu\text{g/ml}$) for 14 days, and the positive cells were detected by western blot analysis.

Expression plasmids. Wild-type pcDNA3.1/UCHL1 expression plasmid against UCHL1 was a gift from Dr Julia Shackelford. The pcDNA3.1/UCHL1-C90S mutant was generated by inserting a specific mutation at the Cys 90 site in wild-type pcDNA3.1/UCHL1 plasmid by overlap PCR.

Cell growth assay by MTT. Cells were seeded in triplicate into 96-well plates with 3×10^3 cells/well. Cells were maintained for 5 days, and then 20 μl of MTT (Sigma, USA) solution (5 mg/ml) was added and cells were further incubated at 37°C for 4 h. Absorbance at 492 nm was determined with a microplate reader. All MTT assays were repeated three times.

In vitro cell invasion and migration assays. Cell invasion and migration assays were carried out in a chemotaxis chamber (Millipore). Cells (1×10^5) suspended in 200 μl of serum-free medium were loaded into the upper chamber and allowed to pass through an 8- μm -pore polyethylene terephthalate filter. The lower chamber was filled with complete medium. The chamber was either pre-coated with Matrigel (1:8; BD Biosciences, Bedford, MA, USA) for invasion assays or left uncoated for migration assays. Cells that failed to pass through the filters were removed by scrubbing with cotton swabs after 20 h (migration assay) or 24 h (invasion assay). Cells on the undersurface were fixed in methanol and stained with 0.5% crystal violet, then photographed and quantified in 5 random fields per membrane. Each sample was assayed in triplicate.

Tumor growth and metastasis in nude mice. For the tumor growth assay, cells (40 μl , 4×10^6 cells) were collected and inoculated subcutaneously into the nail pad of 4-week-old male nude mice. Experimental and control groups had 5 mice each. Mice were sacrificed 38 days later. Tumor growth curves were calculated. After tumor excision, the tissues were fixed in 10% buffered formalin. All formalin-fixed and paraffin-embedded samples were carefully examined after staining with hematoxylin and eosin (H&E) and UCHL1 antibody.

For the tumor metastasis assay, cells (50 μl 2.5×10^6 cells) were collected and injected into the tail vein of 4-week-old male nude mice. Each group included 6 mice. Mice were sacrificed 4 weeks later and examined for metastasis development. Lungs and visibly observable lymph nodes were excised. All lungs were fixed with Bouin's fluid. All lymph nodes were formalin-fixed and paraffin-embedded, then carefully examined after staining with H&E and UCHL1 antibody.

All animal experiments were conducted according to the institutional and national guidelines for animal experiments.

Immunohistochemical staining. Immunohistochemical (IHC) staining was performed as previously described (16). Specimens were incubated with anti-UCHL1 (1:500, Abcam) overnight at 4°C, and then incubated with a biotinylated second antibody (goat anti-rabbit/IgG 1:100 dilution) for 30 min at 37°C.

Half-life analysis. Cells were treated with cyclohexamide (CHX, 100 $\mu\text{g/ml}$; Sigma) for 0, 3 or 6 h to inhibit further

protein synthesis. Then cells were harvested and analyzed by western blot analysis.

Protein preparation and western blot analysis. For whole-cell protein extraction, cells were lysed with lysis buffer [50 mmol/l Tris-HCl, pH 7.4; 150 mmol/l NaCl; 1.5% NP-40; 0.1% SDS; 50 $\mu\text{g/ml}$ phenylmethylsulfonyl fluoride (PMSF); with freshly added proteinase inhibitor cocktail] for 40 min on ice, followed by centrifugation at 14,000 \times g for 15 min at 4°C. The supernatants were collected as total protein.

For preparation of cytoplasmic and nuclear protein, cells were collected and rinsed with cold phosphate-buffered saline (PBS) twice, then centrifuged at 3000 \times g for 5 min at 4°C. The pellets were suspended in buffer A (10 mM HEPES, pH 7.9; 1.5 mM MgCl_2 ; 10 mM KCl; 0.5 mM DTT; 0.2% NP-40; 50 $\mu\text{g/ml}$ PMSF; with freshly added proteinase inhibitor cocktail) and kept for 15 min at 4°C, then centrifuged at 5,000 \times g for 5 min. The supernatants (cytoplasmic protein) were harvested. The insoluble pellets were then centrifuged at 14,000 \times g for 1 min, again after being washed with cold buffer A. The pellets were lysed in lysis buffer and kept for 40 min at 4°C, then centrifuged at 14,000 \times g for 10 min. The supernatants were harvested as nuclear protein. Western blot analysis was performed using conventional protocols as described previously (16).

Statistical analysis. All statistical analyses were performed using SPSS 13.0 software (SPSS Inc., Chicago, IL, USA). Data were expressed as means \pm SD. The Student's t-test was used for statistical comparison. P-values < 0.05 indicated statistical significance.

