

Nitidine chloride inhibits proliferation and induces apoptosis in colorectal cancer cells by activating the p53 signaling pathway

Qiao Zheng

Hospital of Chengdu University of Traditional Chinese Medicine

Yan Zhang

State Key Laboratory Basis of Xinjiang Indigenous Medicinal Plants Resource Utilization, Chinese Academy of Sciences

Lanlan Zhang

University of Chinese Academy of Sciences

Nianzhi Chen

Hospital of Chengdu University of Traditional Chinese Medicine

Gang Zhang

Hospital of Chengdu University of Traditional Chinese Medicine

Maoyuan Zhao

Hospital of Chengdu University of Traditional Chinese Medicine

Wenhao Liao

Hospital of Chengdu University of Traditional Chinese Medicine

Zhongzhen Zeng

Hospital of Chengdu University of Traditional Chinese Medicine

Yujie Wei

Hospital of Chengdu University of Traditional Chinese Medicine

Gang Zhou

NMPA

Jianyuan Tang (✉ tangjy@cdutcm.edu.cn)

Hospital of Chengdu University of Traditional Chinese Medicine

Ziyi Zhao

Hospital of Chengdu University of Traditional Chinese Medicine

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Abstract

Background: Colorectal cancer, as a general malignancy of the digestive system, has long constituted a threat to the health of the population and the development of the social economy. It has been widely recognized that exploiting a drug with weaken toxic side effects and excellent antitumor activity can be a viable and promising strategy. Nitidine chloride is a typical bioactive base extracted from the dried roots of the *Zanthoxylum nitidum* (Roxb.) DC, has been clinically evidenced to exhibit robust immune efficacy against several types of tumor cells. However, the underlying function mechanism of the anti-colorectal-cancer effectiveness is still in its exploratory stage and thereby deserves further in-depth investigations.

Objective: To examine the anti-cancer activity of Nitidine chloride against colorectal cancer in vitro and in vivo and its mechanism of effect.

Methods: In this work, we combined a series of cellular characterization techniques to examine the inhibitory effect of Nitidine chloride (NC) on colorectal cancer cells, including RKO, HCT116 and HT29 cells. In addition, the potential signaling pathways were clarified via transcriptome sequencing technique. Moreover, we proposed a reliable biomedical mechanism towards the inhibition effects of the NC against colorectal cancer on the basis of qRT-PCR and western blot.

Results: At first, we examined the toxic effect of NC on several colorectal cancer cells (RKO, HCT116 and HT29), within which the value of IC₃₀ and IC₅₀ were determined to be 2 μ M and 3 μ M for RKO; 3.5 μ M and 5 μ M for HCT116; and 6 μ M and 10 μ M for HT29, respectively. Through transcriptome sequencing, it is acquired that the p53 pathway and downstream signaling was prominently activated, indicating that NC is strongly correlated with cell proliferation and apoptosis. We also reveal that NC was able to suppress malignant behaviors, including proliferation, migration, invasion and tumor formation in RKO, HCT116 and HT29 cells. Besides, NC treatment significantly modified EMT-related hall marker proteins, including E-cadherin, N-cadherin and MMP9, which is indicative of the inhibition of EMT. Furthermore, the IC₅₀ of NC significantly upregulated the pro-apoptosis-related factors, including Bax, Noxa, and caspase-3 and down-regulated the apoptosis-inhibiting factor Bcl2.

Conclusion: In conclusion, the above experimental results and data analysis pointed out that colorectal cancer can be considerably suppressed by NC treatment, which effectively activates p53 signaling. Our approach should hold substantial promise for therapeutic applications in the therapy of colorectal cancer.

1 Introduction

Colorectal cancer, one of the typical malignant tumors of the digestive system, has continuously bring detriment to the human health and public hygiene. According to the International Agency for Research on Cancer (IARC) Global Cancer Statistics, the number of newly diagnosed colorectal cancer cases worldwide in 2020 is estimated to be 1.9 million (the 3rd highest prevalence) and the number of deaths due to colorectal cancer is estimated to be approximately 935, thousand (the 2nd highest mortality)[1]. The current treatment is dominated by endoscopic and surgical local excision, supplemented by radiation

therapy, ablative therapy, biological agents, as well as the immunotherapy, among others[2]. Although these therapeutic options have resulted in increased survival rates, 60% of patients still experience recurrence and metastasis even after surgical resection. Consequently, this also emphasizes the necessity of early diagnosis of colorectal cancer and exploration of new treatment strategies.

Previous studies have shown that the occurrence of colorectal cancer is associated with many oncogenes, including p53, a transcription factor and tumor suppressor encoded by the TP53 gene, which is usually mutated in most tumor types and is the most commonly mutated gene in many types of cancer[3–5]. The mutated p53 promotes the survival of cancer cells, which contributes to the development of cancer and is often associated with a poor prognosis[6, 7]. The p53 regulatory target genes involved in a variety of cellular responses, including cell cycle arrest[8, 9], DNA repair[10], apoptosis[11], metabolism, autophagy, mRNA translation and feedback mechanisms, among others[12]. Many existing studies have confirmed that p53 has been recognized as an oncogenic protein targeting the substrates Bcl-2, NOXA, and CCNB1[13, 14]. Therefore, activation of p53 may be a new approach for effective treatment of colorectal cancer.

NC is an alkaloid isolated from the roots of the plant *Zanthoxylum nitidum* (Roxb.) DC. Its chemical formula is displayed in Fig. 1A, has been verified to behave various biological activities including anti-inflammatory, paroxysmal, antioxidant, and antibacterial[15–17]. Several studies have proven that NC has anti-cancer properties in a variety of tumors. It has been shown that NC inhibits the proliferation, migration and invasion of non-small cell lung cancer cells via activation of the Hippo signaling pathway[18]. Fang et al. discovered that NC exerts its anti-metastatic effect on kidney cancer cells by inhibiting the AKT signaling pathway[19]. While these studies have initially confirmed the antitumor activity of NC, the anticancer mechanism of NC in colorectal cancer has not been clarified. In the current study, we investigated that NC could restrain the proliferation, migration and invasion of colorectal cancer cells (RKO, HCT116 and HT29), induce apoptosis and block the G2 phase of cells. Through in vivo and in vitro experiments, we demonstrated that NC activated p53 to target related substrates to exert oncogenic effects. Finally, it reveals the cancer inhibitory effect of NC on colorectal cancer and provides new ideas to advance NC and related agents.

2 Materials And Methods

2.1 Chemicals and Reagents

Nitidine chloride (molecular weight:382.828g/mol, molecular formula: $C_{21}H_{18}ClNO_4$) was provided by Chengdu Must Bio-Technology Co.,LTD and dissolved in DMSO to an initial concentration of 10mM. Both primary and secondary antibodies were purchased from Beyotime Institute of Biotechnology (Shanghai, China). CCK-8 was obtained from AbMole Bioscience (Houston, TX, USA). And the Annexin V-FITC Apoptosis Detection Kit was given by BD Biosciences (Franklin Lakes, NJ, USA).

