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PTEN inhibits the invasion and metastasis of gastric cancer via downregulation of FAK expression

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ABSTRACT

The tumor suppressor gene phosphatase and tensin homolog (PTEN) is essential in inhibiting tumor growth and metastasis. However, the mechanism by which PTEN restricts gastric cancer progression and metastasis remains largely elusive. Here we demonstrated that PTEN overexpression or knockdown in gastric cancer cells led to the downregulation or upregulation of focal adhesion kinase (FAK), and decreased or increased cell invasion, respectively. Moreover, FAK overexpression could rescue the inhibition of cell invasion by PTEN. These results were further confirmed in orthotropic gastric cancer nude mice model. In addition, in human gastric cancer tissues, PTEN protein level was conversely correlated with FAK protein level. Mechanistically, we found that PTEN inhibited PI3K/NF-κB pathway and inhibited the DNA binding of NF-κB on FAK promoter. Taken together, our data reveal a novel mechanism that PTEN inhibits the growth and invasion of gastric cancer via the downregulation of FAK expression and suggest that exploiting PTEN/PI3K/NF-κB/FAK axis is a promising approach to treat gastric cancer metastasis.

1. Introduction

PTEN (phosphatase and tensin homolog deleted on chromosome 10) is located on human chromosome 10 q23, a locus that is highly susceptible to loss of heterozygosity (LOH), and is recognized as one of the most frequently mutated tumor suppressors in a variety of human cancers [1,2]. In addition, germline mutation and somatic mutation of PTEN are associated with cancer predisposition syndromes, suggesting that the inactivation of PTEN plays an important role in tumorigenesis [3–5]. PTEN is known to negatively regulate PI3K signaling pathway due to its lipid phosphatase activity, thereby inhibiting the activation of downstream components such as AKT/protein kinase B (PKB) and the transcription factors including nuclear factor B (NF-κB) [3,6–8]. A large number of studies have indicated that PTEN-mediated tumor inhibition is related to the suppression of PI3K/AKT pathway, which is abnormally activated in human cancer to stimulate cancer cell proliferation, growth and survival [9–11].

However, several lines of evidence suggest the potential role of PTEN in tumor invasion and metastasis. Focal adhesion kinase (FAK) is a key molecule implicated in integrin signaling and contributes to cancer progression, invasion and metastasis [4,6,12,13]. PTEN could interact with and dephosphorylate FAK, leading to the inhibition of integrin-mediated cell spreading, cell migration and focal adhesion formation [4,10,14–16]. In addition, the suppression of FAK phosphorylation by PTEN is correlated with the inhibition of AKT phosphorylation [15]. Nevertheless, the detailed mechanisms by which PTEN regulates FAK and inhibits tumor invasion and metastasis are not completely understood.

In the present study, we used gastric cancer as the experimental model to investigate the role of PTEN in the regulation of FAK expression and function in tumor invasion and metastasis. Our results showed that overexpression of wild type PTEN downregulated FAK expression at both protein and mRNA levels, and suppressed gastric cancer cell invasion in vitro and in vivo. In addition, we found that these inhibitory effects of PTEN were mediated by the inhibition of PI3K/NF-kappaB pathway.

2. Materials and methods

2.1. Antibodies and reagents

Antibodies to β-actin, IKB were purchased from Santa Cruz Biotechnology. Monoclonal antibody (mAb) to FAK was from Millipore. AKT, PTEN, and P65 mAb were from Cell Signal Tech. FAK Tyr-397 (pY397), AKT Tyr-473 (pY473), IKB and phosphospecific antibodies were from Invitrogen. LY294002, MG132 and PFT-alpha P53 inhibitor were from

Abbreviations: PTEN, phosphatase and tensin homolog; FAK, focal adhesion kinase; AKT, protein kinase B; NF-κB, nuclear factor B or NF-kappaB; ChIP, chromatin immunoprecipitation; EMSA, electrophoretic mobility shift assay; DAPI, 4′,6-diamidino-2-phenylindole.

