Regulatory mechanism of lncRNA HCP5 in nasopharyngeal carcinoma cell proliferation and apoptosis via the miR-140-5p/NPR3 axis

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Research

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Abstract

Objectives

Nasopharyngeal carcinoma (NPC) is malignant tumor frequently occurring in east and southeast Asia. This study investigated the underlying mechanism of lncRNA HCP5 in NPC cell proliferation and apoptosis.

Methods

LncRNA HCP5 expression in NPC cells and tissues was detected. SUNE-1 cells were transfected with HCP5 silencing and CNE-1 cells were transfected with overexpressing HCP5, and then cell proliferation and apoptosis were measured. Subcellular localization of HCP5 was analyzed. The binding relationship between lncRNA HCP5 and miR-140-5p, and miR-140-5p and NPR3 were predicted and verified. Expressions of miR-140-5p and NPR3 in NPC cells were detected. SUNE-1 cells and CNE-1 cells were transfected with miR-140-5p mimic, and si-HCP5-transfected SUNE-1 cells were treated with miR-140-5p inhibitor. Then the proliferation and apoptosis of NPC cells were detected.

Results

LncRNA HCP5 was elevated in NPC tissues and cells. HCP5 silencing suppressed NPC cell proliferation and enhanced apoptosis. HCP5 was mainly located in the cytoplasm of NPC cells. HCP5 promoted NPR3 expression by competitively binding to miR-140-5p. Overexpression of miR-140-5p weakened proliferation and facilitated apoptosis of NPC cells. miR-140-5p inhibitor partially restored the effects of HCP5 silencing on NPC cells.

Conclusion

LncRNA HCP5 upregulated NPR3 expression via sponging miR-140-5p, thereby suppressing NPC cell proliferation and promoting apoptosis. This study may offer new insights into NPC treatment.

Introduction

Nasopharyngeal carcinoma (NPC) represents a squamous cell carcinoma in the epithelium of the nasopharynx [1]. The clinical manifestations of NPC patients encompass epistaxis, obstruction, hearing loss, tinnitus, headache, facial pain and numbness, and neck mass [2]. Multiple factors including Epstein-Barr virus infection, host genetics and environmental factors are responsible for the progression of NPC [3]. The prevalence of NPC shows imbalance in geographical distribution, with South China and South Eastern Asia confronting with a remarkably high incidence [1]. Conventional treatment for NPC is confined to radiotherapy or chemotherapy, and surgical resection is usually the last choice for advanced

and metastatic patients [4]. Therefore, exploring the potential molecular regimens of NPC and identifying new therapeutic targets constitute practical issues for the management of NPC.

Long non-coding RNAs (LncRNAs) are a class of RNAs with transcriptome length of over 200 nucleotides and lack of protein-coding capacity [5]. LncRNAs represent the major regulators of gene expression and participate in biological functions and cancer progression [6]. LncRNA HCP5 is concerned with the occurrence of certain autoimmune diseases and cancers, indicating that HCP5 works as a promising target for antineoplastic drugs [7]. Liang et al. have revealed that HCP5 is enhanced in follicular thyroid carcinoma cells, and overexpression of HCP5 facilitates the proliferation, invasion and angiogenesis of follicular thyroid carcinoma cells [8]. However, the mechanism of lncRNA HCP5 in the progression of NPC still needs further investigation.

Competitive endogenous RNA (ceRNA) is a newly proposed mechanism in RNA field, which links the function of protein-coding genes (mRNAs) with that of the non-coding RNAs (including lncRNAs, microRNAs (miRs), transcribed pseudogenes and circular RNAs) and affects the progression of multiple human tumors [9, 10]. The existing researches have indicated that lncRNA HCP5 can serve as a ceRNA and thus regulates tumor progression via sponging miRs [11–13]. miRs function as oncogenic factors or tumor suppressors by regulating tumorigenesis-associated processes such as inflammation, stress response, apoptosis and invasion [14]. miR-140-5p is demonstrated to be an anti-tumor factor in a variety of carcinomas, such as tongue squamous cell carcinoma [15], gastric cancer [16] and hepatocellular carcinoma [17]. Whether lncRNA HCP5 has influences on the progress of NPC by binding to miR-140-5p remains unclear. Based on the previous findings, we explored the effect of lncRNA HCP5/miR-140-5p on NPC cells, along with its underlying downstream gene, which shall shed lights on the targeted gene therapy for NPC.

Materials And Methods

Ethics statement

The study got the approval of the Clinical Ethical Committee of he People's Hospital of Guangxi Zhuang Autonomous Region, Nanning, Guangxi Zhuang Autonomous Region. Informed consent was obtained from each eligible participant.

Tissue samples

Cancer tissues and adjacent normal tissues were obtained from 60 cases of NPC patients from April 2019 to June 2020 in he People's Hospital of Guangxi Zhuang Autonomous Region, Nanning, Guangxi Zhuang Autonomous Region. All the cases were operated for the first time without radiotherapy or chemotherapy before the operation. The patients were aged 20–65 years old, excluding pregnancy, lactation, liver and kidney dysfunction. All the specimens were pathologically diagnosed. The tissue samples were kept in liquid nitrogen at -80 °C.