2.2 Cell culture

RKO, HCT116 and HT29 cells were all obtained from Chinese Academy of Science Cell Bank (Shanghai, China) and cultured with DMEM culture medium (10% fetal bovine serum and 1% penicillin-streptomycin) at a temperature of 37°C in 5% CO₂. Cells were passaged every 3 days.

2.3 Cell viability assay

Cell viability was detected with the classical CCK-8 assay. We inoculated the cells (RKO, HCT116 and HT29 cells) in 96-well plates with 5×10^3 cells per well, and 6 replicate wells were arranged for each group of experiment. When the cells were plastered, various concentrations (0–80 µM) of NC then added to the cells for 24 h. DMEM medium and CCK-8 reagent were blended in accordance with the 9:1 ratio. The 96-well plates were continually incubated for 2 h at 37°C in 5% CO₂. It was finally determined by absorbance at 450 nm using an enzyme marker (BK-EL10C).

2.4 Transwell assay

Transwells with 8 µm pore size were chosen for migration and invasion experiments. We then put 100 µL of cell suspension into the upper chamber of Transwell and 500 µL of medium containing 10% FBS into the lower chamber, and incubated for 48 h in an incubator at 37°C. We set 0, 2 µM and 3 µM complete medium for RKO cells; 0, 3.5 µM and 5 µM complete medium for HCT116 cells; and 0, 6 µM and 10 µM complete medium for HT29 cells. After fixed with 4% paraformaldehyde for 20 min, the cells were stained with 0.1% crystal violet for 20 min. Finally, we observed the number of cells in the chambers with a fluorescence microscope (OLYMPUS TH4-200). In contrast with the migration assay, the invasion assay required the pre-addition of 50 µL of diluted Matrigel in the upper chamber and incubation at 37°C for 4 h to solidify it into a gel before adding the cell suspension.

2.5 Colony formation assay

Take logarithmically grown cells and inoculate them in 12-well plates at a density of 500 cells per well. 0, 2 µM and 3 µM complete medium were set to treat RKO cells; 0, 3.5 µM and 5 µM complete medium were set to treat HCT116 cells; 0, 6 µM and 10 µM complete medium were set to treat HT29 cells, with three replicate wells for each experimental group. Let the cells incubate in the incubator for 7–14 days, and change the liquid treatment every 2 days. We also observed the cell status using a microscope. In the end, 4% paraformaldehyde was added to fix the cells for 20 min and 0.1% crystal violet was used for staining for 5 min. The number of colonies that were formed was counted under the microscope.

2.6 Cell apoptosis assay

The cells were inoculated into 6-well plates at a cell density number of 1×10^5 cells per well. We set 0, 2 µM and 3 µM complete medium for RKO cells; 0, 3.5 µM and 5 µM complete medium for HCT116 cells; and 0, 6 µM and 10 µM complete medium for HT29 cells, with three replicate wells for each experimental group. After 24 h of administration, cells in the intervention and control groups received treatment with trypsin and two washes with PBS. After centrifugation at 3000 r/min for 5 min, we added 1×Annexin V Binding Solution to prepare 500 µL cell suspension, which was supplemented with 5 µL of Annexin V-

FITC conjugate and 5 µL of PI solution. Cell suspensions were placed in the dark at room temperature for 15 minutes and analyzed using a flow cytometer (Navios).

2.7 Cell cycle analysis assay

The effect of Nitidine chloride on cell cycle was examined by flow cytometry (FCM). It was set up for RKO cells: 0, 2 µM complete medium; for HCT116 cells: 0, 3.5 µM complete medium; for HT29 cells: 0, 6 µM complete medium. After 24 hours of the administration, the cells were fixed overnight in 70% anhydrous ethanol, followed by staining with 50 mg/ml propidium iodide (PI, Sigma) at a temperature of 4°C for 30 minutes. The final evaluation was performed with BD FACSCanto II from BD Bio-Sciences (San Jose, CA, USA). Cell phases were indicated by from G0 - G1, S as well as G2 -M.

2.8 Western blotting analysis

The NC-treated HCT116 or RKO cells were gathered and 1×10^6 of them in 200 µL RIPA buffer were suspended. The cells were lysed and total protein was extracted from the cells. BCA protein assay (Sigma) was applied to measure the protein concentration. By loading the proteins onto 10% SDS-PAGE, the protein samples were separated by electrophoresis, followed by transferring the proteins on the gel to a PVDF membrane. Protein antigens were blocked with 10% skimmed milk prepared with 0.1% TBST at room temperature for 60 min. The membranes were then incubated overnight at 4°C with the corresponding primary antibodies, which included p53 (cat.no.AF5258), MDM2 (cat.no.AF7449), Bax (cat.no.AF1270), Bcl2 (cat.no.AF6285), Noxa (cat.no.114C307), caspase-3 (cat.no.AF1213), Gadd45A (cat.no.AF6954), MMP9 (cat.no.AF5234), N-cadherine (cat.no.AF5237), E-cadherine (cat.no.AF6759), P48 (cat.no.AF6681), p53R2 (cat.no.AF7920) at a dilution ratio of 1:2000. After washing three times with TBST, the secondary antibody was used to incubate for 60min, and finally the protein signal was shown with ECL luminol.

2.9 Real-Time Quantitative PCR

Following the instructions, we extracted total RNA samples by TRIzol reagent (Life Technologies, Grand Island, NY, USA). When the RNA was isolated, the concentration of purified RNA was measured by a UV spectrophotometer (Thermo Fisher). Reverse transcription to cDNA was performed in the extracted total RNA using a reverse Transcriptase Kit (RiboBio, Guangzhou, China). For the analysis of mRNA, SYBR Green Master Mix (Life Technologies, Grand Island, NY, USA) was applied to an ABI 7500 system (Applied Biosystems Foster City, CA, USA)) at the following conditions: 95°C for 10 minutes, 60 cycles of 95°C for 15 seconds, and 60°C for 1 minute. Typical primers used in this study were as follows: GAPDH forward:5'-GCACAGTCAAGGCCGAGAAT-3' ; reverse:5'-GCCTTCTCCATGGTGGTGAA - 3'; p53 forward:5'-CTGAGGTTGGCTCTGACTGT-3'; reverse:5'-TCAAAGCTGTTCCGTCCCAG-3'; Bax forward:5'-TGTCGCCCTTTTCTACTTTGC-3'; reverse:5'-GAAGTCCAATGTCCAGCCCA-3'; Gadd45A forward5'-GTGCTGGTGACGAATCCACA-3'; reverse:5'-ATCCATGTAGCGACTTTCCCG-3'; P48 forward:5'-TCGATGGAACTCAGGGAAG-3'; reverse:5'-TGGCTCCAGATGAGAATGTG'; p53R2 forward:5'-GAGAGGTCTTATGCCAGGACTC-3'; reverse:5'-TCAACAATGATCTCCCTGACC;Noxa forward: 5'-ACATGGGGATTTTGTATAACCAGG reverse: 5'- CAAGGAGCATTTTCCGAACCT-3'.

