IGF-1 and PTEN regulate the proliferation and invasiveness of colon cancer cells through opposite effects on PI3K/Akt signalling

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Abstract

Introduction: The tumour suppressor protein phosphatase and tensin homologue deleted on chromosome 10 (PTEN) is a critical tumour suppressor which acts by suppressing tumour cell invasiveness and proliferation and promoting apoptosis through its antagonism of PI3K. Despite its importance, the underlying mechanism of the molecule which regulates phosphorylated PTEN expression is still undefined.

Material and methods: We herein investigate the mechanism by which IGF-1 affects cell proliferation (WST-1 cell proliferation assay) and invasion by examining its potential suppression of PTEN activity (with RNA interference) and its interaction with the PI3K/PTEN/Akt/AP-1 signalling pathway in colon cancer cells (HT-29, WiDr, CaCo-2 and Colo320). In addition, we also examine how the knockdown of PTEN influences proliferation and invasion and correlates with IGF-1/IGF-1R/PI3K/Akt/AP-1, and determine PTEN up/downstream targets that preferentially contribute to tumourigenesis.

Results: Blockage of PTEN phosphorylation led to a stronger enhancement of cell proliferation and invasion upon stimulation with IGF-1 via its activation of the PI3K/Akt/AP-1 signalling pathway. Furthermore, knockdown of PTEN by siRNA transfection was also found to enhance the activation of the PI3K/Akt/AP-1 pathway, thereby promoting cell invasion and proliferation. IGF-1 induced transcriptional down-regulation of activated PTEN and this signalling pathway promotes cell survival.

Conclusions: The IGF-1/PI3K/Akt/AP-1 cascade may be critical for colon cancer cells to metastasize. Based on our results, we suggest that the modification of IGF-1, PTEN, or PI3K function might be promising new therapeutic approaches to inhibit the aggressive spread of colon cancer.

Key words: AP-1, colorectal cancer, IGF-1, PTEN.

Introduction

Colorectal cancer accounts for almost one million new cancer cases and causes half a million deaths annually worldwide [1-3]. It is the second most common type of newly diagnosed cancer in both males and females and ranks second as a cause of cancer-related death after lung cancer in Europe [4]. The present treatment for colorectal cancer is surgical ablation, but many colorectal cancers are diagnosed at a late stage, when surgical intervention

is no longer effective at curing the disease. At least 40% of patients with colorectal cancer develop metastases [5], and there are no highly effective approaches against disseminated colorectal cancer. Therefore, new, non-surgical therapeutic strategies are urgently needed for the treatment of advanced or metastatic colorectal cancer.

Among the several genetic changes and signalling pathways known to be involved in the development and progression of cancer [6, 7], one of the most common is mutation in the tumour suppressor PTEN (phosphatase and tensin homologue deleted on chromosome ten), which encodes a protein and lipid phosphatase. The mutant of PTEN is unable to dephosphorylate phosphatidylinositol 3,4,5-triphosphate (PIP_3), which is produced by PI3K, resulting in elevated intracellular PIP_3 levels. PIP_3, as an important messenger, transduces signals from growth factors, hormones and extracellular matrix components. One of the best-studied downstream targets of PIP_3 is Akt, also known as protein kinase B. When cells are stimulated, Akt is recruited by PIP_3 to the plasma membrane, where Akt is phosphorylated and activated. PI3K/Akt signalling is involved in promoting cell survival, proliferation and migration [8, 9]. PI3K and Akt can themselves also become hyperactivated due to gene amplification or PTEN inactivation. Consequently, the downstream targets of PI3K/Akt can be abnormally activated, thereby promoting proliferation and survival of cancer cells during carcinogenesis. Nuclear factor κB (NF-κB) and activator protein 1 (AP-1) are the transcription factors and the targets of the Akt pathway whose activation is most strongly correlated with carcinogenesis [10]. PTEN inhibits downstream functions mediated by the PI3K pathway, such as cell growth, survival, migration, and invasiveness [11], and cell cycle progression, through the regulation of the expression of the cyclin-dependent kinase inhibitor protein $p27^{kip1}$ [12], which is induced by PTEN in various cell types [13]. PTEN contains a sequence motif that is highly conserved in the members of the protein tyrosine phosphatase family [14]. PTEN is frequently affected in cancer, and inherited PTEN mutation causes cancer-susceptibility conditions such as Cowden syndrome. PTEN is also frequently mutated in other human cancers, including breast, lung, prostate, bladder and glioblast cancer [8, 15-17]. PTEN mutations have been mapped to the conserved phosphatase catalytic domain, suggesting that the phosphatase activity of PTEN is required for its tumour suppressing function.

Insulin-like growth factor 1 (IGF-1) is an important mitogen for many cell types and acts in an autocrine-paracrine manner [18]. Insulin-like growth factor 1 is a polypeptide which exerts effects on cell proliferation, differentiation, apoptosis and transformation [19]. The biological effects of IGF-1 are mediated through two major signalling pathways downstream of the type 1 IGF-1 receptor (IGF-1R), PI3K and the mitogen-activated protein (MAP) kinase pathways [20]. Insulin-like growth factor 1 stimulates the growth of HT-29, LS411NLS513, SW480 and WiDr human colorectal cells [22]. Colorectal cancers have a 10 to 50-fold increase in the levels of IGF-1 and IGF-1R when compared to adjacent uninvolved colonic mucosa [23, 24]. Furthermore, an IGF-1 D domain analogue peptide can induce apoptosis of human colon cancer cell line HT-29 [25]. The accumulated data demonstrate that IGF-1 plays an important role in development of colonic cancer.

AP-1 is an inducible eukaryotic transcription factor containing products of the jun and fos oncogene families. The inducible AP-1 complexes are composed of Jun-Jun or Jun-Fos dimers [26]. Many stimuli are able to induce AP-1 activity, including the phorbol ester TPA and epidermal growth factor (EGF), which are strong inducers of cellular transformation of many different cell types and animal models [27]. Increased AP-1 activity is associated with malignant transformation and cancer development by UV radiation, growth factor, phorbol esters, and transformation oncogenes [28]. In contrast, several phytochemicals, such as curcumin, capsaicin, resveratrol, and green tea catechins, have been shown to suppress AP-1 activation [29]. An AP-1 blockade has been shown to interfere with the transmission of proliferative signals from both peptide growth factors and steroid growth factors, such as oestrogens [30].