Cell culture and transfection

Immortalized nasopharyngeal epithelial cells (NP69) and human NPC cell lines (CNE-1, CNE-2, C666-1, HNE-1, SUNE-1 and HONE-1) were obtained from Shanghai Institute of Biochemistry and Cell Biology, CAS (Shanghai, China). Human NPC cell lines were maintained in RPMI-1640 medium containing 10% fetal bovine serum (FBS), and NP69 cells were maintained in Keratinocyte Serum Free medium containing 10% FBS at 37 °C with 5% CO_2. Cells were passaged after 2–3 days of incubation. Interference vector of HCP5 (si-HCP5) and its control (si-NC), overexpression vector of HCP5 (ov-HCP5) and its control (ov-NC), miR-140-5p mimic, miR-NC, miR-140-5p inhibitor and inhibitor NC were constructed y GenePharma (Shanghai, China). The transfection was conducted using Lipofectamine 3000 (Thermo Fisher Scientific, Waltham, MA, USA). After 48 h, the subsequent experiments were conducted.

RT-qPCR

Total RNA was extracted from cells and tissues using TRIzol reagent (Invitrogen Inc., Carlsbad, CA, USA) and reverse transcribed into cDNA using the reverse transcription kit (Themo Fisher Scientific). RT-qPCR was conducted using the SYBR Green PCR kit (Applied Biosystem, Carlsbad, CA, USA). PCR was performed on the following conditions: pre-denaturation at 95 °C for 10 s; 40 cycles of denaturing at 95 °C for 10 s, annealing at 60 °C for 30 s and extension at 72 °C for 30 s. Primer sequences are illustrated in Table 1. The relative gene expression was examined by $2^{-\Delta\Delta Ct}$ method, with GAPDH as the internal reference.

Table 1
Primer sequence for RT-qPCR

Gene	Sequence 5'-3'
LncRNA HCP5	F: CCTATCCCTGTGAAGATGAACC
	R: CTGCCACCTCTAAATGTCCTACT
miR-140-5p	F: CCAGTGCAGGGTCCGAG
	R: GATCCGAAACCCAGCAGACAATGTAGCTTTTTT
NPR3	F: CTTCCCTCATCCTCCTGCTAC
	R: ACAAACTGGGTAAAGGTGATGG
si-HCP5	F: GCUGAUGAGUAGGACAUUUTT
U6	F: CTCGCTTCGGCAGCACA
	R: AACGCTTCACGAATTTGCGT
GAPDH	F: ACCATCTTCCAGGAGCGAGA
	R: GACTCCACGACGTACTCAGC

Cell counting kit-8 (CCK-8) assay

Single cell suspension was seeded into the 96-well plates (2×10^3 cells/100 μL/well) and cultured for 24, 48 and 72 h. Then, cells in each well were treated with 10 μL CCK-8 solution (Dojindo Molecular Technologies, Kumamoto, Japan) and cultured for 2 h at 37 °C with 5% CO_2. The optical density of each well at 450 nm was evaluated using a microplate reader.

Colony formation assay

Cells in logarithmic phase were detached with trypsin, blown into single cells and seeded into 6-well plates (300 cells/well). Cells were cultured for 10–15 days to observe the formation of cell colonies. Cells were fixed with methanol for 10 min, stained with crystal violet for 5 min, and then washed with double distilled water. Next, the 6-well plates were inverted and dried, and the cell colonies were counted.

Flow cytometry

Cells in each group were cultured for 48 h, centrifuged and detected using Annexin V-FITC apoptosis detection kit (BD Bioscience, Franklin Lakes, NJ, USA). After that, cells were stained with 1.0 mL propidium iodide for 15 min at 4 °C in the dark. Cell apoptosis was detected on the flow cytometer (BD Bioscience).

Fluorescence in situ hybridization (FISH)

The distribution of HCP5 was predicted by online software LncATLAS (http://lncatlas.crg.eu/). Subcellular localization of HCP5 was analyzed using FISH Tag™ RNA Green Kit with Alexa Fluor™ 488 dye kit (Thermo Fisher Scientific). SUNE-1 and CNE-1 cell slides were fixed, permeabilized and dehydrated; and the samples were hybridized at 42 °C overnight, washed, stained with 4’,6-diamidino-2-phenylindole (DAPI), sealed and observed under the confocal microscope.

Dual-luciferase reporter gene assay

The binding sites of lncRNA HCP5 and miR-140-5p, miR-140-5p and NPR3 were predicted by Starbase (http://starbase.sysu.edu.cn/index.php) and TargetScan (http://www. targetscan.org/vert71/). The binding sequence and mutation sequence of lncRNA HCP5 and miR-140-5p, as well as those of miR-140-5p and NPR3 were cloned to the pmirGLO luciferase vector (Promega, Madison, WI, USA). The wild type (WT) vectors (HCP5-WT/NPR3-WT) and the mutant type (MUT) vectors (HCP5-MUT/NPR3-MUT) were constructed and transfected into HEK293T cells (Shanghai Institute of Biochemistry and Cell Biology, CAS, Shanghai, China). Luciferase activity was examined after 48 h.

Western blot analysis

Total protein of cells was extracted in radio-immunoprecipitation assay buffer (Beyotime, Shanghia, China). The concentration of protein was tested using the bicinchoninic acid assay kit (Beyotime). Then, the protein was separated on electrophoresis and transferred onto polyvinylidene difluoride membranes. The electro-transferring lasted 2 h with 70V in a 4 °C cold chamber. The membranes were blocked in 5% skim milk-tris buffered saline tween(TBST) for 1 h and incubated with the primary antibody rabbit- anti human NPR3 (1/1000, ab37617, Abcam Inc., Cambridge, MA, USA) at 4 °C overnight. Following TBST

washing, the membranes were incubated with the secondary antibody goat anti-rabbit immunoglobulin G (IgG) H&L (1/5000, ab205718, Abcam) at 37 °C for 1 h. Subsequently, the membranes were developed and visualized using the enhanced chemiluminescence reagent. The protein band was observed with GAPDH (1/2000, ab9485, Abcam) as the internal reference.