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Sigma. Plasmids of FAK (WT), FKA-F397, FAK-F925 were gifts from Prof. David D. Schlaepfer. Control–siRNA and PTEN–siRNA were from Santa Cruz Biotechnology.

2.2. Cell culture

MKN-28 human gastric cancer cells were obtained from China Center for Type Culture Collection (Wuhan, China). Early passage cells were used for all experiments and they were not re-authenticated. All cells were cultured in Dulbecco's Modified Eagle's Medium (DMEM) supplemented with 10% fetal bovine serum (FBS), 1 mM non-essential amino acids, 2 mM glutamine, 100 U/mL penicillin and 100 μg/mL streptomycin.

PTEN overexpression cell line (MKN-28–GFP–PTEN) and the control cell line (MKN-28–GFP) were generated by transfection of GFP–PTEN and GFP plasmid, respectively, followed by selection with 500 μg/mL G418 for 4 weeks. The individual colonies were isolated and expanded, and the overexpression of PTEN in these clones was confirmed by Western blot analysis.

2.3. Western blot analysis

Cells were homogenized in ice-cold lysis buffer (1× PBS, 1% Igepal CA-630, 0.5% sodium deoxycholate, 0.1% SDS, 10 mg/mL phenylmethylsulfonyl fluoride, 100 mM sodium orthovanadate, 60 μg/mL aprotinin, 10 μg/mL trypsin inhibitor and 10 μg/mL leupeptin), and then lysed on ice for 1 h. The lysate was centrifugated at 10,000 *g* for 10 min at 4 °C, the supernatants were collected and the protein concentration of the supernatant was measured using BCA protein assay (Pierce). Supernatants (20 μg) were separated on a 10% SDS-PAGE and immunoblot analysis was performed as described previously [17].

2.4. Real-time PCR

The total RNA was extracted by using Trizol (Invitrogen) according to the manufacturer's protocol and treated with RNase-free DNase (Promega) to eliminate genomic DNA contamination. cDNA was synthesized as described previously [18]. mRNA level was quantified with the real-time PCR system: 1× of IQ sybrGreen supermix (Bio-Rad) 200 nmol/L of each primer and 2.5 μL cDNA in a 25 μL reaction system. The primer sequences were as follows: FAK forward primer 5′AGCAGG CGGCCCAGGTTTACTGA3′, reverse primer 5′TGGGCTTGGGCGGTGCTT CATC3′, and PTEN forward primer 5′CGACGGGAAGACAAGTTCAT3′, reverse primer 5′AGGTTTCCTCTGGTCCTGGT3′. The reactions were repeated three times.

2.5. Immunohistochemistry

Immunohistochemistry was performed as described previously [19]. Briefly, tissues were deparaffinized by xylene and tissue cores were rehydrated. Antigen retrieval was performed by boiling in 10 mM sodium citrate (pH 6.0) for 5 min. Endogenous peroxidase activity was quenched with 0.3% hydrogen peroxide for 10 min, blocked, and then incubated with affinity-purified rabbit polyclonal FAK and PTEN antibodies overnight at 4 °C, followed by incubation with biotinylated goat anti-rabbit IgG for 30 min at room temperature.

2.6. In vitro invasion assay

Cells were suspended in DMEM containing 10% fetal bovine at a concentration of 1×10^5 cells/mL and seeded into the upper chamber of a 12-well chemotaxis chamber (Neuroprobe, Gaithersburg, MD). The in vitro invasion assay was performed as described previously [17].