In this study, we hypothesized that the observed biological effects of IGF-1 involve the suppression of PTEN phosphorylation and that its ability to increase the invasiveness of human colon cancer cells occurs via effects on the IGF-1/IGF-1R/PI3K/PTEN/Akt/AP-1 signalling pathway. We show here that the mechanism of IGF-1 action involves inactivation of the PTEN tumour suppressor. Dephosphorylation of PTEN by IGF-1 decreases its activity, elevates PI3K levels and increases signalling through Akt and its downstream targets. Furthermore, we inhibited PTEN function with PTEN siRNA and investigated the impact on cellular proliferation and invasion in colon cancer cells. Thus, we demonstrate that IGF-1 not only enhances proliferation and invasion, but also suppresses PTEN activity in colon cancer cells.

Material and methods

Reagents and antibody

Recombinant human IGF-1 and anti-human IGF-1 antibody were provided by R&D system Inc. (Minneapolis, MN, USA). LY294002 (PI3K inhibitor)

was ordered from Cell Signaling Technology (Beverly, MA, USA). Akt inhibitor was purchased from Bio Vision (Mountain View, CA, USA). The monoclonal antibodies (mAbs) PTEN antibody, phospho-PTEN (ser380) antibody, IGF-1 receptor β (111A9) rabbit mAb, Akt antibody, phospho-Akt (ser473), PI3K p85 antibody, phospho-PI3K p85 (Tyr 458)/p55 (Tyr199) antibody, c-jun antibody, and phosphor-c-jun (Ser63) II antibody were purchased from Cell Signaling Technology.

Cell culture

Four cell lines derived from human colon carcinoma were examined: HT-29, WiDr, CaCo-2 and Colo320. All cell lines were obtained from the American Type Culture Collection (Rockville, MD, USA). The HT-29 was cultured in McCoy's medium supplemented with 10% fetal bovine serum (FBS). WiDr and CaCo-2 were maintained in Eagle's minimum essential medium (Sigma Chemical Co., St. Louis, MO, USA) with high glucose and 10% FBS. Colo320 was maintained in RPMI-1640 medium (Sigma Chemical Co.) supplemented with 10% FBS. All cells were incubated at 37°C in a humidified atmosphere of 5% CO_2 in air. Cells were used at passages 4-8 after their receipt from the supplier.

RT-PCR analysis

Total RNA was extracted from colon carcinoma using an Isogen kit (Nippon Gene, Tokyo, Japan), and quantities determined spectrophotometrically. Total RNA aliquots (5 µg) were pre-treated with random hexamers and dNTP mix, incubated at 65°C for 5 min, chilled on ice, and then reverse-transcribed into cDNA in a cDNA Synthesis Mix containing 10 × RT buffer, 25 mM $MgCl_2$, 0.1 MDTT, RNaseOUT and 200U SuperScript RT (Invitrogen, San Diego, CA, USA) at 50°C for 50 min. The reaction was terminated at 85°C for 5 min. 1 µl of reaction mixture aliquots was used as templates for PCR. The following pairs of forward and reverse primer sets were used: PTEN, 5'-ACCAGGACC-AGAGGAAACCT-3' and 5'-GCTAGCCTCTGGATTTG ACG-3' and IGF1R, 5'-GAAGTGGAACCCTCCCTCTC-3' and 5'-GTTCTCGGC TTCAGTTTTGG-3'. Amplification reactions were performed using a DNA Thermal Cycler (Model TP3000; Takara PCR Thermal Cycle MP). The PCR conditions for PTEN were 35 cycles of denaturation at 94°C for 30 s, annealing at 58°C for 30 s, and extension at 72°C for 60 s. Amplified DNA fragments were resolved by electrophoresis on 2% agarose gels containing ethidium bromide.

Real-time quantitative RT-PCR

PCR was performed using LightCycler apparatus. Freshly isolated RNA was converted to cDNA using the PrimeScrip™ TR Regent kit (Takara Bio Inc., Shiga, Japan), and the PCR reaction was performed using a TaqMan® Gene Expression Assay Kit (Applied Biosystems, Foster City, CA, USA). In brief, 7 µl of water containing 1 µl of total RNA was added to 1 µl oligo dT primer (50 µM), and the mixture was incubated at 37°C for 15 min at 85°C for 5 s to deactivate reverse transcription. The PCR was carried out in a 20 µl final volume containing the following: H_2O up to 20 µl, 10 µl TaqMan® Universal PCR Master Mix, No AmpErase® UNG $(2\times)^2$ – ordered separately, 1 µl 20× TaqMan® Gene Expression Assay Mix; and 9 µl cDNA diluted in RNase-Free water. After an initial denaturation step at 95°C for 10 s, temperature cycling was initiated. Each cycle consisted of denaturation at 95°C for 10 s, hybridization at 60°C for 30 s, and elongation at 72°C for 30 s. The fluorescence signal was acquired at the end of the hybridization step. A total of 40-50 cycles were performed. Melting curves were obtained for the temperature range 65 to 95°C, read every 0.2°C, held for 5 s, then incubated at 65°C for 60 s. Cycling conditions for GAPDH were the same as mentioned above. For each run, a standard curve was constructed from serial dilutions of cDNA from the HT-29 cell line. The level of expression of PTEN mRNA is given as relative copy numbers normalized against GAPDH mRNA and shown as mean ± standard deviation (SD). Relative PTEN mRNA expression was calculated using the formula $A/G \div A_0/G_0$, where A is the relative copy number of PTEN mRNA, G is the relative copy number of GAPDH mRNA, A_0 and G_0 are relative PTEN and GAPDH mRNA from the standard cDNA dilutions as a non-template control.

ELISA for phospho-PTEN

Each colon cancer cell line was seeded at a density of 1×10^6 cells/5 ml in 35-mm dishes containing medium with 5% FBS and cultured overnight. Medium was exchanged and the cells were treated with or without different concentrations of IGF-1 and cultured for a further 8 h. The cells were rinsed with ice-cold PBS, lysed in lysis buffer (20 mM Tris pH 7.5, 150 mM NaCl, 1 mM EDTA, 1 mM EGTA, 1% Triton X-100, 2.5 mM sodium pyrophosphate, 1 mM β-glycerophosphase, 1 mM Na_3VO_4, 1 µg/ml leupeptin and 1 mM PMSF), the dishes were incubated on ice for 5 min, cells were scraped off the plate and the lysate was sonicated on ice, then microcentrifuged for 10 min at 4°C to remove particles. The supernatants were frozen at –80°C until use in ELISA. The concentration of phospho-PTEN in the cell lysate per 1×10^6 cells was measured using a PathScan® Phospho-PTEN (Ser380) sandwich ELISA kit (Cell Signaling Technology) according to the manufacturer's instructions.

