Int J Clin Exp Med 2019;12(6):7249-7256
www.ijcem.com /ISSN:1940-5901/IJCEM0091658

Original Article

Norcantharidin nanocapsules inhibit the invasion and metastasis of esophageal cancer by inhibiting microRNA-206 via IGF1R-PI3K-AKT pathway

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Received January 22, 2019; Accepted May 8, 2019; Epub June 15, 2019; Published June 30, 2019

Abstract: To investigate the effect of norcantharidin nanocapsules (NCTD-NPs) on the growth of esophageal cancer EC109 cells by inhibiting miR-206-mediated IGF1R-PI3K-Akt signaling pathway. Esophageal cancer (ESCA) cell line EC109 was cultured. Proliferative, invasive and metastatic capacities of EC109 cells were detected by cell counting kit-8 (CCK-8), would healing and transwell assay, respectively. Cell apoptosis was detected by flow cytometry. Western blot and quantitative real time-polymerase chain reaction (qRT-PCR) were conducted to determine expressions of relative genes. The model of in situ transplantation tumor of ESCA in nude mice was established. Meanwhile, fluorescence in vivo imaging was observed. Proliferative capacity of microRNA-206 overexpressing EC109 cells remarkably decreased after NCTD-NPs treatment in time- and dose-dependent manners. NCTD-NPs treatment increased apoptotic rate of microRNA-206 overexpressing EC109 cells compared with that of EC109 cells. After treatment with different doses of NCTD-NPs or NCTD, the migratory capacity of microRNA-206 overexpressing EC109 cells remarkably decreased than that of EC109 cells. Transwell assay indicated that 25 nmol/L NCTD-NPs could markedly inhibit invasion of microRNA-206 overexpressing EC109 cells. Western blot results elucidated downregulation of p-AKT (Ser473) and p-AKT (Thr308) in EC109 cells and microRNA-206 overexpressing EC109 cells. Fluorescence in vivo imaging showed better inhibitory effect of NCTD-NPs on ESCA metastasis than NCTD in nude mice. NCTD-NPs inhibited invasion and metastasis of ESCA both in vitro and in vivo. The inhibitory effect of NCTD-NPs on ESCA development is related to IGF1R-PI3K-AKT pathway regulated by microRNA-206.

Keywords: NCTD-NPs, microRNA-206, IGF1R-PI3K-AKT pathway, ESCA

Introduction

Esophageal cancer (ESCA) is one of the common malignancies of the digestive tract. A great number of ESCA patients have progressed to the advanced stage at the time of diagnosis, with the 5-year survival rate of only 15%-25% [1]. The key to effectively treat ESCA and improve the prognosis is to prevent invasion and distant metastasis [2]. Invasion and metastasis of ESCA is a multi-factor, multi-stage dynamic process involving the interaction between tumor cells, host cells and extracellular matrix [3]. Therefore, study on the potential molecular mechanism of invasion and metastasis of ESCA cells is of great significance for finding new treatment methods.

Norcantharidin (NCTD) is a derivative of cantharidin extracted from Chinese traditional antitumor medicine. NCTD has been widely applied in tumor treatment and postoperative chemotherapy. Multiple studies have proved the antitumor effect of NCTD on liver cancer, breast cancer and cholangiocarcinoma [4, 5]. However, therapeutic effect of NCTD on ESCA is rarely reported. NCTD is strictly administrated because of its fast degradation in body and toxic to the urinary system [6]. Lac-NCTD-NPs (lactosy norcantharidin nanoparticles) is actively liver-specific since the galactose residue on Lac-NCTD can be specifically recognized by ASGP-R on the liver cell membrane. Besides, lac-NCTD-NPs also present passive-targeting characteris-

tics to improve the anti-tumor efficacy and reduce toxicity [7-9].

Previous studies have suggested the regulatory effects of NCTD on invasion and metastasis of liver cancer cells [10]. The specific mechanism and therapeutic targets have not been fully elucidated yet. It is reported that microRNA-206 is differentially expressed in various tumor tissues, and its mechanism is closely related to the regulation of IGF1R-PI3K-AKT pathway [11-13]. This study investigated whether NCTD-NPs could inhibit invasion and metastasis of ESCA by regulating microRNA-206-mediated IGF1R-PI3K-AKT pathway. Our results provide theoretical basis for ESCA treatment.

