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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

stable cell lines. The single colonies were picked up for detection of let-7a expression. Meanwhile, WSU-HN6 cells transfected empty vector were also generated as control. The stably expressing let-7a cell line (hereafter named HN6-let-7a) was preserved for the following assays.

2.9. Western blot assay

Whole cells were harvested and lyzed in RIPA buffer (Applygen Technologies, Beijing, China) with protease inhibitors (Roche Diagnostics). Then, 30 μg of protein per each sample was loaded and separated by sodium dodecyl sulfate-polyacrylamide gel. Proteins were transferred to polyvinylidene difluoride membrane. After blocking for 1 h at room temperature in 5% nonfat milk and probed with antibodies against phosphorylated (p)-STAT3 (1:1000 dilution, Genetex, Irine, CA, USA), total (t)-STAT3 (1:1000 dilution, GeneTex), cyclin-dependent kinase (CDK) 2 (1:1000 dilution, Cell signaling Technology, USA), CDK4 (1:1000 dilution, Cell signaling Technology), mini-chromosome maintenance protein (MCM) 2 (1:1000 dilution, Cell signaling Technology), MCM7 (1:1000 dilution, Cell signaling Technology), and GAPDH (1:1000 dilution, Santa Cruz Biotechnology, CA, USA). Following HRP-linked secondary antibody incubation, immunoreactive bands were detected with an ECL detection system (Applygen Technologies, Beijing, China).

2.10. Statistical analysis

Data were presented means ± standard deviation (SD) obtained from three independent experiments. Statistical analysis was performed with GraphPad Prism v5.0 software. The differences between groups were examined using an analysis of one-way analysis of variance (ANOVA), followed by post hoc Bonferroni correction tests. $p < 0.05$ was considered statistically significant.

3. Results

3.1. Niclosamide inhibits cell proliferation of WSU-HN6 and Tca83

To determine the effects of niclosamide on the growth of WSU-HN6 and Tca83 cells, cell viability was evaluated by CCK-8 assay. After treatment with vehicle DMSO or the indicated doses of niclosamide for 24 h, CCK-8 results showed that niclosamide markedly inhibited the proliferation of WSU-HN6 (Fig. 1A) and Tca83 cells (Fig. 1B) in a dose-dependent manner. Niclosamide at 10 μM had the most inhibition effects in both two OSCC cell lines.

3.2. Niclosamide induces cell cycle arrest in G1 phase

To examine whether niclosamide inhibits OSCC cells growth through cell cycle arrest, cell cycle analysis was performed using flow cytometry. The results revealed that niclosamide could cause G1 phase arrest in WSU-HN6 and Tca83 cell lines, respectively, compared with corresponding untreated controls. Meanwhile, the percentage of S phase was markedly decreased by niclosamide. The peak value was found at 2.5 μM niclosamide treatment in WSU-HN6 cells (Fig. 2A) and at 5 μM in Tca83 cells (Fig. 2B).

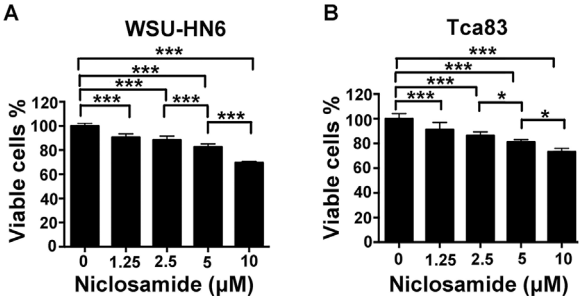

Fig. 1. Niclosamide inhibits WSU-HN6 and Tca83 cells proliferation. A-B. WSU-HN6 and Tca83 cells were treated with DMSO or different doses of niclosamide (1.25–10 μM) for 24 h. Cell proliferation was detected by CCK-8 assay. The results show that niclosamide significantly inhibits both WSU-HN6 and Tca83 cell lines growth in a dose-dependent manner. Data are expressed as the means ± SD of three independent experiments (n = 3, *$p < 0.05$, ***$p < 0.001$).

3.3. Niclosamide downregulates MCM2, MCM7, CDK2 and CDK4, thus causing cell cycle arrest in G1 phase

To investigate the underlying mechanisms regarding the change of cell cycle by niclosamide, we did western blot assay to detect key proteins related to cell cycle. As shown in Fig. 2C, niclosamide could downregulate cell cycle-associated proteins MCM2, MCM7, CDK2 and CDK4 in both two OSCC cell lines. MCMs are involved in the initiation of eukaryotic genome replication. CDK2 and CDK4 activity are restricted in G1-S phase. Therefore, cell cycle was arrested in G1 phase obviously owing to the downregulation of accelerated cell cycle proteins, thus finally leading to growth inhibition caused by niclosamide (Fig. 2C).