Results

UCHL1 promotes proliferation, migration and invasion capabilities of HCT8 cells in vitro. As we previously reported (16), HCT8 did not express endogenous UCHL1. Thus, we used HCT8 to generate the UCHL1 stable cell line. The HCT8/UCHL1 stable cells expressed high exogenous UCHL1, and the HCT8/pcDNA3.1 cells were used as empty vector control (Fig. 1A). HCT8/UCHL1 cells showed increased cell proliferation, migration, and invasion capabilities *in vitro* compared with control cells ($P < 0.05$) (Fig. 1B-D). These results implied that UCHL1 promoted the proliferation, migration, and invasion capabilities of HCT8 cells *in vitro*.

UCHL1 promotes tumorigenesis and metastasis of HCT8 cells in vivo. To investigate UCHL1 functions *in vivo*, we injected HCT8/pcDNA3.1 and HCT8/UCHL1 cells into nude mice through the nail pad. Mice were humanely sacrificed and dissected for tumors 38 days later. Tumor nodules resected from the HCT8/UCHL1 group showed positive UCHL1 staining (Fig. 2D). In addition, the nodules from this group were larger and heavier than those from the control group ($P < 0.05$) (Fig. 2A-C). However, we did not find lymph node metastasis or lung metastasis in either group in this model.

To investigate whether UCHL1 promotes tumor metastasis *in vivo*, we injected HCT8/pcDNA3.1 and HCT8/UCHL1 cells into nude mice via tail veins. Mice were sacrificed 4 weeks later. In the HCT8/UCHL1 group, 2 mice died before the

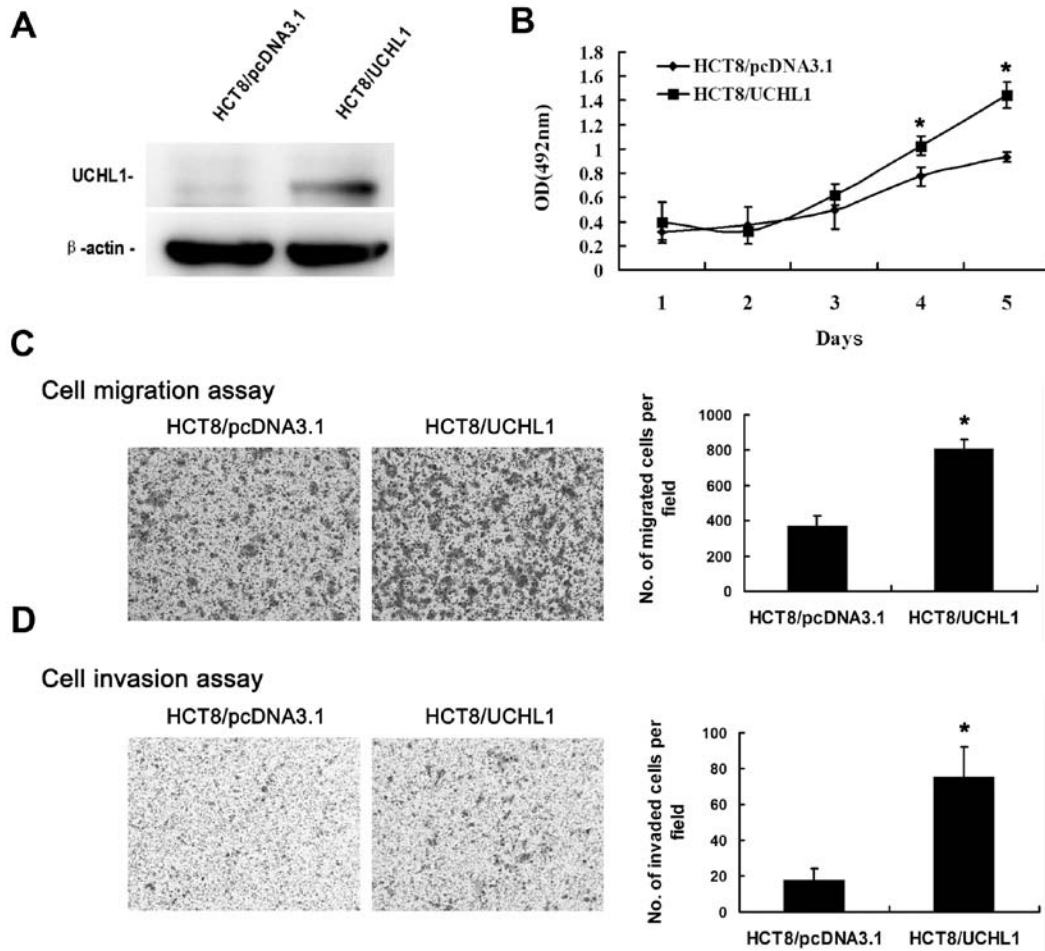


Figure 1. UCHL1 promotes proliferation, migration and invasion capabilities of HCT8 cells *in vitro*. (A) HCT8 cells were transfected with UCHL1 expression vector or empty vector and maintained in the presence of G418 for 14 days. UCHL1 expression was identified by western blot analysis; β -actin served as control for sample loading. (B) Cell growth curve measured by the MTT assay. Each point represents optical density \pm SD of three independent experiments. (C) Cell migration and (D) invasion assays were performed by using chemotaxis chambers. Cell number quantified data were shown as means \pm SD (n=3) (magnification, $\times 100$). *P<0.05, Student's t-test.

