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Original article

Loss of periplakin expression is associated with the tumorigenesis of colorectal carcinoma

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ABSTRACT

Periplakin (PPL), a member of the plakin protein family, has been reported to be down-expressed in urothelial carcinoma. The role of PPL in human colorectal cancer, however, remains largely unknown. Also little is known about the contribution of PPL to the malignant property of colorectal cancer and the intracellular function of PPL. In this study, we demonstrated that PPL was apparently down-expressed in colon carcinomas compared with normal and para-carcinoma tissues, which was correlated with the tumor size. Enforced expression of PPL in HT29 cells inhibited its proliferation evidenced by decreased expression of phosphorylated ERK and PCNA. Furthermore, PPL overexpression could reduce metastasis and epithelial-mesenchymal transition (EMT) of HT29 cells, with decreased expression of N-cadherin, Snail, Slug and α-SMA while increased expression of E-cadherin. On the contrary, the PPL knockdown could promote the cell proliferation, migratory, invasive and EMT ability of HT29 cells. Moreover, enforced expression of PPL induced G1/G0 cell cycle arrest, with decreased cyclin D1, p-Rb and increased expression of $p27^{kib}$, which could be reversed by PPL knockdown. In addition, PPL overexpression inhibited the growth of colon cancer allograft in vivo. Taken together, acted as a tumor suppressor in colon cancer progression, PPL could be a new biomarker or potential therapeutic target in colon cancer.

1. Introduction

Colorectal cancer is the first among the most three commonly diagnosed cancers worldwide, with more than 1.2 million new cases and half a million cases died every year [1,2]. Although much work has been done, the treatment of colon cancer is still dissatisfactory. Surgery alone always leads to tumor metastasis because of recurrence. And 5-Fluorouracil (5-FU) was the first chemotherapeutic agent widely used to treat colorectal cancer. However, high frequency of drug resistance limits its clinical efficacy [3]. Consequently, there is still an urgent need to seek for new targets and agents for colorectal cancer therapy.

Periplakin (PPL) is a 195 kDa membrane-associated protein and a member of the plakin protein family [4], which is mainly localized in the desmosomes and interdesmosomal plasma membranes of differentiated epidermal keratinocytes [5]. PPL is expressed in keratinized and nonkeratinized epithelial cells of the epidermis, the urinary bladder, the oral, the esophageal, and the colorectal [4]. It is reported that PPL is one of the candidates for a tumor marker for urothelial carcinoma [6].

On the other hand, PPL was reported to be significantly down-regulated in cancer tissues and scarcely expressed in advanced-stage of human esophageal cancers [7]. Also, the loss of PPL expression was associated with pathological stage and cancer-specific survival in patients with urothelial bladder cancer [8]. Moreover, the molecular mechanism underlying the regulation of PPL expression in the esophageal squamous cell carcinoma might be related to the aberrant DNA hypermethylation [9].

However, it was reported that plakin families function "molecular bridges" of cells that link the intracellular cytoskeleton and cell-cell junctions. PPL as an adhesion molecule plays a role in cellular movement and attachment in pharyngeal squamous cancer cell [10]. Although, PPL down-regulation is associated with

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cancer progression. Its role in colorectal tumorigenesis has not been studied yet. The intracellular function of PPL is also not clear.

In this study, we assessed PPL expression in normal human colon tissue (N), para-carcinoma tissue (P) and tumor tissue (T). We have determined the potential clinical correlations to assess the loss of PPL if it could be a useful clinical phenomenon during cancer formation. We also investigated the antitumor effects and mechanism of PPL to determine its potential to be a candidate for colorectal carcinoma therapy.

2. Materials and methods

2.1. Reagents

3-(4, 5-dimethyl-2-thiazyl)-2, 5-diphenyl-2H-tetrazolium bromide (MTT) and puromycin were purchased from Sigma-Aldrich (St Louis, MO). TRIzol reagent and Lipofectamine® 3000 transfection reagent and cell culture products were obtained from Life technologies (Carlsbad, CA, USA). Propidium iodide (PI) was purchased from BD Biosciences (San Jose, CA). Streptavidin-HRP and DAB substrate were obtained from Genetech Company (Shanghai, China). Antibodies against phospho-Rb (Ser 807/811), Rb, cyclin D1, p21, $p27^{kip}$, N-cadherin, phospho-ERK1/2, ERK1/2 and Flag were purchased from Cell Signal Technology (Beverly, MA). Antibodies against α-SMA, PPL, PCNA, Slug, Snail and Actin were obtained from Santa Cruz Biotechnology (Santa Cruz, CA). PPL plasmid was obtained by Nanjing Jinuomei Biotech Company (Nanjing, China). Lentivirus vector and shRNA-PPL were obtained from ABM Biotechnology Company (Nanjing, China).

2.2. Human colorectal cancer samples

The primary colorectal cancer tissues (T) and their matching, adjacent normal colon tissue (N) tissues and para-carcinoma tissue (P) were collected from 50 colorectal cancer patients undergoing surgery at the First Affiliated Hospital of Nanjing Medical University, Nanjing, China. Informed consent was given in all patients examined. All samples were confirmed by pathological examination. Histological grade was defined according to the World Health Organization classification.