Western blot analysis

The cells were lysed in lysis buffer [25 mM Tris (pH 7.8) with H_3PO_4, 2 mM CDTA, 10 mM DTT, 10% glycerol, 1% Triton® X-100, 2 mM PMSF, 1 mM sodium orthovanadate, and 10 µM leupeptin]. The protein concentrations were measured with a BCA protein assay kit (Pierce, Rockford, USA). The amounts of samples were 30 µg per lane. The lysates were separated by 10% SDS-polyacrylamide gel electrophoresis, and transferred to polyvinylidene membrane (Immobilo PVDF; Nihon Millipore Ltd, Tokyo, Japan). The membrane was incubated in the blocking buffer for 60 min at room temperature. The blocking buffer consisted of 5% non-fat dry milk dissolved into Tris buffered saline containing 0.1% Tween 20 (TBS-T). After washing the membrane with TBS-T, the membrane was immunoblotted with each primary antibody diluted into 1 : 1000-2000 overnight at 4°C. Afterward, membranes were washed with TBS-T three times, and subjected to HRP-conjugated secondary antibody for 60 min at RT temperature. Protein antibody complexes were visualized with an ECL Western blotting detection and analysis system (Amersham Biosciences, Buckinghamshire, UK). Beta-actin Western blots served as controls.

Proliferation assay

Colon cancer cells were seeded at a density of 2×10^3 cells/100 µl into 96-well flat-bottomed plates and cultured overnight. The media were changed, and the cells then cultured in the medium alone (control) or in the medium containing different concentrations of IGF-1 and LY294002, and after 72 h incubation, 10 µl WST-1 reagent was added to each well and cells were incubated for another 4 h at 37°C, then the cell proliferation was measured by the WST-1 Cell Proliferation Assay System (Takara Bio Inc, Shiga, Japan). The absorbance was determined using a microplate reader (Molecular Devices, Sunnyvale, CA, USA) at a test wavelength of 450 nm and reference wavelength of 690 nm.

Invasion assay

The invasive capability of human colon cancer cell lines was determined by Matrigel-coated invasion chambers (Becton Dickinson, Bedford, MA, USA). This system is separated by a PET membrane coated with Matrigel Matrix such that only invasive cells can migrate through the membrane to the reverse side. After rehydration for 2 h in a humidified incubator at 37°C with 5% CO_2, cells were seeded at a density of 1×10^5 cells/well into the inner chambers of a cell culture insert and incubated at 37°C with 5% CO_2 for 24 h with various concentrations of IGF-1 and LY294002. After 24 h incubation, no-filtering cells were removed from the upper surface of the membrane by scrubbing gently with cotton-tipped applicators. The cells that invaded to the reverse side of the membrane were fixed with 70% ethanol, then stained with Giemsa solution, and were counted in five random fields of the low filter surface under a microscope at 200× magnification.

Measurement of caspase-3 activity

Caspase-3 activity was measured by the CaspACE™ colorimetric assay system (Promega, Madison, WI, USA) according to the manufacturer's instructions. Briefly, all four colon cancer cell lines were treated with or without IGF-1, LY294002 and IGF-1 antibody and incubated for 24 h. The cells were harvested and resuspended in the cell lysis buffer at a density of 1×10^6 cells/ml. After lysis, cell extracts (50 µg protein) were mixed with 32 µl of assay buffer and 2 µl of 10 mM DEVD-pNA substrate. After incubating at 37°C for 4 h, absorbance was measured using a microplate reader at 405 nm. Absorbance of each sample was determined by subtraction of the mean absorbance of the blank from that of the sample.

RNA interference (siRNA)-induced gene silencing

Colon cancer cells were transfected with siRNA for PTEN and with control non-specific siRNA using Stealth™ siRNA Duplex Oligoribonucleotides (Invitrogen). Human colon cancer cell lines were plated at 2×10^5 cells per 35-mm dish in medium with 10% FBS and without antibiotic for 24 h before transfection, grown to 90% confluence the day of transfection. We diluted 200 pM of Stealth™ PTEN siRNA oligomer or PTEN siRNA control in 500 µl of Opti-MEM® I Reduced Serum Medium (Invitrogen), then diluted 10 µl of Lipofectamine™ 2000 (Invitrogen) in 500 µl Opti-MEM® I Reduced Serum Medium. These were mixed gently and incubated for 5 min. After incubation, the diluted siRNA and diluted Lipofectamine™ 2000 were combined, gently mixed and allowed to incubate for 20 min at room temperature. The siRNA: Lipofectamine™ 2000 mixture was added directly to the cells. After 4 h incubation, complete medium with 10% FBS was added and cells were cultured for another 24-48 h.

Extraction of nuclear protein

Cells were plated on 35-mm dishes at a density of 2 ± 10^6 cells/dish. After a 12 h attachment period, cells were treated with or without 50 µM of LY294002 and Akt inhibitor in the serum-free medium for the indicated time and then with 100 ng/ml of IGF-1 for 30 min. These cells were washed twice with ice-cold PBS, followed by isolation of the cytoplasmic or nuclear proteins from the cells using NE-PER®

Nuclear and Cytoplasmic Extraction Reagents according to the manufacturer's instructions (Pierce, Rockford, IL, USA). The nuclear lysates were used in Western blot as the extract. Protein concentrations were determined using a BCA protein assay kit (Pierce) and stored at –80°C until analysis.

Statistical analysis

Statistical comparisons were made using Student's *t*-test for paired observations or one-way ANOVA with a *post hoc* test (Dunnett multiple comparison) for multiple group comparisons. Statistical significance was indicated by $p < 0.05$. Data are presented as mean ± SD. Each experiment was carried out in triplicate.

Results

Expression of PTEN and IGF-1R mRNA in colon cancer cell lines

Expression of PTEN and IGF-1R mRNA was detected in all four colon cancer cell lines using RT-PCR (Figure 1A). Similarly, by immunoblotting analysis all colon cancer cell lines were found to express PTEN and IGF-1R protein (Figure 1B). We previously determined the liver-metastatic capability of human colon cancer cell lines by intrasplenic liver metastatic assay and classified them into either the high liver metastatic group (HT-29, WiDr) or the low liver metastatic potential group (CaCo-2, Colo320) [31]. In the present study, we found that there was a negative relationship between the relative quantity of PTEN mRNA and metastatic potential. In other words, expression of PTEN mRNA by the high metastasis group (HT-29 and WiDr) was significantly lower than that in the low metastasis group ($p < 0.01$ compared with HT-29, Figure 1C).

