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Research Article

Down-regulation of PINCH-1 Reduces Expression of EGFR, ERK1/2 and c-Myc, and Leads to Loss of Cell Viability in Colon Cancer Cells

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Abstract

Particularly interesting new cysteine-histidine rich protein (PINCH) is related to poor outcome in colorectal cancers. Here, the relationship between PINCH-1 and cell survival in colon cancer cells was analyzed and the signaling pathways regulated by PINCH-1 by using PINCH-1 siRNA. KM12C cells were treated with PINCH-1 siRNA or control siRNA. Cell number was analyzed by crystal violet staining and caspase-3 activity was assessed using a fluorescent substrate. PINCH-1 extra- and intracellular pathways in KM12C cells were investigated, using phospho-kinase/phospho-receptor tyrosine kinase (RTK) antibody arrays. The expression of c-Myc was evaluated by Quantitative real-time PCR (qPCR) and Western blot analysis. Cell number was significantly decreased ($P=0.003$) and the caspase-3 activity increased ($P=0.019$) in PINCH-1 depleted KM12C cultures compared to siRNA cultures. In PINCH-1 silenced KM12C cells, the levels of EGFR and ERK1/2 were significantly decreased ($P=0.008$, $P=0.003$, respectively) compared to their controls, as were the c-Myc mRNA and protein expressions ($P=0.0073$, $P=0.0002$, respectively). Down-regulation of PINCH-1 reduced the cell survival and lowers the levels of EGFR, ERK1/2 and c-Myc in colon cancer cells. PINCH-1 is essential for cell survival, and may be a future target for anticancer therapy.

Keywords: PINCH; Cell survival; Colon cancer; EGFR; ERK; c-Myc

Introduction

Particularly interesting new cysteine-histidine rich protein (PINCH, also known as LIMS1) is connected to integrins on the cell surface and forms a complex

with integrin-linked-kinase (ILK) and parvin. The expression of PINCH increases from normal mucosa to tumor to metastasis, and is related to worse outcome in colorectal cancers [1,2]. By binding to downstream effectors, PINCH regulates cell proliferation, spreading, motility and survival [3-5].

The epidermal growth factor (EGF) is a well-known growth factor that plays an important role in tumor development and regulates cell division, proliferation and apoptosis by binding to its receptor EGFR.

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Recently, it was shown that EGFR was associated to PINCH via non-catalytic region of tyrosine kinase adaptor protein 2 (Nck-2) [6]. Binding of EGF to the EGFR activates the tyrosine kinase on the cytoplasmic domain of the EGF receptor which in turn activates the downstream intracellular rat sarcoma/extracellular signal regulated kinase (Ras/ERK) pathway. Previously, it was shown by Chen et al (2008) that PINCH was associated to Ras/ERK by studying fibrosarcoma cell lines [7]. PINCH has also been shown to be connected to other intracellular effectors as protein kinase B/Akt (PKB/Akt) who plays an important role in tumor cell proliferation and progression [8,9]. Signal transducer and activator of transcription (STAT) mainly function as a DNA binding cofactors for transcription in the nucleus [9]. The STAT proteins are activated by many cytokines and growth factors, and play a key role in cell survival, proliferation, and differentiation. In tumors, a dysfunction of STAT leads to increased angiogenesis, enhanced cell survival, and immunosuppression [10].

C-Myc controls key cell functions like proliferation, differentiation and apoptosis and has been shown to play an important role in tumor carcinogenesis [11]. As far as we know, only one previous study has analyzed the relationship between PINCH and c-Myc in tumors. In that study, performed by ours on colon cancer cells the expression of PINCH and c-Myc tended to covary, but no significant interaction was found [12]. Since both PINCH and c-Myc is involved in the regulation of cell proliferation and apoptosis, the relationship between PINCH-1 and c-Myc was further analyzed in this study on PINCH-1 depleted colon cancer cells.

Several studies have shown strong relationships between different extra- and intracellular proteins and PINCH in relation to apoptosis and cell survival [3,6,7,13], but no previous study has systematically analyzed the connection between the PINCH-1 expression and the phosphorylation status of a large number of cell signaling proteins. In order to simultaneously analyze several extra- and intracellular proteins for their relationship with PINCH-1, phospho-kinase/phospho-receptor tyrosine kinase (RTK) membrane-based antibody arrays were used in this study.

