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Proliferating cell nuclear antigen promotes cell proliferation and tumorigenesis by up-regulating STAT3 in non-small cell lung cancer

Liuxin Wang, Weixiang Kong, Bing Liu, Xueqing Zhang*

Department of Respiration, Jining First People's Hospital, Jining, Shandong, China

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ABSTRACT

Proliferating cell nuclear antigen (PCNA) functions as a bridging molecule, which targets proteins that have distinct roles in cell growth. The expression of PCNA is dysregulated in some tumors and takes part in the progression of oncogenesis. However, the roles of PCNA in the progression of non-small cell lung cancer (NSCLC) remain unknown. The present study aimed to investigate the function of PCNA in the occurrence and development of NSCLC and its underlying molecular mechanisms. Western blotting, RT-PCR, and immunohistochemistry assays were used to detect the expression pattern of PCNA in NSCLC tissues and cells. A log rank test was performed to compare the overall survival (OS) of patients with high/low expression of PCNA. Besides, the relationship between PCNA and signal transducer and activator of transcription-3 (STAT3) proteins were evaluated. Then, MTT, flow cytometry, clonal formation, and in vivo xenograft assays were conducted to investigate the effects of PCNA/STAT3 on cell growth, clonal formation, apoptosis, and tumorigenesis. Results showed that PCNA expression was elevated in NSCLC tissues and cells and it could combine with STAT3 and increased its expression and phosphorylation. Moreover, the expression of PCNA showed a positive correlation with the TNM grade and occurrence rate of the lymphatic metastasis and poor prognosis of NSCLC patients. Overexpression of PCNA promoted cell proliferation, clonal formation, and tumorigenesis in lung cancer cells and inhibited cell apoptosis. In contrast, these effects were inhibited when knockdown of STAT3. In conclusion, this study demonstrates that PCNA functions as an oncogene in the progression of NSCLC through up-regulation of STAT3. These findings point to a potentially new therapeutic strategy for NSCLC.

1. Introduction

Lung cancer is a leading cause of cancer-related deaths, with about 1.4 million deaths worldwide each year [1]. Non-small cell lung cancer (NSCLC) accounts for almost 80% of all lung cancer cases. Although there have been some advances in treatments for NSCLC, the prognosis of this disease has remained virtually unchanged in recent decades, with 5-year OS of just 16% in the U.S. and less than 10% in the U.K. [2]. Thus, enhanced understanding of NSCLC is needed, in addition to new treatment approaches and better biomarkers for early stratification of the disease [3,4].

PCNA, also known as cyclin or auxiliary protein of DNA polymerase δ [5] and DNA polymerase ε [6,7], is a highly conserved 36 kDa acidic nuclear protein [7]. Increasing evidence suggests that PCNA acts as a central coordinator of DNA transactions by providing a multivalent interaction surface for factors involved in DNA replication, repair, chromatin dynamics, and cell cycle regulation [8,9]. PCNA is involved in the progression of oncogenesis, which expression is dysregulated in some tumors [10]. A systematic review conducted by Lv et al. [11] suggested that PCNA expression was significantly associated with poor 5-year survival, advanced-stage disease, and a higher WHO grade. They proposed that it might serve as a useful prognostic and diagnostic biomarker or an effective therapeutic target in various cancers, including cervical cancer and gliomas. Besides, PCNA binds to p21 (CDK inhibitor) to promote cell cyclin and interacts with inhibitor of growth 1 to regulate cell apoptosis [12,13]. Thus, PCNA can function as a bridging molecule, which targets proteins that have distinct roles in cell growth [12,13]. Recently, Wang et al. [14] reported that miR-363-3p, a tumor suppressor, inhibited tumoral growth by down-regulation of PCNA expression in lung adenocarcinomas and suggested that PCNA might play an unfavorable role in NSCLC progression.

Signal transducer and activator of transcription-3 (STAT3), a key mediator of intracellular signaling, is stimulated by various cytokines, hormones, growth factors, and oncoproteins [15]. Once activated, STAT3 translocates to the nucleus where it binds to consensus STAT3-binding sequences located in the promoters of target genes, thereby

* Corresponding author at: Department of Respiration, Jining First People's Hospital, Jining 272000, Shandong, China.
E-mail address: wjy2013313@163.com (X. Zhang).

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inducing transcriptional activation of various growth-promoting genes [15]. STAT3 could also regulate cell cycle, migration, invasion, and angiogenesis and enables immune system evasion [16–18]. Although the literature suggests that both abnormal activation of the STAT3 pathway and dysregulated expression of PCNA may play vital roles in tumorigenesis, additional investigations are needed to shed light on the mechanisms underlying the expression of PCNA and STAT3 in NSCLC.

To identify the functional role of PCNA in NSCLC, we firstly determined the expression pattern of PCNA in NSCLC tissues and cells. We then investigated the effects of PCNA on cell proliferation and apoptosis and explored the relationship between PCNA and STAT3. Finally, we studied the function of PCNA/STAT3 signaling in the progression of NSCLC, which might provide clinical insights into an efficacious therapeutic strategy for NSCLC.

2. Material and methods

2.1. Patients and sample preparation

Sixty paired samples of fresh NSCLC and adjacent tissues were obtained from patients with NSCLC who had undergone a radical pneumonectomy without chemotherapy or radiation. The study was approved by the Human Research Committee of Jining First People's Hospital and was performed in accordance with the Helsinki Declaration.