2.10 Animal models

BALB/c nude mice with an age of 4 weeks and a body weight of 18–20 g were provided by Viton Lever Laboratory Animal Technology Co. The mice were fed in SPF-grade animal barriers at a controlled temperature of 24°C, with relative humidity maintained at 50% and fresh air changes of 10 times per hour. All of the animal experiments were carried out under the approval of the Chengdu University of TCM Animal Care and Utilization Ethics Committee. 5×10^5 HCT116 cells were injected subcutaneously into the right flank via nude mice to establish a subcutaneous graft tumor model. After 7 days of modeling, the nude mice were randomly divided into control and 5 mg/kg NC groups, with 5 mice in each group. The NC treatment was administered 5 days per week, with weight and tumor size measured every 7 days until week 4.

2.11 Statistical analysis

GraphPad Prism 9.0 was applied to perform the analysis of the data, all of which was displayed as mean $\pm$ standard deviation. The approach followed for the comparison of statistical differences among the groups was one-way ANOVA, and the t-test was chosen for the comparison between the two groups. As a guarantee of the scientific validity of the data, all these experiments were repetitively performed at the very least three times in this research. At a p-value < 0.05 , we regarded the probability of a difference due to chance as relatively low and the difference as statistically significant.

3 Results

3.1 NC suppresses cell survival of CRCs

The cell proliferation inhibition rate was measured by CCK-8 after NC treatment on CRCs (RKO, HCT116 and HT29 cells), as shown in Fig. 1B, NC had an apparent proliferation inhibitory effect on Colorectal cancer cell lines. For the RKO cells, when the NC concentration reached 2 μM , the cells reached 30% inhibition rate (IC₃₀); When the NC concentration reached 3 μM , the cells reached a half inhibition rate (IC₅₀). The cell proliferation rate decreased dramatically with the increase of NC concentration, and the maximum inhibition rate reached 12% with a statistical difference. In contrast, for HCT116 and HT29 cells, the IC₃₀ and IC₅₀ were 3.5 μM and 5 μM , 6 μM and 9 μM , respectively, with a maximum inhibition rate of 21% and 28%, respectively. Accordingly, the proliferation inhibitory effect of NC on Colorectal cancer cells has been shown to be concentration-dependent. Cell mortality was measured using the Calcein-AM/PI double staining method. The results showed that the proliferation of RKO, HCT116 and HT29 cells was inhibited by NC, and the ability to suppress cell proliferation was stronger as the concentration of NC increased (Fig. 5B)

3.2 p53 is a potential target of NC in treating colorectal cancer

After screening the sensitivity of NC to colorectal cancer cells, we further explored the signaling pathways that may play a role in the treatment of colorectal cancer by transcriptome sequencing. The control cells and NC-treated cells were cultured under the same conditions for 24 h, and then their RNA was extracted for transcriptome sequencing. KEGG is a knowledge base for systematic analysis of gene function and linking genomic information to functional information. The bubble map showed (Fig. 2A) that the p53 signaling pathway was enriched with the most differential genes. The results showed (Fig. 2B) that colorectal cancer emerged among the predicted human diseases; the p53 signaling pathway was shown to have the greatest impact in the cellular process. Figure 2C showed the clustering heat map analysis of colorectal cancer cells with relatively significant differences in gene expression compared to the control group. We inferred that the addition of NC may contribute to apoptosis by activating the p53 pathway in colorectal cancer cells, thereby suppressing tumors.

3.3 NC suppresses colony formation

The cancer cells, in contrast to normal cells, are capable of unlimited proliferation proceeding to destroy normal cells in the body. Therefore, colony formation assays were performed to reveal the inhibitory effect of NC on the ability of individual colorectal cancer cells to proliferate indefinitely. The number of RKO cell colonies was remarkably reduced in the NC group at 2 μ M and 3 μ M compared to the control group with a large number of purple colored cell colonies (Fig. 3A and B). When the concentration of NC was 3 μ M, the inhibition rate was about 50%. For the HCT116 cells, the 5 μ M NC group exhibited a colony inhibition rate of about 48%. However, NC had a significant colony inhibition for HT29 cells (~ 82% inhibition, $P < 0.01$).

3.4 NC invokes cell cycle block

Via the consequential cytometry analysis, we were able to determine the spreading of the cell cycle at each divisional stage after drug treatment. The experimental results revealed that the cells all underwent G2/M blockade at the time of NC of IC30 concentration exposure. With respect to the control group, the G2/M phase of RKO cells subjected to 3 μ M NC treatment increased from 7.83% in the control group to 24.3%; HCT116 cells exposed to 4.5 μ M NC showed an elevation of G2/M phase from 22.02% in the control group to 39.72% and an increase in G2/M phase from 4.6% in the control to 20.06% in HT29 cells after 9 μ M NC processing (Fig. 3C). All of the above data illustrates that NC possesses a cycle blocking function on CRC cells.

3.5 NC diminishes motivational properties of CRC cells

With the aim of exploring the migration and invasive ability of colorectal cancer cell lines after NC treatment, we performed Transwell experiments with NC of IC30 and IC50. The results demonstrated that the number of the invasion of RKO cells was significantly lower in both 2 μ M and 3 μ M NC groups compared to the cellular control group ($P < 0.05$) (see Fig. 4A and B). Moreover, the NC treatment group also markedly inhibited the migratory ability of RKO cells, with a maximum antimigration rate of approximately 82% ($P < 0.05$). The NC treatment group also exhibited a significant ability to inhibit the invasion and migration of HCT116 and HT29 cells, giving maximum anti-migration rates of 95% and

81%, respectively. Coincidentally, the suppressive effect of NC on the invasion and migration ability of colorectal cancer cells displayed a concentration dependence. After that, we tested the expression of EMT-related proteins (N-Cadherin, E-Cadherin and MMP-9) by WB. The findings indicated that N-Cadherin and MMP-9 protein expression decreased with increasing NC concentration ($P < 0.05$). The expression of E-Cadherin protein was upregulated and statistically significantly different in the NC group compared with the control group (Fig. 4C).

3.6 NC causes apoptosis in CRC cells

To assess the effect of NC intervention on apoptosis in colorectal cancer cells, we treated each colorectal cancer cell line separately with NC of IC₅₀ concentration for 24 h and analyzed the apoptosis rate using Annexin V-FITC/PI dual fluorescence staining combined with flow cytometry. The experimental results suggested that NC significantly promoted apoptosis in colorectal cancer cells (Fig. 5A). The apoptosis rate of RKO cells increased from 7.7–44.6% in the control group with 3 μ M NC intervention, while that of HCT116 cells increased from 9–44% in the control group with 5 μ M NC intervention and that of HT29 cells increased from 8.6–34.9% in the control group with 10 μ M NC intervention. To verify that NC can induce apoptosis in colorectal cancer cells, we measured the expression of apoptosis-related proteins (Bax, Noxa, Bcl2, Caspase-3) by WB. In Fig. 5C and D, Bax, Noxa and Caspase-3 protein expression were upregulated in each experimental group in comparison to the control group. It also appeared a more pronounced concentration dependence but statistically insignificant difference in HCT116 cells. With respect to Bcl2 protein, the expression decreased with increasing NC concentration in both RKO cells and HCT116 cells ($P < 0.05$).

