ISSN 1672-9145 **Acta Biochimica et Biophysica Sinica** 2006, **38**(12): 883–892 **CN** 31-1940/Q

Effect of DPC4 Gene on Invasion and Metastasis of Colorectal Carcinoma Cells

De-Sheng XIAO, Ji-Fang WEN*, Jing-He LI, Kuan-Song WANG, Zhong-Liang HU, Jian-Hua ZHOU, Zheng-Hao DENG, and Ying LIU

Department of Pathology, Xiangya School of Medicine, Central South University, Changsha 410078, China

Abstract To investigate the effect of DPC4 gene on invasion and metastasis of colorectal carcinoma cells, the expression of DPC4 was detected in sixty-three samples of colorectal tumors and seven cases of colorectal mucosa. The biological behavior of tumors expressing DPC4 was evaluated (including tumor staging, differentiation degree and metastasis). pcDNA3.1-DPC4 plasmid was constructed and transferred into HCT116 cells not expressing DPC4. The cell models (DPC4$^+$-HCT116) steadily expressing DPC4 were obtained. Compared with HCT116 and pcDNA3.1-HCT116 cells, the doubling time of DPC4$^+$-HCT116 cells was lengthened obviously (P<0.01), the apoptosis rate of DPC4$^+$-HCT116 cells was significantly increased (P<0.01), the cloning efficiency, cell adherency, migration and invasion ability of DPC4$^+$-HCT116 cells were dropped obviously (P<0.01). The number of cancer nodules was decreased significantly in abdominal cavity and liver of the nude mice inoculated with DPC4$^+$-HCT116 cells. The activity of MMP-9 and MMP-2 was detected by gelatin zymography. In comparison with HCT116 and pcDNA3.1-HCT116 cells, the activity of MMP-9 was decreased in DPC4$^+$-HCT116 cells. Therefore, the down-regulation of DPC4 expression may be associated with the carcinogenesis of colorectal carcinoma. DPC4 may inhibit the proliferation of colon cancer cell by restraining growth and inducing apoptosis, and the invasion and metastasis of colorectal carcinoma cells. MMP-9 may be one of the downstream target genes regulated by DPC4.

Key words DPC4; colorectal carcinoma; invasion; metastasis; MMP9

A high frequency of LOH (loss of heterzyousity) on chromosome 18q has been recognized in the progression of carcinomas of colon and other tissues, including pancreas [1,2]. Since DPC4 gene was isolated from the same region as a tumor suppressor gene for pancreatic cancer, mutation analysis of this gene has been carried out on cancers in various other organs. DPC4 plays an important role in TGF-β signal transduction pathway, and was found to be functionally inactivated in about 50% of pancreatic carcinomas [3–6], 30% of colon carcinomas and a subset of biliary tract carcinomas [7,8]. DPC4 was thought as a tumor suppressor gene [9]. Despite the extensive research of DPC4, we still knew little of how the loss of DPC4 function contributes to the tumorigenic process. To determine the effect of DPC4 on the invasion and metastasis of colorectal carcinoma cells, expression of DPC4 was investigated in seventy cases including colon normal mucosa and tumor tissue, pcDNA3.1-DPC4 plasmid was re-constructed, and transferred into HCT116 cells, and a cell model steadily expressing DPC4 was obtained. On the base of experiment platform, the effect of DPC4 on cell proliferation, invasion and metastasis were investigated.

Materials and Methods

Materials

Sixty-three cases of surgically resected primary colorectal tumors were collected over the past five years from Department of Pathology of Xiangya Hospital (Changsha, China) and seven cases of normal colorectal tissues were obtained from the same patients as well with permission. Clinical and pathological data were obtained

Accepted: June 9, 2006 Received: September 6, 2006

*Corresponding author: Tel, 86-731-2650400; Fax, 86-731-2650408; E-mail, Jifangwen@hotmail.com

DOI: 10.1111/j.1745-7270.2006.00233.x

from the patients' medical records, including age, sex, grade, and lymph nodal or distant metastases. All specimens were fixed and imbedded by 40 g/L formalin and paraffin respectively. The groups include: normal colon mucosa (n=7), adenoma (n=11), well-differentiated adenocarcinoma (n=17), poorly differentiated adenocarcinoma (n=35); Dukes A, B stage (n=38), C, D stage (n=14), 14 cases of the total had presence of lymph nodal or liver metastases. Sections were sliced about 5 μm thickness consecutively.

NIH3T3, HCT116, HT29, LS174T, SW480 and SW620 cell lines were purchased from the Cell Bank of Type Culture Collection of Chinese Academy of Sciences, Shanghai Institute for Biological Sciences, Chinese Academy of Sciences (Shanghai, China). Nine nude mice were purchased from Shanghai experimental animal center of Chinese Academy of Sciences (Shanghai, China).

Immunohistochemistry

Sections of 5 μm were cut from the paraffin block selected for each case and deparaffinized by routine techniques [10]. Sections were washed with PBS for 5 min. Endogenous peroxidase was blocked with 3% H_2O_2. Sections were steamed for 20 min at 80 ºC, then were cooled for 5 min and incubated in a solution containing 10% normal goat serum before incubating with a 1:50 dilution of monoclonal antibody against DPC4 protein using the automated stainer. Finally, anti-DPC4 antibody was detected by adding biotin-conjugated goat anti-mouse IgG followed by streptavidin peroxidase and DAB coloring reagent. Sections were counterstained with hematoxylin. Based on positive cell population and staining intensity [11, 12], four grades are distinguished according to positive cell population and staining intensity. Briefly, negative, weakly positive, positive, strongly positive were scored as 0, 1, 2, 3; and positive cell populations 25%, 25%–50%, 50%–75%, >75% were scored as 0, 1, 2, 3 respectively. Then the total score of a visual field was obtained by the combination of these two facts. Ten high power visual fields were selected randomly and scored, and their average scores were regarded as the staining results of each slice. Normal pancreas tissue and substitute PBS solution were used for positive control and negative control respectively.

Plasmid construction

pCMV5-DPC4 plasmid was a kind gift from Prof. Joan MASSAGUE (Howard Hughes Medical Institute, Memorial Sloan-Kettering Cancer Center, New York, USA); pcDNA3.1(+) plasmid and *Escherichia coli* (JM109) were stored in our department.

