Gynecologic Oncology 139 (2015) 529–535

Contents lists available at ScienceDirect

Gynecologic Oncology

journal homepage: www.elsevier.com/locate/ygyno

PIAS3, SHP2 and SOCS3 Expression patterns in Cervical Cancers: Relevance with activation and resveratrol-caused inactivation of STAT3 signaling

Peng Zhang [a], Bin Yang [b], Yuan-Yuan Yao [a], Li-Xia Zhong [c], Xiao-Yan Chen [a], Qing-You Kong [a], Mo-Li Wu [a], Cong Li [a], Hong Li [a,*], Jia Liu [a,*]

[a] Liaoning Laboratory of Cancer Genetics and Epigenetics and Department of Cell Biology, College of Basic Medical Sciences, Dalian Medical University, Dalian 116044, China
[b] Department of Gynaecological Surgery, Sheng-Jing Hospital, China Medical University, Shenyang 110004, China
[c] Department of Clinical Oncology, Second Affiliated Hospital of Dalian Medical University, Dalian 110042, China

HIGHLIGHT

- PIAS3 reduction is negatively correlated with STAT3 activation in cervical cancers (CCs).
- PIAS3 up-regulation and STAT3 inactivation are evidenced in resveratrol-suppressed CC cells.
- Increased PIAS3 level may reflect promising therapeutic outcome of resveratrol and better prognosis of CCs.

ARTICLE INFO

Article history:
Received 14 July 2015
Received in revised form 21 September 2015
Accepted 28 September 2015
Available online 4 October 2015

Keywords:
Cervical cancers;
Resveratrol;
SHP2;
PIAS3;
SOCS3;
STAT3 signaling pathway

ABSTRACT

Objective. Resveratrol inhibits cervical cancer (CC) cells by blocking STAT3 signaling. However, the mechanism of resveratrol-induced STAT3 inactivation remains largely unknown. SHP2, PIAS3, and SOCS3 are STAT3 negative regulators; therefore, their statuses in cervical adenocarcinoma (HeLa) and squamous cell carcinoma (SiHa and C33A) cell lines without and with resveratrol treatment and their correlation with STAT3 activation in CC specimens were investigated.

Methods. MTT and TUNEL assays were used to check the resveratrol sensitivity of CC cells, and immunocytochemical staining, Western blotting, and RT-PCR were used to analyze SHP2, PIAS3, and SOCS3 expression and the intracellular distribution of STAT3. Tissue microarray based immunohistochemical staining was performed to investigate potential correlations between SHP2, PIAS3, and SOCS3 expression and STAT3 activation.

Results. PIAS3 and SOCS3 were found to be weakly expressed in CC cells and upregulated by resveratrol; this was accompanied by inhibition of STAT3 signaling. The SHP2 level remained unchanged in all three cell lines after resveratrol treatment. STAT3 nuclear translocation was more frequent in adenocarcinomas and squamous cell carcinomas than that of their noncancerous counterparts. The SOCS3 level and detection rate were higher in noncancerous squamous cells (but not in glandular epithelia) compared with their cancerous counterparts. The phospho-SHP2 detection rate was similar in noncancerous and tumor tissues of squamous and glandular origins; however, PIAS3 levels were distinct.

Conclusions. Of the three STAT3 negative regulators, PIAS3 correlated most negatively with STAT3 nuclear translocation and may inhibit STAT3 signaling in both histological CC subtypes. PIAS3 responsiveness may reflect greater resveratrol sensitivity and improved therapeutic outcome in CCs.

1. Introduction

Cervical cancer (CC) is one of the most common malignancies in women [1,2]. In developing countries, it is the second leading cause of cancer-related death in women because of delayed diagnosis and the high risk of metastasis [3,4]. Consequently, patients must receive chemotherapy to prevent tumor relapse and metastasis [5]. Cisplatin is commonly used to treat CC but it has serious side effects [6,7] and

* Corresponding authors at: Liaoning Laboratory of Cancer Genetics and Epigenetics and Department of Cell Biology, College of Basic Medical Sciences, Dalian Medical University, 9 West Section, Lvshun South Road, Dalian 116044, China.
E-mail addresses: lihongmcn@dlmedu.edu.cn (H. Li), jialiudl@aliyun.com (J. Liu).

http://dx.doi.org/10.1016/j.ygyno.2015.09.087
0090-8258/

drug resistance is common [8]. It is therefore necessary to develop more effective and less toxic anti-CC agents to improve patient management.

