

Association of clinicopathological features with UbcH10 expression in colorectal cancer

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Abstract

Purpose UbcH10 is the cancer-related E2 ubiquitin-conjugating enzyme, and its overexpression has been demonstrated in a variety of malignancies. The aim of this study is to investigate the association of UbcH10 gene expression with the carcinogenesis and tumor progression of colorectal cancer.

Methods The expression levels of UbcH10 in human malignant colorectal carcinoma tissues and their adjacent normal tissues were examined using real-time quantitative RT-PCR and immunohistochemical analysis. The correlations of UbcH10 expression to the clinicopathologic characteristics of the colorectal cancer were analyzed. Cell proliferation and Matrigel invasion assays were performed in HT-29 cells transfected with UbcH10 expression plasmid pcDNA3.1-UbcH10, UbcH10 RNA interference vector pUbcH10-RNAi as well as their control vectors.

Results Our study demonstrated that the expression of UbcH10 in colorectal carcinoma tissues was significantly higher than that in non-cancerous tissues ($P < 0.01$), and the UbcH10 overexpression was related to the degree of tumor differentiation and lymph node metastasis of colorectal cancer patients ($P < 0.05$). In vitro, the overexpression of UbcH10 promoted cell proliferation and tumor invasiveness, but the downregulation of UbcH10

expression significantly reduced the growth rate and the invasiveness activity of tumor cell line.

Conclusions Our study suggests that the overexpression of UbcH10 gene plays a critical role in the carcinogenesis and tumor progression of colorectal cancer. It may be a new marker in diagnosis and prognosis of colorectal cancer, and the inhibition of UbcH10 may be a therapeutic potential for the treatment of colorectal cancer.

Keywords UbcH10 · Gene expression · Colorectal cancer · Carcinogenesis · Tumor progression

Introduction

UbcH10 is the cancer-related E2 ubiquitin-conjugating enzyme (Okamoto et al. 2003). In ubiquitin-proteasome system, ubiquitin-conjugating enzyme (E2) together with ubiquitin ligase (E3) transfers ubiquitin to the specific substrate protein (Hershko and Ciechanover 1998; Joazeiro and Weissman 2000; Burger and Seth 2004). The ubiquitin-proteasome system plays a pivotal role in protein homeostasis and is critical in regulating the normal and cancer-related cellular processes. UbcH10 is not only one important member of ubiquitin-proteasome system, but also one of the key regulators of cell-cycle progression. Current studies have suggested the critical role of UbcH10 in the spindle assembly checkpoint and the subsequent accurate separation of sister chromatids (Reddy et al. 2007; Rape and Kirschner 2004), which is orchestrated by a series of molecular interactions governed by the complex and diverse cell-cycle machinery (Yu et al. 1996; Townsley et al. 1997). Recently, aberrantly high UbcH10 expression has been demonstrated in a variety of malignancies, such as thyroid carcinoma,

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astrocytic tumors, breast cancer and lymphomas (Lee et al. 2008; Jiang et al. 2008; Chen et al. 2006; Berlingieri et al. 2007; Troncone et al. 2009; Fujita et al. 2009). However, its role in colorectal cancer carcinogenesis and tumor progression has not been well defined.

Colorectal cancer is a one of the most common malignancies in developed countries and the second-leading cause of cancer-related deaths in Europe (Boyle and Ferlay 2005). Symptoms of colorectal cancer are often non-specific and occur late in the course of the disease (Grabon et al. 2009). Patients with colorectal cancer are at a high risk for metastases. Therefore, the searching of highly sensitive and specific markers has been an area of vigorous studies.

In the present study, we examined the expression levels of UbcH10 in human malignant colorectal carcinoma tissues and their adjacent normal tissues in 45 patients with malignant colorectal carcinoma by real-time quantitative RT-PCR and immunohistochemical analysis. We constructed the UbcH10 expression plasmid pcDNA3.1-UbcH10 and UbcH10 RNA interference vector pUbcH10-RNAi, then transfected them to human colon cancer cell line HT-29 as well as their control plasmids, respectively. And then, we evaluated the properties of tumor cell growth and invasiveness by cell proliferation and Matrigel invasion assays. All the studies were conducted to better understand the association of UbcH10 with colorectal cancer carcinogenesis, and to look for a possible prognostic marker in these neoplasias.