Statistical analysis

Data analysis was introduced utilizing SPSS 21.0 (IBM Corp., Armonk, NY, USA) and GraphPad Prism 8.0.1 (GraphPad Software Inc., San Diego, CA, USA). Data are expressed as mean ± standard deviation. The *t* test was adopted for analysis of comparisons between two groups. One-way analysis of variance (ANOVA) or two-way ANOVA was employed for the comparisons among multi-groups, followed by Tukey's multiple comparison test. The *p* value was obtained from a two-tailed test, and $p < 0.01$ meant a statistically significant difference.

Results

LncRNA HCP5 was elevated in NPC tissues and cells

LncRNA HCP5 is elevated in many cancer samples, which may exert effects on cancer cell functions and cancer progression [18, 19]. We detected HCP5 expression in NPC tissues and adjacent tissues using RT-qPCR, and found that HCP5 expression in NPC tissues was notably higher than that in normal tissues (Fig. 1A; $p < 0.01$). Then, we detected HCP5 expression in human NPC cells and immortalized nasopharyngeal epithelial cells (NP69), and found that HCP5 was elevated in NPC cells in comparison with NP69 cells (Fig. 1B; $p < 0.01$). Upregulation of HCP5 was concerned with the progression of NPC.

Inhibition of lncRNA HCP5 suppressed NPC cell proliferation and promoted apoptosis

To determine the role of HCP5 in NPC, we transfected si-HCP5 into SUNE-1 cells with relatively high expression of HCP5, and transfected ov-HCP5 into CNE-1 cells with relatively low expression of HCP5. RT-qPCR revealed that HCP5 expression in si-HCP5-transfected SUNE-1 cells was effectively decreased, and that in ov-HCP5-transfected CNE-1 cells was increased (Fig. 2A; $p < 0.01$). SUNE-1 cell proliferation in the si-HCP5 group was notably slower than that in the si-NC group, while CNE-1 cell proliferation in the ov-HCP5 group was faster than that in the ov-NC group (Fig. 2B; $p < 0.01$). The colony number of si-HCP5-transfected SUNE-1 cells was reduced, while that of ov-HCP5-transfected CNE-1 cells was promoted (Fig. 2C; $p < 0.01$). Inhibition of HCP5 repressed NPC cell proliferation.

The apoptosis of SUNE-1 cells transfected with si-HCP5 and CNE-1 cells transfected with ov-HCP5 was analyzed using flow cytometry. The apoptosis rate of si-HCP5-treated SUNE-1 cells was notably enhanced in contrast to that of si-NC-treated cells, and the apoptosis rate of ov-HCP5-treated CNE-1 cells was repressed in contrast to ov-NC-treated cells (Fig. 2D; $p < 0.01$). Downregulation of HCP5 promoted the apoptosis of NPC cells *in vitro.*

LncRNA HCP5 regulated NPR3 expression via sponging miR-140-5p

The mechanism of lncRNA depends on its subcellular localization. LncATLAS and FISH assay showed that HCP5 was mainly located in the cytoplasm of NPC cells (Fig. 3A/B), suggesting that lncRNA HCP5 might play a role in NPC through the ceRNA network. Then, the ceRNA network of HCP5 was predicted by Starbase. There were binding sites between HCP5 and miR-140-5p, and miR-140-5p and NPR3 (Fig. 3C). Previous literarures have exhibited that upregulation of miR-140-5p facilitates the proliferation and apoptosis of NPC cells [20] and NPR3 is implicated in colorectal cancer cell proliferation [21]. We speculated that HCP5 regulated NPR3 expression via sponging miR-140-5p, thereby affecting the progression of NPC. The binding relationship between lncRNA HCP5 and miR-140-5p, and miR-140-5p and NPR3 were verified (Fig. 3D; $p < 0.01$). Then, expression of miR-140-5p and levels of NPR3 mRNA and protein were detected. The results demonstrated that SUNE-1 cells in the si-HCP5 group had upregulated miR-140-5p expression and downregulated NPR3 mRNA and protein levels, while CNE-1 cells in the ov-HCP5 group showed the opposite trends (Fig. 3E/F; $p < 0.01$). LncRNA HCP5 regulated NPR3 expression via sponging miR-140-5p.

Overexpression of miR-140-5p weakened proliferation and enhanced apoptosis of NPC cells

To further study the function of miR-140-5p in NPC cells, we transfected miR-140-5p mimic into SUNE-1 and CNE-1 cells. miR-140-5p expression was promoted in NPC cells transfected with miR-140-5p mimics (Fig. 4A; $p < 0.01$). Overexpression of miR-140-5p repressed the cell proliferation (Fig. 4B; $p < 0.01$), and reduced the number of cell colonies (Fig. 4C; $p < 0.01$). The apoptosis rate of miR-140-5p mimic-treated cells was elevated in contrast to miR-NC-treated cells (Fig. 4D; $p < 0.01$). Overexpression of miR-140-5p inhibited NPC cell proliferation and facilitated apoptosis.

miR-140-5p inhibitor partially restored malignant behaviors of NPC cells suppressed by HCP5 silencing

To confirm that HCP5 modulated proliferation and apoptosis of NPC cells via the miR-140-5p/NPR3 axis, we transfected si-HCP5-treated SUNE-1 cells with miR-140-5p inhibitor. miR-140-5p expression of cells in the si-HCP5 + miR-140-5p inhibitor group was reduced compared with that in the si-HCP5 + inhibitor-NC group (Fig. 5A; $p < 0.01$). Cells in the si-HCP5 + miR-140-5p inhibitor group showed accelerated proliferation ability and declined apoptosis rate compared with those in the si-HCP5 + inhibitor-NC group (Fig. 5B-D; $p < 0.01$). miR-140-5p inhibitor restored the effects of HCP5 silencing on cell proliferation and apoptosis. HCP5 modulated proliferation and apoptosis of NPC cells by competitively binding to miR-140-5p with NPR3.