2.3. Reverse transcription-polymerase chain reaction and Real-time PCR

Total RNA from tissue samples was isolated using TRIzol reagent (Invitrogen, Carlsbad, CA, USA), according to the supplier's instructions. Approximately 1 μg total RNA was reverse transcribed into cDNA using Superscript II enzyme and oligo (dT) (Invitrogen). Real-time PCR was performed as follows: the cDNA was subjected to quantitative PCR, which was performed with the BioRad Real-Time PCR Detection System (Bio-Rad, CFX Connect TM SYBR® Green Supermix) (Bio-Rad, 1708880), and threshold cycle numbers were obtained using BioRad CFX Manager Software. The program for amplification was 1 cycle of 95 °C for 2 min followed by 40 cycles of 95 °C for 10 s, 60 °C for 30 s, and 95 °C for 10 s glyceraldehyde-3- phosphate dehydrogenase (GAPDH) was used as an internal control. The primer sequences used in this study are listed below, Primer: GAPDH, forward 5′-CGCATCTTCTTTTGCGTCGCC-3′, reverse 5′-TCCACGACGTACTCAGCGCCA −3′. PPL, forward 5′-AGTGACCTCCTTGGTGTCGT-3′, reverse 5′-AGG GTGAATGATGGTTGGG-3′. Snail, forward 5′-TCGGAAGCCTAACTACAGCGA −3′, reverse 5′-AGATGAGCATTGGCAGCGAG −3′. Slug, forward 5′-TCTCTGATCCCTCAATTTGGTCT −3′, reverse 5′-CCACACACAGGGTTAAAGTGTC−3′. Twist, forward 5′- GTCCGCAGTCTTACGAGGAG −3′, reverse 5′- GCTTGAGGGTCTGAATCTTGCT −3′.

2.4. Immunohistochemistry

Formalin-fixed paraffin-embedded tissue sections were deparaffinized in xylene, rehydrated through graded ethanol and then boiled for 10 min in citrate buffer (10 mM, pH 6.0) for antigen retrieval. Endogenous peroxidase activity was suppressed by exposure to 3% hydrogen peroxide for 10 min. Slides were then blocked with 3% goat serum (Life Technology, 16210-064), incubated with diluted PPL primary antibodies for 2 h at room temperature at 37 °C and then incubated with streptavidin-HRP (Shanghai Gene Company, GK500705) for 40 min, then stained with DAB (Shanghai Gene Company, GK500705) substrate and counter-stained with hematoxylin. Images were acquired by microscopy (Olympus FV1000). The immunohistochemical staining intensity was scored by immunoreactive score (IRS) and integral optical density (IOD).

2.5. Cell culture and cell proliferation assay

The human colon cancer cell HT29 and mouse colon cancer cell CT26 were purchased from the Shanghai Institute of Cell Biology (Shanghai, China). Cells were maintained in RPMI 1640 medium and DMEM medium (Gibco, USA) supplemented with 10% (v/v) fetal bovine serum (FBS) (Gibco, USA), antibiotics (100 U/ml penicillin and 100 μg/ml streptomycin), at 37 °C in a humidified atmosphere of 5% CO^2. Cell proliferation was determined by MTT assay as previously reported [11].

2.6. Plasmid transfection

Transient transfection of HT29 cells was performed using the Lipofectamine® 3000 (Invitrogen, Carlsbad, CA, USA) reagents by following the manufacturer's instructions. For stable expression, CT26 colon cancer cells were transfected with 4 μg of PPL plasmid (The backbone vector of this plasmid is pcDNA3.1), and the expression of PPL was confirmed by western blotting. At 48 h post transfection, the cells were selected with 600 mg/ml of Kanamycin, and the positive clones were picked up for further expansion.

2.7. Lentivirus infection

The lentivirus harboring human or mouse shRNA-PPL and vector (pLV-IRES-Puro) were purchased from ABM Biotech Company. The cells were infected with lentivirus with the MOI 5:1. The stable cell line was selected with 10 μg/ml of puromycin, and the positive clones were picked up for further expansion.

2.8. Transwell migration assay

Cells (1×10^5/well) were plated into the top chamber and 10% FBS-containing medium was placed into the bottom chamber. After incubation at 37 °C in 5% CO^2 for 24 h, the cells remaining on the upper surface of the membrane were removed with a cotton swab. The cells that migrated through the 8-mm sized pores and adhered to the lower surface of the membrane were fixed with 4% paraformaldehyde, stained with 0.1% crystal violet and photographed by microscopy. The number of migration cells was assessed in 10 randomly selected fields under a microscope.

2.9. Colony-formation assay

Cells were harvested and seeded into the six-well plate (1000 cells/well) and incubated at 37 °C in a 5% CO^2 humidified incubator for 10 days. The medium was changed at 3-days interval. At the end of the incubation period, the cultures were fixed with 4% paraformaldehyde, stained with crystal violet and photographed.