Materials and methods

Cell lines and reagents

Human ESCA cell line EC109 was obtained from Cell Bank, Chinese Academy of Sciences (Shanghai, China); NCTD and NCTD-NPs were purchased from Sigma-Aldrich (St. Louis, MO, USA); Primary and secondary antibodies were obtained from Cell Signaling Technology (Danvers, MA, USA), including p-AKT (Thr308), p-AKT (Ser473), pan-AKT, IGF1R and β-actin; LY294002 was purchased from Selleck Chemicals (Houston, TX, USA).

Cell culture and transfection

EC109 cells were cultured in RPMI-1640 medium (Roswell Park Memorial Institute-1640) (Gibco, Rockville, MD, USA) containing 10% FBS (fetal bovine serum) (Gibco, Rockville, MD, USA) and maintained in a 5% CO_2 incubator at 37°C. For cell transfection, EC109 cells were pre-seeded in the 6-well plates. After overnight culture, 20 μL solution containing hsa-microRNA-206 negative control or has-microRNA-206 lentivirus was added, followed by 5 mg/L polybrene addition in each well. Culture medium was replaced the other day. GFP-positive cells were sorted by flow cytometry.

In situ tumor bearing mouse model of ESCA

30 male BALB/C nude mice (4-week-old, 20±2 g) were obtained from Shanghai Laboratory Animal Center (Shanghai, China). Mice were randomly assigned into microRNA-206 overexpression group and control group. Briefly, EC109 cells and microRNA-206 overexpressing EC109 cells were prepared for single cell suspension at a density of 1×10^7/mL. 0.2 mL of suspension was injected into mice of microRNA-206 overexpression group and control group through tail vein. 2 weeks later, mice in both groups were intraperitoneally injected with 0.2 mL of NCTD, NCTD-NPs or 0.9% saline, respectively. Drug administration was performed for 5 consecutive days a week, followed by 2 days of rest, for a total of 6 weeks.

Real time-polymerase chain reaction (RT-PCR)

TRIzol kit (Invitrogen, Carlsbad, CA, USA) was used to extract the total RNA. Expression level of microRNA-206 was detected using Bulge-Loop method. Relative gene expression was calculated by $2^{-\Delta\Delta Ct}$.

Cell counting kit-8 (CCK-8) assay

EC109 cells were seeded in the 96-well plates at a density of 2×10^4 per well. After cell treatment, 10 μL of CCK-8 solution (Dojindo, Kumamoto, Japan) was added for 1 h incubation. The optical density was measured at the wavelength of 450 nm using microplate reader.

Cell apoptosis detection

EC109 cells were seeded in the 24-well plates at a density of 5×10^4 per well. After treatment, cells were collected and resuspended in binding buffer. After incubation with 2.5 μL of Annexin V-FITC and 5 μL of propidium iodide (PI) in dark for 15 min, cells were collected for apoptosis detection. Morphology was observed and captured as well.

Wound healing assay

EC109 cells were seeded into 24-well plates at a density of 5×10^5 per well. When the cell confluence was up to 80%, a sterile 10 μL micropipette tip was used to vertically scratch the cell plate. After removing the exfoliated cells with phosphate buffered saline (PBS), serum-free medium was placed for 48 h incubation. The cell migration was observed under an inverted microscope (Nikon, Tokyo, Japan), and the width of the scratch was measured and photographed.

Transwell assay

EC109 cells were centrifuged and resuspended in serum-free RPMI-1640 at a density of 5.0×10^4/mL. Transwell chambers pre-coated with Matrigel were placed in 24-well plates.

100 μL of cell suspension and 600 μL of medium containing 10% FBS were added in the upper and lower chamber, respectively. After culture for 48 h, cells were fixed with 4% paraformaldehyde for 15 min and stained with crystal violet for 15 min. Cells in the upper chamber were carefully cleaned. Penetrating cells were captured in 5 randomly selected fields of each sample.

Western blot

The RIPA (radioimmunoprecipitation assay) protein lysate (Beyotime, Shanghai, China) was used to extract the total protein in each group of cells. The BCA (bicinchoninic acid) method was performed to quantitate the protein concentration. Protein samples were electrophoresed on polyacrylamide gels and then transferred to polyvinylidene fluoride (PVDF) membranes (Merck Millipore, Billerica, MA, USA). After blocking with 5% skimmed milk, the membranes were incubated with primary antibody (Cell Signaling Technology, Danvers, MA, USA) at 4°C overnight. The membrane was incubated with the secondary antibody after rinsing with the buffer solution (TBST). Chemiluminescence was used to expose the protein bands on the membrane.