Discussion

NPC is a geographically distributed head and neck epithelial tumor with poor clinical efficacy and high metastasis tendency in advanced stage [22]. LncRNA HCP5 is reported to commonly act as an oncogenic role in human malignant tumors [23]. We elucidated the regulatory role of lncRNA HCP5 in NPC cell proliferation and apoptosis via the miR-140-5p/NPR3 axis.

Dysregulation of lncRNA HCP5 are responsible for various biological processes of malignant tumors, including proliferation, apoptosis, invasion and migration [18, 19]. For example, Yun et al. have exhibited that HCP5 facilitates proliferation and migration of colon cancer cells, facilitating the colon cancer progression [24]. We exhibited that HCP5 was elevated in NPC tissues and cells, and enhancement of HCP5 might be concerned with the occurrence and development of NPC. Next, NPC cells were treated with si-HCP5 or ov-HCP5 to probe into the specific role of HCP5 in NPC. NPC cells transfected with si-HCP5 showed reduced proliferation and promoted apoptosis, while NPC cells transfected with ov-HCP5 showed the opposite trend. Consistently, Chen et al. have revealed that HCP5 expression is notably upregulated in anaplastic thyroid cancer cell lines, and knockdown of HCP5 decreases survival rate of cancer cells and increases apoptosis rate [25]. Hu et al. have clarified that downregulating HCP5 restrains prostate cancer cell proliferation and facilitates apoptosis [26]. Briefly, inhibition of lncRNA HCP5 suppressed NPC cell proliferation and promoted apoptosis.

Mechanically, lncRNA HCP5 exerts pivotal effects on the progression of human tumors as an oncogene by modulating the functions of targeting miRs, thus affecting the expression of downstream target genes [27]. The deregulation of miRs in NPC can lead to tumor-promoting effects, and miRs provide insights into the biological and clinical behaviors of NPC [28]. A previous literature has demonstrated that lncRNA HCP5 facilitates proliferation and metastasis of clear cell renal cell carcinoma via sponging miR-140-5p [29]. Our experiments showed that HCP5 could sponge miR-140-5p. Importantly, elevation of miR-140-5p is reported to reduce the drug resistance of NPC cells, restrain NPC cell colony formation and boost cell apoptosis [20]. Consistently, we exhibibted that overexpressing miR-140-5p repressed proliferation of NPC cells and facilitated apoptosis.

Subsequently, we shift to investigating the target gene regulated by HCP5/miR-140-5p. A recent study has exhibited that patients with NPR3-positive tumors can show remarkably declined progression-free and overall survival [30]. Jorge Martinez-Romero et al. have also indicated that NPR3 is upregulated in colorectal cancer samples and considered as a marker of poor outcome [31]. The targeting relationship between miR-140-5p and NPR3 was verified in the current study. Furthermore, we revealed that the si-HCP5-transfected NPC cells exhibited upregulated miR-140-5p expression and downregulated NPR3 expression, while the ov-HCP5-transfected NPC cells showed an opposite trend. It was suggested that HCP5 worked as a ceRNA to competitively bind to miR-140-5p with NPR3 and regulated the expression of NPR3. In addition, we conducted functional rescue experiments to verify that HCP5 modulated proliferation and apoptosis of NPC cells via the miR-140-5p/NPR3 axis. The proliferation of NPC cells transfected with si-HCP5 and miR-140-5p inhibitor was significantly accelerated and the apoptosis was reduced. It was confirmed that HCP5 modulated proliferation and apoptosis of NPC cells by competitively binding miR-140-5p with NPR3.

To sum up, lncRNA HCP5 repressed proliferation and facilitated apoptosis of NPC cells via the miR-140-5p/NPR3 axis. The study may hint the possibility of lncRNA HCP5 and miR-140-5p as potential targets for clinical therapies and prognosis of NPC. In the future, we shall conduct researches on the regulation mechanism of lncRNA HCP5 in NPC from the perspective of epigenetics. Additionally, more prospective trials on the feasibility and safety of lncRNA HCP5 in the treatment of NPC are needed, so as to transform lncRNA HCP5 from a gene tool into a clinical means.

Abbreviations

LncRNA: Long non-coding RNA; NPC:Nasopharyngeal carcinoma; ceRNA:competitive endogenous RNA; FBS:fetal bovine serum; RT-qPCR:Reverse transcription quantitative polymerase chain reaction; GAPDH:glyceraldehyde-3-phosphate dehydrogenase; CCK-8:Cell counting kit-8; PI:propidium iodide; FISH:Fluorescence in situ hybridization; DAPI:4',6-diamidino-2-phenylindole; IgG:immunoglobulin G; ANOVA:analysis of variance.

Declarations

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Conflict of Interest

The authors declare that they have no conflicts of interest.

Availability of data and materials

All the data generated or analyzed during this study are included in this published article.

Authors' Contributions

BH is the guarantor of integrity of the entire study; YLL contributed to the study concepts, study design, definition of intellectual content, literature research, manuscript preparation and manuscript editing and review; YFS contributed to the clinical studies; ZXD and GPLcontributed to the experimental studies and data acquisition; QZ contributed to the data analysis and statistical analysis. All authors read and approved the final manuscript.

Ethics approval and consent to participate

Not applicable.