3.4. Niclosamide induces the apoptosis of WSU-HN6 and tca83

To determine whether niclosamide induces WSU-HN6 and Tca83 cells growth inhibition through apoptosis, apoptosis assay was performed. As shown in Fig. 3A, when cells were treated with 0, 1.25, 2.5, 5 and 10 μM niclosamide for 24 h, respectively, the percentage of apoptotic cells was 1.49 ± 0.11%, 3.48 ± 0.28%, 3.07 ± 0.35%, 5.17 ± 0.35% and 8.8 ± 0.65% in WSU-HN6, and 3 ± 1.16%, 5.74 ± 0.04%, 7.76 ± 0%, 8.41 ± 1.18%, 10.46 ± 0.53% in Tca83 ($p < 0.05$, Fig. 3A–B). The results indicated that niclosamide could significantly promote OSCC cells apoptosis.

3.5. Niclosamide upregulates p53 and cleaved caspase-3 expression, leading to cell proliferation inhibition and apoptosis

To investigate the underlying mechanisms regarding the growth inhibition and apoptosis of OSCC cells by niclosamide, we did western blot assay to detect key proteins related to cancer cell proliferation and apoptosis. As shown in Fig. 3C, niclosamide upregulated apoptosis-related proteins p53 and cleaved caspase-3 expression, which provided a reasonable explanation and evidence for apoptotic results.

3.6. Niclosamide suppresses migration and invasion of WSU-HN6 and Tca83

One characteristic of tumor metastasis is the increased ability of tumor cell migration. To assess whether niclosamide inhibits OSCC migration and invasiveness, we did wound healing and Transwell invasion assays. Wound healing results showed that the relative speed of wound edge in 0, 1.25, 2.5, 5 and 10 μM of niclosamide treatment groups were 1.00 ± 0.23 μm/h, 0.17 ± 0.07 μm/h, 0.11 ± 0.03 μm/h, 0.10 ± 0.06 μm/h and 0.08 ± 0.05 μm/h in WSU-HN6, and 1.00 ± 0.04 μm/h, 0.71 ± 0.02 μm/h, 0.68 ± 0.05 μm/h, 0.64 ± 0.09 μm/h and 0.74 ± 0.05 μm/h in Tca83, respectively ($p < 0.05$, Fig. 4A–D). The results showed that compared with corresponding controls, niclosamide significantly reduced the migration of the WSU-HN6 and Tca83 cells.

Meanwhile, the Transwell invasion assay showed that the number of invading cells in 0, 1.25, 2.5, 5 and 10 μM of niclosamide were 327 ± 16.97, 319 ± 12.73, 299 ± 36.77, 166.5 ± 9.19, 67.5 ± 6.36 in WSU-HN6 and 140 ± 7.52, 134.4 ± 5.57, 102 ± 7.04, 92.2 ± 6.72, 71.8 ± 12.34 in Tca83, respectively ($p < 0.05$, Fig. 4E–H). Compared with the number in the corresponding control cells, niclosamide significantly reduced the invasiveness of the WSU-HN6 and Tca83 cells. These results indicated that niclosamide inhibited the invasion