Materials and methods

Tissue specimens

Tumor tissues and their adjacent normal tissues (the distance from normal tissue to tumor was beyond 5 cm) used in this study were obtained from surgical specimens and verified by histological examination. All the tissue samples were collected with the informed consent of all patients and approval of the ethics committee of General Hospital of Jinan Military Region. Tumors ranged from well differentiated to poorly differentiated, and included various histotypes of colorectal cancer. Most of colorectal cancers are of adenocarcinoma type.

Real-time RT-PCR analysis of UbcH10 mRNA in tissues

Total RNA was isolated using the Trizol reagent (Invitrogen, USA) according to the manufacturer's recommendations. A 5 µg of total RNA from each sample was incubated

with random primers and Superscript II reverse transcriptase (Invitrogen, USA) to yield cDNA. Real-time RT-PCR was carried out on cDNA using SYBR[®] Premix Ex Taq[™] (TaKaRa, Japan). The human GAPDH was used as an endogenous control. PCR was performed using the following program: 95°C for 5 min; and 40 cycles of 95°C for 10 s and 60°C for 20 s. Specific primers are as follows: (1) for UbcH10, 5'-TGATGTCTGGCGATAAAGGG-3' (forward primer), 5'-TGATAGCAGGGCGTGAGGAA-3' (reverse primer); (2) for GAPDH, 5'-GAAGGTGAAGGTCGGAGTC-3' (forward primer), 5'-GAAGATGGTGA TGGGATTTTC-3' (reverse primer). All the primers were designed with Primer Premier 5.0 software. The relative expression level of UbcH10 gene was calculated by comparing the threshold cycle (Ct) values of sample to that of the reference.

Immunohistochemistry analysis of UbcH10 protein in tissues

Cellular distribution of UbcH10 protein was assessed by immunohistochemical analysis (streptavidin-peroxidase method, viz. S-P method). Slides of deparaffinized tissue sections were placed in citrate buffer and treated with microwave heat for 20 min. UbcH10 primary monoclonal antibody [(M01), clone 9D3; Santa Cruz, Abnova] with 1:300 dilution were added afterwards and the sections were incubated overnight at 4°C. After washing in PBS, the sections were incubated with biotin-conjugated secondary antibody for 10 min at 37°C, then with peroxidase-conjugated biotin-streptavidin complex for 10 min, and finally visualized with 3, 3'-diaminobenzidin and counterstained with hematoxylin. The staining intensity was graded as follows: -, <5% positive cells; +, 5–25% positive cells; ++, 25–50% positive cells; and +++, >50% positive cells.

Construction of UbcH10 expression plasmid

To obtain full-length human UbcH10 cDNA, total RNA prepared from colorectal cancer patients tumor tissue was reversely transcribed using oligo(dT) and Superscript II reverse transcriptase (Invitrogen, USA). The subsequent PCR was performed with the following oligonucleotide primers: UbcH10 sense, 5'-GGAATTCAATGCTTCCCAAAACCGCG-3'; and UbcH10 antisense, 5'-CCCAAGCTTATCAGGGCTCCTGGCTGGT-3'. These sense and antisense oligonucleotide primers were synthesized based on the nucleotide sequence databases (GenBank and European Molecular Biology Laboratory) and contained an engineered *EcoRI* and *HindIII* restriction site (underlined), respectively. All the primers were designed with Primer Premier 5.0 software. The specific amplified fragment was

gel purified (QIAGEN QIAquick Spin), digested completely with *EcoRI* and *HindIII*, and subcloned into the identical restriction sites of the pcDNA3.1 expression plasmid to construct pcDNA3.1-UbcH10. The resultant expression plasmid was sequenced to confirm the UbcH10.

Construction of UbcH10 RNAi vector

For RNA interference (RNAi) experiments, the following double-stranded RNA oligos specific for UbcH10 coding region were used: 5'-AACCTGCAAGAAACCTACTCA-3' as Wagner KW described (Wagner et al. 2004). As negative control, we used a corresponding scrambled sequence as follows: 5'-AACTAACACTAGCTCAAGA CC-3'. The expression plasmids pGPU6/GFP/Neo/UbcH10-RNAi (pUbcH10-RNAi) and negative control plasmid pGPU6/GFP/Neo/RNAi-NC (pRNAi-NC) were constructed by GenePharma Company (Shanghai, China).