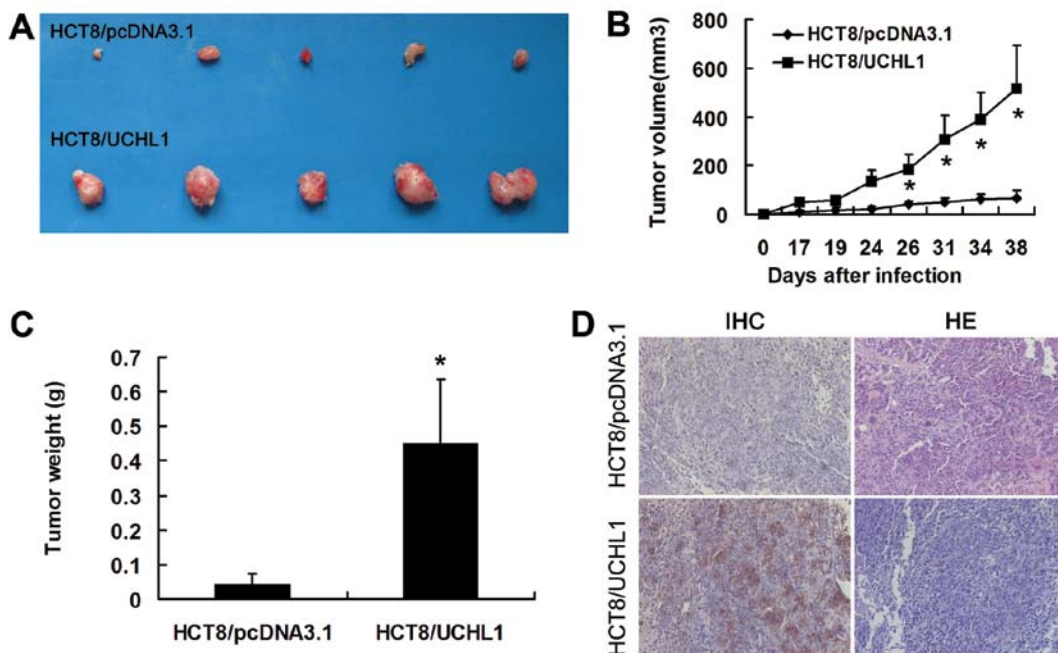


Figure 2. UCHL1 promotes tumor xenografts of HCT8 cells in nude mice. (A) Gross observation of tumor xenografts in different groups. (B) Tumor volume after injection in nude mice. Each point represents tumor volume \pm SD. (C) Tumor weight in different groups at the end of the study. Data are expressed as means \pm SD. (D) IHC and H&E staining of tumor xenografts in different groups (magnification, $\times 100$). *P<0.05, Student's t-test.