3.7 NC restrains the tumor tissue growth in vivo

Herein, human-derived HCT116 cells were selected for subcutaneous implantation, and the number of inoculated cells was $2 \times 10^6/200$ μ L. The therapeutic effect of NC on mice against tumors was assessed by observing the volume of tumors. The experimental group was given the intervention using a dose of 5 mg/kg. By the 28-day measurement and recording, the tumor volume in the experimental group was much smaller than that in the control group (Fig. 6A and C). In addition, the adverse effects of NC were evaluated by changes in body weight of mice during the treatment cycle. The results suggested that NC had no dramatic effect on the body weight of mice, which indicated that NC had no significant side effects (Fig. 6B). The TUNEL and Ki67 staining detected the apoptosis and proliferation of tumor cells before and after drug administration. The Ki67 results revealed that the proliferation of colorectal cancer cells was significantly reduced after NC intervention compared to the model group (Fig. 6E and G). The TUNEL results demonstrated that apoptotic cells were significantly increased in the NC group (Fig. 6F and G). H&E staining showed irregular morphology of tumor cells and more cavities in the NC group (Fig. 6D). The above data suggest that NC has the effect of inhibiting tumor cell proliferation and promoting apoptosis in tumor tissues.

3.8 Treatment of NC activates the p53 signaling pathway

In the present study, we found that NC could induce apoptosis and further explored the molecular mechanism of NC-induced apoptosis, and a p53 signaling pathway activation was observed to be required. We next verified the results in sequencing by qRT-PCR and WB. qRT-PCR results showed (Fig. 7A and B) that genes associated with the p53 pathway were significantly higher than the control group after NC treatment. We then examined the changes of p53 signaling pathway-related nodes in colorectal cancer cells treated with NC by WB (Fig. 7C and D), and found that the expression of p53, p53R2, P48 and GADD45 was increased while the expression of MDM2 was decreased in vitro. The results indicated that NC activated the p53 signaling pathway and its induced upregulation of p53, p53R2, P48, GADD45 expression and downregulation of MDM2 expression might co-operate with the upregulation of apoptosis-related proteins Bax, Noxa and Caspase-3 and the downregulation of Bcl2 to cause apoptosis in colorectal cancer cells.

4 Discussion

Colorectal cancer is a common malignant tumor formed in the digestive system. By 2020, the IARC claimed that the incidence and mortality rate of colorectal cancer rank third and second among all types of cancers, respectively[1]. Conventional treatment methods such as surgery and chemotherapy have shortcomings including postoperative metastasis and recurrence and weakened immunity[2]. Traditional Chinese medicine (TCM) has long been regarded as a promising treatment for cancer owing to its low toxicity side effects and multi-target regulation[20]. For example, Astragali Radix[21], Solanumnigrum L[22]and Hedyotis diffusa[23]can effectively inhibit the malignant behavior of cancer cells and improve the rehabilitation rate of patient. These facts provide theoretical support for the anti-tumor functionality of TCM. NC is a kind of alkaloid extracted from the roots of the medicinal plant *Zanthoxylum nitidum* (Roxb.) DC., which was previously proven to have a wide range of biological activities including anti-inflammatory, anti-fungal, and antioxidant. Several studies have confirmed the anti-cancer effects of NC on a variety of tumors, including colorectal cancer[24], lung cancer[25], kidney cancer[26], oral cancer[27], liver cancer[28]and other common malignancies, and thus NC has been identified as a potential anti-tumor compound. In this case, systematic investigations of the biomedically anti-tumor mechanism of NC are of great significance in both theoretical research and clinical applications.

In previous studies, NC has been used to inhibit the proliferation of ovarian cancer cells and promote their apoptosis by activating the Fas pathway[29]. Additionally, NC can efficiently inhibit EMT in glioblastoma through regulating the JAK/STAT3 signaling pathway[30]. In the study of colorectal cancer cells, H. ZHAI et al. have observed that NC could suppress cell proliferation and promote its apoptosis[24]. Furthermore, in this study, we have verified that NC have effects on inducing apoptosis, inhibiting tumor cell proliferation and metastasis, and facilitating G2 stage block in cells. More specifically, we took a step further to unveil the molecular mechanism by which NC affects colorectal cancer cells, and proposed that the activation of p53 signaling pathway constituents a crucial link in the NC treatment of colorectal cancer cells. The p53 signaling pathway, which is a potential pharmacological target in tumor target therapy research, has been extensively studied[31]. It is confirmed that the p53 signaling pathway plays a vital role in tumor progression by inhibiting cell proliferation and facilitating apoptosis[32]. Regarding the

p53 activation effects on apoptosis, previous studies have demonstrated that activation of the p53 pathway improves the apoptotic susceptibility of cancer cells[33]. In our study, it is revealed that NC could enable the upregulation of p53 protein content within colorectal cancer cells, showing a dose-dependent manner. Notably, the expression of Bax, Noxa and caspase-3 remarkably increased in p53 signaling pathway, while a distinct decrease was observed for the Bcl2, after NC treatment. The above findings indicate that the p53 signaling pathway was activated during NC-induced proliferation inhibition and pro-apoptosis, and induced final transitions of the target substrates. Moreover, our results also demonstrate that NC inhibits the motility of colorectal cancer cells. EMT is considered to be a key process that promotes tumor cell division, invasion and metastasis[34]. Increased N-cad expression and decreased E-cad expression in tumor cells are typical biomarkers during the EMT process[35]. Interestingly, similar change trends of the N-cad and E-cad were observed in NC-treated cells, which further illustrate the positive impact of NC on colorectal cancer cell invasion and tumor metastasis. Inspired from the study of the DNA damage and repair, we found that the expression of p53R2, P48 and Gadd45 proteins were significantly improved in cancer cells of NC-treated group, with respect to that of the untreated ones in control group. It is reasonable to conclude that DNA damage and repair factors were participated in NC-induced inhibition of cell proliferation, cell cycle arrest, and promotion of apoptosis.

In summary, our in vitro experiments elucidate that NC inhibits both proliferation and metastasis processes of colorectal cancer cells, such as RKO, HCT116 and HT29, and concomitantly giving rise to cell cycle arrest and apoptosis. The results point out that NC can be utilized as a potential candidate component to suppress tumors, wherein the p53 signaling pathway was activated during NC treatment. In this sense, this study opens the door to NC treatment of colorectal cancer and lays the theoretical foundation for accelerating the application of NC in clinical treatment of tumors, and thus enriches and extends the scientific insights of TCM in the remedy of malignant tumors.