Primers aiming directly at DPC4 gene fragment were designed, and synthesized by Shanghai BoYa company (Shanghai, China): forward, 5′-TCCCTGGCCCAGGATCAGTA-3′; reverse, 5′-CTGATAAGGTTAAGGGCCCCAAC-3′.

Restriction enzyme *Hin*dIII, *Bam*HI, RPMI 1640 medium, 100 bp DNA marker, neomycin (G418), Lipofectamin and bovine serum albumin (BSA) were obtained from Gibco (Carlsbad, USA); fibronectin (FN) and artificial basal membrane (Matrigel) was purchased from Collaborative Res company (Essex, USA); MilliCell was from Millipore (Bedford, USA); mouse anti-DPC4 monoclonal antibody Ab-1 (primary antibody) was from Neomarkers (Fremont, USA); biotin-conjugated goat anti-mouse IgG (secondary antibody), streptavidin peroxidase, SP kit and DAB coloring reagent were from Beijing Zhongshan Company (Beijing, China).

Plasmids pCMV5-DPC4 and pcDNA3.1 were transferred into competent *E. coli* (JM109) respectively. Transferred *E. coli* (JM109) was cultured to amplify these two plasmids. Plasmids were digested with *Hin*dIII and *Bam*HI, and identified by routine agarose gel electrophoresis. The DPC4 sequence was massively cut, extracted, purified, and cloned into pcDNA3.1 plasmid [13]. The construction was confirmed by agarose gel electrophoresis and direct sequencing. Empty vector pcDNA3.1 was used for control transfection.

Cell culture and screening

Human colon carcinoma cell lines HCT116, HT29, LS174T, SW480 and SW620 were maintained in RPMI 1640 medium supplemented with 10% fetal calf serum in a CO_2 incubator (5%CO_2 and 95% air) at 37 ºC. On one hand, the expression of the DPC4 was analyzed by Western blot of lysates. Briefly, cells were harvested with a cell scraper and were lysed in lysis buffer (20 mM Tris-HCl, pH 7.4, 150 mM NaCl, 0.5% Triton X-100, 1 mM EDTA) containing a proteinase inhibitor cocktail. Proteins were resolved by sodium dodecyl sulfate-10% polyacrylamide gel electrophoresis and transferred to immobile membranes. DPC4 was detected using a primary anti-DPC4 monoclonal antibody with a 1:100 dilution, followed by exposure to peroxidase-conjugated secondary antibody and developed using the enhanced chemoluminescent detection system. On the other hand, DPC4 mRNA was isolated from monolayer cells. Reverse transcription from 100 ng of mRNA was performed by using 18-mer oligo(dT) and Superscript II transcriptase (Fermentas, Vilnius, Lithuania). The transcript and PCR primer set used for amplification

was: forward, 5′-TCCCTGGCCCAGGATCAGTA-3′; reverse, 5′-CTGATAAGGTTAAGGGCCCCAAC-3′[14].

Gene transfer

HCT116 cells were maintained in RPMI 1640 medium supplemented with antibiotics and 10% fetal calf serum. Cells were transfected with pcDNA3.1-DPC4 and pcDNA3.1 plasmids respectively by a standard lipofectamine reagent kit method [15]. Positive clones were obtained after 3 weeks of cultivation in medium containing 0.8 g/L G418 [14]. Pools of geneticin resistant clones were passaged, or single colony was isolated with cloning cylinders and expanded for Western blot analysis and immunohistochemistry.

Growth inhibition studies

The standard cell proliferation assay was performed as described previously. Briefly, the cell growth inhibition was determined by thiazolyl blue (MTT) dye assay. A total of 2×10^4 cells per well was seeded in 96-well plates for one, two, till seven days respectively. Forty microliters of Thiazolyl Blue solution (2 mg/L) were added to each well, and plates were incubated at 37 ºC for 4 h. Wells were aspirated, and 100 μl of DMSO per well were added. Plates were read at 570 nm in a spectrophotometer.

Clone formation experiment

For analysis of cloning efficiency, 100 cells were seeded in 6-well plates respectively. When cell clones appeared, they were discontinued cultivating, washed twice with PBS solution, fixed with 2 ml of pure methanol for 15 min and stained with Giemsa for 20 min. The result was evaluated. The experiments were repeated three times.

Flow cytometry analysis

1×10^6 cells were digested with 2.5 g/L pancreatic enzyme, washed with D-hanks solution, and centrifuged at 1500 rpm. The cell sediment was washed by PBS solution, centrifuged twice and fixed with 700 mg/L ethanol. Percentage of cell cycle and apoptosis rate of HCT116, pcDNA3.1-HCT116 and DPC4$^+$-HCT116 cells were determined by flow cytometry [16,17].

Evaluation of adherency ability

Fifty milligrams per liter Matrigel solution diluted with asepsis double distilled water (1:8) were prepared, added in 96-well plates (50 μl per well) and incubated for 12 h at 4 ºC. Ten grames per liter BSA were used for control. After abandoning remnant liquid, no-serum culture solution containing 10 g/L BSA (50 μl per well) was added and incubated at 37 ºC for 30 min. Tumor cells were digested with 2.5 g/L pancreatic enzyme and modulated to 1×10^5/ml of density. One hundred microliters of cell suspension were seeded in invested Matrigel per well. Every group had four samples at equal pace. The cells were cultivated with RPMI 1640 medium containing 10 g/L BSA and 10% fetal bovine serum for 1 h at 37 ºC, then the absorbance (*A*) was decided by MTT colorimetric method. Cell adhesion rate of Matrigel group was calculated by the formula as **Equation 1**.

$$\text{Adhesion rate}=[(A_{exp}/A_{BSA})-1]\times100\% \quad (1)$$

Evaluation of migration ability of transfected cells

NIH3T3 cells were cultured in RPMI 1640 containing 10% fetal bovine serum. The cells spread in culture flasks were washed twice and cultured for 24 h with no-serum culture solution, then centrifuged. The supernatant was collected as tumor cell chemotactic factor and added into MilliCell outer compartment. One hundred microliters of suspended tumor cells containing 1×10^5 HCT116, pcDNA3.1-HCT116 and DPC4$^+$-HCT116 cells were seeded into MilliCell internal compartment respectively. Then the MilliCells were put into 24-well plates and kept for 8 h. Then the MilliCells were taken out, washed with PBS, fixed by 3% (*V*/*V*) glutaraldehyde and stained with Giemsa. The upper layer cells of microporous membrane were erased carefully with tampon. The subnatant cells of microporous membrane were counted on inverted microscope.