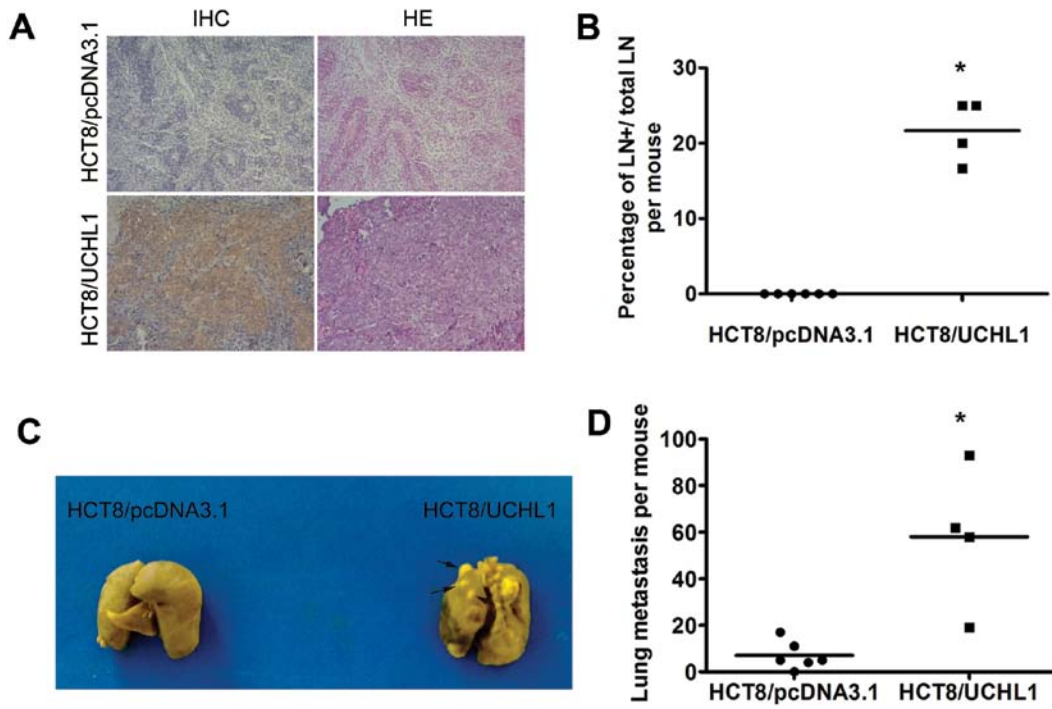


Figure 3. UCHL1 promotes lymph node and lung metastases in nude mice. (A) UCHL1 expression was assessed by immunohistochemistry; H&E was used for histological examination to confirm metastasis. Top row, normal lymph node resected from the HCT8/pcDNA3.1 group showing negative UCHL1 staining. H&E staining depicting normal histological structure. Bottom row, metastatic lymph node resected from the HCT8/UCHL1 group showing strong UCHL1 staining. H&E staining depicting destroyed lymph node histologic structure (magnification, x100). (B) Percentage of metastatic lymph nodes (LN⁺)/total LN per mouse. (C) Lung metastases of each group at 4 weeks after injection. (D) Statistical plot showing the metastatic loci counted on the whole lung surface per mouse. *P<0.05, Student's t-test.