Patient consent for publication

Not applicable.

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Figures

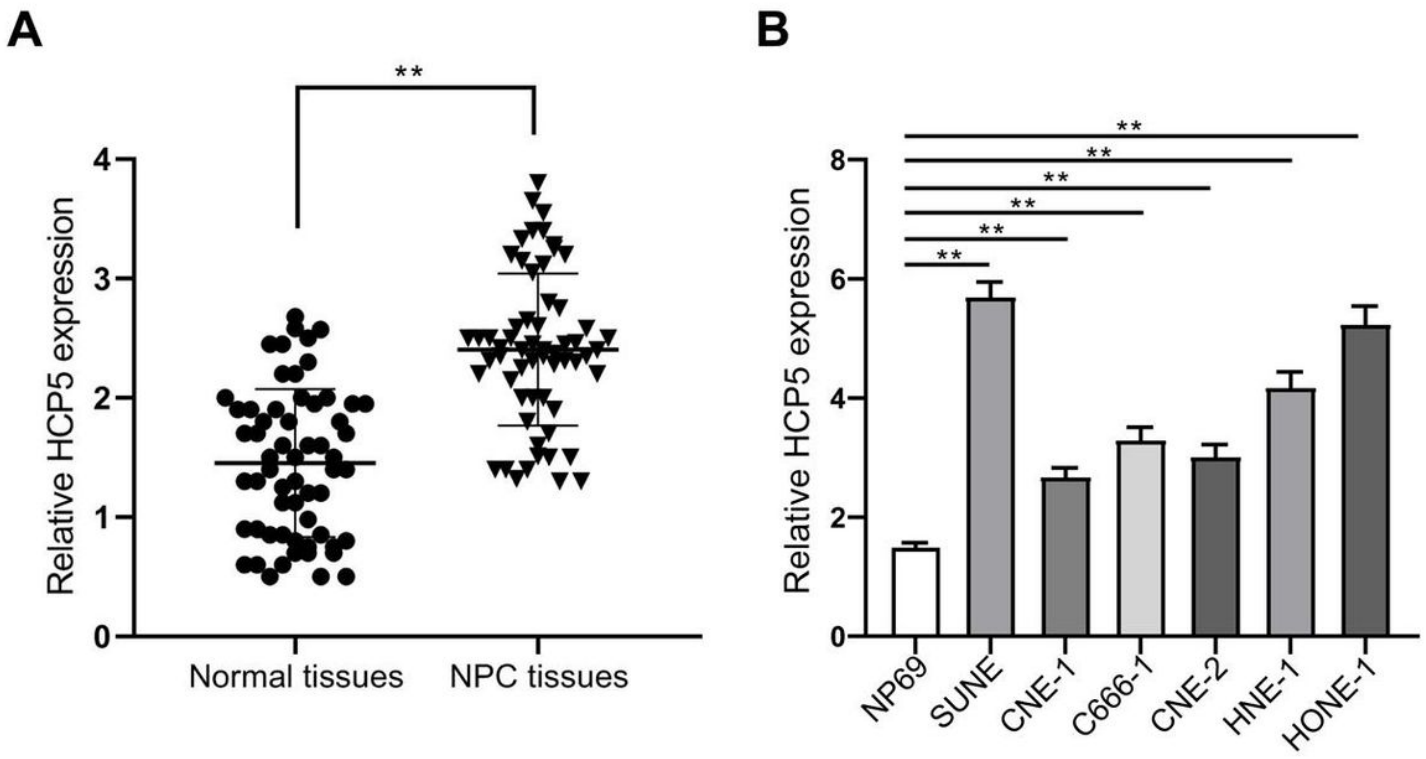

Figure 1

LncRNA HCP5 was upregulated in NPC tissues and cells. A: HCP5 expression in NPC tissues and adjacent normal tissues was detected using RT-qPCR, N = 60; B: HCP5 expression in human NPC cell lines and immortalized nasopharyngeal epithelial cells was detected using RT-qPCR. Cell experiments were repeated three times independently. Data are presented as mean ± standard deviation. Data were analyzed using t test or one-way ANOVA, followed by Tukey's multiple comparison test, **p < 0.01. NPC = nasopharyngeal carcinoma