Evaluation of invasion ability

The bottom of MilliCell was invested with 50 mg/L Matrigel solutions diluted with asepsis double distilled water (1:8) and air-dried (4 ºC). After remnant liquid was suctioned, no-serum culture solution containing 10 g/L BSA was added and kept at 37 ºC for 30 min. Four hundred microliters of culture fluid and the supernatant containing tumor cell chemotactic factor (1:1) were added into MilliCell outer compartment. One hundred microliters of cultures containing 1×10^5 HCT116, pcDNA3.1-HCT116 and DPC4$^+$-HCT116 cells were seeded into MilliCell internal compartment respectively. MilliCells were put into 24-well plates. The cells were cultivated in RPMI 1640 medium containing 10 g/L BSA and 1% fetal bovine serum for 48 h at 37 ºC, and every group has four samples at equal pace. Then MilliCells were taken out, washed with PBS, fixed by 3% (*V*/*V*) glutaraldehyde for 15 min, stained with Giemsa for 15 min. The upper layer cells of microporous membrane were erasured carefully with tampon. The subnatant cells of microporous membrane

were counted on inverted microscope.

Establishment of experimental metastasis model in nude mice

Cells were digested with 2.5 g/L pancreatic enzyme, washed with no-serum culture solution, and centrifuged at 1800 rpm. The cell sediment was washed with serum free culture solution, centrifuged twice and floated with asepsis PBS solution. Two hundred microliters of cell suspension containing 1×10^7 of HCT116, pcDNA3.1-HCT116 and DPC4$^+$-HCT116 cells were seeded into abdominal cavity of nude mice by syringe respectively. The vim, appetite, and defecation of nude mice were observed regularly, and their weight was weighed. After nine weeks, nude mice were executed and observed.

Gelatin zymography assay

HCT116, pcDNA3.1-HCT116 and DPC4$^+$-HCT116 cells were cultured respectively. Three days later, cells were washed by D-hanks solution, and then seeded into serum free culture solution. Twenty four hours later, the supernatant of the cells was collected, and concentrated with Amicon filters (Millipore) to 10% initial volume. Each sample was guaranteed to contain the same amount of total protein, gelatin zymography was performed in 10% (*W*/*V*) polyacrylamide containing 0.1% (*W*/*V*) gelatin. The identification of transparent bands at 92 kDa and 72 kDa on the Coomassie blue background of the slab gel was considered positive for the presence of enzymatic activity.

Statistical Analysis

Results are expressed as mean±SD for experiments with triplicate measurements. Differences between groups were tested with Student's *t* test and $P<0.05$ was considered significant.

Results

Expression of DPC4

The expression of DPC4 is shown in **Fig. 1**. DPC4 positive signal was localized in cytoplasm and nucleus.

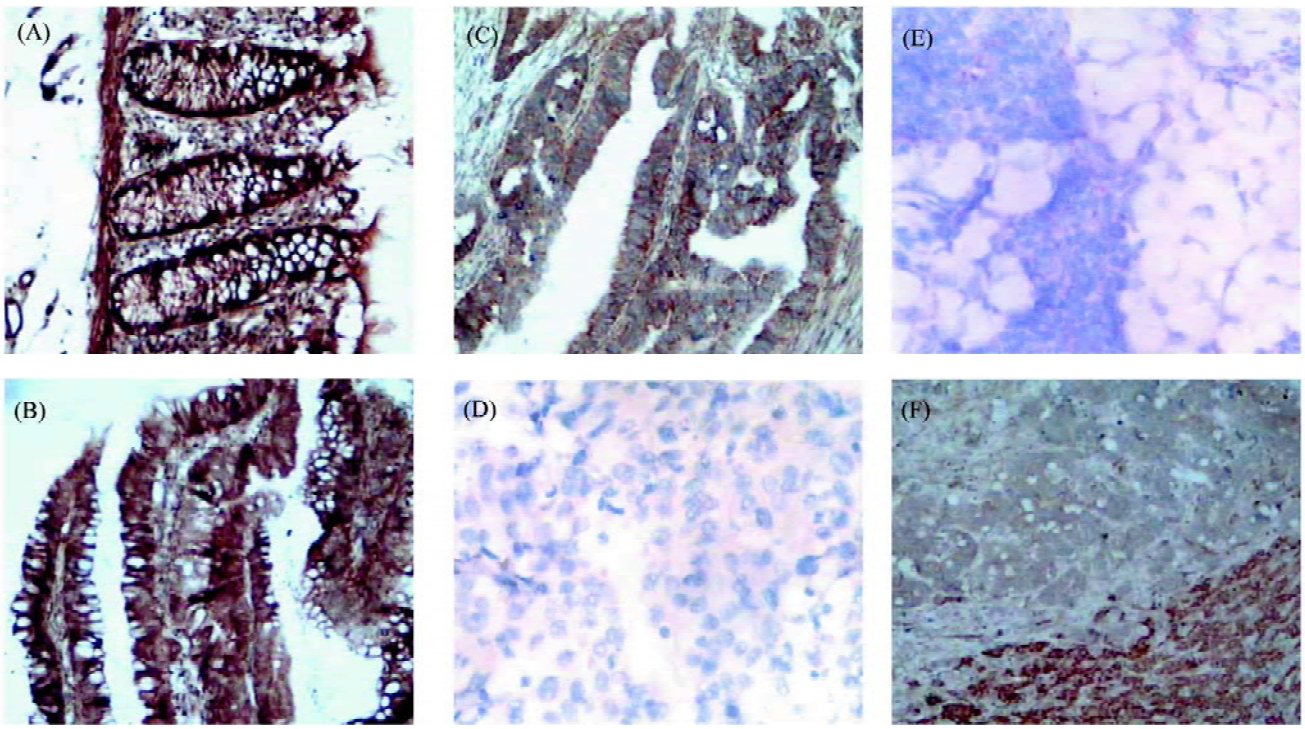

Fig. 1 Expression of DPC4 in colorectal neoplasia

(A) Normal mucosa. (B) Adenoma. (C) Well-differentiated adenocarcinoma. (D) Poorly differentiated adenocarcinoma. (E) Metastatic focus in lymphoid node. (F) Metastatic focus in liver (left superior angle for colon carcinoma tissue, right inferior angle for hepatic tissue). Magnification, 200×.