PTEN siRNA interference strongly downregulates PTEN mRNA and protein expression

The four human colon cancer cells were transfected with siRNA that specifically targets PTEN. Downregulation of PTEN mRNA and protein expression by siRNA treatment were confirmed by real-time quantitative RT-PCR and immunoblotting. The levels of PTEN mRNA were significantly reduced by 90% (HT-29), 84% (WiDr), 93% (CaCo-2) and 91% (Colo320) respectively compared with untreated cells ($p < 0.01$) (Figure 2A). Transfection of siRNA resulted in a near total loss of PTEN expression. An anti-β-actin antibody served as a control (Figure 2B).

IGF-1 downregulated phospho-PTEN

Phosphorylation of PTEN protein in colon cancer cells was measured using a PathScan®Phospho-PTEN sandwich ELISA assay. IGF-1 treatment of all four colon cancer cell lines led to inhibition of PTEN

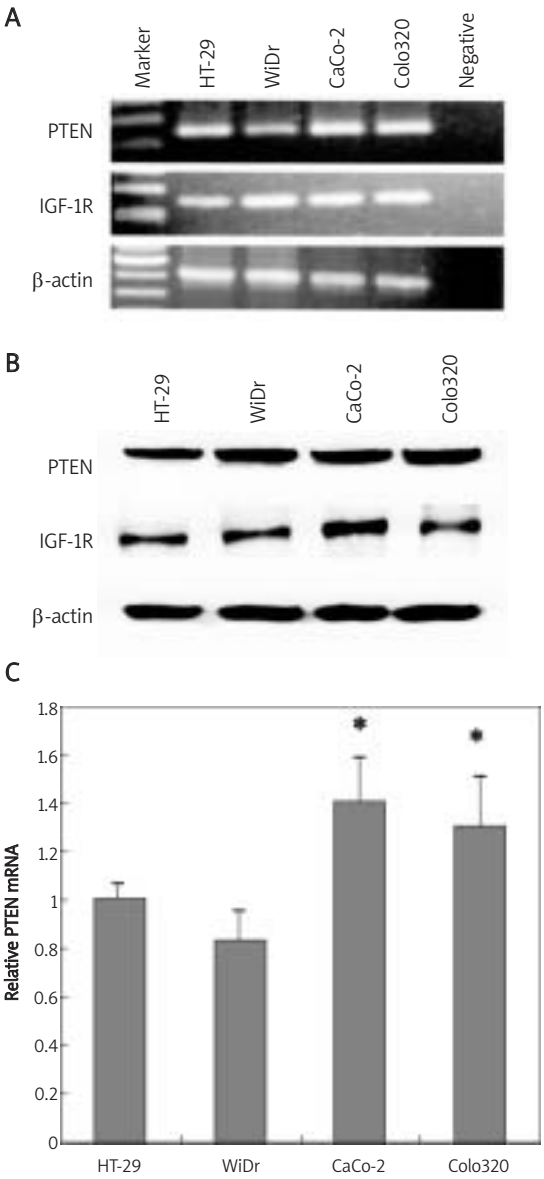

Figure 1. Expression levels of PTEN and IGF-1R in colon cancer cell lines. (A) PTEN and IGF-1R mRNA were detected by RT-PCR in colon cancer cells. PCR-amplified products of reverse-transcribed mRNA (cDNA) from GenBank, using primers specific for PTEN and IGF-1R PCR products, were separated through 2% agarose gels and stained with ethidium bromide. β-actin served as a loading control. (B) The protein expression levels of PTEN and IGF-1R in colon cancer cell lines were determined in whole-cell lysates by Western blotting analysis. Thirty micrograms of total cell lysate was subjected to 10% SDS-PAGE and transferred to polyvinylidene difluoride membrane. The membrane was probed with antibodies to PTEN and IGF-1R. β-actin acted as a loading control. (C) Relative expression of PTEN mRNA in colon cancer cell lines compared to GAPDH was assessed using semi-quantitative RT-PCR. Relative expression of PTEN mRNA is significantly higher in CaCo-2 and Colo320 cells as compared with HT-29 cells (*$p < 0.01$). Multiple comparisons were performed by one-way ANOVA followed by Dunnett test. Bars indicate SD

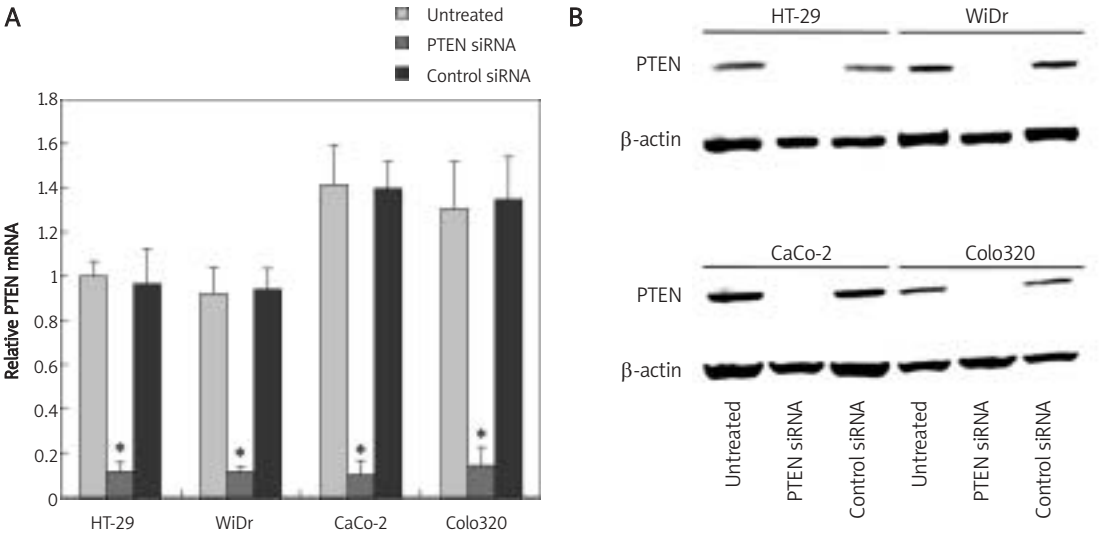

Figure 2. siRNA blockage of PTEN expression. Knockdown of PTEN by PTEN siRNA was confirmed by real-time qPCR (A) and immunoblotting (B) in all four colon cancer cell lines. siRNA duplex oligoribonucleotides were transfected into cells for 12 or 48 h, the total RNA and proteins were extracted, and then real-time PCR and Western blot were performed. Multiple comparisons were made by one-way ANOVA followed by Dunnett test. Bars indicate SD