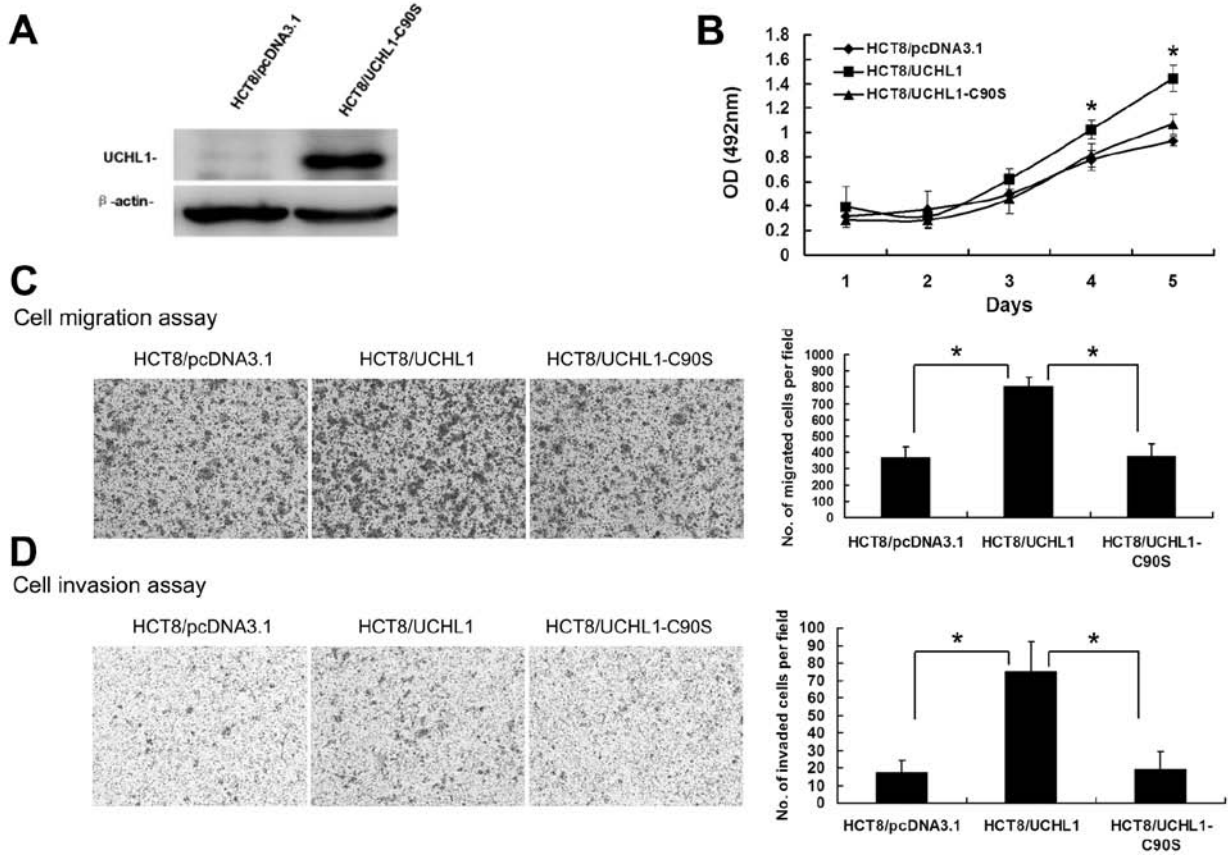


Figure 4. Deubiquitinase activity is crucial for UCHL1 to promote the malignant transformation of HCT8 cells. (A) HCT8 cells were transfected with UCHL1-C90S expression vector or empty vector and maintained in presence of G418 for 14 days. UCHL1 expression was identified by western blot analysis; β -actin served as a control for sample loading. UCHL1-C90S mutant could not promote (B) proliferation, (C) migration and (D) invasion capabilities of HCT8 cells *in vitro*. *P<0.05, Student's t-test.

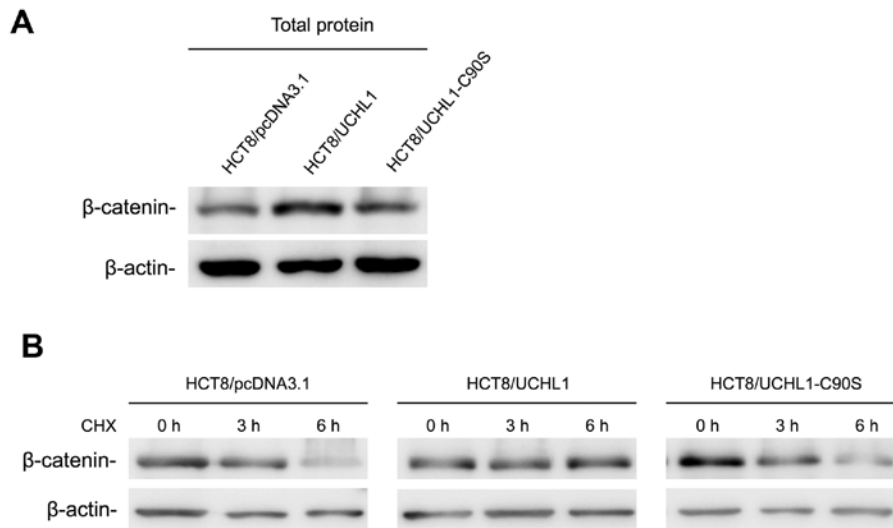


Figure 5. UCHL1 upregulates β -catenin by enhancing its stability. (A) β -catenin increased in HCT8/UCHL1 cells compared with HCT8/pcDNA3.1 and HCT8/UCHL1-C90S cells. (B) Cells were exposed to CHX for different durations. β -catenin protein levels were evaluated by western blot analysis. β -actin served as a loading control.

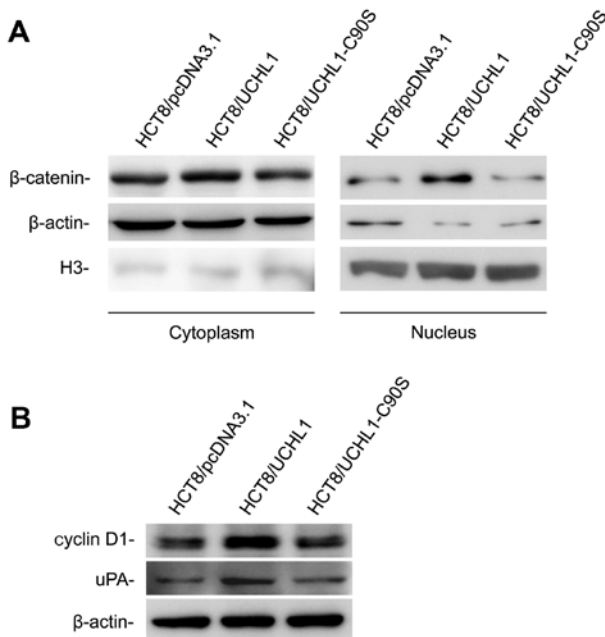


Figure 6. UCHL1 induces the accumulation of β -catenin in nucleus, and induces the expression of cyclin D1 and uPA. (A) The cytoplasmic and nuclear proteins were isolated from cells and subjected to western blot analysis. β -catenin accumulated in the nucleus in HCT8/UCHL1 cells compared with HCT8/pcDNA3.1 and HCT8/UCHL1-C90S cells. β -actin and H3 served as loading control for cytoplasm and nucleus proteins respectively. (B) UCHL1 induced the expression of cyclin D1 and uPA. β -actin served as loading control.

end of the experiment. In the remaining 4 mice, all of them developed lymph node metastases, accompanied with positive UCHL1 staining (Fig. 3A). However, no lymph node metastasis was found in HCT8/pcDNA3.1 group. The percentage of metastatic lymph nodes with respect to the total visible lymph nodes per mouse was significantly higher in the HCT8/UCHL1 group (22%) than in the control group (0%; $P < 0.05$) (Fig. 3B). Furthermore, UCHL1 re-expression dramatically

promoted lung metastasis of HCT8 cells. The metastatic loci in the HCT8/UCHL1 group (58 ± 30) were significantly more than those in the control group (7 ± 6 ; $P < 0.05$) (Fig. 3C-D). These results indicated that UCHL1 promoted tumorigenesis and metastasis of HCT8 cells *in vivo*.