The staining of DPC4 was strong positive in normal colon mucosa and adenoma. The positive cells distributed in the whole colon mucosa [**Fig. 1(A,B)**]. The expression of DPC4 was positive in well-differentiated adenocarcinoma [**Fig. 1(C)**], and weakly positive or negative in poorly differentiated adenocarcinoma and metastatic focus [**Fig. 1(D–F)**]. Compared with that in adenoma, the expression of DPC4 in the normal colon mucosa had no significant difference ($P>0.05$). Compared with the that in well-differentiated, poorly differentiated adenocarcinoma or metastatic focus, the expression of DPC4 in the normal colon mucosa was stronger ($P<0.05$). The expression of DPC4 in the poorly differentiated adenocarcinoma was weaker than that in well-differentiated adenocarcinoma ($P<0.05$). Compared with that in Dukes A, B stages, the expression of DPC4 in Dukes C, D stages was weaker ($P<0.05$). The expression of DPC4 was associated with tumor staging, cell differentiation degree and metastasis (**Table 1**).

Table 1 Expression of DPC4 in colorectal neoplasia

Patho-parameter	Score	*n*
Normal mucous membrane	2.86±0.24	7
Adenoma	2.56±0.45	11
Well-differentiated adenocarcinoma	1.66±0.73 [a,b]	17
Poorly differentiated adenocarcinoma	1.11±0.78 [a]	35
Carcinomatous metastasis	1.00±0.78 [a]	14
Dukes A, B stage	1.49±0.70	38
Dukes C, D stage	1.00±0.79	14

[a] $P<0.05$ versus normal mucous membrane; [b] $P<0.05$ versus poorly differentiated adenocarcinoma.

Identification of recombinant plasmids

Plasmids pcDNA3.1 and pCMV5-DPC4 were cut by *Hin*dIII and *Bam*HI, and electrophoresed by low melting-point agarose gel. pcDNA3.1 and DPC4 fragments were extracted, purified, and re-constructed with T_4 DNA ligase. Recombinant plasmids were transferred, extracted, cut and identified by routine agarose gel electrophoresis (**Fig. 2**).

Cell screening

Five cell lines of colon carcinoma, including HCT116, HT29, LS174T, SW480 and SW620, were screened by RT-PCR and Western blot analysis. RT-PCR amplification

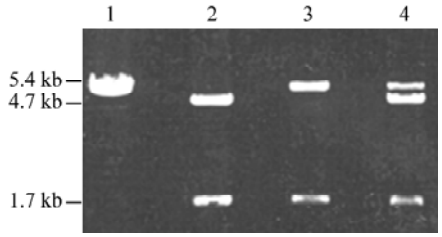

Fig. 2 Identification of recombinant plasmids
1, pcDNA3.1; 2, pCMV5-DPC4; 3, pcDNA3.1-DCP4; 4, pCMV5-DPC4$^+$.

of DPC4 mRNA in HCT116 cell line was not detected [**Fig. 3(A)**]. The staining of DPC4 was negative in HCT116 cell line [**Fig. 3(B)**]. HCT116 was an ideal cell line for this study.

Gene transfection

HCT116 cell clones transfected with pcDNA3.1 and pcDNA3.1-DPC4 respectively were obtained after gene transfection and screening. The expression of DPC4 was the highest in the cell clones 1, 6 and 7 [**Fig. 4(A)**]. In order to avoid the deviation brought about by single cell clone, cell clones 1, 6 and 7 were used simultaneously in the experiment. Immunocytochemistry analysis indicated that the expression of DPC4 was negative in pcDNA3.1-HCT116 and HCT116 cells, but positive in DPC4$^+$-HCT116 cells, and the positive signal was localized in cytoplasm and nucleus, mainly in cytoplasm [**Fig. 4(B)**].

In vitro growth property

The growth curve and cell doubling time of trans-

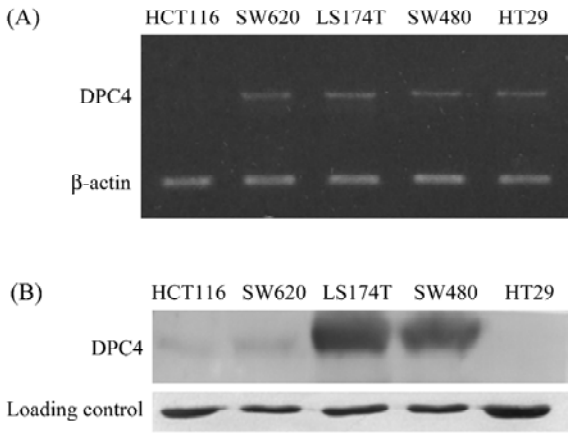

Fig. 3 Expression of DPC4 in HCT116, HT29, LS174T, SW480 and SW620 cell lines
(A) RT-PCR analysis of DPC4. (B) Western blot analysis of DPC4.