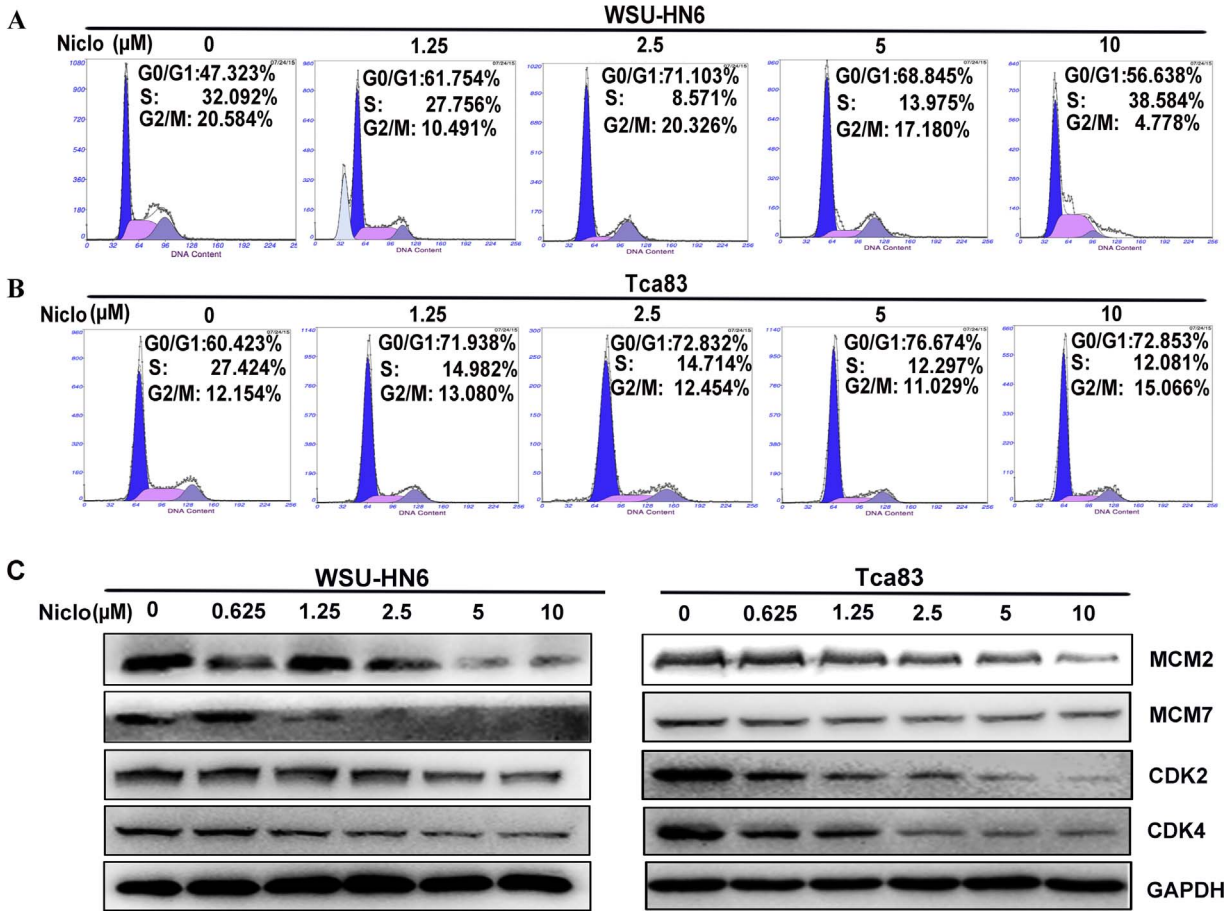

Fig. 2. Niclosamide induces G1 phase cell cycle arrest of WSU-HN6 and Tca83 cells. A-B. WSU-HN6 and Tca83 cells were treated with DMSO or different concentrations of niclosamide (1.25–10 μM) for 24 h. The results show that the percentage of G1 phase is markedly increased and the percentage of S phase is significantly decreased by niclosamide compared with DMSO group in both WSU-HN6 and Tca83 cell lines. The peak value was found at 2.5 μM niclosamide treatment in WSU-HN6 and at 5 μM niclosamide treatment in Tca83. Data are three independent experiments. C. Western blot detected cell cycle-related genes expression at protein level. The results show that niclosamide downregulates cell cycle-related proteins MCM2, MCM7, CDK2, and CDK4 expression.

of the WSU-HN6 and Tca83 cells.

3.7. Niclosamide suppresses p-STAT3 expression, leading to a decrease in migration and invasion of WSU-HN6 and Tca83

To explore the underlying mechanisms which niclosamide inhibits OSCC cells migration and invasion, western blot assay was designed to detect the expression of p-STAT3. The results showed that niclosamide reduced the expression level of p-STAT3 (Tyr-705) in WSU-HN6 and Tca83 (Fig. 4I), which provided an explanation and evidence for wound healing and Transwell invasion results.