Fluorescence in vivo imaging

8 weeks after in situ tumor bearing mouse model construction, fluorescence *in vivo* imaging was detected. First, nude mice were intraperitoneally injected with 150 μg/g D-Luciferin. Then, 20 min later, fluorescence imaging of ESCA was observed and acquired by PerkinElmer IVIS software (Waltham, MA, USA).

Statistical analysis

We used Statistical Product and Service Solutions (SPSS) 13.0 software (IBM, Armonk, NY, USA) for statistical analysis. The quantitative data were represented as mean ± standard deviation ($\bar{x}$±SD). Differences among groups were compared using one-way ANOVA, followed by post-hoc test. $P<0.05$ was considered statistically significant.

Results

NCTDs and NCTD-NPs inhibited proliferative capacity of EC109 cells

EC109 cells stably expressing microRNA-206 were sorted as those of GFP-positive ones. RT-PCR results verified that microRNA-206 expression in microRNA-206 overexpressing EC109 cells was 15-fold higher than those of EC109 NC cells (**Figure 1A**). Proliferative capacity was remarkably inhibited in EC109 cells, EC109 NC cells and microRNA-206 overexpressing EC109 cells after NCTD treatment for 24-72 h. The inhibitory effect showed time- and dose-dependent manners (**Figure 1B-D**). NCTD-NPs treatment also gradually inhibited cell proliferation alongside the increases of treatment time and dose, which was more pronounced than that of NCTD (**Figure 1E-G**). In particular, the inhibitory effect of NCTD-NPs on cell proliferation was similar at 48 h and 72 h. MicroRNA-206 overexpressing EC109 cells presented the most inhibited proliferation by NCTD-NPs treatment.

NCTD and NCTD-NPs induced apoptosis of EC109 cells

Accompanied by the increased NCTD dose, apoptotic rates of EC109 cells, EC109 NC cells and microRNA-206 overexpressing EC109 cells were gradually elevated (**Figure 2A-C**). NCTD-NPs presented similar effect on apoptosis of EC109 cells (**Figure 2D-F**). Moreover, NCTD-NPs remarkably induced apoptosis in microRNA-206 overexpressing EC109 cells than other cells (**Figure 2G**). Cell morphology showed typical apoptotic characteristics after different doses of NCTP-NPs treatment in microRNA-206 overexpressing EC109 cells for 48 h, manifesting as floated particles (**Figure 2H**). Therefore, NCTD and NCTD-NPs promote apoptosis of EC109 cells, and NCTD-NPs appears to be more effective.

NCTD and NCTD-NPs inhibited invasion and metastasis of EC109 cells

EC109 cells were treated with different doses of NCTD-NPs (25 nmol/L-400 nmol/L). Wound healing assay elucidated that the migratory rate in EC109 NC cells decreased in a dose-dependent manner. High-dose NCTD-NPs even resulted in cell death (**Figure 3A**). Transwell assay showed similar results that NCTD and NCTD-NPs remarkably inhibited metastasis, especially in microRNA-206 overexpressing EC109 cells (**Figure 3B**). There were significant differences between the medication groups and the control group ($P<0.01$). The results showed that NCTD-NPs significantly inhibited the invasion of EC109 cells overexpressing