The relationship between PINCH and radiation has been studied by us and by others. In one of our previous studies on rectal cancer we observed a significant

difference in survival between RT treated patients with weak and strong PINCH expression, but no difference was found in patients that did not receive RT, suggesting that there might be a relationship between PINCH, RT and survival [12]. Interestingly, others have shown that PINCH-1 is related to enhanced radio-resistance *in vitro* [8]. In this study, the relationship between PINCH, cell survival and radiation was further investigated in colon cancer cells by using PINCH-1 siRNA.

The aim of this study was to investigate the relationship between PINCH-1 and cell survival in PINCH-1 depleted colon cancer cells, and to further explore the signaling pathways controlled by PINCH-1. Moreover, the association between the expression of PINCH-1 and radiation response in colon cancer cells was studied.

Materials and Methods

Cell culture

The colon cancer cell lines KM12C, KM12SM and KM12L4a [14,15], was kindly provided by Prof. IJ Fidler (MD Anderson Cancer Center, Texas University, Houston, TX, USA). Initially, a western blot analysis was performed, which showed that PINCH was equally expressed in KM12C, KM12SM and KM12L4a [12]. The KM12C cell line, which is derived from a patient with stage II colon cancer, was selected for further siRNA analyses since it is the cell line that mostly resembles primary tumor cells. The KM12C cells were cultured in Eagle's minimum essential medium (EMEM) supplemented with 50 IU/ml penicillin, 50 µg/ml streptomycin (GIBCO, Invitrogen, Paisley, UK) and 10% fetal bovine serum (FBS) (ATCC, Manassas, USA) in T-75 cm² flasks at 37°C, 5% CO₂.

Small interfering RNA (siRNA) transfection

The KM12C cells were seeded in wells (100 mm diameter) 2×10⁶ cells/well in 10 ml complete medium without antibiotics and transfected 24 h later with 25 nM PINCH-1 siRNA (FlexiTube, Hs_LIMS1_7, Gene accession NM_001193482, QIAGEN) or 25 nM AllStars negative control siRNA (QIAGEN), by using 2

μl/ml of the DharmaFECT² (Thermo Scientific, Basel, Switzerland) transfection reagent. The cultures were further incubated. Down-regulation of PINCH-1 was confirmed by quantitative real-time PCR (qPCR) and Western blot analysis at 72 h after transfection. For assessment of the influence of PINCH-1 silencing on cell survival/proliferation and response to radiotherapy, cultures were trypsinized and reseeded at 500 cells/well into 12-well plates at 24 h after transfection.

Crystal violet assay

Fourteen days after siRNA treatment (after changing medium at day 10) the relative cell number was assessed by crystal violet staining. The cells were fixed in 4% paraformaldehyde (PFA) for 20 min at room temperature and stained with 0.04% crystal violet in 1% ethanol (20 min, room temperature). The plates were then washed extensively under running tap water and air dried. After solubilization of the samples in 1% SDS (800 μl/well), the absorbance at 550 nm was measured using a microtiter plate reader Synergy 2/BioTek (BioTek instruments, Winooski, VT, USA). By each measurement there were 3 empty wells with no cells, the empty wells were fixed and stained in the same way as the wells including cells. These wells were used as blank to reduce background. All experiments were performed 3 times in triplicates.

Caspase-3 activity

Caspase-3 activity was measured, using the fluorescent substrate Ac-DEVD-AMC (BD Pharmingen, BD Biosciences, San Jose, CA, USA) according to the manufacturer's instructions. Cell lysates were harvested at 24 and 48 h after siRNA transfection and incubated with the substrate for 1 h at 37°C. Thereafter, the fluorescence was analyzed at an excitation wavelength of 380 nm and an emission wavelength of 460 nm in a Victor X4 plate reader (PerkinElmer, Upplands Väsby, Sweden). The fluorescence was correlated to total protein as determined by the Pierce[®] BCA protein assay (Thermo Scientific). All experiments were repeated 3 times.

