

## Original Article

# Side population were reduced by Nitidine chloride through suppressing STAT3/c-myc signaling via miR-17-5p in gastric cancer

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**Abstract:** Objective: To explore the changes of side population (SP) after treated with Nitidine chloride (NC) in human gastric cancer cells MKN-45 and OCUM-2MD3, and mechanism of it, specially, to find which proteins and miRs were involved in this process. Methods: MKN-45 and OCUM-2MD3 cells were treated with varying concentrations of NC for 72 hours. The changes of SP were detected by flow cytometry. The related proteins were tested through Western blot analysis and the miRs were detected by stem-loop RT-PCR, respectively. Among them STAT3, c-myc and miR-17-5p were observed to play an important role in SP cells after NC treatment. Results: The SP cells were obvious reduced after NC treatment in MKN-45 and OCUM-2MD3. NC suppressed the expression of STAT3 and c-myc and resulted in great increased miRs, especially miR-17-5p which was one of the mostly changed miRs and its level was increased about times. More interestingly, the decrease of SP was caused by the down regulation of c-myc via inhibited STAT3. Furthermore, the experiment of above two cell lines with knock down STAT3 or c-myc and miR-17-5p or miR-17-5p inhibitor treatments proved the over expression of miR-17-5p reduced SP by inhibited STAT3/c-myc, and so we think it was an upstream gene of STAT3. Conclusions: Down regulation of STAT3 and c-myc and over expression of miR-17-5p reduced SP after NC treatment. Subsequently, NC reduced SP through suppressing STAT3/c-myc signaling via miR-17-5p in gastric cancer cells MKN-45 and OCUM-2MD3.

**Keywords:** NC, SP, STAT3, c-myc, miR-17-5p, gastric cancer

## Introduction

Nitidine chloride, a natural bioactive phytochemical alkaloid derived from *Zanthoxylum nitidum*, has displayed many biological properties, such as anti-malarial remedy, anti-microbial, anti-HIV [1-3]. In recent years, NC has shown the effects on anti-metastasis in breast cancer by suppressing c-Src/FAK associated signaling pathway and anti-inflammatory via MAPK and NF-kappa B pathway in RAW 264.7 cells [4-7]. Inhibition of the angiogenesis and growth in human gastric cancer via suppressing STAT3 signaling pathway was also reported [8, 9]. Considering the growing evidence of NC on anti-cancer therapy, further research should be needed to elucidate the underlying mechanism.

Cancer stem-like cells (CSCs), are a rather small population of cells in tumors that have the dys-regulated properties, have been considered to be responsible for tumor initiation, treatment resistance, distant metastasis and recurrence [10, 11]. In gastric cancer (GC), c-myc, oct-4, sox-2, CD44, CD133, ALDH and the side population (SP) have been reported by different laboratories to designate population of CSCs [10, 12]. The SP cells, a small population of efficient Hoechst 33342 dye excluding cells from GC tissues and cell lines, have been shown to display increased oncogenicity when transplanted into immunocompromised mice [10, 12]. ATP-binding cassette (ABC) family of transporter protein was responsible for SP cells on the differential ability of to efflux the Hoechst 33342 dye [10]. MicroRNAs (miRs), 19-25 nucleotide

non-protein coding RNAs, act as powerful regulators of gene expression. They target specific mRNA degradation or suppression or activation of translation at the post-transcriptional level [13]. MiRs take part in many biological processes including growth, development, metastasis and apoptosis in cancer. Recently studies have showed more and more miRs abnormal expression in GC [14-17]. Based on this background, the aim at this study was to investigate the anti-tumor function of NC in GC cell lines (MKN-45 and OCUM-2MD3), and the change of miRs and SP in this process.

### Materials and methods

#### *Cell line and cell culture*

Human gastric cancer cell lines MKN-45 and OCUM-2MD3 were purchased from Shanghai Cell Biology Institute of the Chinese Academy of Sciences. These two cells were cultured in DMEM media supplemented with 10% fetal bovine serum, 100 U/ml penicillin, and 50 in/ml streptomycin at standard conditions (37°C, 5% CO<sub>2</sub>, 95% humidity). Plasmid (Plasmid 26596: pSIH1-puro-STAT3 shRNA; Plasmid 29435: Lenti-sh1368 knockdown c-myc) were purchased from Addgene. MKN-45 and OCUM-2MD3 cells were transfected with pSIH1-puro-STAT3 shRNA purified by Puromycin. MKN-45 and OCUM-2MD3 cells were transfected with Lenti-sh1368 knockdown c-myc were isolated by fluorescence-activated cell sorting (FACS). MiR-17-5p inhibitor (purchased from genepharma, China) and plasmid were transfected into cells with Lipofectamine 2000 (Invitrogen, USA).