2.7. Immunofluorescent staining

Immunofluorescent staining was performed as described previously [19]. Briefly, the cells were plated at low density onto 1% gelatin-coated glass coverslips until colonies of ~30 cells were formed (48–60 h). After overnight serum starvation, cells were transfected with plasmids by using Lipofectamine 2000 reagent (Invitrogen), according to the manufacturer's instructions. 24 h later, cells were fixed with 3.7% cold paraformaldehyde in PBS for 10 min, permeabilized with 0.5% Triton X-100 in PBS for 5 min, and blocked with 5% bovine serum albumin in PBS for 1 h. Then coverslips were incubated with primary antibody for FAK at 4 °C overnight and visualized using Alexa-fluor-488 goat anti-mouse IgG, Texas Red-phalloidin. The nuclei was stained with 4′,6-diamidino-2-phenylindole (DAPI).

2.8. Luciferase reporter gene constructs and luciferase assay

The 1220 base pair 5′-UTR of FAK gene was cloned into the empty luciferase reporter vector pEZX-PG04 (GeneCopoeia, Rockville, MD), generating a wild-type FAK UTR luciferase reporter construct pEZX-WT-UTR (P-1173 CCS-HPRM11594-PG04, GeneCopoeia). Truncations were made as follows: -723- +47 bp (P-723, CCS-HPRM11594-PG04-1, GeneCopoeia), -480- +47 bp (P-480, CCS-HPRM11594-PG04-2, GeneCopoeia), -350- +47 bp (P-350, CCS-HPRM11594-PG04-3, GeneCopoeia), -280- +47 bp (P-280, CCS-HPRM11594-PG04-4, GeneCopoeia), and -90- +47 bp (P-90, CCS-HPRM11594-PG04-5, GeneCopoeia). Mutations in the 5′-UTR of FAK were designed for two NF-κB binding sites of P-1173 and named as NF-κB up mutant (CCS-HPRM11594-PG04-6), NF-κB low mutant (CCS-HPRM11594-PG04-7) and NF-κB double mutant (CCS-HPRM11594-PG04-8), respectively. All constructs were verified by DNA sequencing. For the dual luciferase assay, Lovo cells were plated in triplicate into 12-well plates and co-transfected with 1 μg of the reporter construct and 15 pmol of pEZX-PG04-NC together with siRNA of other plasmids by using Lipofectamine 2000 (Invitrogen). Transfected cells were cultured and 24 h later, the supernatants were collected for luciferase assay using Dual Luminescence assay kit (GeneCopoeia MD) according to the manufacturer's instructions.

2.9. Electrophoretic mobility shift assay (EMSA)

EMSA was performed using the LightShift® Chemiluminescent EMSA Kit (Pierce, USA) according to the manufacturer's protocol. Biotin end-labeled oligonucleotides were synthesized and annealed to obtain double-stranded DNA fragments. The oligonucleotide sequences were described by Golubovskaya et al. [20]. DNA binding reactions were performed in 20 μL samples containing 20 fmol of biotin-labeled oligonucleotides, 5 mg of nuclear extracts, 2 mg of d(I-C), 2 μL of 10× binding buffer, 0.1 mM of EDTA, and 10% glycerol at room temperature for 20 min. For competitive EMSA, 100-fold dilutions of unlabeled oligonucleotides were added prior to the addition of the labeled probe. The samples were run on 6% non-denaturing polyacrylamide gels with 0.5 × TBE buffer and transferred to a nylon membrane (Amersham Biosciences, Pharmacia, UK) (presoaked it in 0.5 × TBE for at least 10 min.). A chemiluminescent detection method was used as described by the manufacturer, and the membranes were scanned by the Image Station 4000R (Kodak, USA).

2.10. Chromatin immunoprecipitation assay (ChIP)

ChIP assay was performed using the EZ ChIP Kit (Millipore), according to the manufacture's protocol. Solubilized chromatin was prepared from a total of 1×10^7 MKN-28 and cross-linked using 1% paraformaldehyde. After washing with PBS, the cells were resuspended in 300 μL of lysis buffer. DNA was sheared to small fragments by sonication, then incubated with Protein G agarose at 4 °C for 1 h. The recovered

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