**p < 0.01 compared with control. Re-probing with an anti-β-actin antibody served as a control*

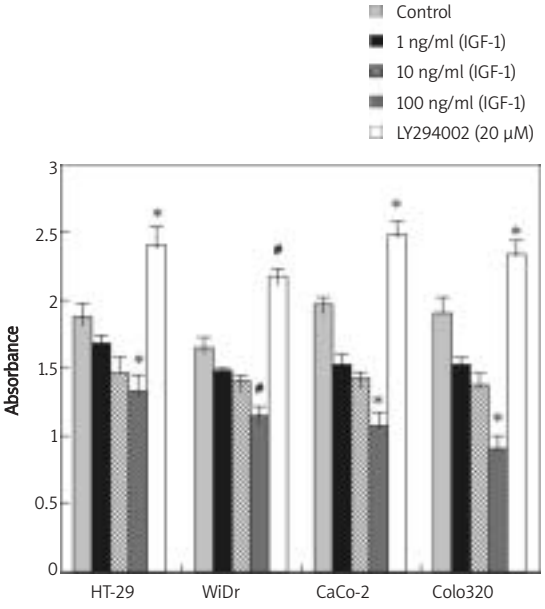

Figure 3. IGF-1 effect on PTEN phosphorylation. IGF-1 inhibition of the phosphorylation of PTEN protein in cultured medium by colon cancer cell lines was examined by ELISA assay. Cells were cultured without IGF-1 (control, light columns), and with 1 ng/ml (black columns), 10 ng/ml (light striped columns), 100 ng/ml of IGF-1 (black striped columns) and 50 μM of PI3K inhibitor LY294002 (striped columns). Multiple comparisons were performed by one-way ANOVA followed by Dunnett test. Bars indicate SD

*#p < 0.05, *p < 0.01 compared with control*

phosphorylation in a concentration-dependent manner; 100 ng/ml of IGF-1 significantly lowered phospho-PTEN levels in the HT-29, CaCo-2 and Colo320 cell lines ($p < 0.01$), as well as in the WiDr cell line ($p < 0.05$), as compared with the control. In contrast, PTEN phosphorylation was significantly enhanced by treatment with the PI3 kinase inhibitor LY294002 in HT-29 and CaCo-2 cells ($p < 0.01$) when compared with control cells (Figure 3).

Effect of IGF-1 on the proliferation of human colon cancer cells

We next investigated colon cancer cell proliferation with and without treatment by PTEN siRNA. We also examined the proliferative effects of IGF-1 over a range of concentrations. We found that IGF-1 enhanced proliferation of the four cell lines in a dose-dependent manner. The growth of both PTEN siRNA transfected cancer cells and untransfected cells was significantly enhanced by 100 ng/ml of IGF-1 when compared with controls ($p < 0.01$) (Figures 4). The addition of LY294002, an inhibitor of PI3K, inhibited the proliferation of cancer cells (HT-29, CaCo-2 and Colo320, $p < 0.01$ and WiDr, $p < 0.05$ compared with control) (Figure 4A). For cells pre-treated with PTEN siRNA, the proliferative capability was enhanced more than siRNA control cells ($p < 0.01$ for HT-29 and $p < 0.05$ for WiDr and Colo320, Figure 4B).

The roles of IGF-1 and PTEN in the invasive behaviour of colon cancer cells

After pre-treatment (or no treatment) with either PTEN siRNA or control siRNA, colon cancer cells were cultured with or without IGF-1 and with or without LY294002 for 24 h. At that point, the invasive capability was assessed. IGF-1 was found to enhance the invasiveness of colon cancer cells in a concentration-dependent manner. IGF-1 at 100 ng/ml was the most effective ($p < 0.01$, Tables IA and IB). Invasive capability was higher in PTEN siRNA transfected cells than either untreated cells or control siRNA-treated cells ($p < 0.01$ in HT-29, and CaCo-2 cells $p < 0.05$, Table IB). On the other hand, the invasive ability was blocked by LY294002 in PTEN siRNA cancer cells. There was a statistical difference between the colon cancer cells HT-29 and WiDr cells ($p < 0.05$) or Colo320 cells ($p < 0.01$) compared with siRNA control cells (Table 1B).

Table IA. IGF-1 effect on invasion by colon cancer cells

Cell line	Relative number of invading cells [%]			
	Untreated	IGF-1 [ng/ml]		
		1	10	100
HT-29	100 ±9.2	112.3 ±8.7	136.7 ±17.3#	159.0 ±13.4*
WiDr	100 ±12.2	107.3 ±16.8	121.5 ±20.3	162.6 ±14.9*
CaCo-2	100 ±11.5	139.3 ±16.4	146.9 ±11.2	152.0 ±27.2*
Colo320	100 ±11.1	107.5 ±10.0	117.5 ±12.9	148.4 ±23.5*

Colon cancer cells were treated with different concentrations of IGF-1 and incubated for 24 h. Cell invasion was then measured by the Matrigel assay. Statistical significance was tested by one-way ANOVA followed by the Dunnett test. Statistical significance was inferred if $p < 0.05$. All data are expressed as mean ± SD
*#$p < 0.05$, and *$p < 0.01$ when compared with untreated cells*

Table IB. Effect of PTEN siRNA and IGF-1 on invasiveness of colon cancer cells

Cell line	Relative number of invading cells [%]					
	PTEN siRNA	Control siRNA	LY294002	PTEN siRNA + IGF-1 [ng/ml]		
				1	10	100
HT-29	147.3 ±16.7	100 ±11.3	70.6 ±13.2#	124.7 ±17.3	132.5 ±17.3	189.0 ±21.3*
WiDr	128.5 ±9.63	100 ±12.0	75.1 ±10.7*	117.2 ±10.2	123.0 ±16.3	176.3 ±22.2*
CaCo-2	134.0 ±18.0	100 ±16.4	61.3 ±10.1*	131.2 ±15.2	130.2 ±12.8	153.7 ±30.6*
Colo320	111.9 ±9.14	100 ±10.4	59.1 ±10.1*	113.1 ±20.3	109.6 ±21.5#	156.4 ±15.0*

After transfection with PTEN siRNA or control siRNA, colon cancer cells were treated with different concentrations of IGF-1 and incubated for 24 h, followed by measurement of cell invasion by a Matrigel assay. Statistical significance was tested by one-way ANOVA followed by the Dunnett test. Statistical significance was inferred if $p < 0.05$. All data are expressed as mean ± SD
*#$p < 0.05$, and *$p < 0.01$ when compared with the control siRNA group*