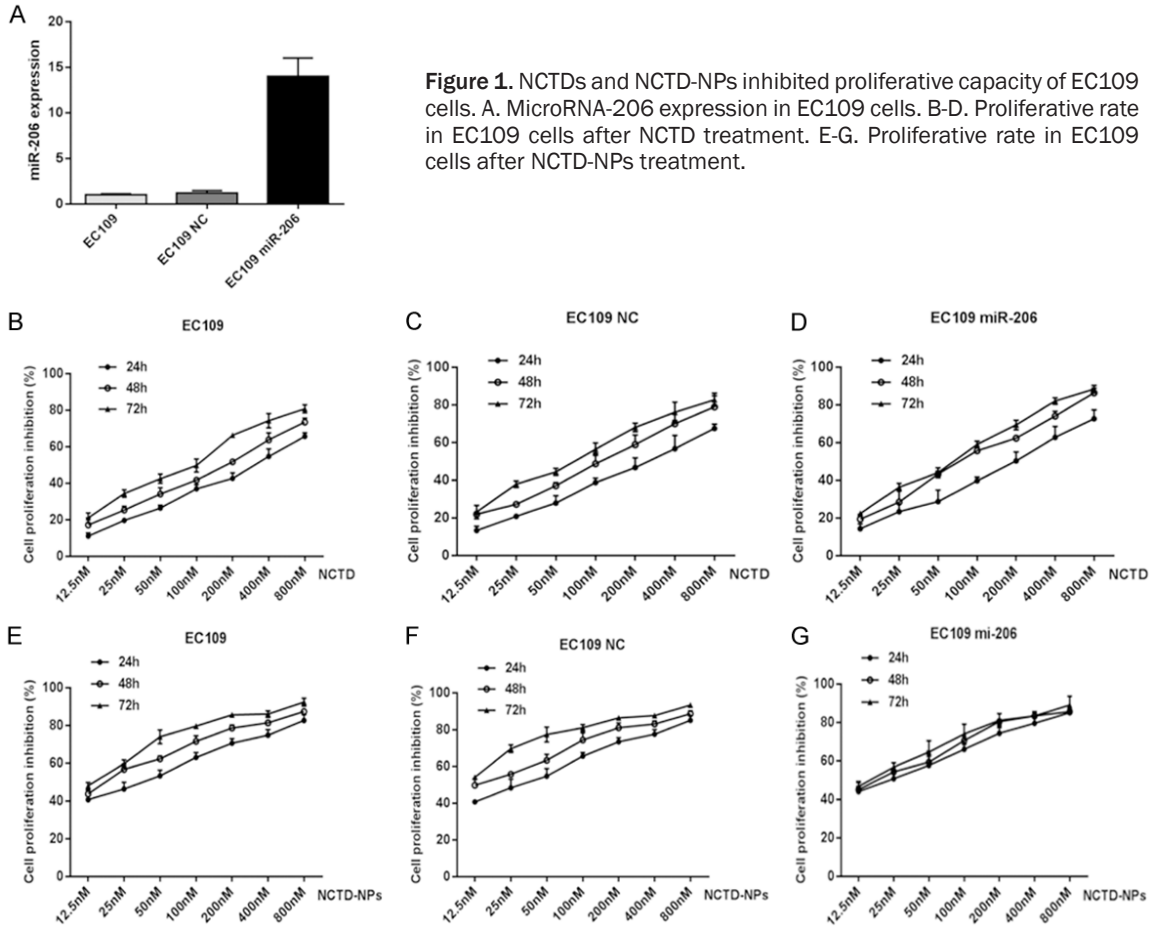

Figure 1. NCTDs and NCTD-NPs inhibited proliferative capacity of EC109 cells. A. MicroRNA-206 expression in EC109 cells. B-D. Proliferative rate in EC109 cells after NCTD treatment. E-G. Proliferative rate in EC109 cells after NCTD-NPs treatment.

miR-206 at 25 nmol/L. In conclusion, NCTD-NPs significantly inhibits the invasion and metastasis of EC109 cells.

NCTD-NPs inhibited IGF1R-PI3K-AKT pathway in ESCA

Western blot results showed that the protein expression of IGF1R in EC109 cells was down-regulated after treatment of NCTD-NPs in a concentration dependent manner. Additionally, protein expressions of p-AKT (Ser473) and p-AKT (Thr308) in EC109 cells were decreased after treatment of LY294002 and NCTD-NPs, respectively. However, no significant changes of pan-AKT protein expression were observed (**Figure 4A**). These results indicated that NCTD-NPs could suppress the proliferation and metastasis of esophageal cancer cells by inhibiting the IGF1R-PI3K-Akt signaling pathway.

NCTD and NCTD-NPs inhibited in vivo metastasis of ESCA

Fluorescence in vivo imaging showed that fluorescence uptake in EC109 cells after treatment of NCTD-NPs was significantly lower than that in control group and NCTD group, respectively. In addition, EC109 cells after treatment of NCTD-NPs and overexpression of miRNA-206 obtained the least fluorescence uptake (**Figure 4B**). All these results suggested that both NCTD and NCTD-NPs can significantly inhibit tumor metastasis, and the tumor suppressive effect of NCTD-NPs is significantly better than NCTD. The combined use of NCTD-NPs and miR-206 has a better effect on metastasis inhibition of esophageal cancer.