3.8. Let-7a is involved in the anti-cancer effects of niclosamide through downregulation of p-STAT3 expression

To explore whether non-coding RNA is involved in the anti-cancer effects of niclosamide, real-time PCR assay was carried on, and we found that let-7a was significantly upregulated by 5 μM niclosamide in WSU-HN6 cell line (Fig. 5A). To further explore the roles of let-7a, we generated stably overexpressing let-7a cell line in WSU-HN6. Real-time PCR showed that let-7a was highly expressed in HN6-let-7a cell line (Fig. 5B), which indicated that stable cell line HN6-let-7a was established successfully. HN6-let-7a as well as empty vector control cell line (HN6-miR-ctrl) was used to detect the expression of p-STAT3, and then to do migration and invasion assays to determine whether niclosamide exerts its anti-cancer effects through let-7a/STAT3 axis. Western blot assay showed that let-7a, like niclosamide, could downregulate the expression of p-STAT3 (Fig. 5C). Meanwhile, wound healing and invasion results showed that HN6-let-7a markedly reduced the migration (Fig. 5D–E) and invasion (Fig. 5F–G) compared with control.

4. Discussion

In this study, we demonstrated an effective anti-OSCC agent niclosamide. OSCC has become a major worldwide problem that afflicts a large number of populations. What is more, the incidence of OSCC seems to be increasing. The complex mechanisms of OSCC remain largely unclear, which indicates the need for developing novel OSCC therapeutic strategies. Recently, a number of studies have shown that niclosamide has a potential anticancer effect both *in vitro* and *in vivo* through regulating multiply signaling pathways [22]. In this study, we find new anticancer mechanisms that niclosamide inhibits OSCC cells proliferation through cell cycle arrest in G1 phase, decreases the ability of migration and invasion through let-7a/STAT3 axis in two OSCC cell lines.

Niclosamide is a Food and Drug Administration (FDA)-approved drug, which has a good safety profile and exhibits low toxicity even after long-term exposure in animals [29]. The broad clinical applications of niclosamide for anti-cancer have been studied recent years. Many studies showed that niclosamide alone [30] or combined with cisplatin [31] could significant inhibit cancer cell growth even at a low concentration, thus implying that niclosamide may be a therapeutic agent for cancer. In our study, through CCK-8, cell cycle and apoptosis assays, we clearly find that niclosamide not only inhibits OSCC cell