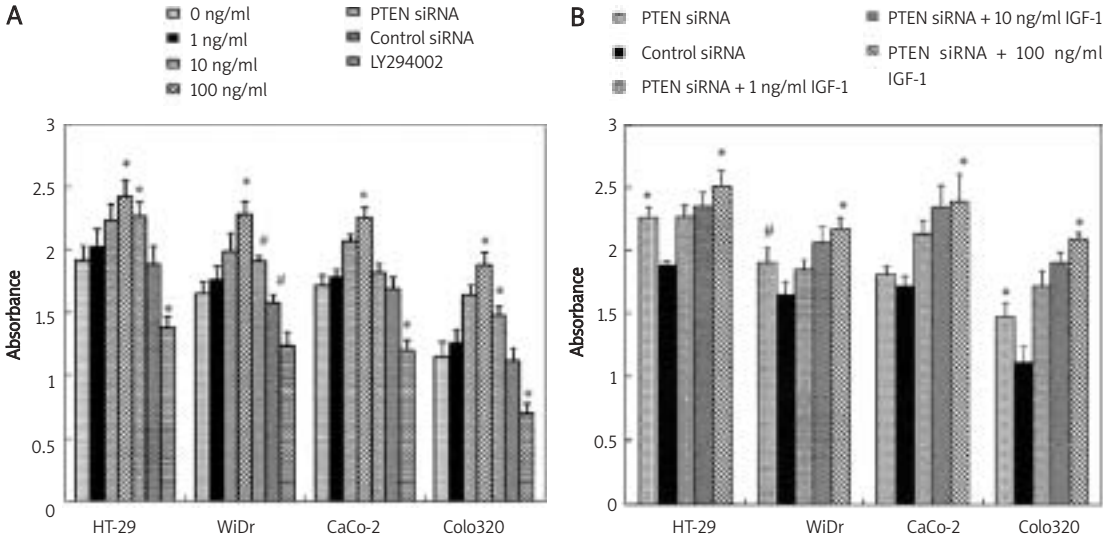

Figure 4. IGF-1 enhanced the proliferation of colon cancer cells. (A) All five colon cancer cell lines were cultured in the presence or absence of IGF-1 and/or the PI3K inhibitor LY294002 for 72 h. Cell proliferation was determined by the Premix WST-1 Cell Assay System and absorbance was read at 450 nm. The reference wavelength is 690 nm. (B) Colon cancer cells which were transfected with siRNA duplex oligoribonucleotides for 48 h, then cultured in medium without IGF-1 (control, light columns), or with IGF-1 at 1 ng/ml (black columns), 10 ng/ml (light striped columns), 100 ng/ml (black striped columns), 50 μM of PI3K inhibitor LY294002 (striped columns) for 72 h. Multiple comparisons were performed by one-way ANOVA followed by the Dunnett test. Bars indicate SD
*#$p < 0.05$, *$p < 0.01$, compared with control (0 ng/ml)*

Effect of IGF-1 antibody on colon cancer cell apoptosis

Colon cancer cells were treated with IGF-1 antibody, LY294002, PTEN siRNA or control siRNA and incubated for 24 h, at which point apoptosis was measured by the CaspACE™ assay. IGF-1 antibody and LY294002 significantly increased apoptosis of colon cancer cells ($p < 0.01$ and $p < 0.05$, respectively, when compared with control). In contrast, the apoptosis of colon cancer cells was inhibited by PTEN siRNA transfection ($p < 0.01$ in CaCo-2 and $p < 0.05$ in HT-29, WiDr and Colo320 cells compared with siRNA control cells) (Figure 5).

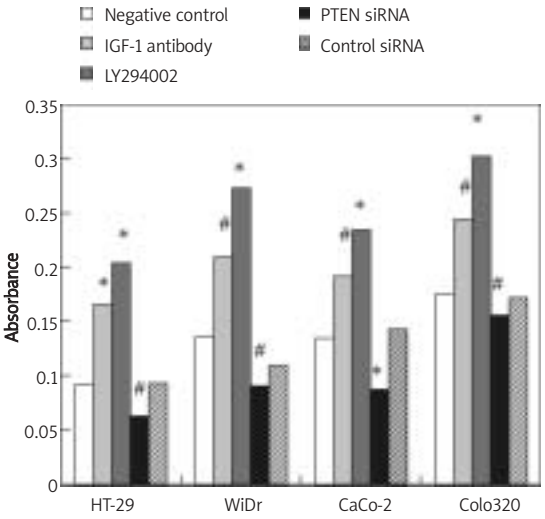

Figure 5. Effects of anti-IGF-1 antibody, LY294002 and PTEN siRNA on apoptosis in colon cancer cells. Colon cancer cells were treated with anti-IGF-1 antibody, PI3K inhibitor (LY294002), PTEN siRNA, or control siRNA and incubated for 24 h, followed by detection of apoptosis using the CaspACE™ Assay System, with absorbance being measured in the wells at 405 nm. Statistical significance was tested by one-way ANOVA followed by Dunnett test. The *p*-values indicate statistical significance between control and experimental data sets. Bars indicate the SD
*#p < 0.05, *p < 0.01 compared with control*

Activation of the PI3K and Akt signalling pathway after IGF-1 stimulation in human colon cancer cells

We used the colon cancer cell lines to examine the activation of the PI3K/Akt signalling pathway, a downstream target of IGF-1. IGF-1 treatment increased PI3K phosphorylation in a dose-dependent manner in HT-29 cells (Figure 6A). We also examined the response to IGF-1 in HT-29 cells which had been transfected with PTEN siRNA. The data indicate that phosphorylation of PI3K was enhanced in PTEN siRNA transfected cells more than in untransfected cells (Figure 6B). This result demonstrates that PTEN protein could reduce PI3K phosphorylation in colon cancer cells.

The Akt kinase activity of colon cancer cells was remarkably enhanced by IGF-1 stimulation in a time-dependent manner (Figure 7A). Stronger activation of Akt kinase activity was observed in HT-29 cells which had been transfected with PTEN siRNA (Figure 7B). In contrast, LY294002 suppressed Akt kinase activation (Figure 7C). Not only does IGF-1 stimulate Akt kinase activity, but that stimulation is enhanced by treatment with PTEN siRNA.