Cell culture and transfection

Human colon cancer cell line HT-29 obtained from the Chinese Academy of Sciences, were maintained in RPMI 1640 medium supplemented with 10%(v/v) FBS (Gibco, USA). One day before transfection, equal numbers of HT-29 cells (5.0×10^4 per ml) were seeded in 12-well tissue culture plates supplemented with 1640 media containing 10% FBS. When cells reached ~80% confluency, they were transfected with pcDNA3.1-UbcH10, pUbcH10-RNAi and control plasmids by LipofectaminTM 2000 (Invitrogen), respectively. The cells were allowed to incubate at 37°C in a humidified atmosphere of 5% CO₂ for 5 h followed by the replacement of RPMI 1640 containing 10% FBS. The successfully transfected cells could express enhanced green fluorescent protein (GFP) which were verified by fluorescent microscopy.

Western blot analysis of UbcH10 protein

Proteins were extracted 3 days after treatment. Cells were washed with ice-cold PBS twice, and lysed by lysis buffer supplemented with protease inhibitor cocktail. Protein concentrations were determined with the BCA Protein Assay Reagent Kit (Beyotime, CA, USA). A total of 40 µg of protein was resolved in 12.5% sodium dodecyl sulfate-polyacrylamide gel, and then transferred to NC membranes (Millipore, Bedford, USA). Immunoblots were performed with primary antibodies against UbcH10 [(M01), clone 9D3 1:800 dilution; Santa Cruz, Abnova] and β-actin (Chemicon International 1:5,000 dilution; Temecula, CA, USA) followed by horseradish peroxidase-conjugated anti-mouse or anti-rabbit secondary antibodies (Chemicon

1:10,000 dilution; Temecula, CA). Signals were visualized using enhanced chemiluminescence detection reagent from Millipore, and captured by the ECL Western blotting detection system (Bedford, MA, USA).

Cell proliferation assay

To observe the effect of the UbcH10 gene expression on cell proliferation in vitro, Cell Counting Kit-8 (Beyotime, CA) was employed to draw cell growth curves. HT-29 cells were plated onto 96-well cell culture plates at a density of 4×10^3 cells/well in 100 µl of culture medium containing 10% FCS and grown overnight. On the next day, the cells were transfected with pcDNA3.1-UbcH10, pcDNA3.1, pUbcH10-RNAi and pRNAi-NC, respectively. All experiments were performed in quintuplicate. At 0, 24, 48, 72 and 96 h, cell proliferation was measured by absorbance at 450 nm.

Matrigel invasion assay

Cell invasion capability was assessed using a Matrigel invasion chamber (Becton-Dickenson, Bedford, USA) according to the manufacturer's instructions. Matrigel was diluted to 1 mg/ml in serum-free RPMI 1640 medium; 60 µl of 1 mg/ml Matrigel was placed into each insert (8.0 µm pore size, Costar), in a 24-well plate (Costar). The inserts were incubated at 37°C for 1 h to allow gel polymerization. Cells were harvested and washed with the appropriate serum-free medium, then 100 µl of the cell suspension containing 5×10^4 cells was added to each insert and 500 µl of appropriate medium containing 15% FCS was added to the well underneath the insert. Cells were incubated at 37°C for 24 h. After this time, the inner side of the insert was wiped with a wet swab to remove the cells while the outer side of the insert was gently rinsed with PBS and stained with 0.25% crystal violet for 10 min, rinsed again and then allowed to dry. The inserts were then viewed under the microscope and the numbers of cells per field in five random fields were counted at 200× magnification.

Statistical analysis

The SPSS 10.0 software was applied to complete data processing. Independent-sample *t* test was used to evaluate the differences of numbers of migrated cells between groups with various treatments. All data were represented as mean ± SD of three independent experiments. The χ^2 test, Fisher's exact test and Spearman correlation test were used for statistical analysis of

clinicopathological data. The results were considered statistically significant when the P value was <0.05 .