Resveratrol is found in a variety of natural plants and possesses a variety of potential biological effects including cancer prevention and treatment [9]. More importantly, this compound has little toxicity toward normal cells at the effective anticancer doses [10], suggesting its potential value in cancer chemotherapy. The extremely low intracellular availability due to enzymatic biotransformation is the main obstacle to the clinical use of resveratrol [11]. However, this is not the case for cervical cancers because of the possibility of direct drug administration through the vagina. In this context, resveratrol could be an anti-CC drug candidate if the molecular mechanism(s) of its suppressive effects on CC cells can be further clarified.

Our recent studies demonstrated that resveratrol suppresses the growth of cervical adenocarcinoma (HeLa) and squamous cell carcinoma (SiHa and C33A) cells and inhibited Wnt2, Notch, and STAT3 activation [12]. Further analysis revealed that only selective STAT3 inhibition had similar cellular and molecular consequences in resveratrol-treated CC cells, suggesting a critical role for STAT3 signaling in CC cell growth and survival. Data from other cancer types showed the importance of STAT3 activation on tumor growth and the inhibitory effect of resveratrol on STAT3 signaling [13,14]. Nevertheless, the mechanism(s) underlying resveratrol-mediated STAT3 inhibition is not yet known. It was reported that protein inhibitor of activated STAT protein 3 (PIAS3), SHP2, and suppressor of cytokine signaling 3 (SOCS3) negatively regulate STAT3 signaling [15]. However, no comprehensive study has so far reported the status of these three STAT3 negative regulators and their correlation with STAT3 activation in human CC. The current study thus aims to address these issues by investigating the effect of resveratrol treatment on cervical adenocarcinoma and squamous cell carcinoma tissue specimens and CC cell lines.

2. Materials and Methods

2.1. Cell culture and treatment

Human cervical adenocarcinoma cell line HeLa and human cervical squamous carcinoma cell lines SiHa and C33A were obtained from Cell Bank of Academia Sinica, Shanghai. HeLa cells were cultured in Roswell Park Memorial Institute 1640 Medium (RPMI1640, Gibco Life Science, Grand Island, NY, USA), SiHa and C33A cells were cultured in MEM essential medium containing 10% fetal bovine serum (Gibco Life Science, Grand Island, NY, USA) under 37 °C and 5% CO_2 condition. Trans-resveratrol (Sigma, St. Louis, MO, USA) was dissolved in DMSO (Sigma) to a stock concentration of 100 mM, wrapped in aluminum foil for protection against light, and stored at −20 °C. NEST-DISH (China Patent for Invention: ZL200610047607.0; NEST Biotech., Co., Ltd., Wuxi, China) were used for H/E morphologic and immunocytochemical/ICC staining. The coverslips prepared were separated into normal culture group (Group N) and 48 hours 100 μM resveratrol treated group (Group R).

2.2. 5-Dimethyl-2-thiazolyl-2,5-diphenyl-2-H-tetrazolium bromide/MTT assay

Total cell numbers and cell viability of HeLa, SiHa and C33A cells without and with 100 μM resveratrol treatment were determined in 12 hours intervals and lasted for 48 hours by MTT assay [16]. Briefly, the cells were plated to 96-well plates at a density of 1×10^4/well and then 10 μL MTT solution (15 mg MTT in 3 mL PBS) was added to each of the wells and incubated for 2 to 4 hours until purple precipitate was visible. 100 μL detergent reagent was added individually to the wells at room temperature in darkness, incubated for another 2 hours and read absorbance of the supernatants at 570 nm (Ultraviolet spectrophotometer, Varian, Australia). The results were shown as percentage of cell viability (OD of the experiment samples/OD of the control) or OD values.