Declarations

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Conflicts of interest Qiao Zheng, Yan Zhang, Lanlan Zhang, Ziyi Zhao, Jinghao Zeng, Nianzhi Chen, Gang Zhang, Maoyuan Zhao, Wenhao Liao, Zhongzhen Zeng, Yujie Wei Gang Zhou, Jianyuan Tang declare that they have no conflicts of interest that might be relevant to the contents of this article.

Ethics approval The animal study was reviewed and approved by the Institutional Animal Care and Use Committee of Institute of Chengdu University of Traditional Chinese Medicine.

Consent for publication Not applicable.

Availability of data and material Not applicable.

Code availability Not applicable.

Authors' contributions

Q. Zheng, Y. Zhang, L. Zhang, J. Tang, Z. Zhao designed the experiments and performed most of the experiments included in this study. N. Chen and G. Zhang performed the experiments on processing cells. Q. Zheng, M. Zhao, W. Liao, Z. Zeng, Y. Wei are responsible for data collection and performed the statistical analysis. Q. Zheng and M. Zhao performed the molecular experiments. Q. Zheng and Y. Zhang wrote the manuscript. G. Zhou and J. Tang revised the manuscript. All authors contributed to the article and approved the submitted version.

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Figures

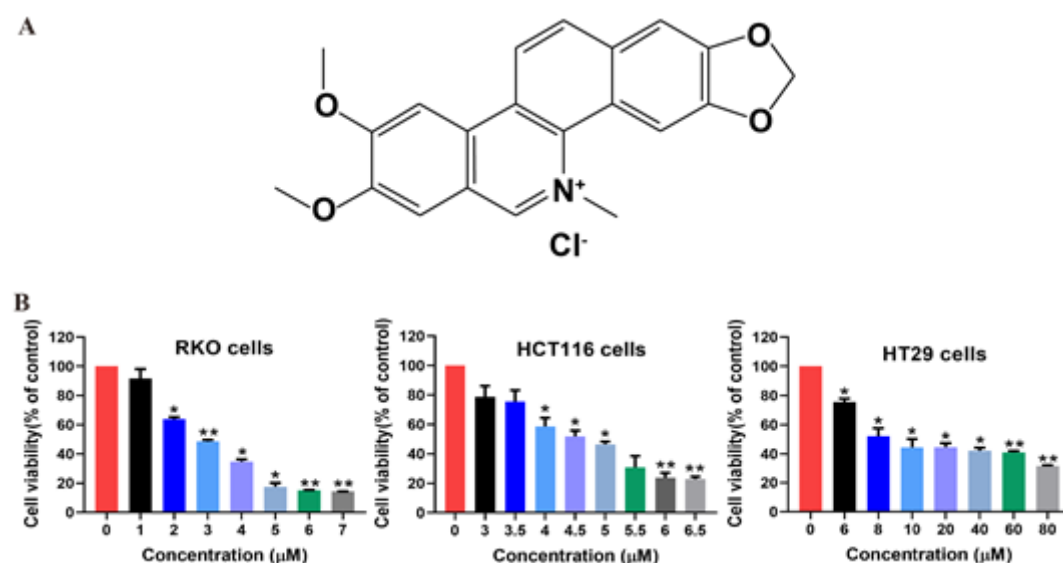


Figure 1

NC suppresses the proliferation of CRC cells. (A) Chemical formula of Nitidine chloride. (B) Colorectal cancer cells were intervened at a variety of concentrations of NC for 24 h and tested by the CCK8 assay. *P<0.05, **P <0.01 vs. control.

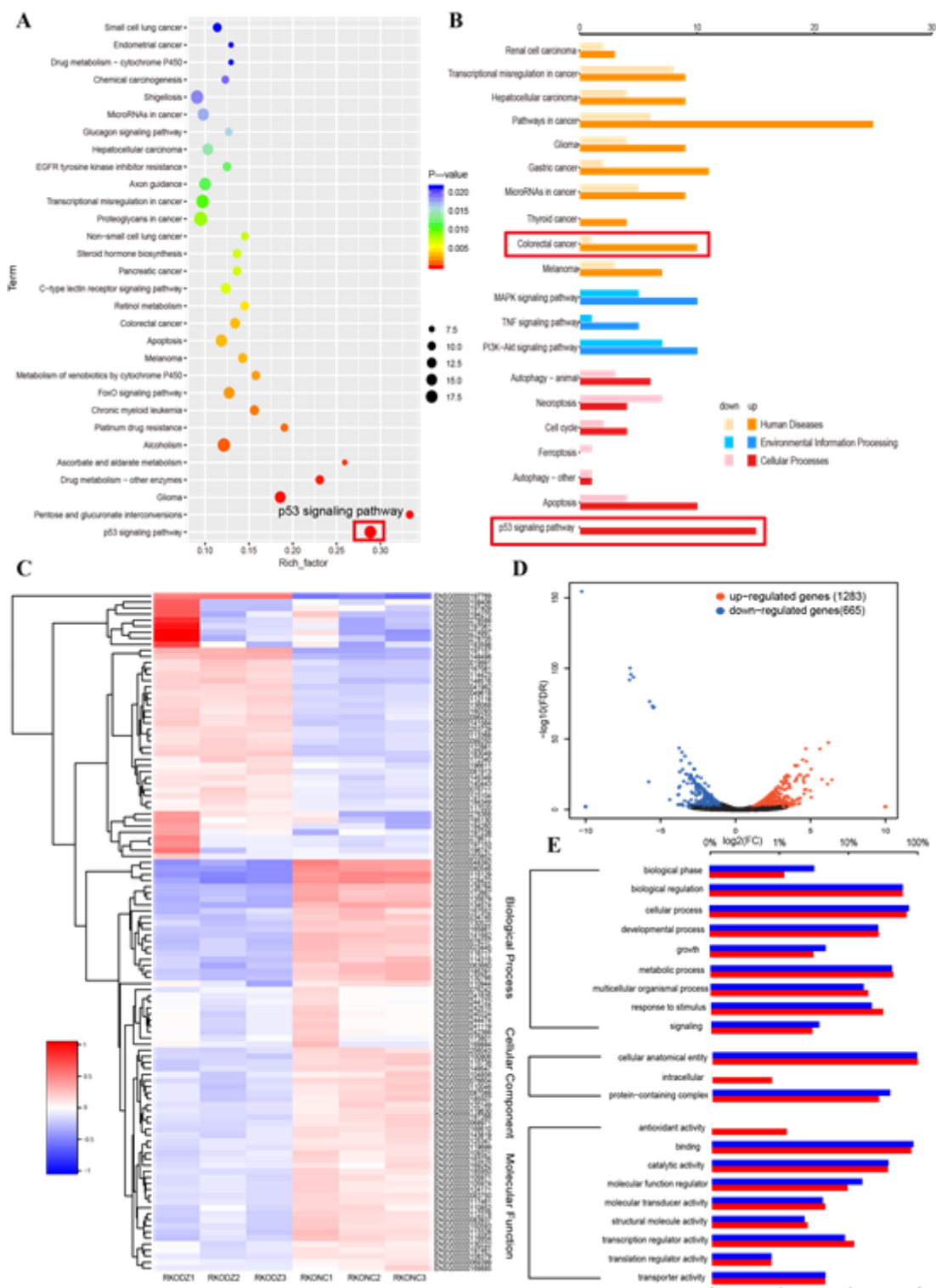


Figure 2

p53 is a potential target of NC in treating colorectal cancer. (A and B) KEGG enrichment analysis of differentially expressed target genes of NC treatment. (C) Cluster heat map analysis of differential gene expression after NC intervention in CRCs. (D) Visualization of differential genes in volcanoes after NC treatment of CRC. (E) Up-down regulation of differential gene GO annotation bar graphs of NC.