#### *MTT assay to test inhibition of cell growth by NC in vitro*

MKN-45 and OCUM-2MD3 cells were respectively seeded into 96-well plates and incubated overnight, and then treated for 72 h with varying concentrations of NC (Shanghai P&T Fine Chemical Co., Ltd. China), and 20ine Che.

3-(4,5)-dimethylthiazolium(-z-y1)-3,5-di-phenyltetrazoliumromide (MTT) (Sigma, USA) solution (5 mg/ml in PBS) were added to each well and incubated for 4 h at 37°C. After the removal of the medium, MTT formazan was dissolved in 150 (Sigma, USA) solution (5 mg/ml in PBS) were added to each well in concentrations

cells were cultured in DMEM media supplemented with 10% fetal bovine

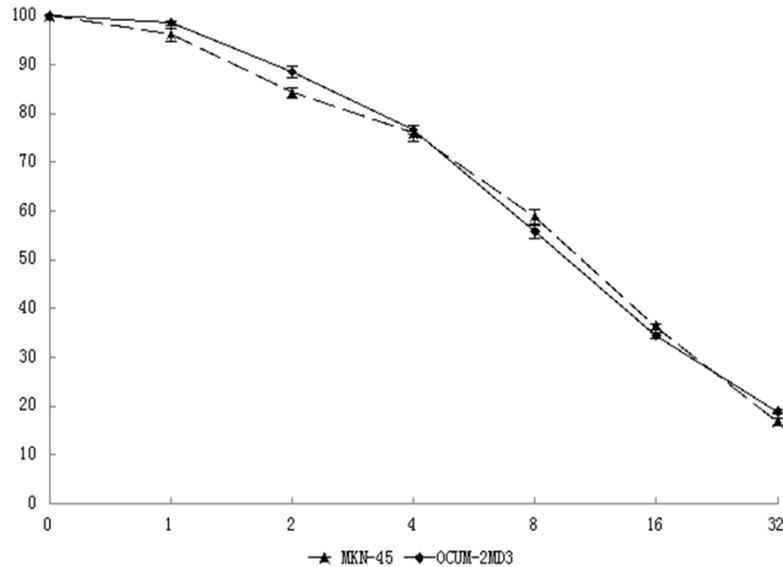
#### *Side population analysis*

Briefly, cells growing in a logarithmic growth phase, were detached from the culture dish, washed twice with PBS and resuspended in culture media supplemented 2% fetal bovine serum at a concentration of 1×10<sup>6</sup> cells/ml. Hoechst 33342 (Sigma, USA) dye was then added at a final concentration of 5 µg/ml in the presence or absence of 50 µg/ml verapamil (Sigma, USA), then incubated at 37°C in a 5% CO<sub>2</sub> for 90 min in the dark with continuous overturn. Later, cells were washed twice with ice-cold PBS, stained with propidium iodide (1 µg/ml, Sigma, USA), and maintained at 4°C for flow cytometry analyses (Beckton Dickson). The Hoechst dye was excited with an UV laser at 351 nm, and the propidium iodide was excited with a green laser at 488 nm.

#### *Real-time PCR to quantify the levels of miRs*

Total RNA was extracted from MKN-45 and OCUM-2MD3 cells, respectively. cDNA was synthesized from total RNA using gene specific primers according to the TapMan MicroRNA Assay protocol (Applied Biosystems) in a 20sp reaction volume with 10 ng of RNA template. Briefly, 10 ng template DNA + gene specific reverse transcription primers (0.05 µmol/l) add RNase-free water to 12 µl, gently blending, incubate at 65°C for 5 min and then at 4°C for 5 min. Then add 4 µl 5\*reaction buffer, 1 µl Ribolock<sup>TM</sup> RNase inhibitor, 2 µl 10 mM dNTP Mix and 1 µl RevertAid<sup>TM</sup> Reverse transcriptase. Reverse transcription was performed using the following program: 60 min at 42°C, 5 min at 72°C and then held at 4°C. Reverse transcription products were diluted 20-fold, and 2 µl was used in a total reaction volume of 20 µl for relative quantification by real-time PCR using an Applied Biosystems 7500 Sequence Detection system. Thermal cycling program used for quantification was as follows: 95°C for 10 min, followed by 40 cycles of 95°C for 15 s and 60°C for 30 s. For normalization, random nonamer-primed cDNA synthesis was made in parallel, and the relative expression of the Ubiquitin gene (UBC) was quantified as previously described. Each measurement was performed in triplicate and no-template controls were included for each assay. Relative expres-

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**Figure 1.** Suppress cells growth effects of NC in gastric cancer. MKN-45 and OCUM-2MD3 cells were treated with varying concentrations of NC for 72 h and cell viability was determined by MTT assay. The experiments were repeated three times. *Errors bars* stand for standard deviations.

sion values were obtained using tenfold dilution curves.

