Indomethacin sensitizes human lung cancer cells to DDP through autophagy activation in a mouse model

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Research

Keywords: Indomethacin, Autophagy, Drug resistance, DDP, Lung cancer

Posted Date: April 15th, 2020

DOI: https://doi.org/10.21203/rs.3.rs-20683/v1

Abstract

Background: Indomethacin is one of the non-steroidal anti-inflammatory drugs (NSAIDs) , and it was considered to have chemosensitization effects on many cancers, however, the mechanism of its effect on chemotherapy is still unclear. In this study, using human lung cancer SPC-A-1 cells, we examined the effects of indomethacin sensitize s human lung cancer cells to cisplatin (DDP).

Methods: Human lung cancer SPC-A-1 cells were inoculated subcutaneously into BALB/c-nu/nu mice, and all the transplanted nude mice were divided into a control group, a DDP group and a indomethacin combined with DDP group , after daily treatment for 4 weeks a ll the mice were sacrificed by cervical dislocation. Immunohisto-chemical staining was employed to determine the expression of PCNA of lung tumor cell in the tumor tissues, western blot was used to detect the expression of autophagy related protein LC3-II and P62 What's more, the formation of antophagosomes was observed through transmission electron microscope.

Results: Compared with the control group and DDP group, we can find that tumor growth is very slow in indomethacin combined with DDP group , and the tumor weight decreased significantly in combined group. H&E staining reveal s that tumor cell necrosis is more obvious in combined group . The immunohistochemical staining results indicated that the expression of PCNA was much lower in combined group . than that in control and DDP group s.More importantly, western blot results show that the expression of LC3-II was significantly up-regulated in combined group than that in control group and DDP group ($P < 0.05$) , and the expression of p62 protein was significantly down-regulated in combined group ($P < 0.05$). Moreover, transmission electron microscope results show that compared with the control group and DDP group, the number of antophagosomes increased significantly in combined group.

Conclusions : Our findings suggested that indomethacin can enhance the chemo s ensitivity of lung cancer cells to DDP, and the underlying mechanism maybe associated with the activation of autophagy.

Background

Lung cancer is one of the most common tumors and it is also one of the leading causes of cancer-related death worldwide [1], among which non-small cell lung cancer (NSCLC) accounts for nearly 85%. The treatment of lung cancer patients includes surgery, radiotherapy, chemotherapy and targeted therapy [2–3]. According to recently studies, traditional chemotherapy and radiotherapy play an irreplaceable role in the whole treatment of lung cancer, however, about 30% of the cancer patients can't benefit from chemotherapy due to chemotherapy resistance[4]. Therefore, it is important and urgent to enhance the chemosensitivity of lung cancer cells.

Autophagy, which was a lysosome-dependent degradation progress, and it is considered an alternative cell death mechanism in many reports [5–6], it has been thought to act as a pro-survival or pro-death response to several stressors, especially for chemotherapy and radiotherapy. According to many previous studies [7–9], autophagy plays an important role in many cancers such as breast cancer, gastric cancer,

colon cancer, lung cancer and so on. A large number of clinical researches have reported that autophagy is controlled by a highly regulated set of signaling events, and it is still controversial whether autophagy activation leads to cell survival or cell death[10], some reports revealed that through enhancers of autophagy can prevent cancer progression in pre-malignant lesions, conversely, other reports also suggest that enhancing autophagy and inhibiting it all can be served as tumor treatment strategies.

Many studies have proved the effectiveness of long-term use of nonsteroidal anti-inflammatory drugs (NSAIDs) in the treatment of many cancers [11–13]. Indomethacin, a non-selective NSAIDs, and it is also a COX-1 and COX-2 inhibitor, previous researches have suggested that indomethacin can slow down colorectal cancer cell growth through reduced mTOR signaling[14], and Tomoko Kato[15] study suggested that indomethacin can induces cellular morphological change and migration via epithelial-mesenchymal transition in A549 human lung cancer cells, so indomethacin may also have anti-tumour activity at other cancers in humans.

In this study, we investigated the role of indomethacin in sensitizing human lung cancer cells to DDP, and to explore the specific molecular mechanism of indomethacin enhancing chemosensitivity of lung cancer. Our results demonstrated that combined indomethacin and DDP treatment can obviously slow down the growth rate of lung cancer, and we also found that indomethacin can significantly enhanced autophagy of lung cancer SPC-A-1 cells in a mouse model. Upon our results, indomethacin may be a new therapeutic approach to enhance the chemosensitivity of cisplatin to lung cancer.

Materials And Methods

Cell culture

SPC-A1 cell line (human NSCLC) was obtained from Genechem Technology Co., Ltd（Shanghai，China）. SPC-A-1 cells were cultured in DMEM media(Gibco, USA) supplemented with 10% fetal bovine serum (HyClone, USA), antibiotics penicillin (50 U/mL) and streptomycin (U mg/mL) (Solarbio, Beijing, China) in 37 °C 5% CO2 incubator (Sanyo, Japan).The cells were recently authenticated by STR profiling, and are free from mycoplasma contamination.

Generation and treatment of xenografts.