Results

UbcH10 mRNA is overexpressed in colorectal cancerous tissues

We performed real-time PCR (RT-PCR) to measure the levels of UbcH10 gene expression in 45 colorectal cancerous tissues and their adjacent non-cancerous tissues. Our results showed that UbcH10 mRNA expression was significantly increased in all of the colorectal carcinoma tissues we examined when compared with their adjacent non-cancerous tissues (Fig. 1), and the average levels of gene expression in colorectal cancerous tissues were more than ten times of their corresponding normal tissues (0.0802 ± 0.0907 vs. 0.0079 ± 0.0088 , $P < 0.01$). The overexpression of UbcH10 (>2 -fold) was observed in 41/45 cases, 17 of which had >20 -fold increases in expression.

UbcH10 protein is expressed at high level in colorectal cancerous tissues

To evaluate whether the overexpression of UbcH10 is a feature of the colorectal cancerous tissues, we performed immunohistochemical analysis using a commercial antibody against UbcH10 protein (Fig. 2). The results showed that normal colorectal tissues were almost completely negative for UbcH10 expression. Only occasionally, single UbcH10-labeled cells could be observed. A higher UbcH10 level was detected in colorectal cancerous tissues

compared with non-malignant normal tissues. Moreover, the UbcH10 expression in the local invasive cells increased obviously (Fig. 2e).

Clinicopathological significance of UbcH10 expression in colorectal cancer patients

To determine the relationship of UbcH10 expression in colorectal cancer to clinical or pathological characteristics, we performed a clinicopathological analysis of UbcH10 in these 45 colorectal cancer patients (Table 1). There were no significant differences in the patients' sex ($P = 0.554$), patients' age ($P = 0.768$) and tumor size ($P = 0.547$) between the UbcH10 high expression (+++) and UbcH10-over expression (+++) cancers. However, statistically significant differences were observed in lymph node metastasis ($P = 0.037$) and degree of tumor differentiation ($P = 0.047$) between UbcH10 high expression and UbcH10 over expression in colorectal cancer cancers.

UbcH10 overexpressed in HT-29/UbcH10, but downregulated in HT-29/UbcH10-RNAi

To confirm that HT-29 was transfected with pcDNA3.1-UbcH10 (HT-29/pcDNA3.1-UbcH10) or pUbcH10-RNAi (HT-29/UbcH10-RNAi), the fluorescence was detected to evaluate the transfection rate and UbcH10 expression was measured by western blot at 72 h after transfection. Our results showed that the transfection was in high efficiency (Fig. 3a) and UbcH10 expression in HT-29/pcDNA3.1-UbcH10 increased obviously, but in HT-29/UbcH10-RNAi reduced obviously (Fig. 3b).

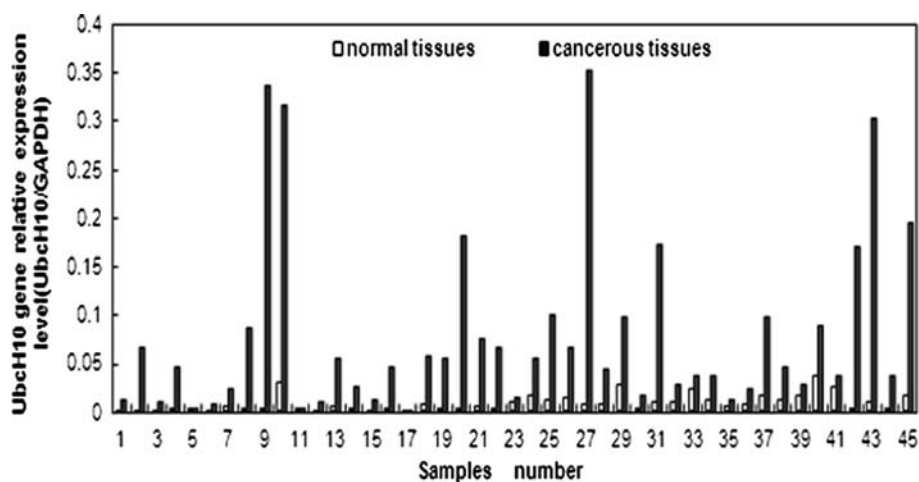


Fig. 1 Real-time RT-PCR analysis of UbcH10 mRNA in colorectal cancerous tissues and their adjacent normal tissues. UbcH10 mRNA relative expression was calculated according to the formula $2^{(CT_U - CT_R)}$, where CT_R , CT_U is the threshold cycle number for

the GAPDH gene and the UbcH10 gene in the sample, respectively. The average levels of gene expression in colorectal cancerous tissues were more than ten times of their corresponding normal tissues (0.0802 ± 0.0907 vs. 0.0079 ± 0.0088 , $P < 0.01$)