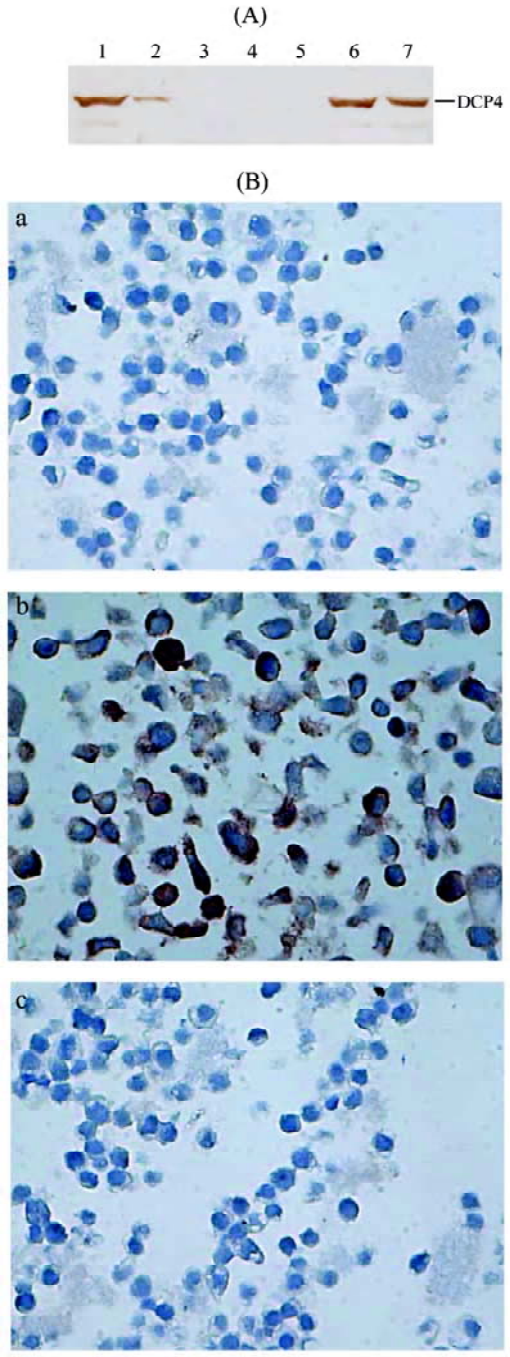

Fig. 4 Expression of DPC4 in transfected cells

(A) Western blot analysis of DPC4 from seven clones. (B) Immuocytochemistry analysis of DPC4. a, HCT116; b, DPC4$^+$-HCT116; c, pcDNA3.1-HCT116.

fected cells were drawn and calculated respectively. The growth curve is shown in **Fig. 5**. Compared with that of HCT116 cells (57 h) and pcDNA3.1-HCT116 cells (52 h), the doubling time of DPC4$^+$-HCT116 cells (93 h) lengthened obviously (P<0.01) (**Fig. 6**). The cloning efficiency of every group cells was calculated. In comparison with that of HCT116 cells (83%) and pcDNA3.1-HCT116 cells (83%), the cloning efficiency of DPC4$^+$-HCT116 cells (15%) dropped obviously (P<0.01) (**Fig. 7**).

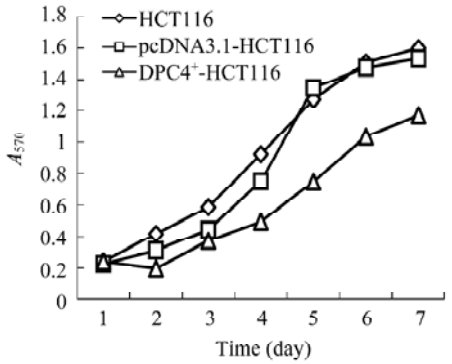

Fig. 5 Cell proliferation in different cell lines

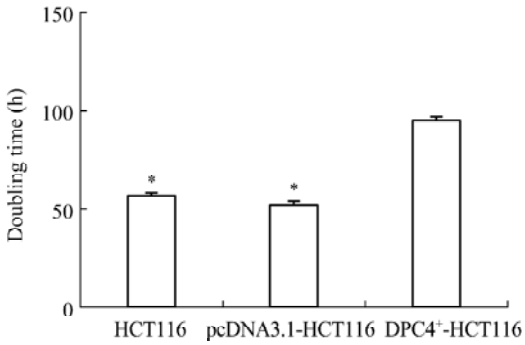

Fig. 6 Comparison of doubling time in different cell lines

*P<0.01 versus DPC4$^+$-HCT116 cells.

Alterations of cell cycle and apoptosis rate

Cell cycle and apoptosis rate of three group cells were determined by flow cytometry. Compared with that of HCT116 and pcDNA3.1-HCT116 cells, the G_0-G_1% of DPC4$^+$-HCT116 cells was much higher and the S% was much lower (P<0.01) (**Fig. 8**). The apoptosis rate of DPC4$^+$-HCT116 cells increased more significantly than that of HCT116 and pcDNA3.1-HCT116 cells (P<0.01) (**Fig. 9**).

Alterations of adherency, migration and invasion ability

A value was decided by MTT colorimetric method and cell adhesion rate was calculated by Equation 1. Compared with that of HCT116 (36%) and pcDNA3.1-HCT116 cells (31%), the cell adherency rate of DPC4$^+$-HCT116 cells (11%) was dropped obviously (P<0.01) (**Fig. 10**). Compared with that of HCT116 (88) and pcDNA3.1-HCT116 cells (88) respectively, the cell migration rate of

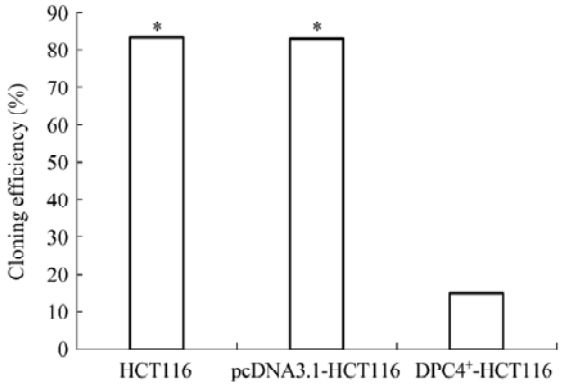

Fig. 7 Cloning efficiencies in different cell lines
*P<0.01 versus DPC4$^+$-HCT116 cells.