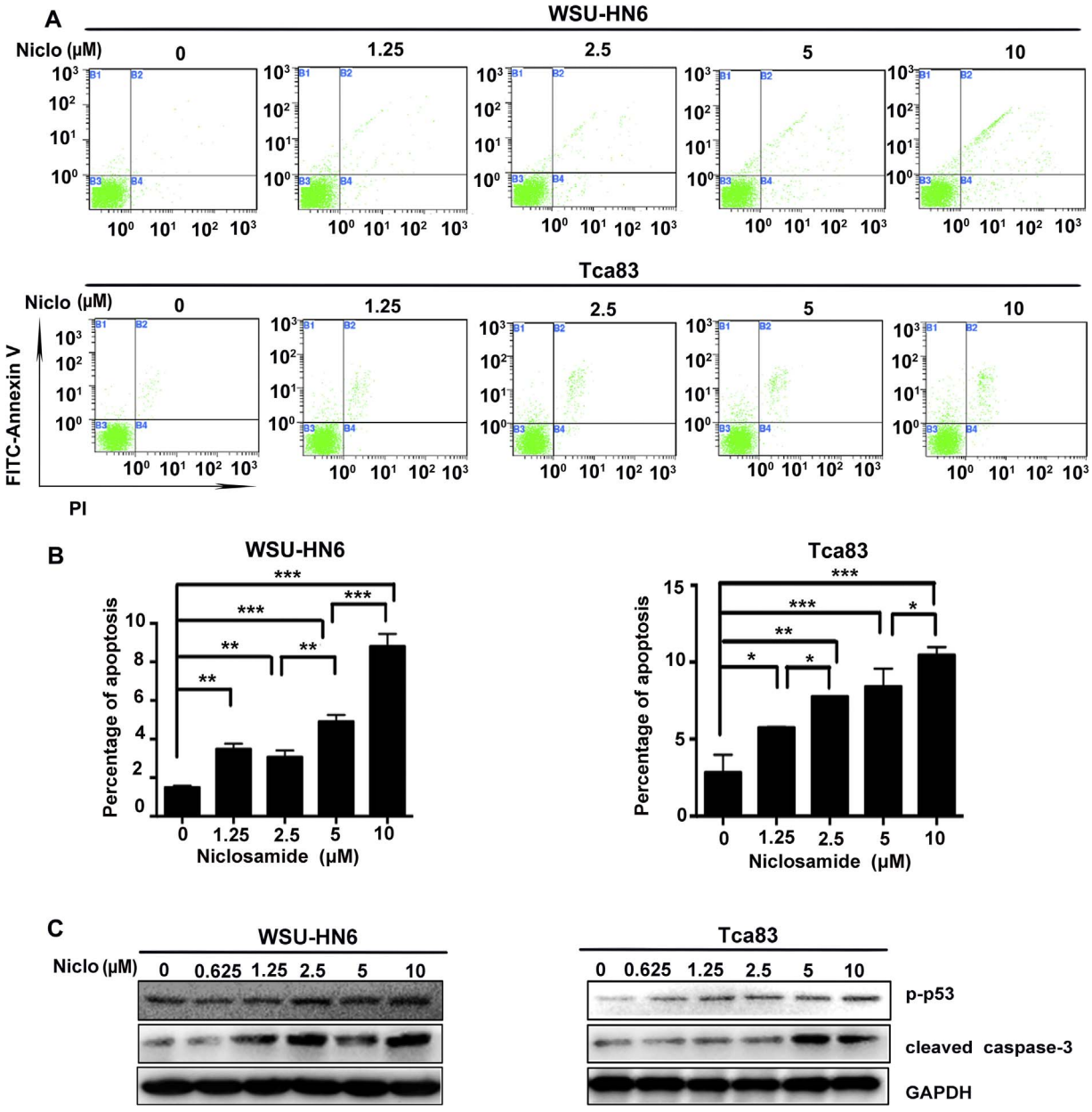

Fig. 3. Niclosamide induces WSU-HN6 and Tca83 apoptosis. A. Two OSCC cell lines were treated with DMSO or different doses of niclosamide (1.25–10 μM) for 24 h, and the level of apoptosis was evaluated using the FITC-Annexin V/PI dual-labeling technique, as determined by flow cytometry. The results show that niclosamide significantly increases the apoptotic cells of WSU-HN6 and Tca83 cell lines. B. Apoptotic data are expressed as means ± SD from three independent experiments (n = 3, $*p < 0.05$, $**p < 0.01$, $***p < 0.001$). C. Western blot detected apoptosis-related genes expression at protein level. The results show that niclosamide upregulates the expression of apoptosis-related proteins p-53 and cleaved caspase-3.

proliferation, but also arrests cell cycle in G1 phase and induces apoptosis in both two OSCC cell lines. This indicates that niclosamide can block tumor cells to pass through G1 phase, finally leading to more dead cells, which provides the explanation for OSCC cells proliferation inhibition by niclosamide. The deduction is in line with CCK-8 data, which shows niclosamide dose-dependently inhibits oral cancer cell growth. Meanwhile, the percentage of S phase was decreased by niclosamide, which indicates that high dose of niclosamide-treated OSCC cells need more time to complete S phase, finally leading to the delayed process of cell cycle and apoptosis. To test the possibility, we did apoptosis assay and found that OSCC cells only could survive at low concentration of niclosamide. High doses of niclosamide markedly induce OSCC cell apoptosis.

Cell cycle is a process from a parent cell to two daughter cells via duplication and division of all components in a cell. The classical cell cycle comprises four phases-G1, S, G2, and M. The cell cycle is tightly controlled by multiple signals and factors, such as cyclin-dependent kinases (CDKs) and their cyclin partners. The cell proliferation occurs in G1 phase, which is controlled by cyclin D-CDK4/6 and cyclin E-CDK2 [32]. Then DNA is replicated in S phase. What is more, the presence of stress, such as DNA damage, can lead to the arrest from S phase to G2 phase. In the normal cell cycle, the levels of CDK2, CDK4, and CDK6 proteins remain relatively constant. However, CDK4/6 activity is frequent alteration of human cyclins in cancer [33]. To further understand molecular changes during niclosamide treatment in OSCC cells, we detect some factors that related to cell cycle, proliferation and apoptosis. MCMs are required in the initiation of eukaryotic genomic DNA replication [34]. In this study, we find MCM2 and MCM7 are markedly downregulated by niclosamide, which indicates that cells need more time to complete their DNA replication in niclosamide-treated OSCC cells. Moreover, we accordingly test two important cell cycle related factors, CDK2 and CDK4, and find their expression levels are