2.3. Immunocytochemistry

Immunocytochemical staining (ICC) was performed on the coverslips obtained from each of the experimental groups. The antibodies used were a rabbit anti-human p-STAT3(1:100; Santa Cruz, CA, USA), a rabbit anti-human survivin polyclonal antibody (1:160; Santa Cruz, CA, USA), a mouse anti-human c-Myc monoclonal antibody (1:100; Santa Cruz, CA, USA), a mouse anti-human cyclinD1 monoclonal antibody (1:50; Dako, Denmark), a rabbit anti-human VEGF polyclonal antibody (1:150; Santa Cruz, CA, USA), a rabbit anti-human p-SHP2 polyclonal antibody (1:150, Santa Cruz, CA, USA), a rabbit anti-human SOCS3 polyclonal antibody (1:120, Santa Cruz, CA, USA), and rabbit anti-human PIAS3 polyclonal antibody (1:100; Santa Cruz, CA, USA). All of the antibodies were used according to the manufacturer's instruction. ICC staining was conducted by the method described elsewhere [17]. The microarray sections lacking incubation with individual primary antibodies were used as background controls. The staining results were evaluated separately by two investigators, with the intensity of immunolabeling scored as negative (−), weakly positive (+), moderately positive (++) or strongly positive (+++).

2.4. RNA isolation and RT-PCR

Total cellular RNA samples were isolated from each of experimental groups using Trizol solution (Life Technologies, Grand Island, NY). Reverse transcription (RT) was performed on RNA samples, followed by polymerase chain reaction (PCR) amplification. For RT, 0.5 μg of the RNA sample was added to 20 μL of RT reaction mixture (Takara, Inc., Ltd., Dalian, China) containing 4 μL of $MgCl_2$, 2 μL of 10 × RNA PCR buffer, 9.5 μL of RNase-free distilled H_2O, 2 μL of deoxyribonucleotide triphosphate mixture, 0.5 μL of RNase inhibitor, 1 μL of AMV reverse transcriptase, and 1 μL of oligo dT-adaptor primer. The reaction was carried out by treating the samples at 55 °C for 30 minutes, at 99 °C for 5 min, and at 5 °C for 5 minutes. PCR was conducted using the primers specific for each of the target genes. Briefly, 2.5 μL of RT products were mixed with 16 μL of PCR-grade water, then with 6.5 μL of PCR working solution containing 1 × PCR buffer, 1 μL of deoxyribonucleotide triphosphate, 2.5 units of Taq DNA polymerase, and 50 pM upstream and downstream primers for human STAT3, survivin, c-Myc, cyclinD1, VEGF, SHP2, SOCS3 and PIAS3 (Supplementary Table). PCRs for individual genes were performed according to the conditions reported elsewhere [18]. The PCR products were resolved on 1% agarose gel containing ethidium bromide (0.5 μg/mL). The bands were visualized and photographed using UVP Biospectrum Imaging System (UVP, Inc., Upland, CA). The PCR products generated from the same RT solution by a pair of β-actin primers were cited as internal quantitative controls.

2.5. Protein preparation and Western blot analysis

Total cellular proteins were prepared from the cells under different culture conditions by the method described previously [19]. For Western blot analyses, the sample proteins (50 μg/well) were separated by electrophoresis in 10% sodium dodecylsulfate-polyacrylamide gel electrophoresis (SDS-PAGE), transferred to polyvinylidene difluoride membrane (Amersham, Buckinghamshire, UK). The membranes were blocked with 5% skimmed milk in TBS-T (10 mM Tris–Cl, pH 8.0, 150 mM NaCl, and 0.5% Tween 20) at 4 °C overnight, rinsed three times (10 minutes each time) with TBS-T, followed by 3 hours of incubation at room temperature with the first antibodies in appropriate concentrations (p-STAT3:1:800, survivin:1:600, c-myc:1:600, cyclinD1:1:500, VEGF:1:500, p-SHP2:1:500, SOCS3:1:500, PIAS3:1:1000), followed by 1 hour incubation with HRP-conjugated anti-mouse or anti-rabbit IgG (ZymedLab, Inc). The bound antibody was detected using the enhanced chemiluminescence system (Roche GmbH, Mannheim, Germany). After removing the labelling signal by incubation with stripping buffer (62.5 mM Tris–HCl, pH 6.7, 100 mM 2-mercaptoethanol, 2% SDS) at