Deubiquitinase activity is crucial for UCHL1 to promote the malignant transformation of HCT8 cells. To investigate if the deubiquitinating capability is essential for UCHL1 function, we constructed a C90S mutant (with cysteine 90 converted to serine), with impaired deubiquitinase activity (17). Then the HCT8/UCHL1-C90S stable cell line was generated (Fig. 4A). The proliferation, migration, and invasion capabilities of HCT8/UCHL1-C90S cells were similar to those of HCT8/pcDNA3.1 cells (Fig. 4B-D). These results indicated that the malignant transformation promoting function of UCHL1 was dependent on its deubiquitinating activity.

UCHL1 upregulates β -catenin by enhancing its stability. To see whether UCHL1 could affect β -catenin expression in HCT8 cells, we detected β -catenin in three stable cell lines. Our results confirmed that β -catenin expression was upregulated by UCHL1, but could not be upregulated by the UCHL1-C90S mutant (Fig. 5A).

To determine whether UCHL1 upregulates β -catenin expression by enhancing its stability, a half-life assay was performed. The data showed that UCHL1 re-expression significantly extended the half-life of β -catenin. In contrast, we failed to observe this function in the UCHL1-C90S mutant (Fig. 5B). These results indicated that UCHL1 upregulated β -catenin by enhancing its stability, depending on its deubiquitinating activity.

UCHL1 induces modification of proliferation, migration and invasion-related genes. To further confirm the effect of UCHL1 on β -catenin/TCF signaling, the immunoblotting analyses were carried out using the protein isolated from the cell cytoplasm and nucleus in three stable cell lines. Our

results demonstrated that UCHL1 re-expression increased the accumulation of β -catenin in cytoplasm, which resulted in increased β -catenin translocation into cell nucleus (Fig. 6A). However, β -catenin remained unchanged in HCT8/UCHL1-C90S cells compared with HCT8/pcDNA 3.1 cells.

Cyclin D1 and serine protease urokinase plasminogen activator (uPA) are transcriptionally induced by β -catenin in the nucleus (18,19). As shown in Fig. 6B, both molecules were upregulated in HCT8/UCHL1 cells, but not in HCT8/pcDNA 3.1 and HCT8/UCHL1-C90S cells. These data suggested that UCHL1 activated the β -catenin/TCF signaling, depending on its deubiquitinating activity.

Discussion

Although much evidence indicates that UCHL1 is related to CRC, the mechanism is still ambiguous. Some investigations have shown that UCHL1 methylation was more frequent in CRC tissues than in normal colorectal tissues (9,10,20), whereas others have indicated that high UCHL1 expression was related to colorectal tumor progression, invasion, lymph node metastasis, and poor clinical outcome (15,21-23). In this study, we confirmed our previous results by establishing that UCHL1 played an oncogenic role by positively regulating the proliferation, invasion, and metastasis of HCT8 *in vitro* and *in vivo*. Nail pad injection of stable HCT8/UCHL1 cells promoted tumor formation in nude mice. These data are consistent with that UCHL1 expression is related to tumor size and tumor stage in CRC (15,16). In addition, tail vein injection of stable HCT8/UCHL1 cells promoted both lymph node and lung metastases in nude mice. These data are consistent with that UCHL1 expression is related to lymph node metastases in clinical colorectal patients (16,23).

The Wnt/ β -catenin pathway is implicated in over 90% of colon cancers. The activation of β -catenin results in constitutive activity of β -catenin TCF transcriptional complex, which drives tumor formation (24). While exploring the mechanism by which UCHL1 promoted the malignant transformation of HCT8, we found that UCHL1 upregulated the expression of β -catenin by decelerating its degradation depending on its deubiquitinating activity. In agreement with our data, a recent study has also demonstrated that UCHL1 upregulated β -catenin signaling in 293 and NIH 3T3 cells (25). It is well known that the β -catenin subcellular distribution is greatly related to its oncogenic transcriptional regulation. The stabilized form of β -catenin accumulates in cytoplasm and is then translocated into the cell nucleus, where it carries out a pivotal role in tumorigenesis by activating the Wnt signaling target genes. In our investigation, UCHL1 induction increased the accumulation of β -catenin in the cytoplasm, which resulted in increased β -catenin translocation into the cell nucleus. Then upregulation of cyclin D1 and uPA were detected. Both cyclin D1 and uPA are target genes of β -catenin (18,19). Cyclin D1 is a major regulator of the progression of cells into the proliferative stage of the cell cycle (26). The increased expression of cyclin D1 can stimulate CDK activity and promote the cell cycle progression. uPA is a protease involved in degradation of the extracellular matrix. Upregulation of uPA promotes tumor progression, particularly tumor invasion and metastasis (27,28). These results agree with the cell behaviors that we

have demonstrated in this study. Thus, we believe that UCHL1 promotes the malignant transformation of HCT8 by activating β -catenin signaling.