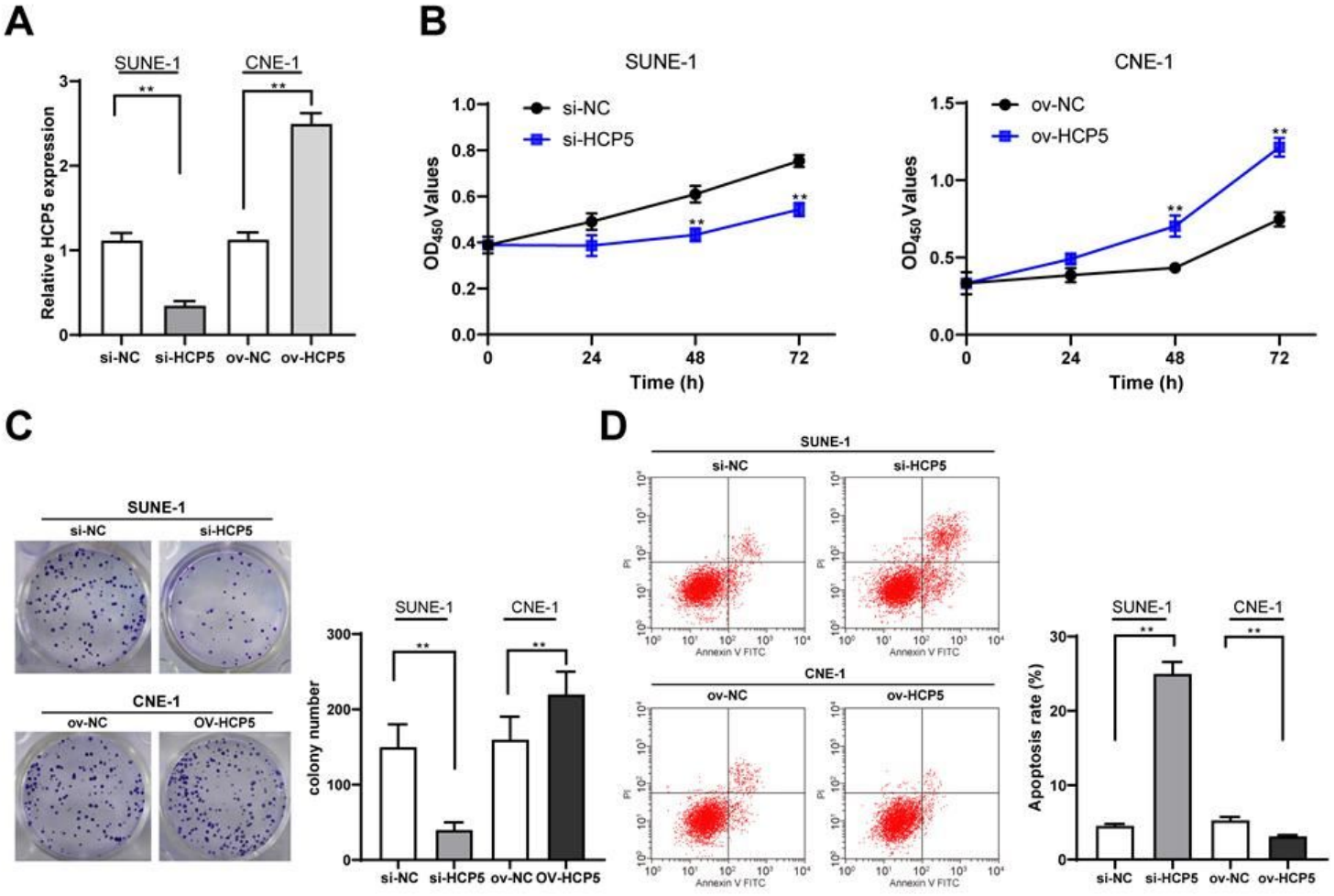

Figure 2

Inhibition of lncRNA HCP5 suppressed proliferation of NPC cells and promoted apoptosis. SUNE-1 cells were transfected with si-HCP5, and CNE-1 cells were transfected with ov-HCP5, with si-NC and ov-NC as the controls. A: HCP5 expression in cells was detected using RT-qPCR; B-C: cell proliferation was measured using CCK-8 assay (B) and colony formation assay (C); D: cell apoptosis was measured using flow cytometry. Cell experiments were repeated three times independently. Data are presented as mean ± standard deviation and analyzed using one-way or two-way ANOVA, followed by Tukey's multiple comparison test, **p < 0.01.

