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Original article

Targeting of cell cycle and let-7a/STAT3 pathway by niclosamide inhibits proliferation, migration and invasion in oral squamous cell carcinoma cells

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ABSTRACT

The low median survival rate of oral squamous cell carcinoma (OSCC) is associated with chemotherapeutic resistance. Niclosamide is an oral anti-helminthic drug, its anti-cancer effect has been reported in recent years. However, the effect of niclosamide on OSCC remains largely unknown. In this study, we, for the first time, investigated the underlying mechanisms from cell cycle arrest and let-7a/STAT3 axis through CCK-8, cell cycle, apoptosis, wound healing, Transwell invasion, generation of stable cell line, real-time PCR, and western blot assays using two OSCC cell lines WSU-HN6 and Tca83. We showed that niclosamide could inhibit OSCC cells proliferation through causing cell cycle arrest in G1 phase and promoting apoptosis, while the cell cycle-related proteins MCM2, MCM7, CDK2 and CDK4 were downregulated and the apoptosis-related proteins p53 and cleaved caspase-3 were upregulated. Furthermore, niclosamide could inhibit migration and invasion of OSCC through upregulation of let-7a expression and downregulation of p-STAT3 expression. What is more, we established the stably expressing let-7a cell line (HN6-let-7a). Like niclosamide, HN6-let-7a could decrease the ability of the cell migration, invasion as well as the expression of p-STAT3. Collectively, our study finds the new mechanisms that niclosamide inhibits OSCC proliferation through causing cell cycle arrest in G1 phase via downregulation of the above cell cycle-related genes; promotes OSCC apoptosis through upregulation of pro-apoptotic genes; decreases migration and invasion of OSCC by let-7a/STAT3 axis, thus providing a preferred therapeutic candidate for OSCC in future.

1. Introduction

Oral cancer occurs in the mouth, lip and tongue, which is one of the most common oral malignancies worldwide [1]. In 2017, according to the American Cancer Society reports, there are 49,670 estimated new oral cancer cases and 9,700 oral cancer deaths in the United States [2]. More than 90% of oral cancer is oral squamous cell carcinoma (OSCC) [1]. OSCC is often diagnosed at an advanced stage. Loco-regional recurrences of OSCC are common [3,4]. Despite the huge progress has been made in surgery, radiotherapy and chemotherapy during the past decades, the overall 5-year survival rate of OSCC patients is still about 50% and has not been significantly improved [5]. The low median survival rate is associated with chemotherapeutic resistance [6]. Therefore, it is a clear necessary to develop new strategy to improve the efficacy of OSCC therapy.

Signal transducers and activators of transcription 3 (STAT3) acts as a signal messenger, which can interact with specific DNA binding elements, thus activating transcription [7]. STAT3 pathway plays a central role in principal cell fate decisions, including regulating cell proliferation, cell cycle progression, apoptosis, angiogenesis and immune evasion [8]. Recent knowledge implicates constitutive activation of STAT3 has been frequency detected in diverse human cancer cell lines and is associated with increased morbidity and mortality in variety of tumors [9]. The fact that STAT3 is considered as an oncogene indicates that inhibition of STAT3 activation may improve the efficacy of OSCC therapy [10].

Abbreviations: OSCC, oral squamous cell carcinoma; CCK-8, Cell Counting Kit-8; STAT3, signal transducers and activators of transcription 3; DMEM, Dulbecco's modified Eagle's medium; RPMI 1640, Roswell Park Memorial Institute 1640 medium; SD, standard deviation; DMSO, dimethyl sulfoxide; MCM, mini-chromosome maintenance; CDK, cyclin-dependent kinase; OD, optical density; ERK1/2, extracellular regulated protein kinases1/2; FBS, fetal bovine serum; GAPDH, glyceraldehyde-3-phosphate dehydrogenase; PBS, phosphate buffer saline; PCR, polymerase chain reaction

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Niclosamide is an oral anti-helminthic drug that is effective against human tapeworms for approximately 50 years [11]. Recently, accumulating evidences show that niclosamide exerts anti-cancer effect in many types of cancers including acute myelogenous leukemia [12], colon cancer [13], prostate cancer [14,15], lung cancer [16,17], breast cancer [18,19] and ovarian cancer [20,21]. However, the effects of niclosamide on OSCC remain largely unknown. Based on the molecular properties of niclosamide, it serves as multiple pathways inhibitor and can modulate Wnt/β-catenin, mTORC1, NF-κB, Notch, and STAT3 pathway [22]. Importantly, STAT3 is constitutively activated in oral cancer, we accordingly deduce that niclosamide perhaps has a potential to exert an anti-cancer effect through STAT3 pathway in OSCC.

Recently, there is emerging evidence indicates that cross-talk occurs between microRNAs (miRNAs) and STAT3 signaling pathway in tumor development and progression [23]. miRNAs as small, non-coding, endogenous RNAs that predominantly bind to within the 3′-untranslated region (3′-UTR) of messenger RNA, thus resulting in increased degradation and inhibition of translation of mRNA. Therefore, miRNAs are likely to be involved in most biologic processes including proliferation, cell cycle regulation, apoptosis, differentiation, and immune response by targeting signaling pathway [24]. miR-21, miR-18b-1, miR-155, miR-125b, miR-17, miR-20a and miR-106a have been shown to play critical roles in STAT3 signaling pathway [25]. The human let-7 family has 9 members including let-7a, let-7b, let-7c, let-7d, let-7e, let-7f, let-7g, let-7i and miR-98, and has been reported as tumor suppressors by targeting certain known oncogenes, such as RAS, high-mobility group A2, and c-Myc [26]. In this article, we investigated whether a microRNA let-7a was involved in the anti-cancer effect of niclosamide through regulating STAT3 pathway. Overexpression of let-7a was shown to inhibit the growth of cancer cell lines [27]. However, no direct connection between let-7a and niclosamide has been described. In addition, the relationship between niclosamide and cell cycle has not been reported to date.