Fig. 2 The immunohistochemical analysis of UbcH10 protein ($\times 100$, UbcH10-positive staining was *brown* mostly located nucleolus). **a** Normal colorectal tissues, **b** well-differentiated colorectal cancerous tissues (Sample 35), **c** mid-differentiated colorectal cancerous tissues (Sample 8), **d** poor differentiated colorectal cancerous tissues (Sample 43), **e** local metastasis tissues (Sample 37)

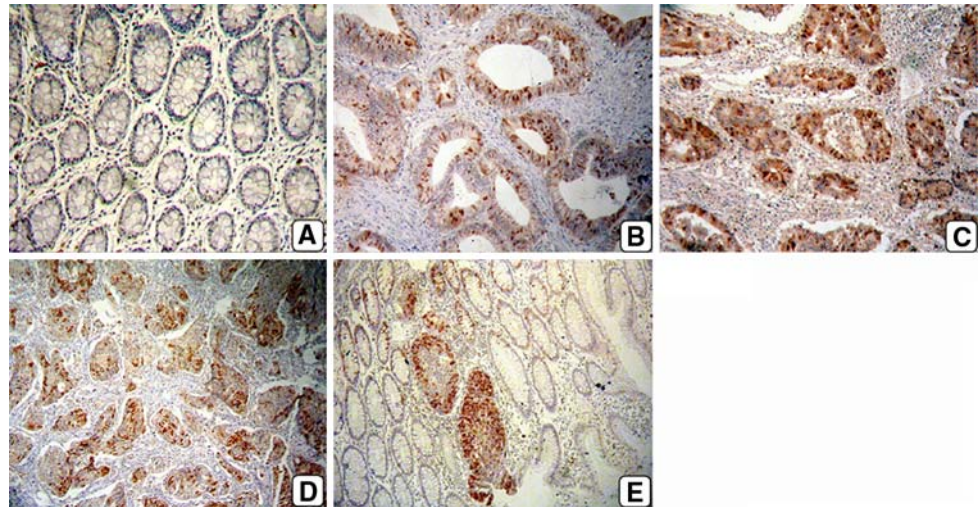


Table 1 Correlations of the UbcH10 expression to clinicopathologic features of colorectal cancer patients

Item	Cases	Expression of UbcH10 [case (%)]		P
		(+--+)	(+++)	
Sex				
Male	25	14 (56.0)	11 (44.0)	NS ^a (0.554)
Female	20	9 (45.0)	11 (55.0)	
Age (years)				
≤60	23	11 (47.8)	12 (52.2)	NS ^a (0.768)
>60	22	12 (54.5)	10 (45.5)	
Tumor size				
≤5 cm	24	11 (45.8)	13 (54.2)	NS ^a (0.547)
>5 cm	21	12 (57.1)	9 (42.9)	
Lymph node metastasis				
N-	27	18 (66.7)	9 (33.3)	0.037 ^b
N+	18	6 (33.3)	12 (66.7)	
Degree of differentiation				
Well differentiated	8	7 (87.5)	1 (12.5)	0.047 ^b
Mid differentiated	27	13 (48.1)	14 (51.9)	
Poorly differentiated	10	3 (30.0)	7 (70.0)	

^a NS not significant at 95% confidence level

^b Significantly different at 95% confidence level

Overexpression of UbcH10 promotes colorectal cancer cell growth and down-regulation of UbcH10 decreases colorectal cancer cell growth

To further investigate a possible contribution of UbcH10 expression to tumor cell growth, we performed cell proliferation assays in HT-29 cells transfected with pcDNA3.1-UbcH10, pcDNA3.1, pUbcH10-RNAi and pRNAi-NC, respectively. The results showed that the overexpression of UbcH10 promoted colorectal cancer cell

growth (Fig. 4a), but the inhibition of UbcH10 expression blocked colorectal cancer cell growth (Fig. 4b).