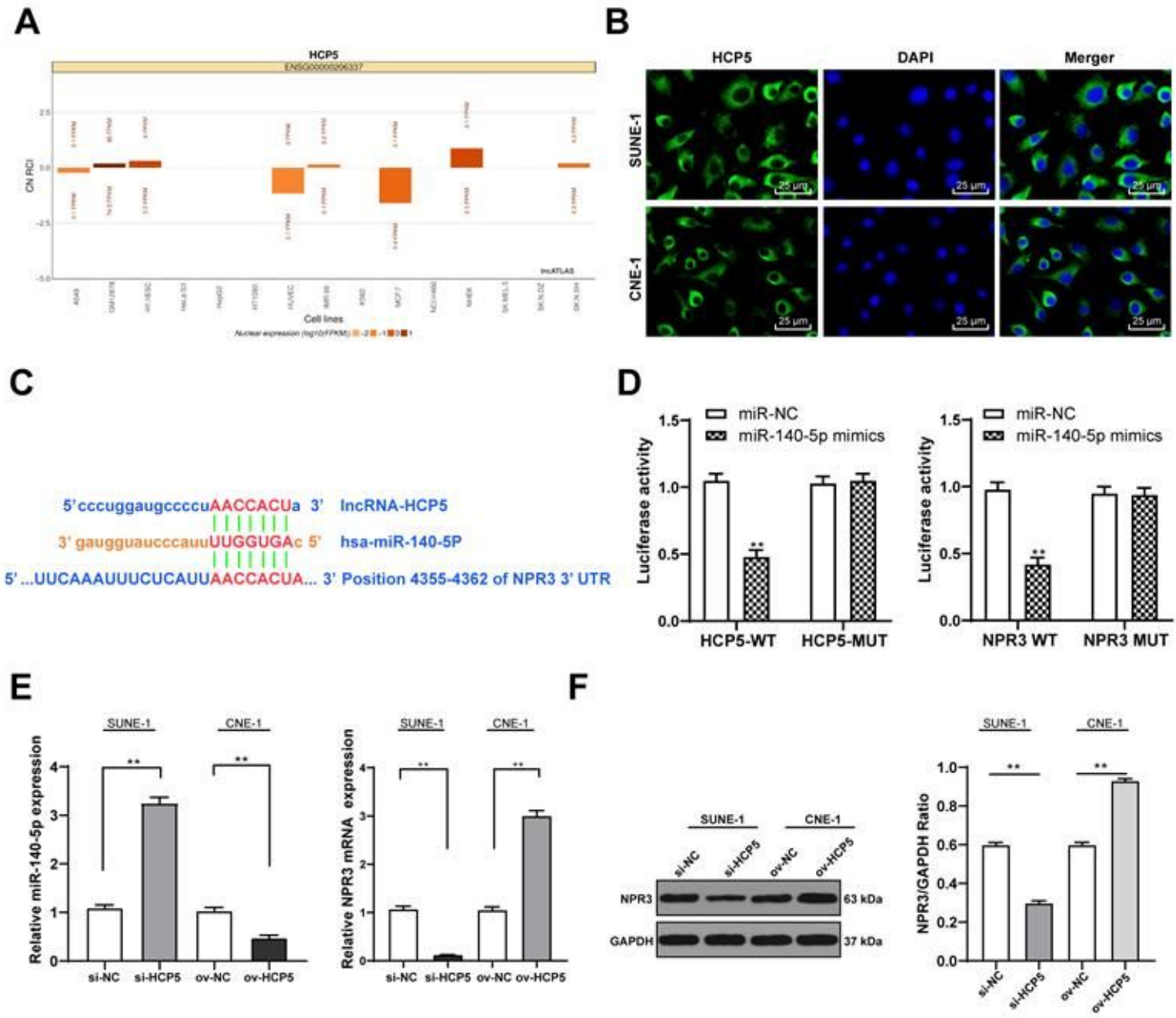

Figure 3

LncRNA HCP5 regulated NPR3 expression via sponging miR-140-5p. A: subcellular localization of HCP5 was predicted by LncATLAS; B: distribution of HCP5 in SUNE-1 and CNE-1 cells was confirmed using FISH assay; C: binding sites between HCP5 and miR-140-5p, and miR-140-5p and NPR3 were predicted by Starbase and TargetScan; D: binding relationships between HCP5 and miR-140-5p, and miR-140-5p and NPR3 were confirmed using dual-luciferase reporter gene assay; E: miR-140-5p and NPR3 expression were detected using RT-qPCR; F: NPR3 protein level in NPC cells was detected using Western blot analysis. Cell experiments were repeated three times independently. Data are presented as mean ± standard deviation and analyzed using one-way or two-way ANOVA, followed by Tukey's multiple comparison test, **p < 0.01.