### *MiRs primer design and target prediction*

The sequences of miRs were acquired from miRBase (<http://www.mirbase.org/>). Real-time quantification of miRs by stem-loop RT-PCR used primer designed by ourselves. To identify potential miR binding sites within the 3'UTR of target genes, the following bioinformatics databases were used: miRDB (<http://mirdb.org>), microRNA.org-targets and expression (<http://www.microrna.org>), Targetscan (<http://www.targetscan.org>), and PicTar (<http://pictar.mdc-berlin.de>).

### *Western blot analysis to test proteins of STAT3 and c-myc*

The cells were trypsinized, washed with PBS, and then lysed with radio immunoprecipitation assay (RIPA) and 1 mM phenylmethanesulfonyl-fluorid (PMSF). The lysate were incubated at 4°C for 30 min and centrifuged at 12,000 rpm for 15 min. Equal amounts of lysate were resolved by SDS-PAGE and transferred to polyvinylidene fluoride (PVDF) membrane (Millipore, USA). The membranes were blocked in 5% Bovine Serum Albumin (BSA)/TBST [20 mM Tris-HCl (PH=7.4), 150 mM NaCl, and 0.1%

Tween-20] at room temperature for 1 h and detected with primary antibodies at room temperature for 2 h. The membranes were then blotted for 1 h at room temperature with an appropriate horseradish peroxidase-linked secondary antibody, followed by enhanced chemiluminescence western blot detection reagents (Amersham Pharmacia Biotech, USA). The primary antibody STAT3 and c-myc were purchased from Cell Signaling (USA).

### *Statistical analyse*

All data were analyzed for statistical significance using SPSS 19.0 software (SPSS Inc., Chicago, IL). All values

are expressed as mean standard error. Results were analyzed by two-tailed student's t test. A p value of less than 0.05 was considered statistically significant.

## Results

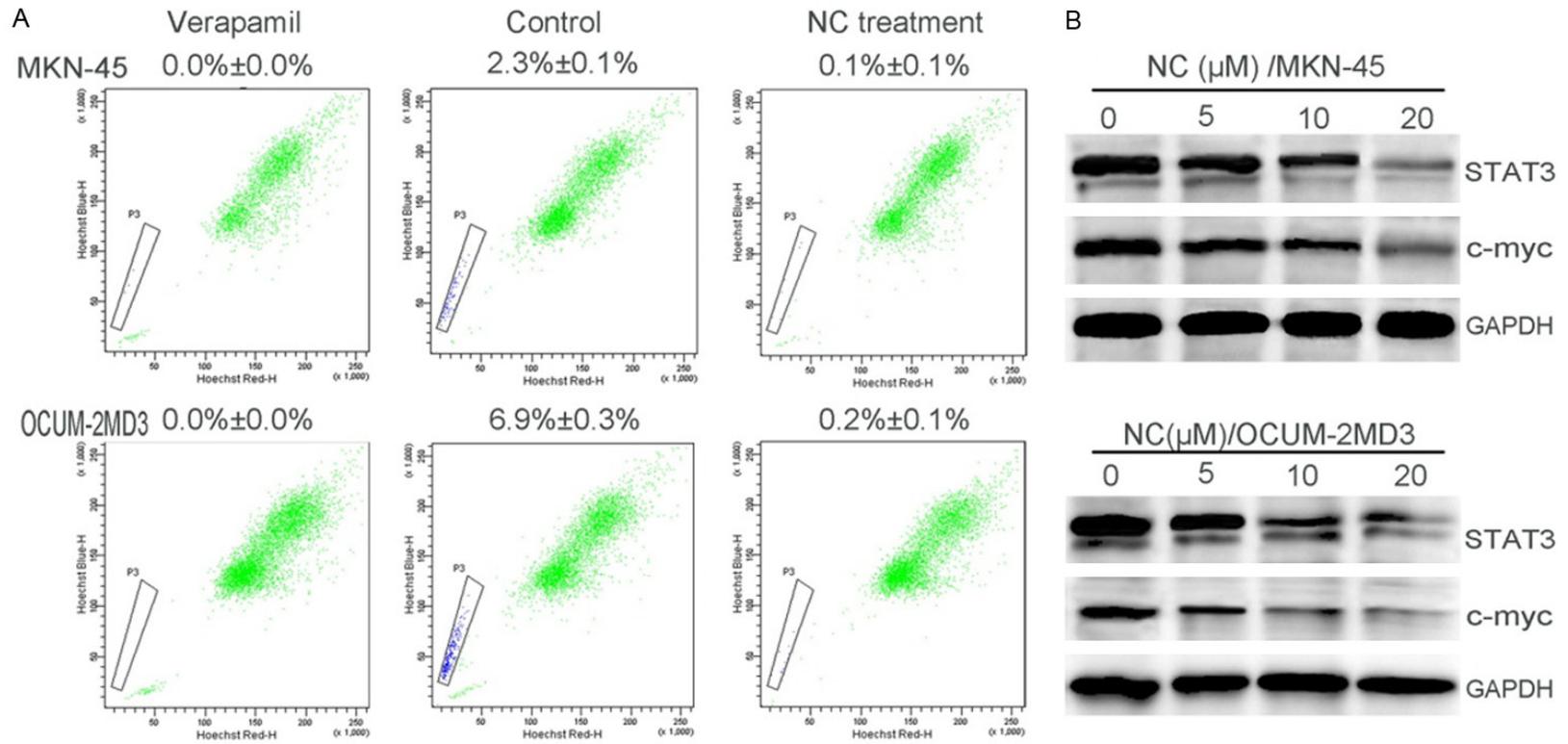
### *NC suppressed the growth of gastric cancer cells in vitro*