Overexpression of UbcH10 increases cells invasion and down-regulation of UbcH10 decreases cells invasion

From Fig. 2e, we found that UbcH10 in the local metastasis tumor expressed obviously. Therefore, we performed the Matrigel assay to evaluate the association of UbcH10 expression with tumor invasiveness in vitro. HT-29 cells (5×10^4 cells per transwell insert) were allowed to invade the Matrigel-coated membrane. The numbers of invading cells were represented as the average of five randomly selected microscopic fields on the underside of the membrane (Fig. 5). As shown in Fig. 5b, in the pUbcH10-RNAi condition only 32 ± 3 cells passed through the membrane when compared with 62 ± 9 cells in the pRNAi-NC condition ($P < 0.01$) and 120 ± 10 cells in the pcDNA3.1-UbcH10 condition passed through the membrane compared with 66 ± 7 cells in the pcDNA3.1 condition and 71 ± 9 cells in the PBS condition passed through the filter ($P < 0.01$). These results clearly demonstrated that the overexpression of the UbcH10 significantly increased cells invasiveness in vitro. In contrast, the inhibition of UbcH10 expression by RNAi significantly decreased cells invasiveness in vitro.

Discussion

Cancer is a complex disease that involves the accumulation of both genetic and epigenetic alterations of numerous genes. It is now generally accepted that colorectal cancer develops by genetic alterations. Analysis of the molecular mechanism makes it possible to develop a

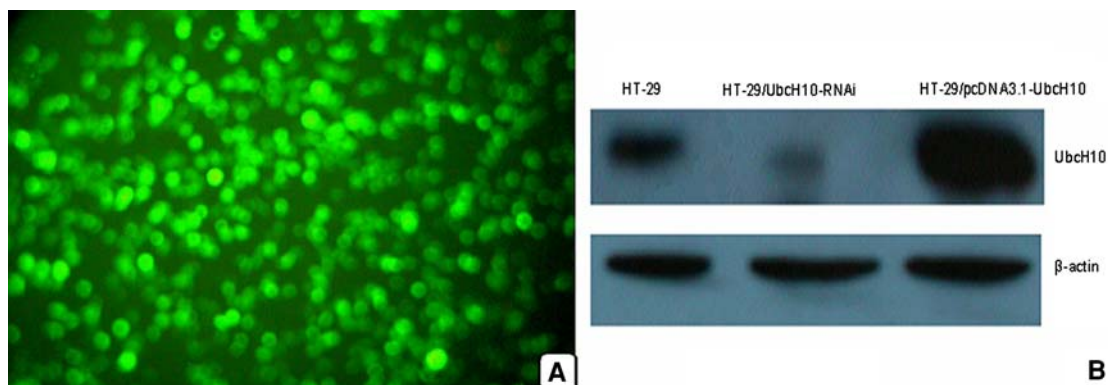
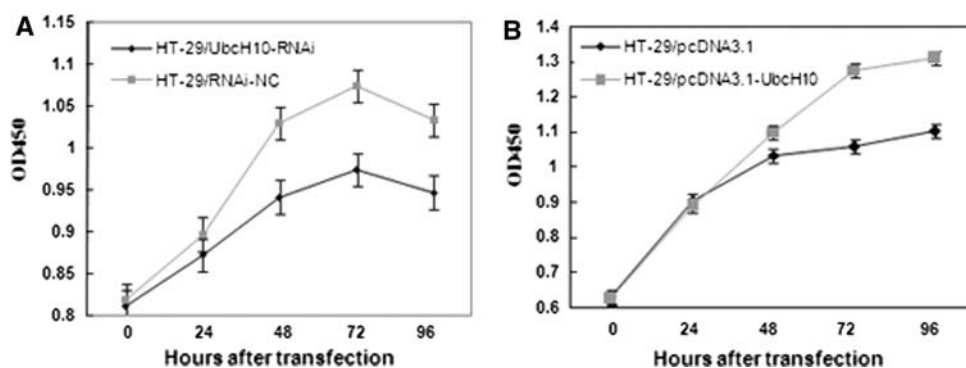