Discussion

NCTD is a monomer extracted from traditional Chinese medicine, which has received extensive attention due to its broad-spectrum anti-tumor effect. However, its shortcomings limit its clinical application. The novel nanocarriers overcome the problem of the clinical application of NCTD [14]. Recent studies showed that NCTD exerts its anti-tumor effect by regulating cell cycle, apoptosis and angiogenesis [15, 16]. A large number of studies have confirmed that

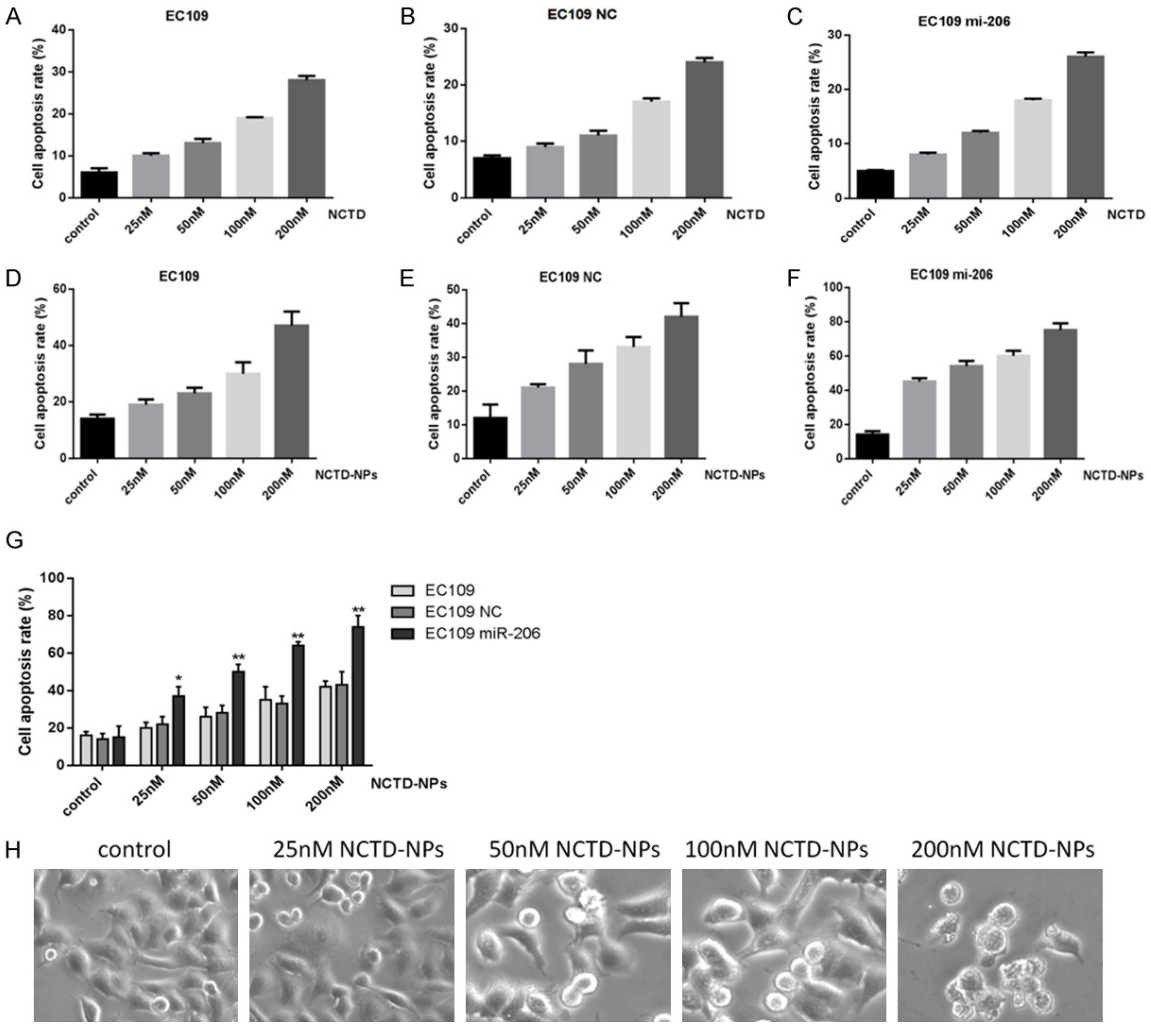

Figure 2. NCTD and NCTD-NPs induced apoptosis of EC109 cells. A-C. Apoptotic rate in EC109 cells after NCTD treatment. D-F. Apoptotic rate in EC109 cells after NCTD-NPs treatment. G. Comparison of apoptotic rate after NCTD-NPs treatment. H. Cell morphology after NCTD-NPs treatment (magnification 200×).