NC was previously shown to suppress the cells growth in gastric cancer [8]. First, we tried to find a suitable concentration of NC which could effectively suppress the growth of gastric cancer cell lines MKN-45 and OCUM-2MD3. We evaluated the anti-growth effects of NC in MKN-45 and OCUM-2MD3 by MTT assay. Cells were treated with indicated concentrations of NC for 72 h. As compared to the untreated cells, the treatment with NC for 72 h significantly suppressed cell viability (**Figure 1**). IC50 of NC in MKN-45 and OCUM-2MD3 cells were 9.73 and 10.34 μM/L, respectively.

### *NC reduced the SP of gastric cancer cells and down-regulated STAT3/c-myc expression in MKN-45 and OCUM-2MD3 cell lines*

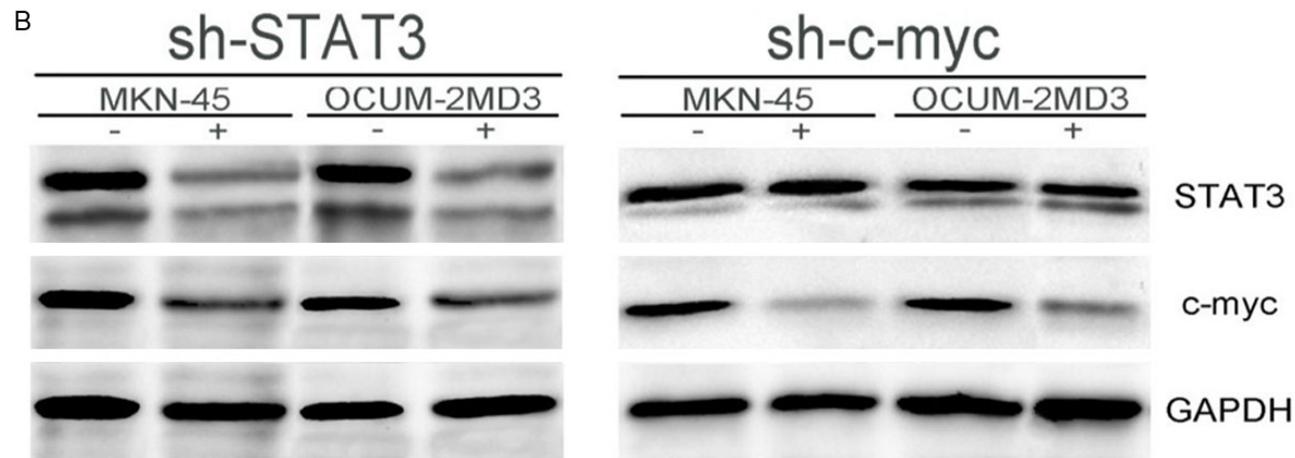