2.2. Immunohistochemistry

Paraffin sections of lung tissues were sliced into sections that were 4 μm thick by a slicing machine. The following routine three-step immunohistochemical staining protocol was then undertaken: sectioning, dewaxing, and hydrating tissues; incubation with 3% H_2O_2 at room temperature for 10 min; antigen retrieval with Tris-EDTA; sealing with 5% goat serum (diluted in phosphate-buffered saline [PBS]); incubation overnight at 4 °C with primary antibody against PCNA at a 1:150 dilution (No. 13110, Cell Signaling Technology, USA); and finally incubation with secondary antibody and rinsing with PBS. Chromogen 3, 3′-diaminobenzidine tetrachloride (Serva, Heidelberg, Germany) was used as a substrate, and the cell nucleus was dyed with Harris's hematoxylin solution.

2.3. Tissue microarrays (TMAs) and immunohistofluorescence staining

We collected 90 cases of NSCLC for immunohistofluorescence staining. Biopsy specimens were assembled and provided to Shanghai Outdo Biotech Company for preparing TMA. Core size was 0.6 cm, and each represented a different tumor sample.

The TMA sections were dewaxed, rehydrated, and blocked with hydrogen peroxide for 10 min. Protein block was applied for 10 min at room temperature to block nonspecific background staining. The tissues were then incubated with polyclonal anti-PCNA (anti-mouse, 1:100) and anti-STAT3 (anti-rabbit, 1:150) overnight at 4 °C, followed by incubation with goat anti-AF568 (red; Life Technologies, USA) and anti-rabbit IgG AF488 (green; Life Technologies, USA) at room temperature in the dark for 1 h. Finally, the tissues were covered with anti-fade mounting medium (Vectashield, Loerrach, Germany) and placed onto microscope slides. The slides were examined under a laser scanning microscope (TCSSP2-AOBS-MP, Leica Microsystems CMS). Two independent pathologists analyzed the same slide. The extent and intensity of immunoreactivity were assessed to determine protein expression levels in tissues.

2.4. Cell culture

A normal human lung cell line (BEAS-2B) and lung cancer cell lines (T790 M, H1975, A549, H157, and NCI-H460) were purchased from the American Type Culture Collection (ATCC, Manassas, VA, USA). BEAS-2B cells were cultured in LHC-8 medium (Gibco, MA, USA); T790 M, H1975, NCI-H460, and H157 cells were cultured in RPMI-1640 medium (Gibco, MA, USA); A549 cells were cultured in F-12 K medium (Gibco, MA, USA). All the culture media were supplemented with 10% fetal bovine serum, 100 μg/ml of penicillin, and 100 μg/ml of streptomycin. All the cells were maintained in a humidified incubator with 5% CO_2 at 37 °C.

2.5. RNA interference and cell transfection

Plasmids, small interfering (si) RNAs targeting the human PCNA gene and STAT3 gene, and their controls were purchased from OriGene (MA, USA). The cells were transfected with siRNAs, plasmids, and their controls using INTERFERin® transfection reagent (Polyplus, France), according to the manufacturer's instructions. In total, 2×10^5 cells were transfected with 110 pmoles of siRNA or 2 μg of DNA. The transfection efficiency was detected by the real-time polymerase chain reaction (RT-PCR) 24 h after transfection and Western blotting 48 h after transfection.

2.6. Western blotting analysis

A549 and H1975 cells were harvested and lysed with ice cold lysis buffer (50 mM Tris-HCl, pH 6.8, 100 mM β-mercaptoethanol, 2% w/v SDS, 10% glycerol). For detection of the phosphorylated proteins, 100X phosphatase inhibitor (No. C500017, Sangon Biotech, Shanghai, China) was diluted in the above lysis buffer to 1×. After centrifugation at 20,000 × *g* for 10 min at 4 °C, proteins in the supernatants were quantified and separated by 10% SDS-PAGE and transferred to PVDF membrane (Millipore, MA, USA). After blocking with 10% nonfat milk in PBS, the membranes were immunoblotted with antibodies as indicated, followed by HRP-linked secondary antibodies (Cell Signaling). Antibodies against PCNA, STAT3, p-STAT3, c-myc, cyclin d1, bcl-2, and GAPDH were purchased from Cell Signaling (MA, USA). Protein levels were normalized to that of total GAPDH using a rabbit polyclonal anti-GAPDH antibody (Santa Cruz, USA).

2.7. RNA extraction and RT-PCR

Total RNA from lung cancer tissues and cell lines was extracted using an RNA pure Tissue Kit (CWBIO, China), according to the manufacturer's instructions and reverse-transcribed into first-strand cDNA using a HiFiScript 1 st Strand cDNA Synthesis Kit (CWBIO, China). RT-PCR analysis was performed in triplicate using a miScript SYBR Green PCR Kit (Qiagen) on a DA7600 Real-time Nucleic Acid Amplification Fluorescence Detection System (Bio-Rad) in a 20 μL reaction system. The expression of mRNAs in cells and tissues was normalized to that of GAPDH. The $2^{-\triangle\triangle Ct}$ method was used to evaluate relative mRNA expression changes. The primers were synthesized by the Beijing Genomics Institute and were as follows:

PCNA forward: AACCTGCAGAGCATGGACTC,
Reverse: TCATTGCCGGCGCATTTTAG,
GAPDH forward: 5′-CATCACCATCTTCCAGGAGCG-3′,
Reverse: 5′-TGACCTTGCCCACAGCCTTG-3′.

2.8. MTT assays

Briefly, 4000 A549 or H1975 cells/well (cells were transfected si-NC, si-PCNA, OE-NC, OE-PCNA, or OE-NC + si-NC, OE-PCNA + si-NC, OE-PCNA + si-STAT3) were plated in triplicate in 96-well plates, and MTT assays were performed according to the manufacturer's protocol. The absorbance at 490 nm was measured using a multi-well plate reader.