Fig. 3 UbcH10 expression in HT-29 cells transfected with pcDNA3.1-UbcH10 and pUbCh10-RNAi. **a** HT-29 cells transfected with pUbCh10-RNAi, **b** Western blot analysis of UbcH10 in HT-29

cells control, HT-29 cells transfected with pcDNA3.1-UbcH10 (HT-29/pcDNA3.1-UbcH10) and HT-29 cells transfected with pUbCh10-RNAi (HT-29/pUbCh10-RNAi)

Fig. 4 Effect of UbcH10 expression on the growth of HT-29 cells. **a** HT-29 cells transfected with pcDNA3.1-UbcH10 and pcDNA3.1, respectively, **b** HT-29 cells transfected with pUbCh10-RNAi and pRNAi-NC, respectively



more targeted approach to the prevention and treatment of this cancer.

UbcH10 was previously identified as a human homolog of the cyclin-selective E2 (E2-c), which belongs to the E2 gene family and codes for a protein of 19.6 kDa that is involved in the ubiquitin-dependent proteolysis (Aristarkhov et al. 1996; Lin et al. 2002). It has been shown that UbcH10 is required for APC-dependent ubiquitination of mitotic cyclins (de Gramont et al. 2006; Bastians et al. 1999), and dominant-negative UbcH10 blocks the ubiquitination as well as the destruction of mitotic cyclins and causes cells to accumulate in mitosis (Townsend et al. 1997). It has also reported that UbcH10 has an unexpected importance in both the G1/S transition and the initiation of anaphase (de Gramont et al. 2006). From the viewpoint of cell-cycle regulation, UbcH10 also can be taken as the focal point in cancer diagnosis and therapy.

In the present study, we evaluated the expression level of the UbcH10 in 45 colorectal cancer patients. We found that the UbcH10 overexpressed in all of the colorectal carcinoma tissues compared with their adjacent normal tissues at mRNA level and protein level. Failure of proliferation control or increase in cell replicative potential is one of the key steps in carcinogenesis. Cell

proliferation assays in HT-29 cells transfected with pcDNA3.1-UbcH10 and pUbCh10-RNAi suggest that the expression of UbcH10 can affect human colon cancer cell proliferation. The results verify that the overexpression of UbcH10 accelerates the carcinogenesis in colorectal cancer and the inhibition of UbcH10 expression may be a promising approach to innovative anticancer therapies.

Although localized tumor growth may cause significant organ dysfunction and even death, metastases cause most human cancer deaths (Hanahan and Weinberg 2000). The ability to metastasize is linked with the ability of cancer cells to invade adjacent tissues, gain access to vascular or lymphatic channels, and survive transit through the bloodstream so that they may extravasate, then reside, and finally colonize heterologous organs or tissues. Cancer cells acquire the capacity of invasion and metastasis through regulated processes related to tissue development and homeostasis. The invasion of lymphatic vessels and its subsequent spread to draining lymph nodes is a key determinant of prognosis in this common and frequently fatal malignancy (Royston and Jackson 2009). At present, histopathology examination is the “golden standard” of diagnosis in lymph node metastasis. However, it can be