Radiation procedure

At 72 h after siRNA treatment the KM12C cells were irradiated with photons from a 6 MeV linear accelerator (Varian Clinac iX, Varian Medical Systems, Palo Alto, CA, USA). The field size was 30 cm×30 cm and the distance between the cells and the radiation source was 100 cm. The cells were exposed to single doses of 0, 2, 4, and 6 Gy at room temperature. Both irradiated and non-irradiated cultures were immediately given fresh medium and re-incubated. At day 14 after siRNA treatment, the relative cell number of irradiated and non-irradiated cultures was assessed by crystal violet staining. All experiments were performed 3 times in triplicate.

Protein arrays

Two membrane-based antibody arrays, the human phospho-RTK array kit (ARY001B) and the human phospho-kinase array kit, ARY003B (R&D Systems, Inc, Minneapolis, MN, USA) were used to study the influence of PINCH-1 silencing on cellular signaling pathways. KM12C cells were harvested 72 h after siRNA treatment according to the array kit instructions, and a 300 μg aliquot of cell lysate was incubated with each membrane. The proteins were visualized, using the Chemi Reagent Mix and detected by X-ray film. All the spots on the human phospho-kinase/phospho-RTK arrays were measured by densitometry. The mean values from the duplicates of each protein were used for further statistical calculations. All experiments were performed 3 times.

Western blot analysis

Cells were washed in cold PBS and lysed in RIPA buffer (65 mM Tris-HCl [pH 7.5], 155 mM NaCl, 1% NP40, 2.5 mM sodium deoxycholate, 1 mM EDTA, 60 μM NaF, 60 μM Na₃Va₄ and 2% protease inhibitor cocktail without metal chelating reagents [cat #:8430; Sigma-Aldrich, Stockholm, Sweden]). The lysate was incubated on ice for 20 minutes and centrifuged at 20800 RFC, 4°C for 10 minutes. Further, the protein concentration was determined with the colorimetric Pierce[®] BCA protein assay reagent (Thermo Scientific). Samples containing 30 μg of proteins were separated

by electrophoresis in a Mini-PROTEAN TGX™ precast 12% gel (Bio-Rad, Hercules, CA, USA) for 55 min at 200 V. The separated proteins were transferred to a 0.2 mm polyvinylidene fluoride transfer membrane (PVDF), using the Trans-Blot® Turbo™ Transfer System (BioRad). The membranes were blocked with 5% milk in tris-buffered saline tween 20 (TBST) for 1 h at room temperature and incubated with a primary anti-PINCH-1 antibody (1 µg/mL; BD Transduction Laboratories, Lexington, KY, USA), as follows: a p-ERK1/2 (1:1000, cat #:9101, Cell Signaling), a c-Myc (1:1000, cat #:5605, Cell Signaling), a p-STAT3^{S727} (1:1000, cat #:MAB4934, R&D Systems), a p-STAT5a/^bY694/Y699 (1:1000, cat #:AF4190, R&D Systems), or ap-AktSer⁴⁷³ antibody (1:1000, cat #:9271, Cell Signaling) in TBST and 1% milk (for PINCH-1), 5% BSA (for ERK1/2, Akt, c-Myc), 5% milk (for STAT3, STAT5b) overnight at 4°C. The membranes were washed and incubated with a horse radish peroxidase (HRP) conjugated polyclonal goat anti-mouse secondary antibody (1:5000) or an anti-rabbit (1:1000) antibody (DakoCytomation, Glostrup, Denmark) for 1 h at room temperature. Proteins were visualized by enhanced chemiluminescence (ECL; Amersham Biosciences/GE Healthcare, Little Chalfont, UK) and detected on X-ray film. To verify equal loading, the membranes were re-probed with a primary mouse monoclonal anti-β-actin antibody (1:5000; Sigma-Aldrich, Steinheim, Germany). For p-ERK1/2 and p-Akt, antibodies for detection of total protein, t-ERK1/2 (1:1000, cat #:9102, Cell Signaling) and t-Akt (1:1000, cat #:9272, Cell Signaling) were used as controls.