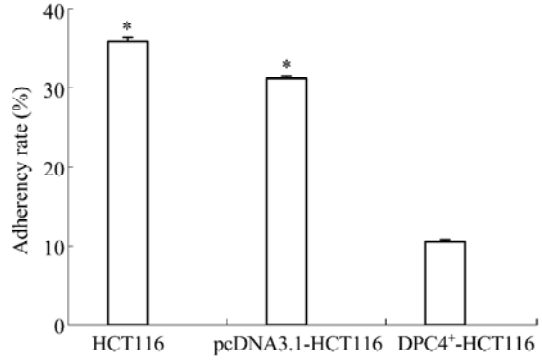

Fig. 10 Cell adherency rate in different cell lines
*P<0.01 versus DPC4$^+$-HCT116 cells.

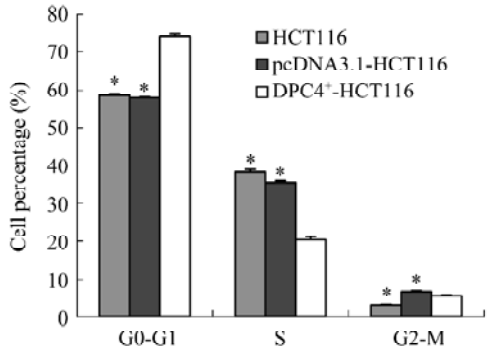

Fig. 8 Flow Cytoeter analysis of cell cycle
*P<0.01 versus DPC4$^+$-HCT116 cells.

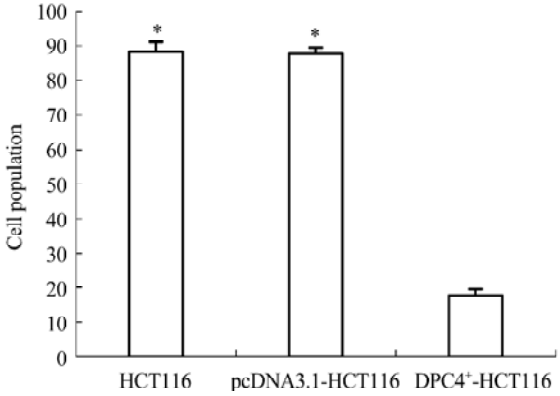

Fig. 11 Cell migration ability in three cell lines
*P<0.01 versus DPC4$^+$-HCT116 cells.

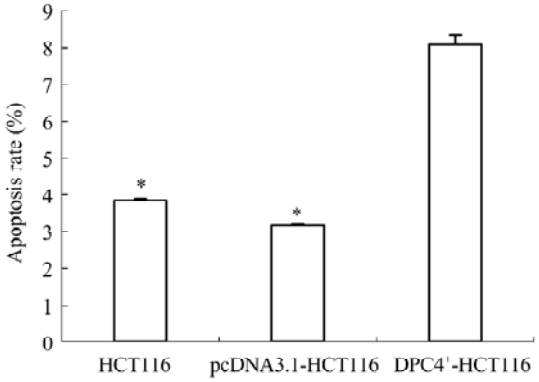

Fig. 9 Apoptosis rate in different cell lines
*P<0.01 versus DPC4$^+$-HCT116 cells.

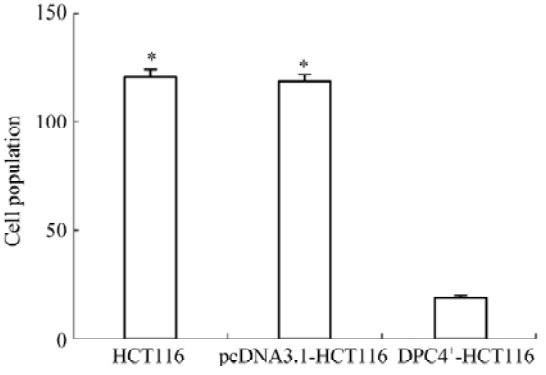

Fig. 12 Cell invasion ability in three cell lines
*P<0.01 versus DPC4$^+$-HCT116 cells.

DPC4$^+$-HCT116 cells (18) dropped obviously (P<0.01) (**Fig. 11**). Compared with that of HCT116 (120) and pcDNA3.1-HCT116 cells (118) respectively, the cell invasion ability of DPC4$^+$-HCT116 cells (18) declined significantly (P<0.01) (**Fig. 12**). Every sample was counted for five different visual fields.

Establishment of experimental metastasis model in nude mice

The metastasis models of colon carcinoma cells were

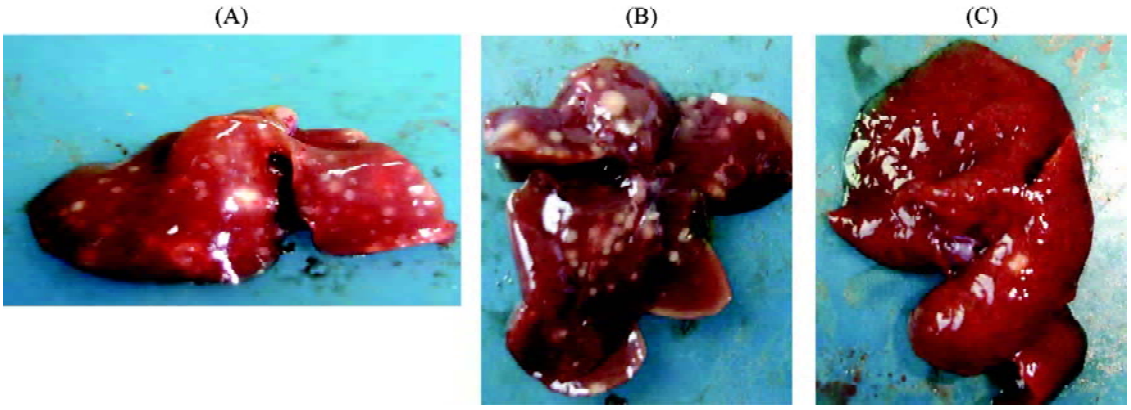

Fig. 13 Livers from nude mice of colon carcinoma cell metastasis model inoculated with HCT116 (A), pcDNA3.1-HCT116 (B) and DPC4$^+$-HCT116 (C) cells

established in nine nude mice. Nine weeks later, the mice were executed and performed exploratory laparotomy. Number and size of metastatic nodules were calculated and measured. There were lots of cancer nodules in abdominal cavity and liver of the nude mice inoculated with HCT116 and pcDNA3.1-HCT116 cells, whereas few cancer nodules in the nude mice inoculated with DPC4$^+$-HCT116 cells (**Fig. 13**).