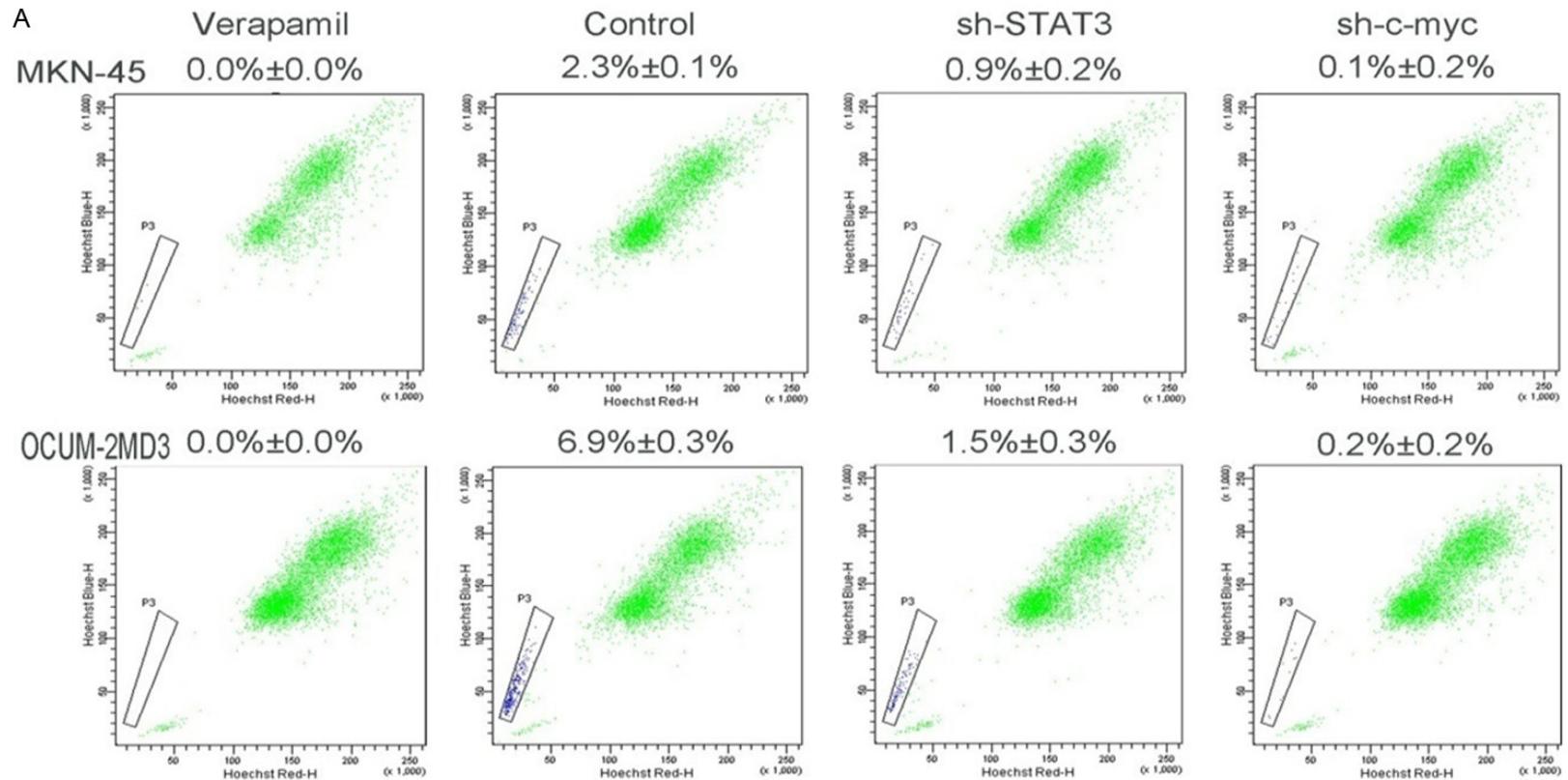
The SP of MKN-45 and OCUM-2MD3 cells display several properties attributable to stem cell-like cancer cells and have been implicated

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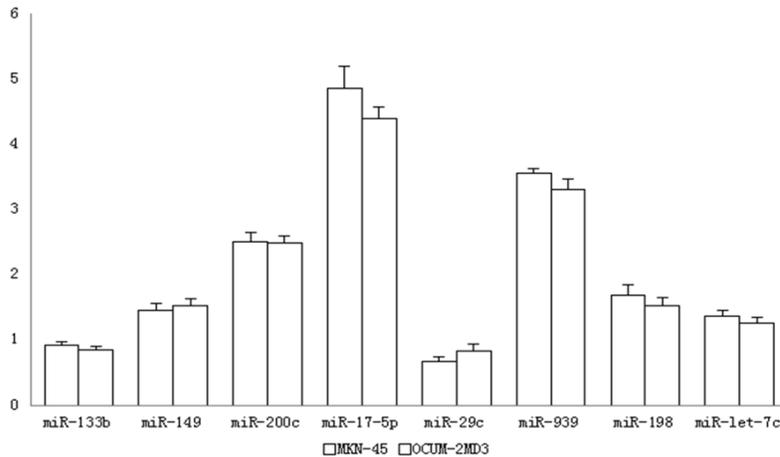
**Figure 2.** NC decreased the SP of gastric cancer cells and down-regulated the expression level of STAT3 and c-myc. A: Flow analysis showed that NC reduced SP in MKN-45 and OCUM-2MD3 cells, which were treated with NC (10 μM/L) for 72 h. B: Western blot showed NC down-regulated STAT3 and c-myc expression in MKN-45 and OCUM-2MD3 cells after treated with varying concentrations of NC for 72 h.