Collectively, in this study, we investigated whether niclosamide inhibited OSCC proliferation through arresting cell cycle and promoting apoptosis; decreased OSCC migration and invasion via let-7a/STAT3 axis, aimed to unravel its mechanisms.

2. Materials and methods

2.1. Cell lines and reagents

Human oral cancer cell lines WSU-HN6 and Tca83 were cultured in Dulbecco's modified Eagle's medium (DMEM, Gibco, Carlsbad, CA, USA) and Roswell Park Memorial Institute 1640 medium (RPMI 1640, Gibco) supplemented with 10% fetal bovine serum (FBS, Hyclone), respectively, at 37 °C in a humidified incubator with 5% CO_2. Niclosamide (Sigma-Aldrich, Louis, MO) was dissolved in dimethyl sulfoxide (DMSO, Sigma-Aldrich) at 0, 1.25, 2.5, 5 and 10 μM.

2.2. Cell proliferation assay

WSU-HN6 and Tca83 cells were harvested and seeded onto 96-well flat-bottomed plates at a density of 5×10^3 cells per well. Subsequent to culturing, the cells were cultured in complete culture medium containing niclosamide at indicated concentrations for 24 h and subjected to Cell Counting Kit-8 (CCK-8) assay according to the manufacturer's instructions. Equal amount of DMSO (1 μl DMSO:1 ml complete medium) was added in each group. The absorbance of each reaction plate was measured at a wavelength of 450 nm using a microplate reader (BioTeK ELx808). Assays were performed in quadruplicate and the experiments were repeated three times.

2.3. Cell cycle analysis

Cells (5×10^5) were cultured in T25-cm^2 flasks with 5 ml of complete medium containing various doses of niclosamide for 24 h, and then harvested by trypsinization, fixed in 70% ethanol at 4 °C overnight. The fixed cells were washed twice with PBS, treated with RNase A (50 μg/ml) for 30 min at room temperature, followed staining with propidium iodide (PI). The stained cells were analyzed for cell cycle by flow cytometry using a Beckman Coulter XL instrument (Beckman Coulter, Brea, CA, USA).

2.4. Apoptotic assay

Following treated with DMSO or various concentrations of niclosamide in 100-mm dish for 24 h, WSU-HN6 and Tca83 cells were trypsinised to get a single-cell suspension, and then subjected to do apoptosis analysis by Cell Death Detection Kit II (Roche). Briefly, Annexin V binding buffer (400 μl) and Annexin V-FITC (5 μl) were added. Cells were incubated in the dark at 4 °C for 15 min. PI (10 μl) was added and incubated for 5 min. Apoptosis was analyzed by flow cytometry according to manufacturer's instruction.

2.5. Wound healing assay

Straight lines were drawn on the back of 6-well plates using a marker pen. Confluent cancer cell monolayer was serum-starved for 30 h. Once the cells reached 90% confluence, wounds scratching across the well were carefully created by a 200-μl pipette tip, from one end to the other end of the well. After removal of floating cells, the remaining cells were treated with indicated doses of niclosamide. At the indicated time point, the monolayer was recorded at 10× magnification for calculating the movement speed of cells. The experiments were performed in triplicate.

2.6. Transwell invasion assay

The upper Transwell chambers (8.0 μm pore size, Millipore, Bedford, MA, USA) were coated with 100 μl of 25 μg/ml Matrigel (BD, Minneapolis, MN, USA) while the bottom chambers were added with 500 μl of culture medium supplemented with 20% FBS. Briefly, cells were starved by serum-free medium for 24 h. And then, a total of 1×10^5 WSU-HN6 and Tca83 cells were seeded onto the upper chambers in 100 μl serum-free medium, then treated with different concentrations of niclosamide. At 20 h after treatment, the cells with membrane were fixed and stained with 1% crystal violet. The cells located on the upper side of the filter were wiped off. Then, the number of migrated cells was counted and photographed under a light microscopy at 20× magnification (Olympus, Tokyo, Japan). The results were expressed as the percentage inhibition of migrated cells by niclosamide. The results obtained from three independent experiments in duplicate.

2.7. Real-time PCR assay

miRNA quantifiation was performed using miDETECT A Track™ miRNA qRT-PCR kit (RiboBio, Guangzhou, China) following the manufacture's instruction. Real-time PCR was performed using the SYBR Green master mix (Roche Diagnostics, Indianapolis, IN, USA) on an ABI 7500 instrument (Applied Biosystems, Foster, CA, USA). Let-7a and U6 primers were ordered from Ribobio Company (Guangzhou, China). U6 severs as miRNA endogenous control. The fold-change was determined as $2^{-\Delta\Delta Ct}$. All real-time PCR reactions were performed in triplicate and repeated three times.

2.8. Generation of stably expressing let-7a oral cancer cell line

WSU-HN6 cells were transfected with pCDNA3-PRI-let-7a plasmids carrying let-7a overexpression cassette, and corresponding empty vector, respectively. pCDNA3-PRI-let-7a was from Addgene (plasmid # 51377) [28]. At 48 h post-transfection, G418 was added for screening