Gelatinase Activity

Gelatin zymography revealed prominent 92 kDa bands (**Fig. 14**), there was activity of MMP-9 in HCT116, pcDNA3.1-HCT116 and DPC4$^+$-HCT116 cells culture supernatant. Compared with that of HCT116 and pcDNA3.1-HCT116 cells, the activity of MMP-9 in DPC4$^+$-HCT116 cells dropped obviously.

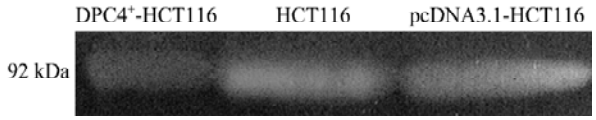

Fig. 14 Comparison of activity of MMP-9 by gelatin zymography assay

Discussion

TGF-β is a member of a large superfamily of structurally related to growth and differentiation factors, which play an important role in modulating immune response, regulating cell differentiation and growth, stimulating extracellular matrix formation, and so on. However, epithelial derived tumor cells has often lost their sensitivity to TGF-β, and lost their growth inhibition [18,19]. A high frequency of DPC4 mutations has been recognized in the progression of carcinomas of the colon. But we knew little of the effect of down-regulation of DPC4 expression on the invasion and metastasis of colon cancer. In this study, we found that the expression of DPC4 was detected in all seventy cases of specimens including normal colon mucosa, adenoma, well-differentiated adenocarcinoma and poorly differentiated adenocarcinoma. Fourteen cases of the tumors had presence of lymph nodal or distant metastases. The results indicated that the DPC4 expression was significantly lower in the colorectal carcinoma than that in the normal colon mucosa, and related to tumor staging, cell differentiation degree and metastasis. The down-regulation of DPC4 expression may be associated with the progression of colorectal carcinoma, and DPC4 may play a role in invasion and metastasis of colorectal carcinoma.

Reintroduction of a candidate tumor suppressor gene in tumor-derived cells, which had been selected for functional inactivation of this gene during the tumorigenic process, is the most direct approach for assessing its tumor suppressor function. Here we report that the DPC4 gene affects on the suppression of the proliferation, invasion and metastasis of the human colon tumor cells by functional reconstitution of DPC4, further analyze the role of DPC4 as a tumor suppressor gene. We screened five colon carcinoma cell lines, constructed successfully pcDNA3.1-DPC4 plasmid, and reconstituted the DPC4 function in HCT116 human colon carcinoma cells defecting DPC4 gene. Similar numbers of G418 resistant clones

were obtained after transfecting pcDNA3.1-DPC4 and pcDNA3.1 plasmids into HCT116 cells. The influence of DPC4 on the proliferation of colorectal carcinoma cells was investigated by the growth curve, cell doubling time and flow cytometry in the experiment. The results indicated the proliferation of cells might be regulated by inhibiting growth and inducing apoptosis of colorectal carcinoma cells.

It has been reported that chromosome 18q21 was deleted in colon carcinomas, and genes of deletion are DCC, DPC4 and SMAD2 chiefly. Many scholars supposed that DPC4 was correlated with invasion and metastasis of tumor [20–28]. But no definite experiment results indicate DPC4 may inhibit the invasion and metastasis of tumor. Our findings suggested that DPC4 probably inhibits the ability of adherency, migration, invasion and metastasis of colorectal carcinoma cells by the MTT and MilliCell methods, and the metastasis models of colon carcinoma cells were established in nude mice.

It has been demonstrated that $P21^{wafl}$ is one of the downstream target genes regulated by DPC4 [29]. Cell adhesion and spreading were associated with the reduced expression levels of the endogenous urokinase-type plasminogen activator (uPA) and plasminogen-activator-inhibitor-1 (PAI-1) genes, the products of which are implicated in the control of cell adhesion and invasion. In patients, the high expression levels of uPA and PAI-1 correlate with poor prognosis. Thus, the reduced expression of uPA and PAI-1 is consistent with suppression of tumorigenicity in DPC4 reconstituted cells [30]. DPC4 restoration influenced angiogenesis, decreasing expression of vascular endothelial growth factor and increasing expression of thrombospondin-1 [8]. Our study showed that the activity of MMP-9 in $DPC4^{+}$-HCT116 cells was inhibited, suggesting MMP-9 may be one of the downstream target genes regulated by DPC4.

In conclusion, down-regulation of DPC4 expression may be associated with the carcinogenesis of colorectal carcinoma. DPC4 reconstituted cells provided ideal material for further investigations on the function of DPC4. These findings shed new light upon physiologically crucial pathways through which DPC4 inactivation may contribute to the invasion and metastasis of colorectal carcinoma cells, with its mechanism mainly through the inhibitory effect on MMP-9. These results support the role of DPC4 in inhibiting the proliferation, invasion and metastasis of colorectal carcinoma cells, and may provide a basis for gene therapy strategies that can restore the signal transduction pathways of TGF-β-mediated growth suppression.

References

1 Hahm KB, Lee KM, Kim YB, Hong WS, Lee WH, Han SU, Kim MW *et al*. Conditional loss of TGF-β signalling leads to increased susceptibility to gastrointestinal carcinogenesis in mice. Aliment Pharmacol Ther 2002, 16: 115–127

2 Biasi F, Tessitore L, Zanetti D, Cutrin JC, Zingaro B, Chiarpotto E, Zarkovic N *et al*. Associated changes of lipid peroxidation and transforming growth factor beta1 levels in human colon cancer during tumour progression. Gut 2002, 50: 361–367

3 Barbera VM, Martin M, Marinoso L, Munne A, Carrato A, Real FX, Fabre M. The 18q21 region in colorectal and pancreatic cancer: Independent loss of DCC and DPC4 expression. Biochim Biophys Acta 2000, 1502: 283–296

4 Schneider G, Schmid RM. Genetic alterations in pancreatic carcinoma. Genetic alterations in pancreatic carcinoma. Mol Cancer 2003, 2: 15