Quantitative real-time PCR (qPCR)

The amount of PINCH-1 and c-Myc mRNA was determined by qPCR. RNA was extracted from KM12C cells, using the RNA Blood Mini Kit (QIAGEN) and cDNA was transcribed using first strand cDNA synthesis kit (Roche, Mannheim, Germany). The expression of PINCH-1 and c-Myc mRNA was determined, using specific primers Hs00757864_m1 for PINCH-1 and Hs00905030_m1 for c-Myc (Applied Biosystems, Stockholm, Sweden). The qPCR reactions were performed in the 7500HT Fast Real-Time PCR System. The cycle threshold (CT) values of the gene of interest and the reference genes β-actin and GAPDH were used for further calculations, using the delta-delta CT

method [16]. Experiments were repeated three times with duplicate samples.

Statistical analyses

An independent *t*-test by group was used to test for the differences in total cell number, caspase activity and radiation response, and to evaluate the differences in the level of phosphorylation of phospho-kinases and phospho-RTKs in KM12C cells treated with PINCH siRNA and siRNA controls. The software program STATISTICA12.1 (StatSoft) was used for the calculations. A *P*-value < 0.05 was considered statistically significant.

Results

Down-regulation of PINCH-1 results in apoptotic cell death and low cell numbers in colon cancer cells

First the influence of siRNA-mediated downregulation of PINCH-1 on KM12C cell survival and proliferation (as assessed by crystal violet staining) was investigated. A reduction of PINCH-1 mRNA by approximately 90%, as well as a significant reduction at the protein level, was achieved by siRNA treatment (Figure 1A). The total number of cells was significantly reduced in cultures treated with PINCH-1 siRNA as compared to cultures treated with control siRNA as shown in Figure 1B (*P*=0.003). Next, caspase-3 activity was analyzed in order to clarify if the reduced cell numbers could be due to apoptotic cell death in response to PINCH-1 depletion. The caspase-3 activity was significantly increased in PINCH-1 siRNA treated KM12C cells compared to their corresponding controls both at 24 and 48 h after transfection (*P*=0.039 and *P*=0.019, respectively; Figure 1C).

Loss of PINCH-1 is associated with lower levels of phosphorylated EGFR, ERK1/2 and STAT5b

The phospho-kinase array (Figure 2A) and the phospho-RTK array (Figure 2B) were used to study the extra- and intracellular pathways downstream of PINCH-1. In PINCH-1 silenced KM12C cells, the

Figure 1: Down-regulation of PINCH-1 results in lower cell numbers and apoptotic cell death in KM12C colon cancer cells. KM12C cells were treated with PINCH-1 siRNA or control siRNA and gene silencing was verified by Western blot and qPCR (A). The relative cell number was assessed by crystal violet staining (B) and apoptotic cell death was measured by Caspase-3 activity (C). Data are expressed as mean SD.

amount of phosphorylated EGFR, ERK1/2 ^{T202/Y204, T185/Y187} and signal transducer and activator of transcription 5b (STAT5b^{Y699}) was significantly increased compared to their corresponding controls ($P=0.008$, $P=0.003$, $P=0.011$, Figure 2A-C). The level of signal transducer and activator of transcription 3 (STAT3^{S727}) was decreased ($P=0.007$), and the amount of phosphorylated Akt^{S473} was increased ($P=0.003$) as compared to their PINCH-1 silenced KM12C cultures. The targets of the arrays that were unaffected by PINCH-1 downregulation are listed in Tables 1 and 2.

Next, the results were verified by Western blot analysis (Figure 2D). It was confirmed that there is a difference in the level of expression of phosphorylated ERK1/2 and STAT5b after PINCH downregulation (Figure 2D). However, the differences in p-STAT3 and p-Akt could not be verified (data not shown). Unfortunately, none of

the four p-EGFR antibodies tested, p-EGFR^{Y1173} (mouse 1:1000, cat #:05-483, Millipore), p-EGFR^{Y1068} (mouse, 1:1000, cat #:2236, Cell Signaling), p-EGFR^{Y1173} (rabbit 1:1000, cat #:4407, Cell Signaling), p-EGFR^{Y1173} (rabbit 1:1000, cat #:44-794, Bio Source), yielded detectable results. Thus, the down-regulation of phosphorylated EGFR in PINCH-silenced cells could not be verified.