Effect of IGF-1 on the activation of the transcription factor c-jun

The Akt signalling pathway is known to activate AP-1, suggesting that control of invasive behaviour in colon cancer cells might also depend on AP-1. To determine whether AP-1 might be involved in mediating some of the effects of IGF-1 in colon cancer cells, we looked at the effects of IGF-1 and/or PI3/Akt kinase inhibitors on the activation of AP-1

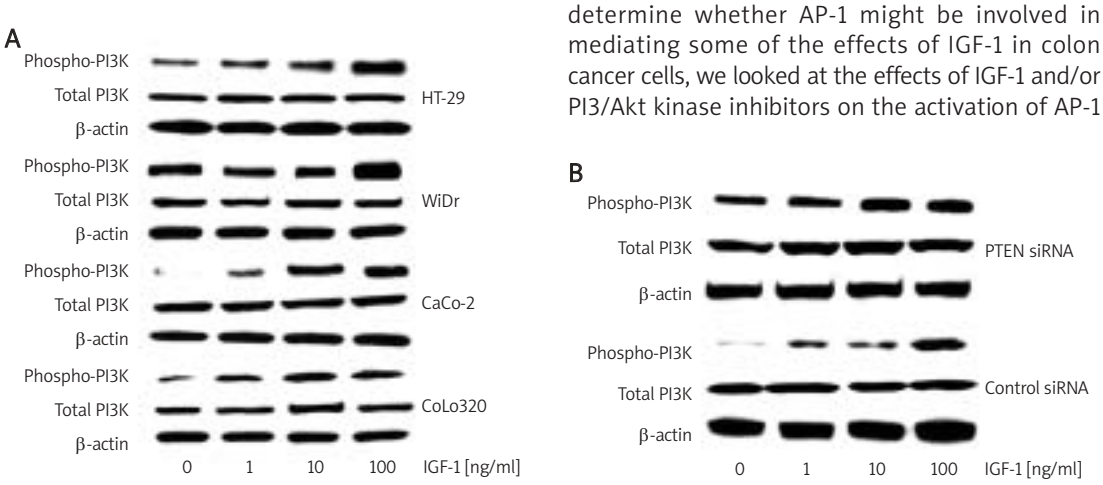

Figure 6. Phosphorylation of Akt in HT-29, WiDr, CaCo-2, Colo320, and PTEN siRNA transfected HT-29 cells. (A) Colon cancer cells were treated with 100 ng/ml of IGF-1 and incubated at 37°C and 5% CO_2 for 15, 30, or 60 min. The cells were collected from each time point, lysed by lysis buffer, and immunoblotted with a phospho-Akt antibody as described in Material and methods. Detection of total Akt levels served as a loading control. (B) Phosphorylation of Akt and total Akt in the PTEN siRNA transfected HT-29 and control siRNA HT-29 cells. β-actin served as a loading control

in these cells. Nuclear extracts were prepared from colon cancer cells which had been pre-treated for 60 min with PI3K/Akt inhibitors and then stimulated overnight with IGF-1 (100 ng/ml). The nuclear extracts were separated by SDS-PAGE, transferred to membranes, and the membranes probed with antibody directed against phospho-c-jun and total c-jun. We found that phosphorylation of c-jun was enhanced in PTEN siRNA transfected cells. Furthermore, the IGF-1-mediated increase in nuclear phospho-c-jun was inhibited by 50 µM PI3K inhibitor (LY294002) and Akt kinase inhibitor. These data indicate that IGF-1 regulates AP-1 nuclear transcriptional activity and suggest that AP-1 could participate in the regulation of invasive behaviour by these cells.

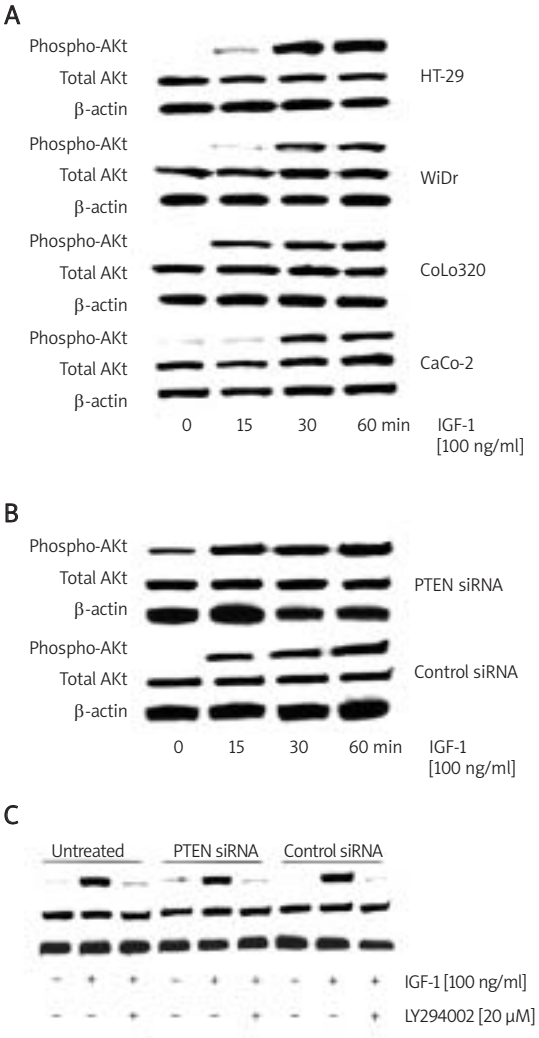

Figure 7. IGF-1-induced phosphorylation of PI3K in human colon cancer cell lines. (A) Ht-29, WiDr, CaC-2 and Colo320 cells were treated with different concentrations of IGF-1 (1, 10 and 100 ng/ml) and cultured for 15 min. The cells were gathered and lysed by lysis buffer. 30 µg of lysed protein were used to do immunoblotting with a phospho-PI3K antibody. Detection of total PI3K levels served as a loading control. (B) Expression of phospho-PI3K and total PI3K in both PTEN siRNA treated HT-29 and PTEN siRNA control treated HT-29 cells. (C) LY294002 inhibited Akt expression in untreated, PTEN siRNA transfected and control siRNA HT-29 cells

Discussion

Epidemiological studies have shown that high levels of IGF-1 are associated with increased risk for several common cancers, including those of breast, prostate, lung and colorectal cancers [32, 33]. Functionally, IGF-1 not only stimulates cell proliferation but also inhibits cell apoptosis. In this study, we demonstrated that IGF-1 induced the suppression of phosphorylated PTEN and enhanced both proliferation and invasion through the IGF-1/PI3K/PTEN/Akt/AP-1 signalling pathway activated in colon cancer cells. Similarly, knockdown of PTEN expression with siRNA interference also led to the enhancement of PI3K/Akt/AP-1 signalling, strongly implicating PTEN as a regulator of Akt signalling in these cells.