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**Figure 3.** The expression level of c-myc is essential to the decreasing SP by NC. A: Flow analysis showed that knock down STAT3 or c-myc reduced SP in MKN-45 and OCUM-2MD3 cells. B: C-myc expressions were more important than STAT3 expressions in the SP changes and NC decreased SP through suppressing STAT3 to down-regulated c-myc expressions.

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**Figure 4.** Change of miRNAs in gastric cancer cells treated by NC for 24 h, standardized to control. MiR-17-5p and miR-939 transcriptional levels notably increased ~4-fold and ~5-fold, compared to control.

in tumor growth, progression, and metastasis [10, 12, 18]. We detected the SP of MKN-45 and OCUM-2MD3 cells, and found similar SP in these two cells as previous reported [10, 18]. Then we examined the change of SP in these two types of cells that were treated with 10  $\mu$ M/L NC for 72 h, and found that NC reduced SP in both MKN-45 and OCUM-2MD3 cells for both cells of control VS NC treatment,  $P < 0.05$  (**Figure 2A**). NC suppressed the angiogenesis and growth of human gastric cancer via inhibition of STAT3 signaling pathway [8]. STAT3 activated the c-myc gene promoter have been demonstrated. So we further detected STAT3 and c-myc by western blot. We found that STAT3 and c-myc protein expression were down-regulated in MKN-45 and OCUM-2MD3 cells after treated with varying concentrations of NC for 72 h (**Figure 2B**).

### *C-myc was essential in the decreasing SP in MKN-45 and OCUM-2MD3 cells*

The expression levels of STAT3 and c-myc were down-regulated in the process of NC reducing the SP of MKN-45 and OCUM-2MD3 cells. The decrease of SP was caused by which protein was unclear. So we knocked down STAT3 and c-myc by human in MKN-45 and OCUM-2MD3 cell lines, respectively. We detect the change of SP and found that the knock down of STAT3 and c-myc led to the decreasing SP in MKN-45 and OCUM-2MD3 cells. Interestingly, the capability of knock down STAT3 to reduce SP was

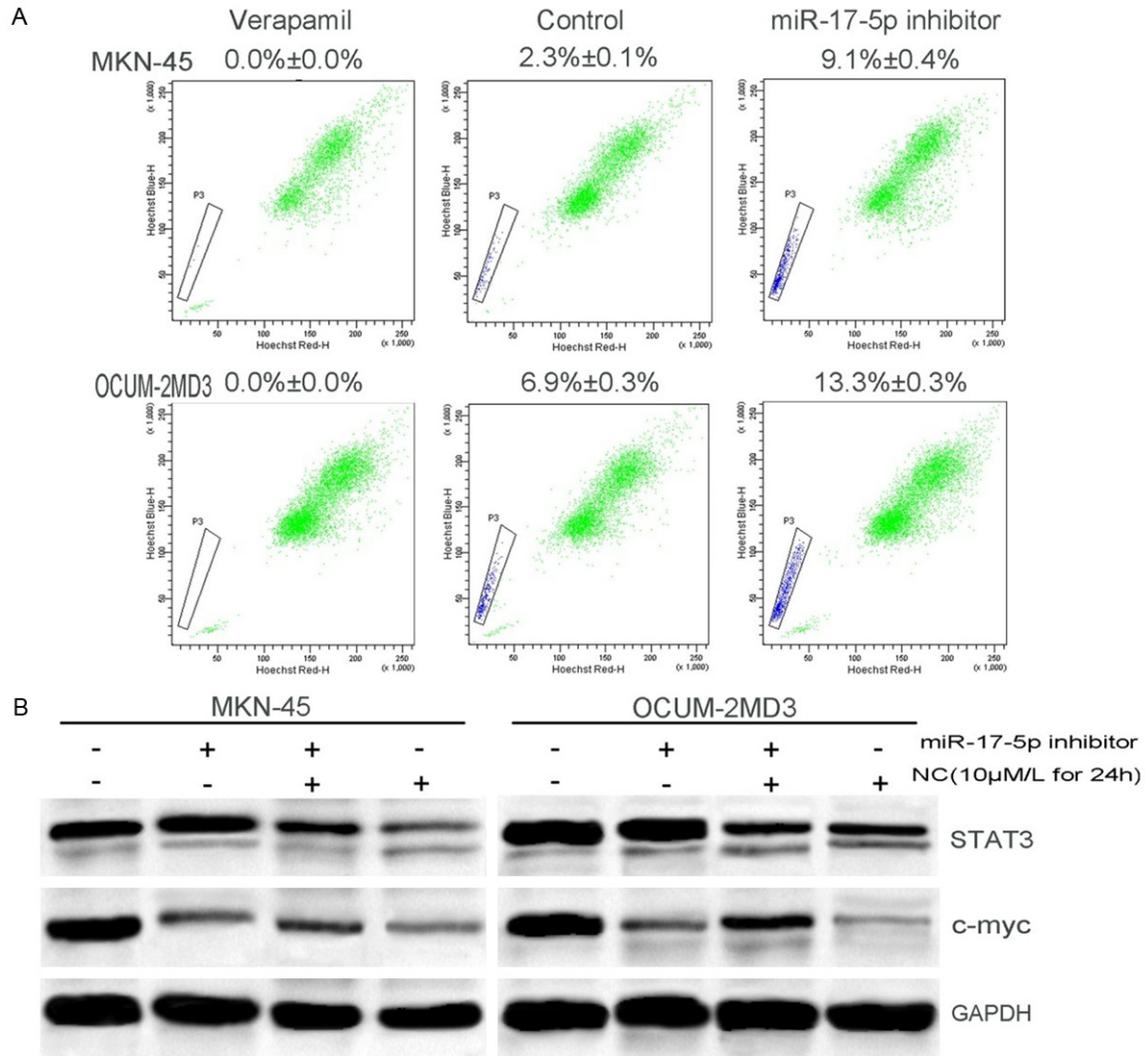
weakened than c-myc for both cells of control VS NC treatment,  $P < 0.05$  (**Figure 3A**). We wondered if c-myc was taken part in reduce SP. Knock down of STAT3 can led to decreasing expression of c-myc. when c-myc was knock down the STAT3 should be kept little change (**Figure 3B**). The above results suggested that c-myc was essential in the reduced SP in MKN-45 and OCUM-2MD3 cells, and NC reduced the SP through suppressing STAT3 to down-regulated c-myc signaling.