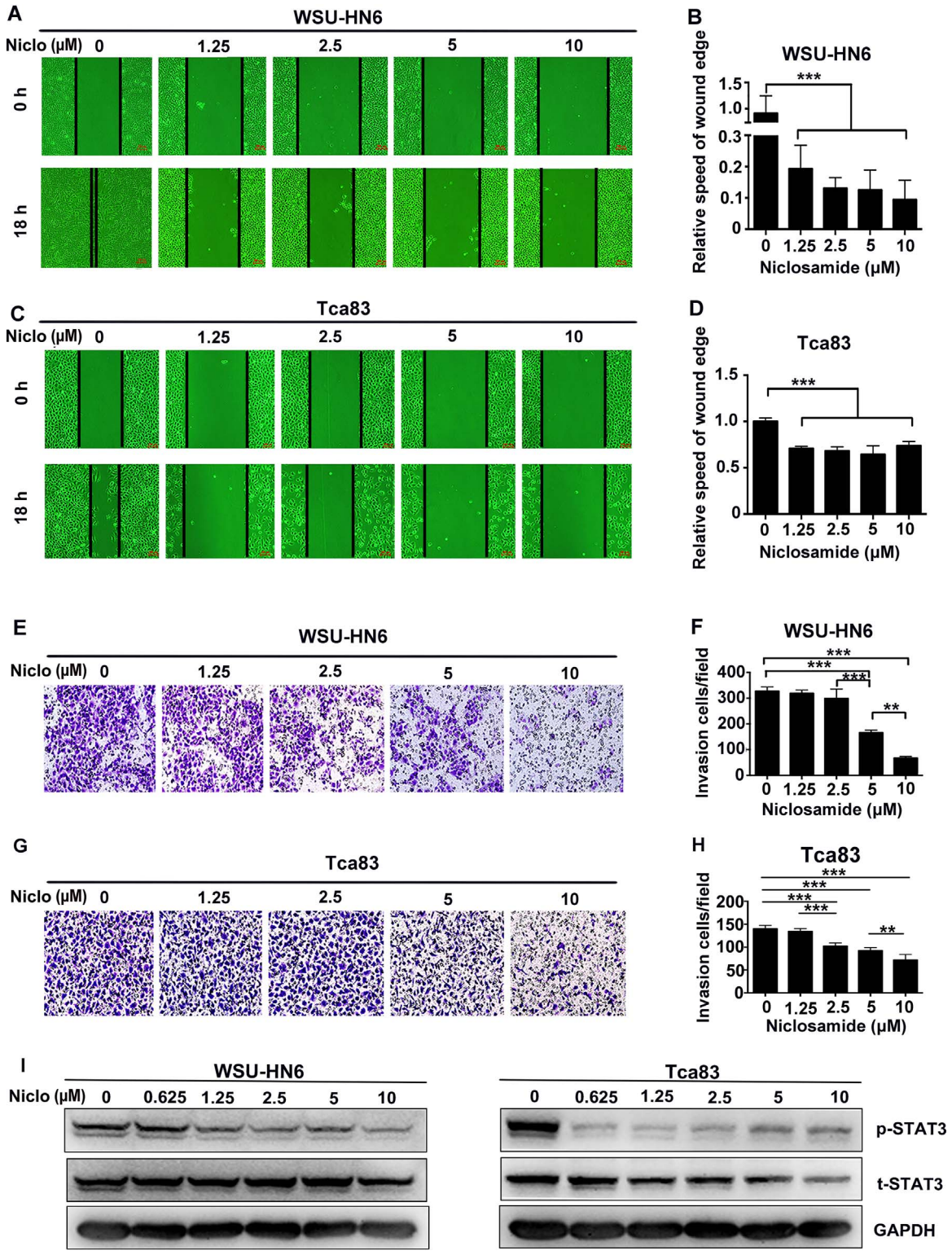

Fig. 4. Niclosamide suppresses WSU-HN6 and Tca83 cell migration and invasion. A & C. Migration results show that niclosamide significantly inhibits the migration of WSU-HN6 and Tca83 cell lines compared with DMSO group. B & D. Quantitative measurement of the speed of wound edge of WSU-HN6 and Tca83 cell lines. The results show that different doses of niclosamide significantly inhibits the migration of WSU-HN6 and Tca83 cell lines. Data are expressed as means ± SD (n = 3, $^{***}p < 0.01$). E & G. Invasion results show that niclosamide significantly inhibits the invading cell numbers of WSU-HN6 and Tca83 cell lines. F & H. Data are expressed as means ± SD (n = 3, $^{**}p < 0.01$, $^{***}p < 0.001$). I. Western blot detected the expression of migration and invasion-related protein p-STAT3 at Tyr705. The result shows that niclosamide significantly inhibits the level of p-STAT3 expression at Tyr705.

significantly reduced in WSU-HN6 and Tca83 cell lines, which indicates that OSCC cells must happen cell cycle arrest. Besides, we also find that pro-apoptosis proteins p53 and cleaved caspase-3 present increased in the two OSCC cell lines.