PTEN functions as a dual-specificity phosphatase that recognizes lipid substrates, the PI3K products PI-3,4P2 and PI-3,4,5P3, as well as protein substrates [34]. Several studies have demonstrated that the overexpression of PTEN inhibits cell growth in a variety of cancer cell lines [7, 12, 35]. Furthermore, it is well known that PTEN is suppressed in a variety of cancers and that PTEN protein plays an important role in the carcinogenesis of multiple

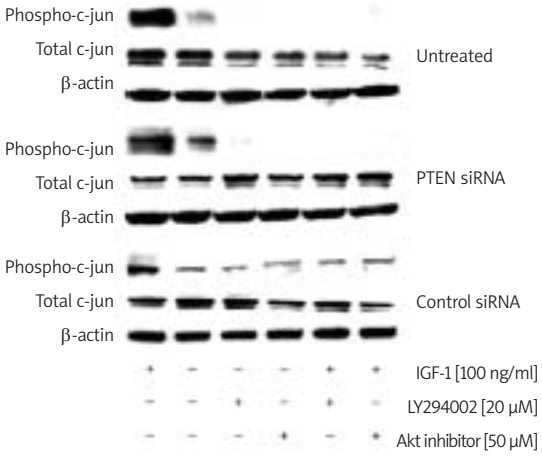

Figure 8. Effect of Akt and PI3K inhibitor on IGF-1-induced AP-1. HT-29, HT-29 transfected with PTEN siRNA, and transfected with control siRNA cells, after being pre-treated with 50 µM Akt inhibitor and 50 µM LY294002 for 1 h, were incubated with 100 ng/ml IGF-1 for 8 h, and nuclear extracts from the cells were immunoblotted using the c-jun antibody. Detection of total c-jun levels served as a loading control

human cancer cells, including colorectal cancer [36]. PTEN modulates cell growth and survival by negatively regulating PI3K/Akt, leading to cell cycle inhibition [37]. PTEN expression is decreased in colorectal cancers compared with its expression in polyps and normal mucosa. This is consistent with evidence suggesting that PTEN expression is decreased in approximately 40% of colorectal cancers, often in association with a PTEN mutation or deletion [38]. In addition to colorectal cancer, the loss or reduced expression of PTEN has been found to occur in other cancers, most notably breast, prostate and gastric carcinomas [39, 40]. Furthermore, the expression of PTEN protein was found to be decreased in the distal colon and rectum in animal studies [41]. Overexpression of PTEN in colorectal cancer cells has been found to result in cell cycle arrest and enhanced cell death through the inhibition of PI3K [42]. It is interesting to speculate whether decreased PTEN expression may contribute to propensity for cancer in the more distal colon and rectum. In our results, the expression levels of PTEN mRNA are significantly reduced in the highly liver metastatic colon cancer cell line HT-29 than in the low liver metastatic colon cancer cell lines CaCo-2 and Colo320 using real-time PCR. In colorectal cancer, this is the first report on the relationship between expression levels of PTEN mRNA and potential of liver metastasis in colon cancer cell lines.

Akt is a downstream target of PI3K and the PI3K/Akt pathway has recently been recognized as one of the most important signals ensuring protection against apoptosis [43]. Consistent with the role of PTEN in the PI3K/Akt signalling pathway, several lines of evidence have suggested that PTEN regulates IGF-1/IGFR-induced Akt activity, thereby modulating IGF-1GFR-mediated cell proliferative and antiapoptotic effects in a variety of cells [4]. In addition to its autocrine/paracrine effects, IGF-1 has been reported to exert an effect on colon cancer by an endocrine mechanism. Long-time prospective studies have clearly shown that high circulating levels of IGF-1 and low levels of IGFBP-3 are associated with a higher risk of developing colorectal cancer [44]. Furthermore, still other studies have demonstrated that PTEN downregulates IGF-1I, IGF-1R and IGF-binding protein expression in hepatoma, prostate and gastric cancer cells, respectively, suggesting that the anti-proliferative effect of PTEN is, at least in part, mediated through the regulation of expression of components of the IGF system [45-47]. These reports suggested that PTEN inhibits the biological function of not only endocrine and paracrine-derived IGF-1, but also autocrine-derived IGF-1. The present study has used RNA interference to systematically examine the role of PTEN in proliferation, invasion and apoptosis in colon cancer cell lines. We found that loss of PTEN can enhance proliferation and invasion and thereby the invasive potential is promoted. This suggests that the lower expression of PTEN in high liver metastatic cell lines may be a key reason those lines are more metastatic. Furthermore, the paracrine-secreted and autocrine-secreted IGF-1 blockage of PTEN activates in a dose-dependant manner; in other words, there is a correlation between the reduction in active PTEN and increase in downstream signalling and behaviour. PTEN suppression results in the activation of PI3K and its downstream target, the serine/threonine kinase Akt, by promoting its phosphorylation at the residue Ser473. Active Akt then phosphorylates and activates the transcription factor AP-1. When stimulated, AP-1 binds to transactivation promoter region TREs [12-*O*-tetradecanoylphorbol-13-acetate (TPA) response elements] and induces transcription of several genes involved in cell proliferation, metastasis, and metabolism [48]. PI3K inhibitor activated PTEN phosphorylation, in turn blocking PI3K and downstream targets, and consequently inhibited the proliferation and invasion in colon cancer cells.

To better investigate the mechanism by which PTEN affects metastatic potential in colon cancer, we evaluated the knockdown of PTEN by RNA interference-induced PTEN gene silencing, and found that blockage of PTEN expression not only enhanced the activity of PI3K and its downstream targets, Akt, and AP-1, but also promoted proliferation and invasion in colon cancer cell lines.

In conclusion, we have demonstrated that activation of the PI3K/Akt/AP-1 pathway by downregulating PTEN leads to enhanced proliferation and invasion in colon cancer cells. Inhibition of IGF-1/IGF-1R signalling may be one approach by which to enhance PTEN phosphorylation, which inhibits colon cancer cell growth. Activation of the IGF-1/IGF-1R system has recently been shown to be a critical event in the development of several murine and human tumours. The results reported herein indicate that inhibition of PI3K phosphorylation may be a major mechanism by which IGF-1 antibody and LY294002 inhibit cancer cell proliferation and invasion and induce apoptosis. Based on our findings, we speculate that PTEN suppresses cell growth, at least in part, through disturbing the function of IGF-1 in colon cancers.

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