NCTD induces apoptosis of tumor cells through activation of mitochondrial pathway. NCTD is also capable of inhibiting tumor growth and promoting apoptosis via JNK, TRAIL/DR5 and TR3-dependent pathways [17, 18]. Few studies have been conducted on exploring IGF1R-PI3K-AKT pathway in the pharmacological action of NCTD.

MiRNA is a type of non-coding RNA with 17-25 nucleotides in length. MiRNA regulates target gene expression by specifically binding to the 3' non-reading region (3'UTR) of the target gene. Functionally, miRNAs participate in the occurrence, progression and metastasis of tumors. IGF1 is the target gene of microRNA-206, which influences the malignant progression of tumors by PI3K/AKT pathway via targeting IGF1R [13]. PI3K/AKT pathway is found to be the downstream of IGF1R [19]. AKT pathways route PI3K-initiated growth factor transport, while AKT is a direct downstream biomolecule when growth factors act through PI3K. AKT is activated by phosphorylation under the action of PI3K and other regulatory genes and further influences cell cycle progression, apoptosis initiation, telomerase activity, tumor angiogenesis, and invasion of tumor cells through downstream pathways.

Thus, phosphorylation of AKT can represent both the activation state of the AKT pathway and the functional state of PI3K [20].

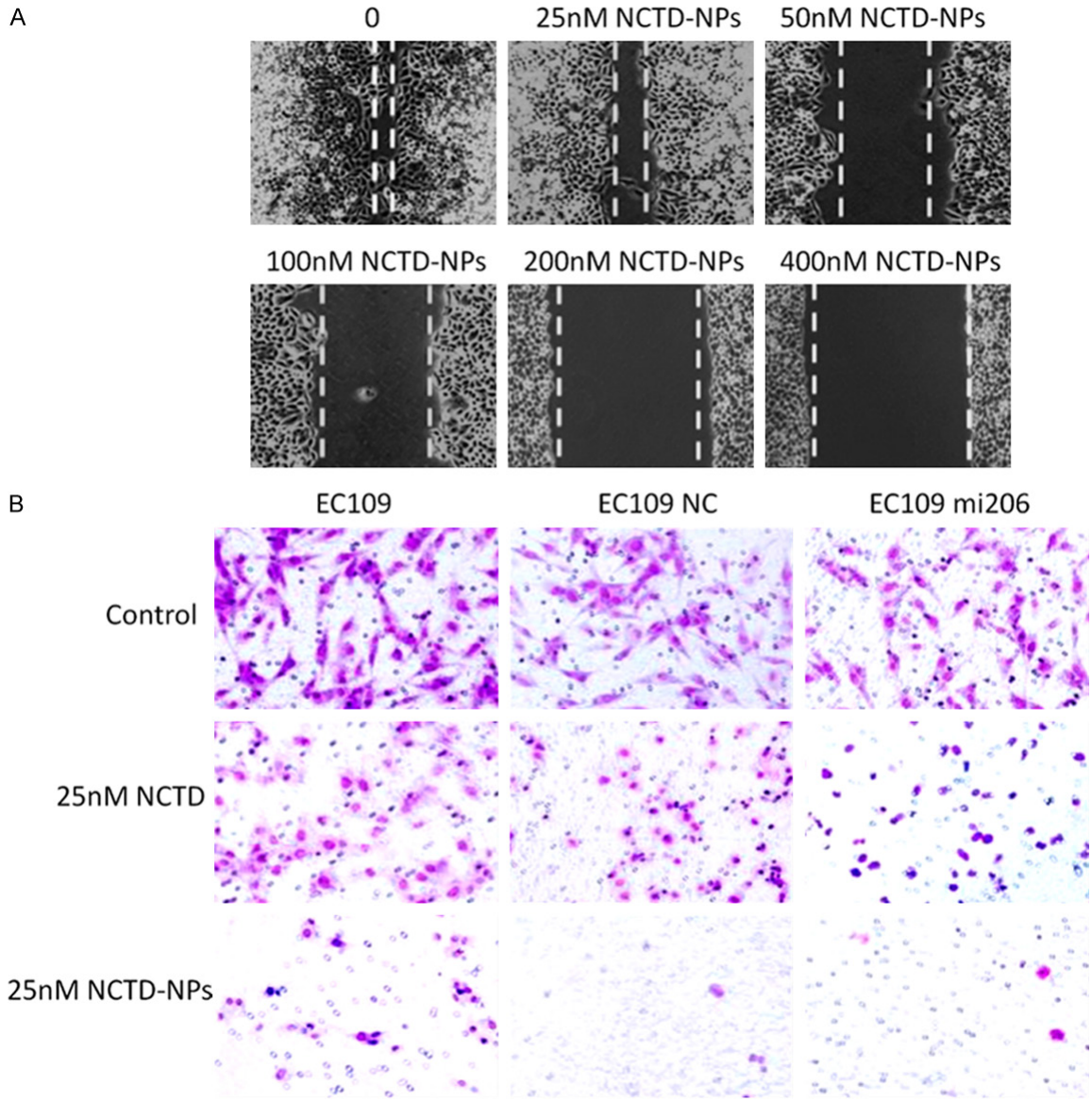