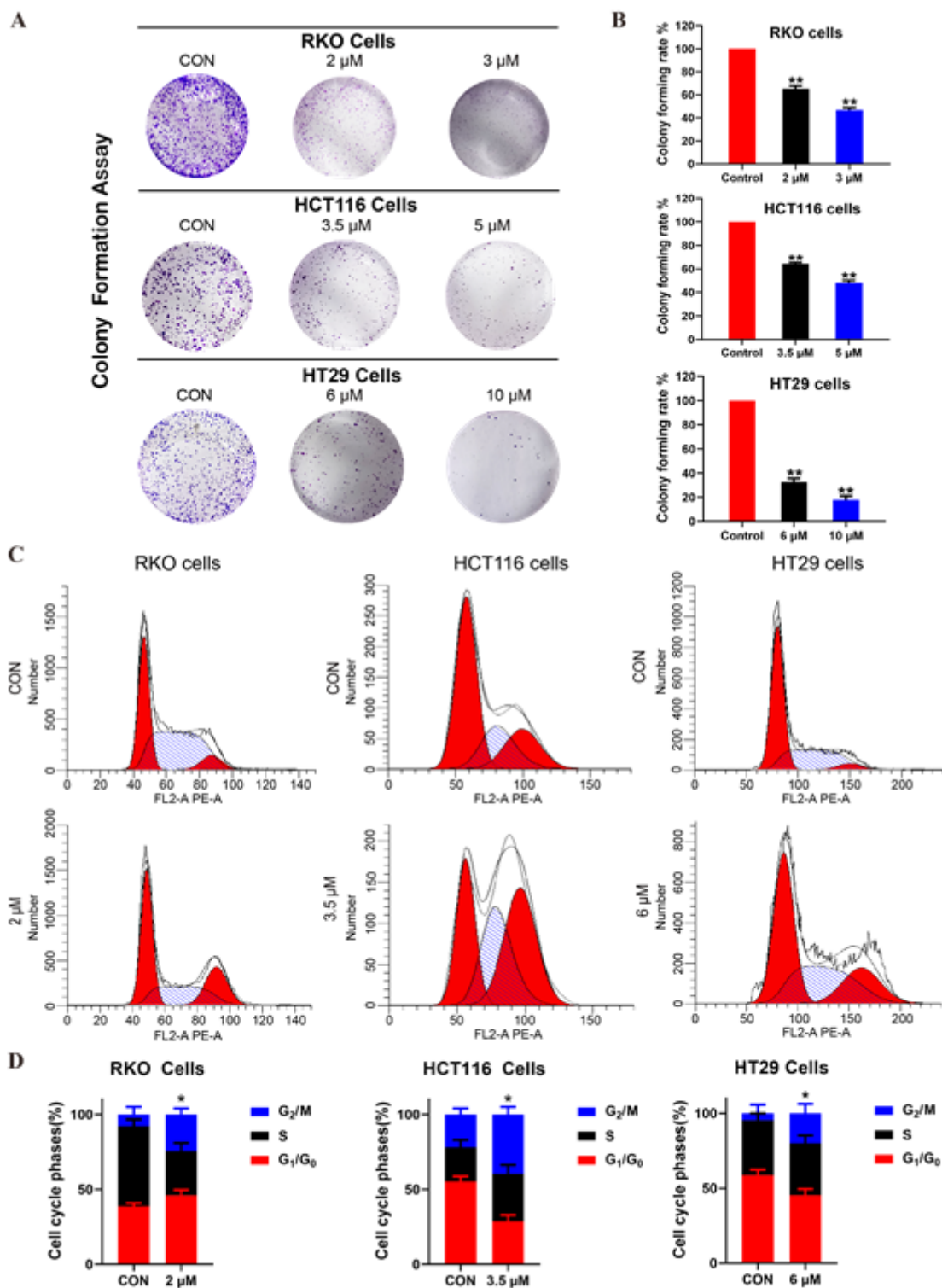


Figure 3

NC inhibits colony formation and blocks the cell cycle. (A) Colony formation experiment was conducted to determine the tumorigenic ability of CRCs exposed to a wide range of NC concentrations. (B) The quantitative field of cloning numbers in (A); ** P < 0.01 versus the control group. (C) After treatment of cells with IC30 concentration of NC for 24 h, the distribution of cell cycle phases was captured by flow cytometry. (D) The percentage of each cell cycle phase of RKO, HCT116 and HT29 cells was illustrated.