### *MiR-17-5p was up-regulated by NC during decreasing SP*

Considering the change of SP was a result of systemic behavior which involved many different signaling proteins, we presumed that NC reduced SP through miRNAs because a single miRNA could regulate hundreds of genes which belonged to different signaling pathways. We used quantitative PCR to profile the changes of eight miRNAs (miR-133b, 149, 200c, 17-5p, 29c, 939, 198, let-7c) which were often dysregulated in gastric cancer [14, 16, 17, 19-21]. As shown in **Figure 4**, these miRNAs responded to NC treatment with different expression patterns, and miR-17-5p transcript increased at least fourfold in MKN-45 and OCUM-2MD3 cells treated with NC for 24 h.

Result of miRNA target prediction and gene classification showed miR-17-5p target genes were involved in Jak-stat signaling pathway, tumor necrosis factor receptor superfamily, mitogen-activated protein kinase signaling, Ras-related GTP binding D, hypoxia inducible factor 1, transforming growth factor, breast cancer metastasis-suppressor 1-like, NF $\kappa$ B inhibitor interacting Ras-like 1, cyclin-dependent kinase inhibitor 1A, Wnt signaling, and tumor susceptibility gene 101. STAT3, MAP3K2, PI3K, HIF1a, Smad7, and CyclinG2 are key targets for miR-17-5p, suggesting that miR-17-5p may play an important role in the occur and development progress of malignant tumors.

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**Figure 5.** MiR-17-5p reduced the SP by cutting down the expression of STAT3 and c-myc in MKN-45 and OCUM-2MD3 cells. A: MiR-17-5p inhibitor increased the SP of MKN-45 and OCUM-2MD3 cells. B: MiR-17-5p inhibitor led to increased expression of STAT3 and c-myc, and NC elucidated the effect of miR-17-5p inhibitor by suppressing STAT3 and c-myc expression.

### STAT3 was regulated by miR-17-5p during NC treatment

To further validate the role of miR-17-5p in reduce SP by NC, expression of STAT3 and c-myc were detected by western blot method. We treated MKN-45 and OCUM-2MD3 cells with miR-17-5p inhibitor alone or combined with NC for 24 h. We found that miR-17-5p inhibitor enhanced expressions of STAT3 and c-myc, and NC played contrary roles (Figure 5A). The expression of STAT3 and c-myc in cells treated with NC and miR-17-5p inhibitor was lower than those in cells treated with miR-17-5p inhibitor, but still higher than those in cells

treated with NC. These data showed that STAT3 and c-myc were regulated by miR-17-5p and NC decreased the SP through miR-17-5p/STAT3/c-myc signaling. In addition, inhibition of miR-17-5p also increase the SP of MKN-45 and OCUM-2MD3 cells (Figure 5B). All results suggested that miR-17-5p was an upstream mediator of STAT3 and c-myc, and played an important role during reduce SP by NC.