Combined with cell cycle arrest, apoptosis data and CCK-8 data, we found that niclosamide-induced cell cycle arrest does not contribute to a dose-dependent growth inhibition effect. But niclosamide-induced apoptosis contributes to the dose-dependent growth inhibition effect of niclosamide.

Considering that tumor proliferation, invasiveness and metastasis

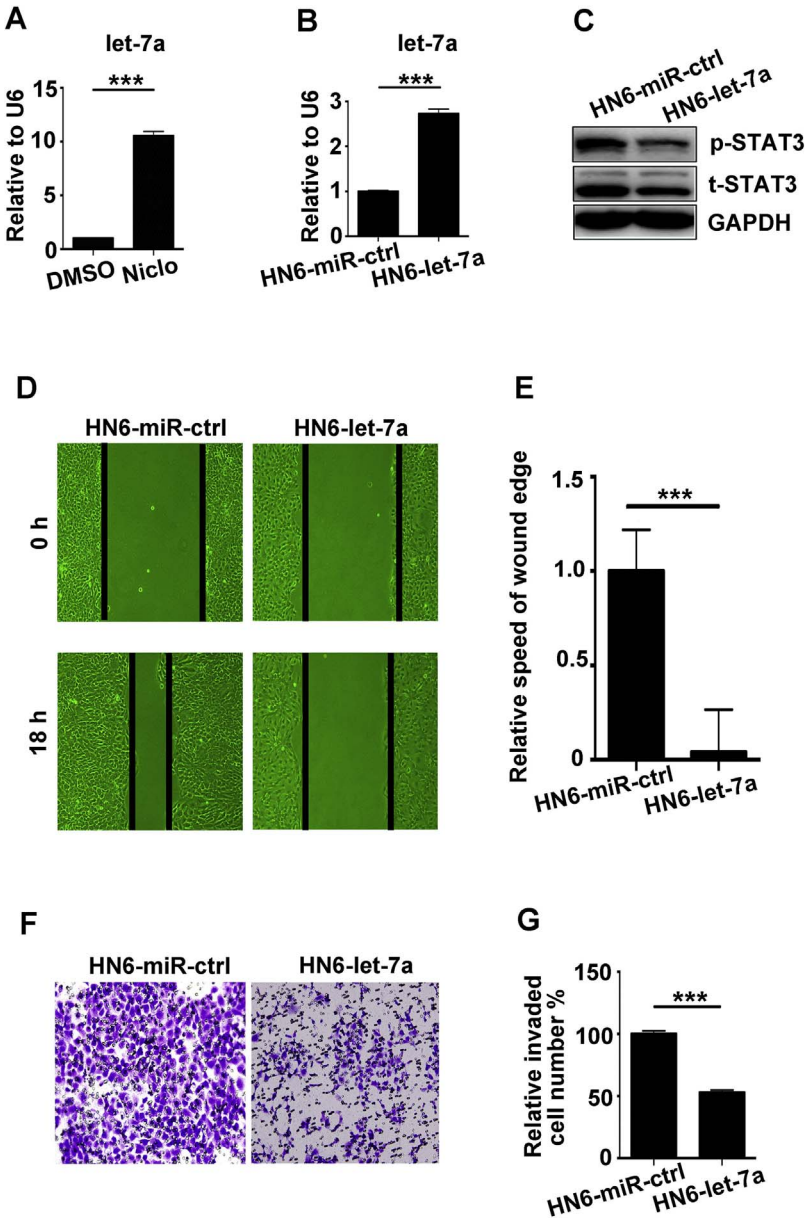

Fig. 5. Niclosamide exerts anti-cancer effects by decreasing migration and invasion through let-7a/STAT3 pathway. A. Real-time PCR assay was done to detect the expression of let-7a under niclosamide treatment. Result shows that let-7a expression is upregulated by 5 μM niclosamide treated. B. HN6-let-7a and HN6-miR-ctrl cell lines were identified by real-time PCR. C. Western blot assay detected the expression of migration and invasion-related protein p-STAT3 at Tyr705. The result shows that HN6-let-7a downregulates the expression of p-STAT3 compared with HN6-miR-ctrl group. D. Wounds scratching result shows that HN6-let-7a has lower migration ability compared with HN6-miR-ctrl. E. Data are expressed as means ± SD (n = 3, ***$p < 0.001$). F. Invasion result shows that HN6-let-7a has lower invasion ability compare with HN6-miR-ctrl. G. Data are expressed as means ± SD (n = 3, ***$p < 0.001$).