Figure 3. NCTD and NCTD-NPs inhibited invasion and metastasis of EC109 cells. A. Invasion of EC109 cells after NCTD-NPs treatment. B. Metastasis of EC109 cells after NCTD-NPs treatment (magnification 200×).

Our experiments used CCK-8, wound healing, transwell test and cell apoptosis detection, and further studied the possible signaling pathways and molecular mechanisms by immunoblotting. We found that NCTD-NPs treatment remarkably inhibits proliferative and invasive capacities of microRNA-206 overexpressing EC109 cells via IGF1R-PI3K-AKT pathway. Furthermore, in vivo experiments found better therapeutic efficacy of NCTD-NPs than NCTD in inhibiting ESCA metastasis in nude mice. Our results provides theoretical basis for the promising application of UCTD and nanomaterials in ESCA treatment.

Conclusions

NCTD-NPs inhibits invasion and metastasis of ESCA both in vitro and in vivo. The inhibitory effect of NCTD-NPs on ESCA development is related to IGF1R-PI3K-AKT pathway regulated by microRNA-206.

Disclosure of conflict of interest

None.

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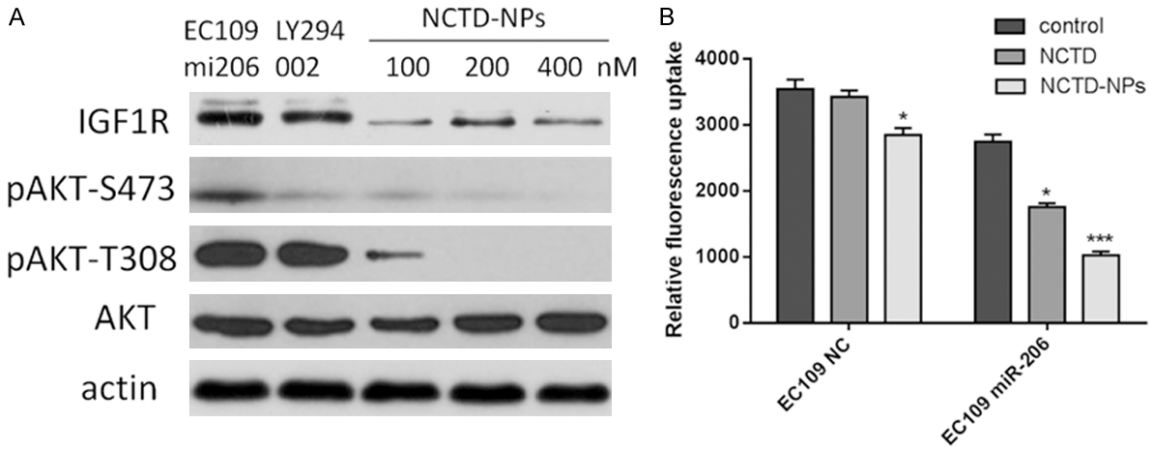

Figure 4. NCTD-NPs inhibited IGF1R-PI3K-AKT pathway in ESCA. A. Protein expressions of relative genes in IGF1R-PI3K-AKT pathway after NCTD-NPs or LY294002 treatment in EC109 cells. B. Fluorescence in vivo imaging of nude mice.

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