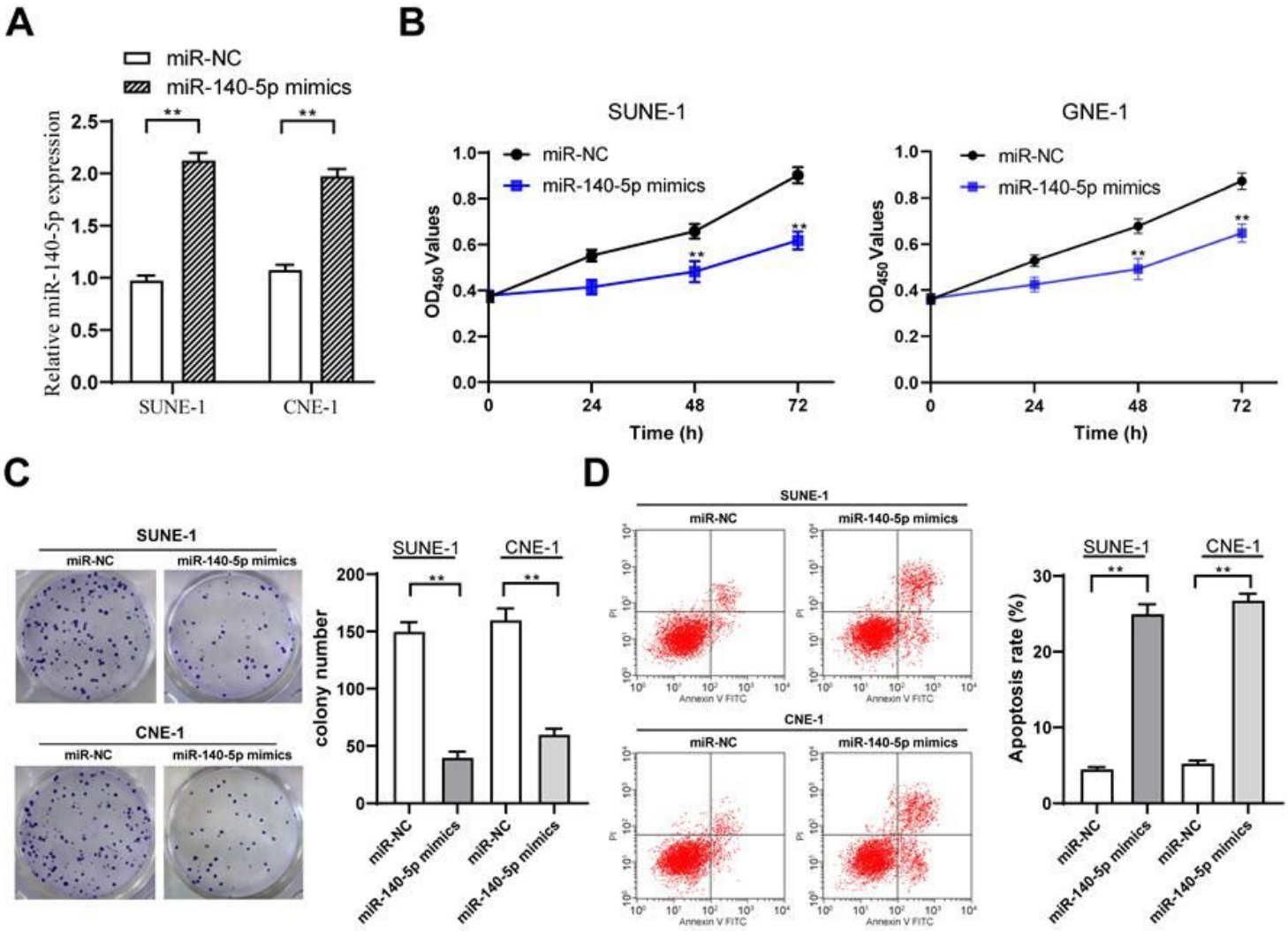

Figure 4

Overexpression of miR-140-5p weakened the proliferation and enhanced the apoptosis of nasopharyngeal carcinoma cells. SUNE-1 and CNE-1 cells were transfected with miR-140-5p mimic, with miR-NC as the control. A: miR-140-5p expression in NPC cells was detected using RT-qPCR; B-C: cell proliferation was measured using CCK-8 assay (B) and colony formation assay (C); D: cell apoptosis was measured using flow cytometry. Cell experiments were repeated three times independently. Data are presented as mean ± standard deviation and analyzed using one-way or two-way ANOVA, followed by Tukey's multiple comparison test, **p < 0.01.

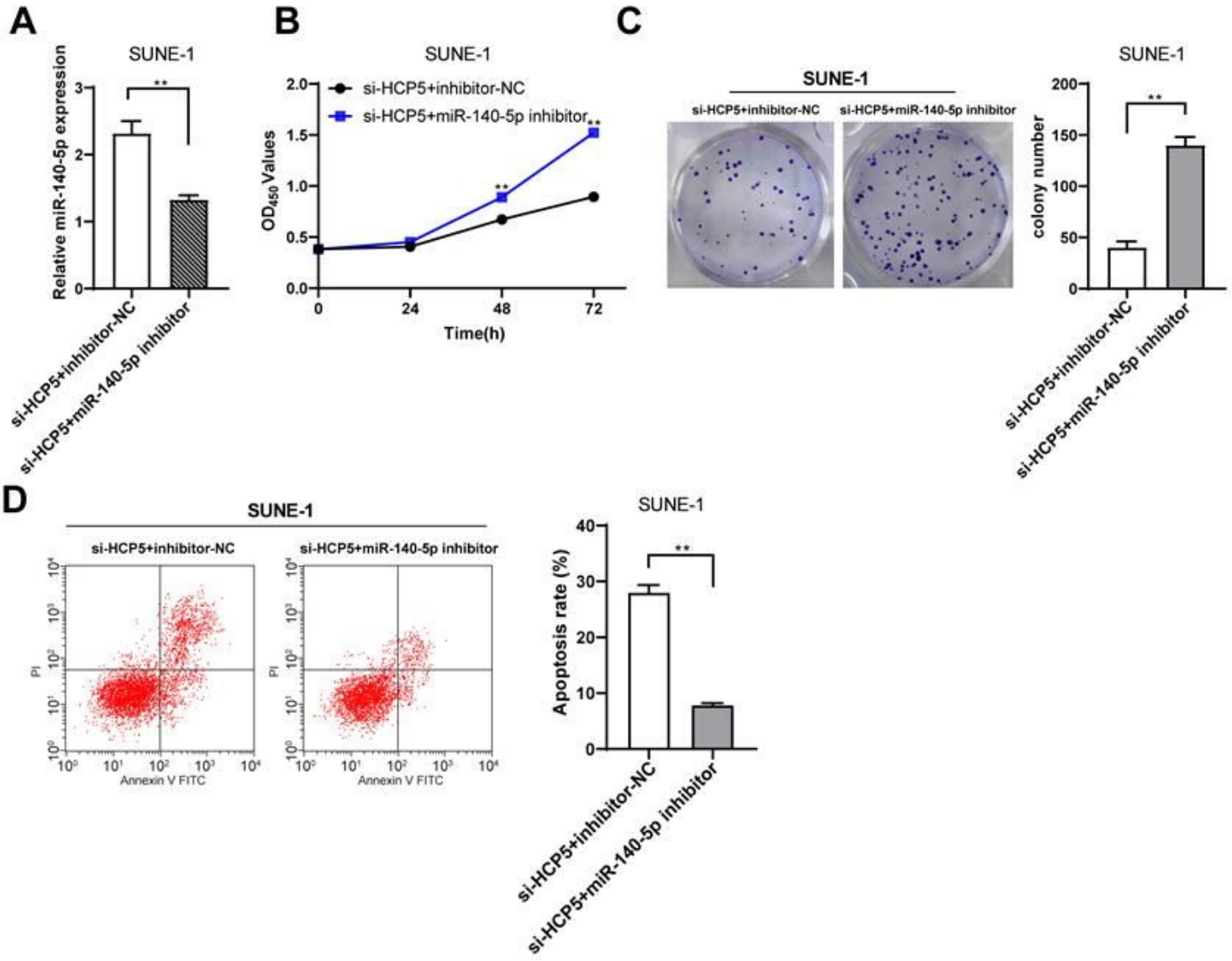

Figure 5

miR-140-5p inhibitor partially restored the malignant behaviors of NPC cells inhibited by lncRNA HCP5 silencing. SUNE-1 cells were transfected with miR-140-5p inhibitor, with inhibitor NC as the control. A: miR-140-5p expression in NPC cells was detected using RT-qPCR; B-C: cell proliferation was measured using CCK-8 assay (B) and colony formation assay (C); D: cell apoptosis was measured using flow cytometry. Cell experiments were repeated three times independently. Data are presented as mean ± standard deviation and analyzed using one-way or two-way ANOVA, followed by Tukey's multiple comparison test, **p < 0.01.