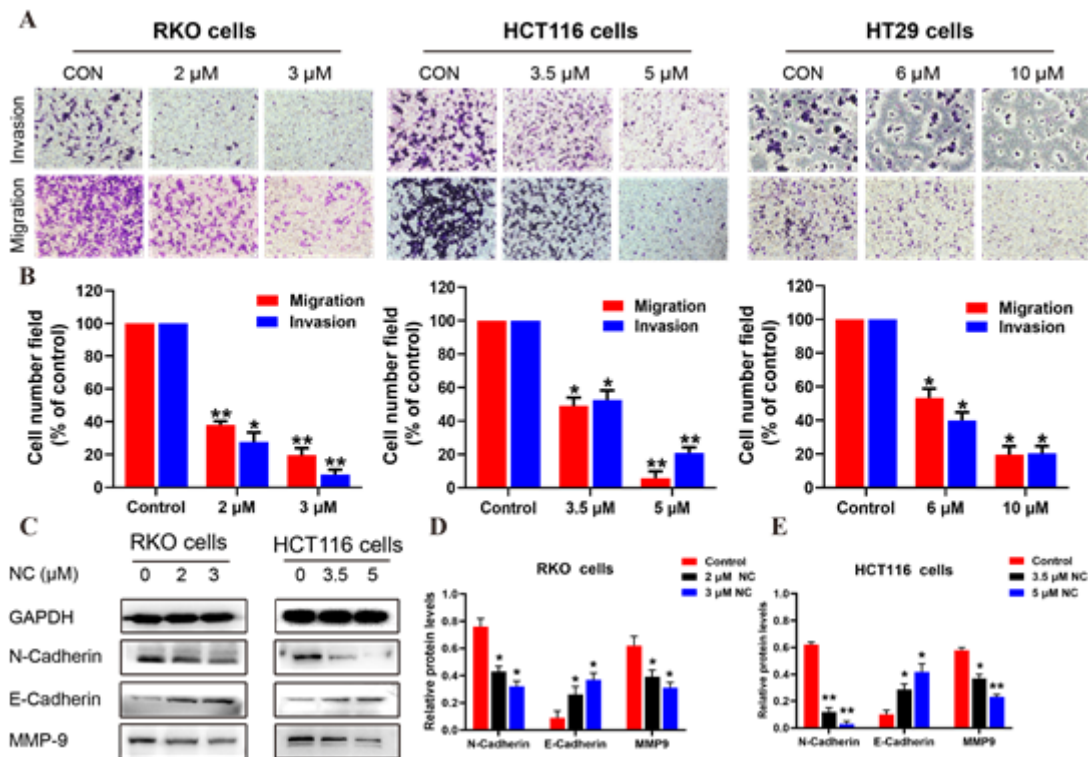


Figure 4

NC inhibits motility of colorectal cancer cells. (A) CRCs were treated with different doses of NC for 24h. Transwell chamber assay was used to detect the migratory and invasive ability of colorectal cancer cells. Take photos of cells that have migrated or invaded to the lower surface in Transwell analysis ($\times 100$). (B) Quantitative analysis was illustrated for the cells of migration and invasion. $**P < 0.01$ vs. control. (C) expression of EMT-related proteins, N-Cadherin, E-Cadherin, MMP9 in RKO and HCT116 cells were detected by Western blotting. (D and E) Quantitative analysis of EMT-related proteins in (C), $*P < 0.05$, $**P < 0.05$ vs. control.

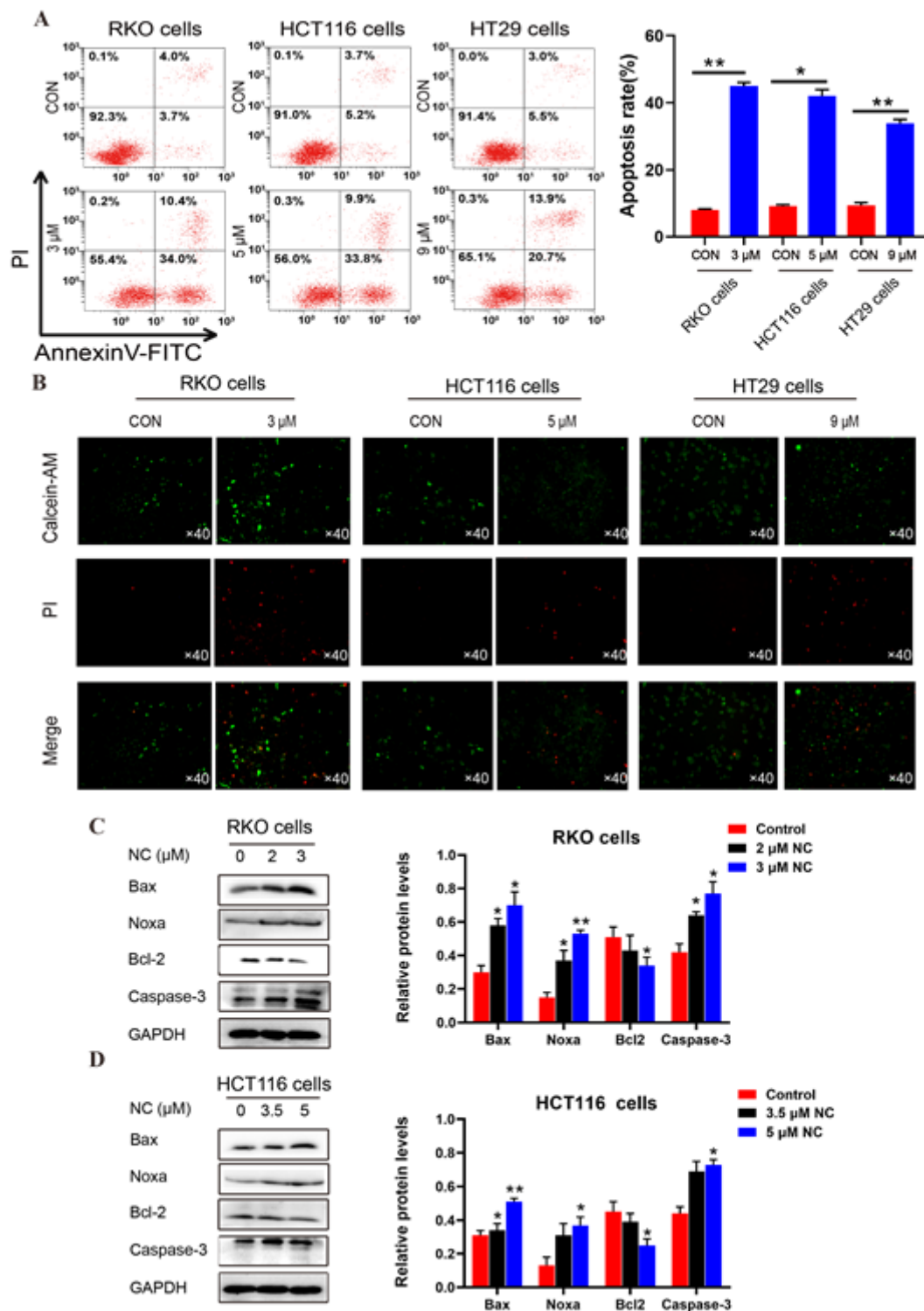


Figure 5

NC induces apoptosis in RKO, HCT116 and HT29 cells. (A) RKO, HCT116 and HT29 cells were treated with IC₅₀ of NC for 24 h, respectively, and stained with Annexin V-FITC/PI double staining. *P < 0.05, **P < 0.01 vs. mock group. (B) Calcein-AM/PI staining was used to detect the cell mortality in CRCs. (C and D) The expression of apoptosis-related proteins Bax, Noxa, Bcl2 and caspase-3 were detected by Western Blotting in RKO and HCT116 cells, *P < 0.05, **P < 0.01 vs. control group.