5 Moore PS, Beghelli S, Zamboni G, Scarpa A. Genetic abnormalities in pancreatic cancer. Mol Cancer 2003, 2: 7

6 Cowgill SM, Muscarella P. The genetics of pancreatic cancer. Am J Surg 2003, 86: 279–286

7 Pizzi S, Azzoni C, Bassi D, Bottarelli L, Milione M, Bordi C. Genetic alterations in poorly differentiated endocrine carcinomas of the gastrointestinal tract. Cancer 2003, 98: 1273–1282

8 Schwarte-Waldhoff I, Volpert OV, Bouck NP, Sipos B, Hahn SA, Klein-Scory S, Luttges J *et al*. Smad4/DPC4-mediated tumor suppression through suppression of angiogenesis. Proc Natl Acad Sci USA 2000, 97: 9624–9629

9 Coelho Siqueira SA, Ferreira Alves VA, Beitler B, Otta MM, Nascimento Saldiva PH. Contribution of immunohistochemistry to small B-cell lymphoma classification. Appl Immunohistochem Mol Morphol 2006, 14: 1–6

10 Maitra A, Molberg K, Albores-Saavedra J, Lindberg G. Loss of Dpc4 expression in colonic adenocarcinomas correlates with the presence of metastatic disease. Am J Pathol 2000, 157: 1105–1111

11 Had ija MP, Kapitanovic S, Radosevic S, Cacev T, Mirt M, Kovacevic D, Cacev T *et al*. Loss of heterozygosity of DPC4 tumor suppressor gene in human sporadic colon cancer. J Mol Med 2001, 79: 128–132

12 Zhou R, Long W, Chen P. Construction of reversed caspase-3 and it's effect on apoptosis induction in human lung adenocarcinoma cell line A549. Zhong Hua Jie He He Hu Xi Za Zhi 2005, 28: 537–540

13 Takahashi H, Oda T, Hasebe T, Aoyagi Y, Kinoshita T, Konishi M, Nakagohri T *et al*. Biologically different subgroups of invasive ductal carcinoma of the pancreas: Dpc4 status according to the ratio of intraductal carcinoma components. Clin Cancer Res 2004, 10: 3772–3779

14 Mikami T, Ookawa K, Shimoyama T, Fukuda S, Saito H, Munakata A. KAI1, CAR, and Smad4 expression in the progression of colorectal tumor. J Gastroenterol 2001, 36: 465–469

15 Yoshida M, Iwasaki Y, Asai M, Nigawara T, Oiso Y. Gene therapy for central diabetes insipidus: Effective antidiuresis by muscle-targeted gene transfer. Endocrinology 2004, 145: 261–268

16 Taketo MM, Takaku K. Gastrointestinal tumorigenesis in DPC4 (Dpc4) mutant mice. Hum Cell 2000, 13: 85–95

17 Ohtaki N, Yamaguchi A, Goi T, Fukaya T, Takeuchi K, Katayama K, Hirose K *et al*. Somatic alterations of the DPC4 and Madr2 genes in colorectal cancers and relationship to metastasis. Int J Oncol 2001, 18: 265–270

18 Shawler DL, Bartholomew RM, Garrett MA, Trauger RJ, Dorigo O, Van Beveren C, Marchese A *et al*. Antigenic and immunologic characterization of an allogeneic colon carcinoma vaccine. Clin Exp Immunol 2002, 129: 99–106

19 Hu JY, Wang S, Zhu JG, Zhou GH, Sun QB. Expression of B7 costimulation molecules by colorectal cancer cells reducestumorigenicity and induces anti-

tumor immunity. World J Gastroenterol 1999, 5: 147–151
20 Xiong B, Gong LL, Zhang F, Hu MB, Yuan HY. World J Gastroenterol 2002, 8: 496–498
21 Yang X, Li C, Herrera PL, Deng CX. Generation of Smad4/Dpc4 conditional knockout mice. Genesis 2002, 32: 80–81
22 Miyaki M, Kuroki T. Role of Smad4 (DPC4) inactivation in human cancer. Biochem Biophys Res Commun 2003, 306: 799–804
23 Cattaneo M, Orlandini S, Beghelli S, Moore PS, Sorio C, Bonora A, Bassi C *et al*. SEL1L expression in pancreatic adenocarcinoma parallels SMAD4 expression and delays tumor growth *in vitro* and *in vivo*. Oncogene 2003, 22: 6359–6368
24 Grady WM, Markowitz SD. Genetic and epi Genetic and epigenetic alterations in colon cancer. Annu Rev Genomics Hum Genet 2002, 3: 101–128
25 Schwarte-Waldhoff I, Schmiegel W. Smad4 transcriptional pathways and angiogenesis. Int J Gastrointest Cancer 2002, 31: 47–59
26 Lin X, Liang M, Liang YY, Brunicardi FC, Melchior F, Feng XH. Activation of transforming growth factor-β signaling by SUMO-1 modification of tumor suppressor Smad4/DPC4. J Biol Chem 2003, 278: 18714–18719
27 Lin X, Liang M, Liang YY, Brunicardi FC, Feng XH. SUMO-1/Ubc9 promotes nuclear accumulation and metabolic stability of tumor suppressor Smad4. J Biol Chem 2003, 278: 31043–31048
28 McCarthy DM, Hruban RH, Argani P, Howe JR, Conlon KC, Brennan MF, Zahurak M *et al*. Role of the DPC4 tumor suppressor gene in adenocarcinoma of the ampulla of Vater: Analysis of 140 cases. Mod Pathol 2003, 16: 272–278
29 Hunt KK, Fleming JB, Abramian A, Zhang L, Evans DB, Chiao PJ. Overexpression of the tumor suppressor g3ene Smad4/DPC4 induces p21waf1 expression and growth inhibition in human carcinoma cells. Cancer Res 1998, 58: 5656–5661
30 Schwarte-Waldhoff I, Klein S, Blass-Kampmann S, Hintelmann A, Eilert C, Dreschers S, Kalthoff H *et al*. DPC4/SMAD4 mediated tumor suppression of colon carcinoma cells is associated with reduced urokinase expression. Oncogene 1999, 18: 3152–3158

Edited by
Ming-Hua XU