Several studies have demonstrated the importance of the deubiquitinating activity of UCHL1 in cancer progression and metastasis (5,13,25). In our study, C90S, a mutant of UCHL1 with impaired deubiquitinating activity, did not induce the activation of β -catenin signaling and it did not promote the proliferation, migration, and invasion capabilities of HCT8 cells. However, identification of other target genes of UCHL1 deubiquitinating activity will provide more insight into the mechanisms of UCHL1 in oncogenesis in CRC.

The conflicting study results of UCHL1 function in CRC may be because of the inadequate sample and the exact subtype of CRC. Besides, UCHL1, which contains two opposing enzymatic activities, deubiquitinating enzyme activity and ubiquitin ligase enzyme activity, may function differently under certain circumstances. Thus, further study is needed to elaborate the exact role of UCHL1, to clarify the function of UCHL1 in the tumorigenic pathway and to assist in the development of novel biomarkers for early detection and accurate diagnosis.

In summary, we provide both *in vitro* and *in vivo* evidence that UCHL1 functions as an oncogene by increasing cell proliferation, migration, and invasion capabilities in HCT8 cells through the β -catenin/TCF pathway depending on its deubiquitinating activity.

Acknowledgements

This research was supported by the National Key Basic Research Program of China (2007CB914700). We are grateful to Dr Julia Shackelford (Lineberger Comprehensive Cancer Center, University of North Carolina at Chapel Hill, Chapel Hill, North Carolina, USA) for providing us with wild-type pcDNA3.1/UCHL1 plasmid.

References

1. Parkin DM, Bray F, Ferlay J and Pisani P: Global cancer statistics, 2002. *CA Cancer J Clin* 55: 74-108, 2005.
2. Levin B and Boyle P (eds): World cancer report 2008. International Agency for Research on Cancer, Lyon, pp374-379, 2008.
3. Osaka H, Wang YL, Takada K, Takizawa S, Setsuie R, Li H, Sato Y, Nishikawa K, Sun YJ, Sakurai M, *et al*: Ubiquitin carboxy-terminal hydrolase L1 binds to and stabilizes monoubiquitin in neuron. *Hum Mol Genet* 12: 1945-1958, 2003.
4. Setsuie R and Wada K: The functions of UCH-L1 and its relation to neurodegenerative diseases. *Neurochem Int* 51: 105-111, 2007.
5. Kim HJ, Kim YM, Lim S, Nam YK, Jeong J, Kim HJ and Lee KJ: Ubiquitin C-terminal hydrolase-L1 is a key regulator of tumor cell invasion and metastasis. *Oncogene* 28: 117-127, 2009.
6. Leiblich A, Cross SS, Catto JW, Pesce G, Hamdy FC and Rehman I: Human prostate cancer cells express neuroendocrine cell markers PGP 9.5 and chromogranin A. *Prostate* 67: 1761-1769, 2007.
7. Tezel E, Hibi K, Nagasaka T and Nakao A: PGP9.5 as a prognostic factor in pancreatic cancer. *Clin Cancer Res* 6: 4764-4767, 2000.
8. Li L, Tao Q, Jin H, van Hasselt A, Poon FF, Wang X, Zeng MS, Jia WH, Zeng YX, Chan AT and Cao Y: The tumor suppressor UCHL1 forms a complex with p53/MDM2/ARF to promote p53 signaling and is frequently silenced in nasopharyngeal carcinoma. *Clin Cancer Res* 16: 2949-2958, 2010.
9. Okochi-Takada E, Nakazawa K, Wakabayashi M, Mori A, Ichimura S, Yasugi T and Ushijima T: Silencing of the UCHL1 gene in human colorectal and ovarian cancers. *Int J Cancer* 119: 1338-1344, 2006.