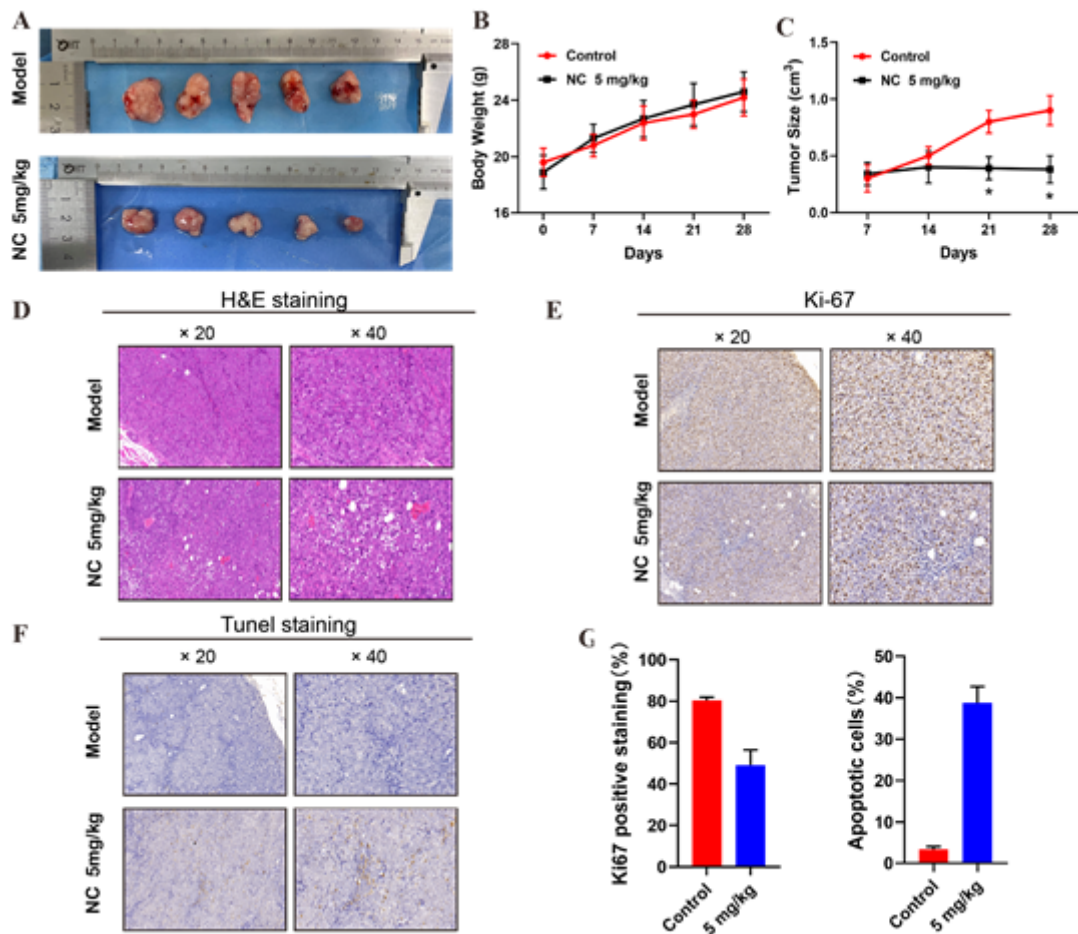


Figure 6

Effects of NC on colorectal cancer tumor tissue in vivo. (A) Representative images for tumors. (B and C) Weight of nude mice and size of seeded tumors were measured at 7, 14, 21 and 28 days after transplantation. * $p < 0.05$, vs. control group. (D) H&E staining characterization of tumor formation. (E) Ki-67 measurements for cell proliferation. (F) The number of apoptotic cells detected by Tunnel staining. (G) Quantitative expression of Ki67-positive cells and TUNEL-positive cells.

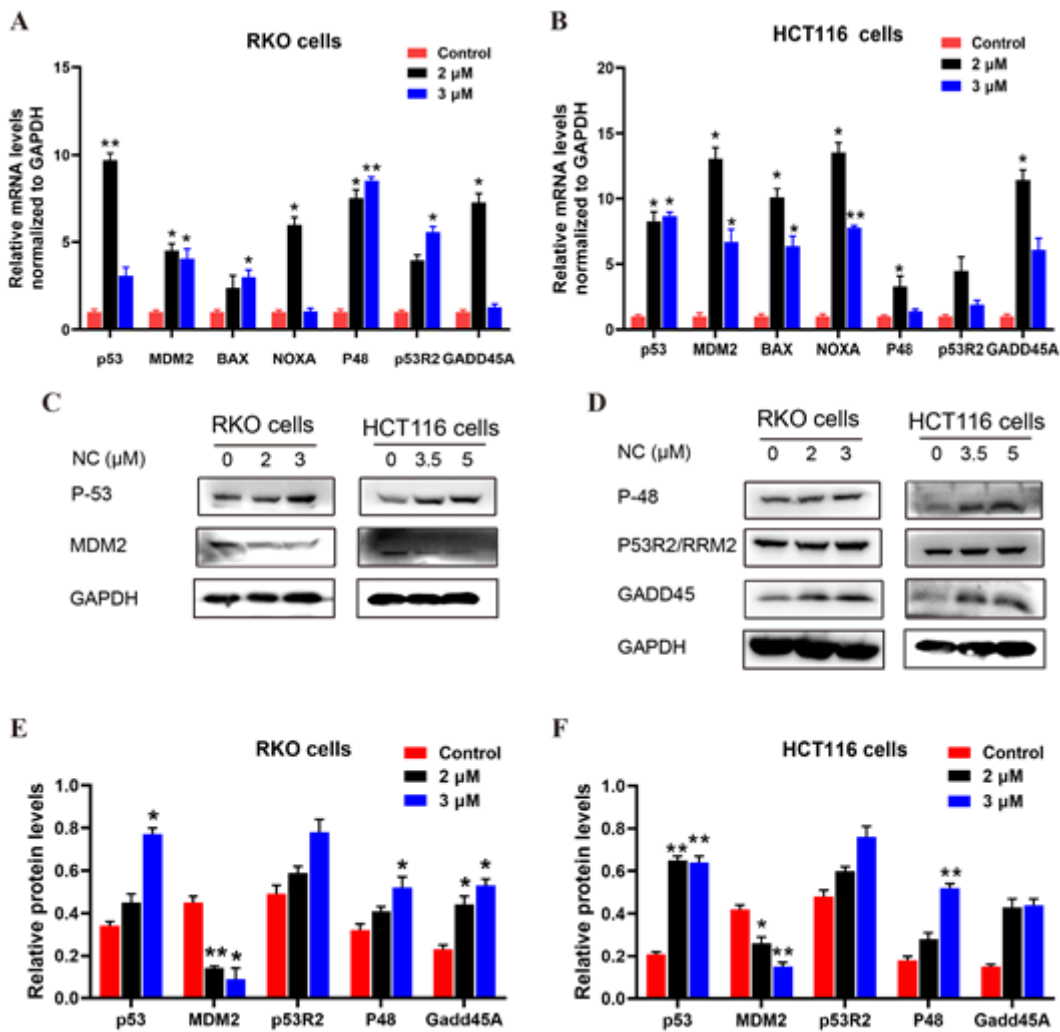


Figure 7

NC activates p53 pathway of CRCs. (A&B) Following different doses of NC treatment in RKO and HCT116 cells for 24h, the mRNA levels of p53, MDM2, Bax, Noxa, P48, p53R2 and GADD45A were evaluated by qPCR. * $P < 0.05$, ** $P < 0.01$ vs. mock group. (C&D) Western blotting examine the expression of key p53 pathway proteins. (E&F) Statistical analysis on relative protein expression of p53 pathway in RKO and HCT116 cells. * $P < 0.05$, ** $P < 0.01$ vs. control.