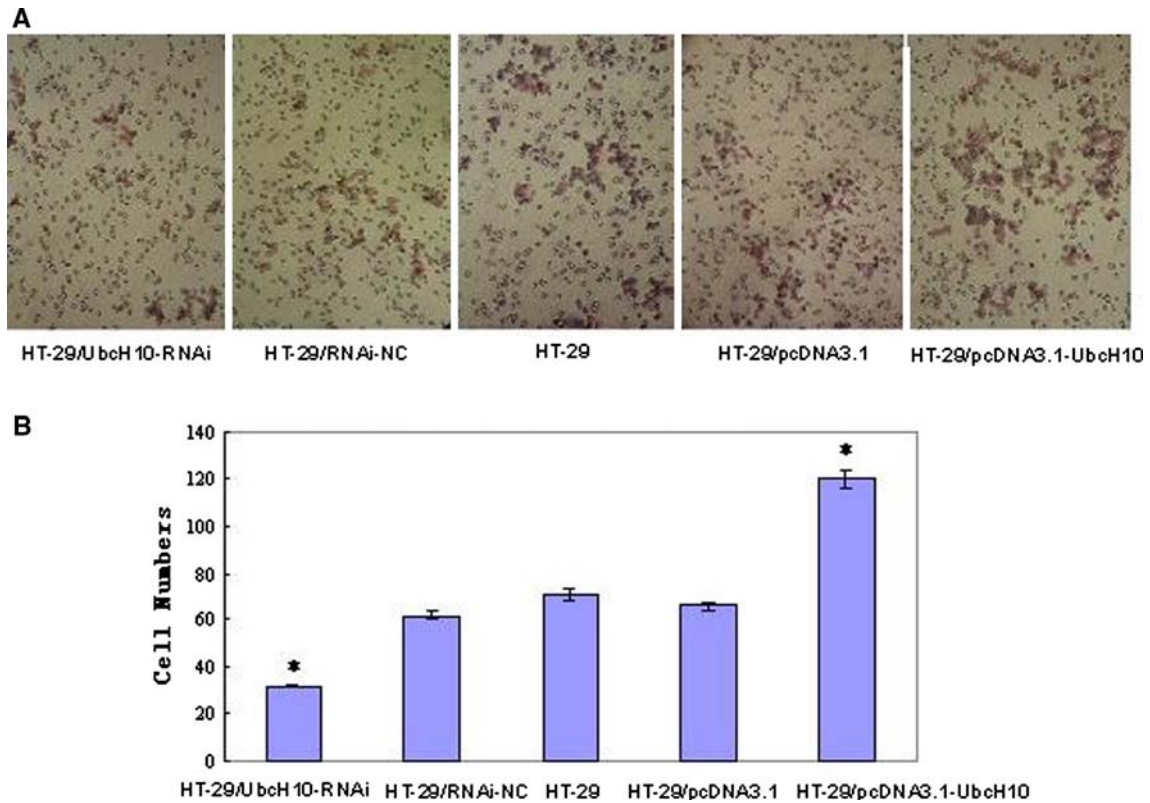


Fig. 5 Effect of UbcH10 expression on tumor invasiveness. **a** HT-29 cells were transfected for 72 h and then allowed to invade transwell inserts (8 μ m pores) coated with Matrigel for 24 h. The cells that invaded through the inserts were stained, counted and photographed

under light microscopy at $\times 200$ magnification, **b** the numbers of cells that invaded through the Matrigel-coated inserts. The data are presented as the mean \pm SD for three separate experiments from each group

affected by some factors, such as the scope of lymph clearing and the objective existence of tiny metastasis. Identification of the key regulators of these processes may provide valuable prognostic information to patients with colorectal cancer as well as opportunities for enhanced intervention. The results of the clinicopathological analysis indicate that the expression of UbcH10 in lymph node metastasis patients is markedly higher than that in no lymph node metastasis ($P < 0.05$) and UbcH10 appears to promote metastasis (Fig. 2e). These data suggest that UbcH10 expression associates with tumor progression in colorectal cancer. Subsequently, in vitro Matrigel invasion assays show that UbcH10 overexpression promotes cellular aggressive behavior and UbcH10 downregulation decreases cellular invasiveness. These findings raise the exciting prospect of future biomarkers of lymphatic metastasis and tumor progression in colorectal cancer and identify potential targets for new generation anti-tumor therapies.

In addition, the results of the clinicopathological analysis indicate that UbcH10 abundance is correlated with the degree of tumor differentiation. Colorectal cancer patients

in poor differentiation show higher levels of UbcH10 expression. However, poor differentiation means a potentially poor prognosis in colorectal cancer patients. Therefore, these results also verify that UbcH10 overexpression involves tumor progression in colorectal cancer. In this regard, our results are consistent with the results of recently reported literature (Fujita et al. 2009). The difference between our studies on colorectal cancer and this recent literature is in the research direction about the effect of UbcH10 overexpression on tumor progression. Fujita et al. verified UbcH10 overexpression accelerated tumor progression from the effect of UbcH10 on cell cycle; in our study, we confirmed the result from the effect of UbcH10 on cell invasiveness.

Taken together, UbcH10 has a substantial function in promoting colorectal cancer carcinogenesis and tumor progression. It could be a potential diagnostic and prognostic marker in patients with colorectal cancer. At the same time, our studies also provide the prospects of a therapy to colorectal cancer based on the suppression of the UbcH10 synthesis and/or function, although many details must be elucidated in its antitumor effect.

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Conflict of interest statement The authors declare that they have no competing interests.

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