Down-regulation of c-Myc mRNA and protein in PINCH-1 silenced cells

The expression of c-Myc by using qPCR and Western blot were studied in KM12C cells treated with PINCH-1 siRNA or control siRNA. Interestingly, the c-Myc expression was significantly decreased both at the mRNA ($P=0.0073$, Figure 3A) and protein level ($P=0.0002$, Figure 3B) in PINCH-1 silenced cells compared to their controls.

Figure 2: Loss of PINCH-1 is associated with lower levels of phosphorylated EGFR, ERK1/2 and STAT 5b. The phosphorylation of several proteins was measured in KM12C cells treated with PINCH-1 siRNA or control siRNA by using phospho-kinase (A) and phospho-RTK (B) antibody arrays. Relative levels of EGFR, ERK1/2 and STAT5b measured by densitometry (C). An altered phosphorylation of ERK1/2 and STAT5b was observed on the blots (D). Data are expressed as mean SEM.

PINCH-1 silencing does not alter the radiotherapy response of KM12C cells

Next, the relationship between PINCH-1 expression and radiotherapy response was evaluated by measuring the total number of KM12C cells after treatment with or without PINCH-1 siRNA and with or without radiation. No significant differences in cell numbers were found between the PINCH-1 siRNA and the control siRNA treated cells irradiated with 2, 4-or 6 Gy, as shown in Figure 4.

Discussion

Here, it is shown that knockdown of PINCH-1 reduces cell number and increases caspase-3 activity in colon cancer cells compared to their corresponding siRNA controls (Figure 1B and C). These data suggest that PINCH-1 acts as a survival factor in these cells, and

that its depletion triggers apoptotic cell death. The relationship between PINCH and cell survival has previously been studied by others. Chen et al studied colon, breast, prostate and hepatocellular cancer cells and showed that the depletion of PINCH markedly increased caspase-3 activity and apoptosis in PINCH-1 silenced cells [7]. Fukuda et al showed that caspase-3 activity and apoptosis were significantly increased in PINCH depleted HeLa cells compared to control cells, suggesting that PINCH-1 was required to protect the cells from apoptosis [3]. Collectively, these data suggest that PINCH plays an important role in the regulation of tumor cell survival.

Recent studies have shown strong relationships between PINCH and several cell signaling components in relation to apoptosis and cell survival [3,6,7,13]. In the present study, an attempt was made to identify the extra- and intracellular pathways that may be implicated

KM12C

Figure 3: Down-regulation of c-Myc mRNA and protein in PINCH-1 silenced cells. The expression of c-Myc in KM12C cells with PINCH-1 siRNA or control siRNA analyzed by qPCR (A) and Western blot (B). Data are expressed as mean SD.

in the reduction in cell survival in PINCH-1 depleted colon cancer cells, using phospho-kinase/phospho-RTK arrays.

EGF is a well-known mitogenic extracellular growth factor that stimulates cell division by binding to its receptor EGFR. Previously, it was shown by Tu et al. [6] that EGFR is associated to PINCH via Nck-2 in human epidermoid carcinoma cells. In line with these findings, our data suggest that PINCH may regulate the activity of EGFR as the amount of phosphorylated EGFR was reduced in PINCH-1 depleted colon cancer cells compared to their corresponding siRNA controls (Figure 2B and C). This result makes us suggest, that the down-regulation of PINCH reduces the activity of phosphorylated EGFR which in turn leads to reduced cell survival and increased apoptosis.

It has also been shown in fibrosarcoma and breast cancer cells that PINCH regulates apoptosis and cell survival via the RAS/ERK pathway [7,13]. This is

consistent with the current results which show that the level of active phosphorylated ERK1/2 was reduced in PINCH siRNA treated colon cancer cells compared to their corresponding siRNA controls (Figure 2A,C and D).

C-Myc has been shown to play an important role in tumor carcinogenesis where it controls key cell functions like proliferation, differentiation and apoptosis [11]. C-Myc is known to act as a transcription factor downstream of ERK signaling, and ERK is known to be regulated by EGFR [11,17]. Recently, it was shown that the expression of PINCH and c-Myc covaried in colon cancer cells, suggesting that there might be some association between PINCH and c-Myc [12]. In this study, it is shown that the expression of c-Myc is significantly decreased in PINCH-1 silenced cells compared to their corresponding controls (Figure 3A and B), suggesting a potential relationship between PINCH and c-Myc. However, further studies are needed to confirm this relationship.