10. Yu J, Tao Q, Cheung KF, Jin H, Poon FF, Wang X, Li H, Cheng YY, Röcken C, Ebert MP, *et al*: Epigenetic identification of ubiquitin carboxyl-terminal hydrolase L1 as a functional tumor suppressor and biomarker for hepatocellular carcinoma and other digestive tumors. *Hepatology* 48: 508-518, 2008.
11. Mandelker DL, Yamashita K, Tokumaru Y, Mimori K, Howard DL, Tanaka Y, Carvalho AL, Jiang WW, Park HL, Kim MS, *et al*: PGP9.5 promoter methylation is an independent prognostic factor for esophageal squamous cell carcinoma. *Cancer Res* 65: 4963-4968, 2005.
12. Miyoshi Y, Nakayama S, Torikoshi Y, Tanaka S, Ishihara H, Taguchi T, Tamaki Y and Noguchi S: High expression of ubiquitin carboxy-terminal hydrolase-L1 and -L3 mRNA predicts early recurrence in patients with invasive breast cancer. *Cancer Sci* 97: 523-529, 2006.
13. Takase T, Hibi K, Yamazaki T, Nakayama H, Taguchi M, Kasai Y, Ito K, Akiyama S, Nagasaka T and Nakao A: PGP9.5 overexpression in esophageal squamous cell carcinoma. *Hepatogastroenterology* 50: 1278-1280, 2003.
14. Wang WJ, Li QQ, Xu JD, Cao XX, Li HX, Tang F, Chen Q, Yang JM, Xu ZD and Liu XP: Overexpression of ubiquitin carboxy terminal hydrolase-L1 induces apoptosis in breast cancer cells. *Int J Oncol* 33: 1037-1045, 2008.
15. Yamazaki T, Hibi K, Takase T, Tezel E, Nakayama H, Kasai Y, Ito K, Akiyama S, Nagasaka T and Nakao A: PGP9.5 as a marker for invasive colorectal cancer. *Clin Cancer Res* 8: 192-195, 2002.
16. Ma Y, Zhao M, Zhong J, Shi L, Luo Q, Liu J, Wang J, Yuan X and Huang C: Proteomic profiling of proteins associated with lymph node metastasis in colorectal cancer. *J Cell Biochem* 110: 1512-1519, 2010.
17. Larsen CN, Price JS and Wilkinson KD: Substrate binding and catalysis by ubiquitin C-terminal hydrolases: identification of two active sites. *Biochemistry* 35: 6735-6744, 1996.
18. Hiendlmeyer E, Regus S, Wassermann S, Hlubek F, Haynl A, Dimmler A, Koch C, Knoll C, van Beest M, Reuning U, *et al*: Beta-catenin upregulates the expression of the urokinase plasminogen activator in human colorectal tumors. *Cancer Res* 64: 1209-1214, 2004.
19. Shtutman M, Zhurinsky J, Simcha I, Albanese C, D'Amico M, Pestell R and Ben-Ze'ev A: The cyclin D1 gene is a target of the β -catenin/LEF-1 pathway. *Proc Natl Acad Sci USA* 96: 5522-5527, 1999.
20. Fukutomi S, Seki N, Koda K and Miyazaki M: Identification of methylation-silenced genes in colorectal cancer cell lines genomic screening using oligonucleotide arrays. *Scand J Gastroenterol* 42: 1486-1494, 2007.
21. Akishima-Fukasawa Y, Ino Y, Nakanishi Y, Miura A, Moriya Y, Kondo T, Kanai Y and Hirohashi S: Significance of PGP9.5 expression in cancer-associated fibroblasts for prognosis of colorectal carcinoma. *Am J Clin Pathol* 134: 71-79, 2010.
22. Hong Y, Ho KS, Eu KW and Cheah PY: A susceptibility gene set for early onset colorectal cancer that integrates diverse signaling pathway: implication for tumorigenesis. *Clin Cancer Res* 13: 1107-1114, 2007.
23. Mizukami H, Shirahata A, Goto T, Sakata M, Saito M, Ishibashi K, Kigawa G, Nemoto H, Sanada Y and Hibi K: PGP9.5 methylation as a marker for metastatic colorectal cancer. *Anticancer Res* 28: 2697-2700, 2008.
24. Bienz M and Clevers H: Linking colorectal cancer to Wnt signaling. *Cell* 103: 311-320, 2000.
25. Bheda A, Yue W, Gullapalli A, Whitehurst C, Liu R, Pagano JS and Shackelford J: Positive reciprocal regulation of ubiquitin C-terminal hydrolase L1 and beta-catenin/TCF signaling. *PLoS One* 4: e5955, 2009.
26. Sherr CJ: Cancer cell cycles. *Science* 274: 1672-1677, 1996.
27. Mazaud S, Guyot R, Guigon CJ, Coudouel N, Le Magueresse-Battistoni B and Magre S: Basal membrane remodeling during follicle histogenesis in the rat ovary: contribution of proteinases of the MMP and PA families. *Dev Biol* 277: 403-416, 2005.
28. Morgan H and Hill PA: Human breast cancer cell-mediated bone collagen degradation requires plasminogen activation and matrix metalloproteinase activity. *Cancer Cell Int* 5: 1, 2005.