are closely related to the prognosis of cancer. Besides, highly extensive invasion into surrounding tissues and metastasis to distant organs are the main reasons to the failure treatment of oral cancer [35,36]. Therefore, we performed wound healing and Transwell invasion assays to detect the ability of migration and transition in two OSCC cell lines and western blot assay to detect p-STAT3 expression. Normally, STAT3 is a latent transcription factor mediates cellular, which can be activated by a variety of cytokines and growth factors [37]. Studies showed that constitutively STAT3 activated could be found at a frequency of 50–90% various human cancers [38]. Aberrant regulation of STAT3 in oral cancer tumorigenesis underlies malignant behaviors by regulating cell cycle progression, survival, apoptosis, invasion, metastasis, and resistance to standard therapies [39]. Thus, targeting the STAT3-inhibiting compounds may be a potency and specificity therapeutic strategy in the treatment of oral cancer. STAT3 is activated primarily through phosphorylation at Tyr705 activation, which seems to be an early step in cancer development and is closely associated with cancer migration and invasion [39,40]. In this study, our data clearly show that niclosamide significantly decreases the migration and invasion of two OSCC cell lines. Meanwhile, we find that p-STAT3 at Tyr705 is downregulated by niclosamide. Accordingly, we assume that niclosamide can inhibit the migration and invasion in OSCC cells through downregulation of p-STAT3 at Tyr705.

Non-coding RNAs are functional important RNAs, which play important roles in both normal physiological and disease processes including cancer by regulation of specific genes through a variety of molecular mechanisms [41,42]. miRNAs are non-coding RNA and serves as endogenous silencers of many target genes [43]. Although scientific reports showed that miRNAs might regulating nearly 90% of human genes, there is limited understanding of their roles in the process of OSCC [44]. miRNA/STAT3 pathway can mediate epigenetic circuits and drive tumor progression in the absence of genetic alterations [23]. Evidences indicate that let-7a could directly target IL-6 mRNA through binding its 3′ UTR. Then, p-STAT3, a downstream target of IL-6, is inhibited by the addition of let-7a [45]. These data support the existence of a second feedback loop between STAT3 and let-7a. What is more, let-7a/STAT3 axis is reported occurred in hepatocellular cancer [46] and cervical cancer [47]. However, there is no evidence show that there exists interaction between let-7a and STAT3 in oral cancer. While our findings have already shown that niclosamide exerts its anti-cancer role

through inactivation of STAT3 pathway, finally leading to inhibition of migration and invasion of OSCC cells. Then, we wonder whether let-7a may be involved in the process of niclosamide-caused STAT3 pathway inactivation. Our results show that let-7a is upregulated by niclosamide. To clarify the target relationship between let-7a and STAT3, we generated the stable cell line HN6-let-7a that highly expresses let-7a, and then detected the expression of p-STAT3. Western blot showed that HN6-let-7a could downregulate the expression p-STAT3 compared with HN6-miR-ctrl. This result indicates that let-7a inhibits p-STAT3 expression. What is more, wound healing and Transwell invasion assays showed that HN6-let-7a has lower migration and invasion ability than HN6-miR-ctrl. The above results indicate that niclosamide can upregulate let-7a, and then dwonregulate the expression of STAT3, finally inhibiting the migration and invasion of two OSCC cell lines.

In summary, this study firstly verified that niclosamide exerts its anti-OSCC effects by cell cycle arrest through regulating cell cycle-related proteins, inhibits migration and invasion by upregulation of let-7a and downregulation of STAT3 in two OSCC cell lines. Considering that niclosamide inhibits the growth, migration and invasion of OSCC cells, it perhaps to be a preferred therapeutic candidate for improving treatment of OSCC patients in future. However, the development of poor bioavailability, the safety needs for cancer patients, and the functional mechanisms analysis of niclosamide are required to be elucidated.

Disclosure

No potential conflicts of interest were noted.

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