Figure 4: PINCH-1 silencing does not affect the radiotherapy response of KM12C cells. KM12C cells treated with PINCH-1 siRNA or control siRNA, subjected to 2, 4 or 6 Gy of radiation. Data are expressed as mean SD.

It is also demonstrated that the down-regulation of PINCH-1 reduces the amount of active phosphorylated STAT5b (Figure A,C and D). The STAT proteins are activated in response to many cytokines and growth factors, and play a key role in cell survival, proliferation, and differentiation [10]. Dysregulation of STAT in tumors leads to increased angiogenesis, enhanced cell survival, and immunosuppression. STAT5b has been shown to be related to EGFR in cancers where EGFR uses the STATs as DNA binding cofactors for transcription in the nucleus [9]. Taken together, the down regulation of PINCH reduces the activity of EGFR and STAT5b which eventually leads to increased apoptosis and reduced cell viability. However, further expanded cell line studies are needed to clarify this issue.

The relationship between PINCH and radiotherapy in rectal cancer patients has previously been studied by ours. In that study it was shown that a strong PINCH expression was related to decreased survival in patients with radiotherapy treatment [2]. No such correlation was found in patients without radiotherapy treatment, indicating that PINCH expression could influence patient response to radiotherapy. PINCH

has also been associated to radio-resistance in *in vitro* studies [8]. Here, the relationship between PINCH-1, cell survival and response to radiotherapy in PINCH-1 silenced colon cancer cells was further studied. No significant differences in the total cell count were found between cultures treated with PINCH-1 siRNA and their corresponding controls after 2, 4, and 6 Gy of radiation, suggesting that the level of PINCH-1 expression does not directly affect the radio-sensitivity of KM12C cells.

In conclusion, the down-regulation of PINCH-1 in colon cancer cells lowers the amount of active phosphorylated EGFR, ERK1/2, and STAT5b, and expression of c-Myc, and this eventually results in loss of cell viability. Our data indicate that PINCH-1 is essential for cell survival in colon cancer cells and may be a future target for anticancer therapy.

Disclosure

The authors declare no conflict of interest.

Table 1: Targets of the ARY003B phospho-kinase array that were un-affected by PINCH-1 siRNA.

Protein (phosphorylation site)	
AMPK α 1 (T183)	p38 α (T180/Y182)
AMPK α 2 (T172)	p53 (S392)
β -Catenin	p53 (S46)
Chk-2 (T68)	p53 (S15)
c-Jun(S63)	p70 S6 Kinase (T389)
CREB (S133)	p70 S6 Kinase (T421/S424)
eNOS (S1177)	PDGF R β (Y751)
EGF R (Y1086)	PLC- γ 1 (Y783)
FAK (Y397)	PRAS40 (T246)
Fgr (Y412)	PYK2 (Y402)
Fyn (Y420)	RSK1/2/3 (S380/S386/S377)
GSK-3 α/β (S21/S9)	Src
Hck (Y411)	STAT2 (Y689)
HSP27 S78/S82	STAT3 (Y705)
HSP60	STAT5a (Y694)
JNK 1/2/3 (T183/Y185, T221/Y223)	STAT5a/b (Y694/Y699)
Lck (Y394)	STAT6 (Y641)
Lyn (Y397)	TOR (S2448)
MSK1/2 (S376/S360)	WNK1 (T60)
p27 (T198)	Yes (Y426)

Table 2: Targets of the ARY001B phospho-RTK array that was un-affected by PINCH-1 siRNA.

Protein		
Axl	HGF R	FGF R1
ALK	Insulin R	FGF R2 α
c-Ret	IGF-I R	FGF R3
Dtk	M-CSF R	FGF R4
DDR1	Mer	TrkC
DDR2	MSP R	VEGF R1
EphA1	MuSK	VEGF R2
EphA2	PDGF R α	VEGF R3
EphA3	PDGF R β	Flt-3
EphA4	ROR1	
EphA5	ROR2	
EphA10	RYK	
EphB3	SCF R	
EphB6	Tie-1	
ErbB2	Tie-2	
ErbB3	TrkA	
ErbB4	TrkB	

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