### Discussion

Increasing data has shown that NC had significant effects in anticancer in gastric cancer, breast cancer and human osteosarcoma cells

[5, 6, 8]. As a traditional Chinese medicine new usage, it was regarded as a great development and application value drug with powerful function to antitumor [22, 23]. Although a few studies have reported that apoptosis signaling, c-Src/FAK associated signaling pathway, NF- $\kappa$ B and MAPK signaling pathways were involved in the regulation of NC [5, 6, 22, 24-29], the mechanism of NC has largely remained unexplored. In our study, NC was purchased from Shanghai P&T Fine Chemical Co., Ltd. in China and composed of light yellow acicular crystal. The purity of it is more than 98%. We proved that NC efficiently suppressed cell growth and decreased SP in gastric cancer cell lines. Importantly, we represented evidence to show that NC decreased SP through suppressing STAT3/c-myc signaling. Moreover, miRs, the most powerful transcriptional factors (especially miR-17-5p), were involved in diminution of SP during NC treatment in gastric cancer.

Cancer stem-like cells are regarded as the cause of tumor formation, recurrence and metastasis [10, 18, 30]. A number of markers, such as CD44+, CD133+, ALDH+ and SP, have proved to be useful for isolation and identification of CSCs in gastric cancer. Moreover, a group of transcription factors, including c-myc, oct-4 and sox-2, have been reported high expression in CSCs [30-34]. SP cells in gastric cancer cell lines (OCUM-2MLN, MKN-45) possessed repopulating capacity and high tumor forming ability in vivo [10, 12, 18]. Our study detected the change of SP cells after treated with NC as a maker of CSCs in gastric cancer cell lines. SP were related to ABC family transporters, which possess of high efflux ability of Hoechst-33342 dye [12, 18]. So we test ABCG2 and MDR by western blot in MKN-45, MKN-45 treated with NC, OCUM-2MD3 and OCUM-2MD3 treated with NC, found that they were very low expression, and had no difference in them.

The interleukin-6 (IL-6)/Janus kinase 2 (JAK2)/signal transducer and activator of transcription 3 (STAT3) was an important signal pathway in mediating the motility, migration and invasion of GC cells, and inhibition of STAT3 may be a useful therapeutic approach for metastasis of gastric cancer [6, 8, 35]. The expression of STAT3 was decreased by NC in GC cells have been report [8]. C-myc played an important role for CSCs, and was regulated by STAT3 by com-

bined with promoter. In the process of decreased of SP by NC, STAT3 and c-myc were decreased, too. The expression of STAT3 and c-myc were knocked down to analysis SP, found that c-myc play an important role in reduced SP. NC decreased the SP through STAT3/c-myc signaling pathway.

Accumulative evidence had shown that miRs were key regulators for majority of signaling and participated in most of cell biological behaviors. In the tumor of GC, many miRs were dysregulated, and promoted progress, invasiveness and metastasis of tumors. MiR-17-92 cluster has been broadly investigated in GC development. MicroRNA-18a, a member of MiR-17-92 cluster, modulates STAT3 activity through negative regulation of PIAS3 during gastric adenocarcinogenesis was report in recent years [21]. MiR-17-5p, highly expressed in (GC) tissues, promotes gastric cancer cell proliferation and inhibits cell apoptosis [36-38]. In our study, several miRs (including miR-200c, 939, 17-5p) were induced by NC, suggesting that NC affected the cell biology by regulating miRs transcript. Gene P130 was proved to be a target gene of miR-17-5p, but the relation of miR-17-5p and STAT3 could be identified by general methods which mainly targeted the 3'UTR sequence of gene mRNA. As far as miR-17-5p was concerned, inhibition of miR17-5p in GC cells resulted in increased expression of STAT3 and c-myc, and notably eliminated the function of NC on these proteins. Considering that miRs can bind to gene mRNA with different modes, miR-17-5p might target gene STAT3 or be an upstream of JAK/STAT3 signaling. These result demonstrated that miR-17-5p was necessary for decreased STAT3 expression by NC, and STAT3/c-myc signaling was a potential target for miR-17-5p.

In this study, we demonstrated, for the first time, that miR-17-5p was an essential mediator in decrease SP by NC in gastric cancer cells. MiR-17-5p suppressed STAT3/c-myc signaling and resulted in gastric cancer cells SP decreased. In addition, many other miRs might be involved in NC regulation; further research was needed to elucidate the underlying mechanism. Nevertheless, our study provided a framework to start understanding the function of NC in anti-cancer via miR-17-5p, suggesting NC was an efficient anti-cancer drug with multiple mechanisms.

In conclusion, Nitidine Chloride can reduce cancer stem cells (side population) through STAT3/c-myc signaling via miR-17-5p in gastric cancer, this imply NC targeting cancer stem cells maybe prevent gastric cancer relapse.

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### Disclosure of conflict of interest

None.

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