Male BALB/c nude mice at 6-8 weeks were purchased from Beijing Vital River Laboratory Animal Technology Co., Ltd. (Beijing, China), and housed in a specific pathogen-free environment. All the animal experiments were approved by the Institutional Animal Care and Use Committee of Institute of Basic Medicine，The First Affiliated Hospital of Shandong First Medical University.SPC-A1 cells（1×10^6 cells） were implanted subcutaneously into into the left lower limbof nude mice.When the tumor volume reached 100 mm^3, mice were randomly grouped as DDP，DDP combined with Indomethacin (DDP+Indo) and negative control.

Mice bearing SPC-A1 tumors were treated with DDP alone (3 mg per kg body weight,ip,once every three dayss), or DDP followed by Indomethacin(DDP+Indo.(50 mg per kg body weight,by Gastric administration,ig)),or vehicle solution(Control) for 3 weeks.

Tumor volume was estimated and plotted against time to evaluate response to treatment.The tumor volume ($V = 1/2$ (length× $width^2$)) was measured every other day. The animals were sacrificed when reached the Mice Welfare Endpoint. The tumor samples were divided into two parts, some of which were fixed in 4% PFA and then embedded in paraffin for H&E staining and Immunohistochemistry staining with PCNA.Another part of the tmour samples were rapidly frozen and retained for Westerm blotting.

Immunohistochemical staining

Tumors were excised for immunohistochemical staining to evaluate cell proliferation by quantifying expression of PCNA by routine methods.. Paraffin sections were dewaxed in xylene and exposed sequentially to decreasing concentration of ethanol (finally to distilled water) following routine methods. Afterward, antigen retrieval by boiling in sodium citrate buffer for 15min. Slides blocked in PBS containing 5% goat serum at 37°C for 1hour. Slides were incubated with the primary antibody PCNA(1:200) at 4 °C overnight, followed by a conjugated secondary at 37°C for 1h. Color development was undertaken with freshly prepared DAB solution and counterstained with hematoxylin. The extent of proliferation was represented by the percentage of viable tumor area occupied by positive nucli.

Transmission electron microscopy (TEM)

The tumor samples were fixed with 2.5% glutaraldehyde at room temperature for 1 h, and then post-fixed with 1% osmium tetroxide buffer for 1 h. Subsequently, an ascending series of alcohol were performed for dehydration before embedding samples in araldite. Ultrathin sections were observed by TEM (JEM-1200 EX electron microscope，Japan).

Westerm blotting

The tumor samples were lysed in RIPA lysis buffer （Biosharp，Shanghai，China，containing protease inhibitor on ice for 30 min, vortex shocking every 6 min. The total protein concentration was measured by Bradford protein assay kit (Bio-Rad Ltd., USA). The proteins were separated with 15% SDS-PAGE （Beyotime，Shanghai，China） and transferred to polyvinylidene fluoride (PVDF) membrane (Millipore Ltd., USA). After blocking with tris buffered saline tween containing 5% non-fat milk for 1 h, the membranes were incubated with primary antibodies (Actin, p62，LC3,and Beclin1, Bioss，Beijing，Chin) at 4 °C overnight. The membranes were then incubated with corresponding secondary antibodies at room temperature for 2 h and the proteins were detected by enhanced chemiluminescence reagent (Milipore Ltd.,USA).Proteins immunoreactivity derived from negative control (Control) was normalized to 1.0.

Values of proteins in DDP or DDP+Indo groups were normalized according to vehicle groups. ` Blotting for Actin was used to control for protein loading

Statistical analysis

All the data were presented as means ± standard deviation (SD). Statistical analysis was carried out by using the SPSS 19.0 statistical software.The significance between different treatment groups was determined by one-way analysis of variance (ANOVA). Student's t-test was utilized to evaluate the differences of 2 groups. $P < 0.05$ was considered to be statistically significant.

Results

Administration of Indo between treatments with chemotherapy.

SPC-A1 cells (1×10^6) were implanted subcutaneously in to the right flanks of male BALB/c nude mice. When the tumor volume reached ~100 mm^3, treatment was initiated as the scheme implied (Fig.1)

Indo increased chemotherapy sensitivity in vivo

DDP (3 mg/kg) alone had limited effects on tumor growth . Indo (50 mg per kg per day, for 3 weeks) given between courses of DDP increased the growth delay of SPC-A1 tumors and the effects seemed additive to those from chemotherapy (Fig.2). DDP significantly inhibited tumor growth, and the combination therapy exhibited synergistic effects (Fig.2). The tumor weight was much lower in DDP group than the control group (2.15 ±0.11g vs 3.34 ± 0.13, $p < 0.05$). The combination therapy further decreased the tumor weight compaed with DDP chemotherapy alone (1.45 ± 0.053 vs 0.026 ± 0.004, $p < 0.05$, Fig. 3).

Effects of Indo on proliferation of SPC-A1 cells in xenografts.

The necrosis of tumor xenografts was evaluated by H&E staining.The hematoxylin-eosin (HE) stained tumor slics showed extensive necrosis in the tumor of DDP and DDP+Indo groups, but not in the control tumorsFigure 4The nuclei of SPC-A1 cells in tumors treated with DDP or DDP+Indo became smaller and condensed (Fig. 4), Significantly histopathological abnormalities were observed, suggesting the combination therapy (DDP+Indo) further **increased chemotherapy sensitivity** (Fig. 4). Furthermore, the combination therapy exhibited much more extensive necrosis than DDP alone. Moreover,. the proliferating cell nuclear antigen-(PCNA) immunostaining showed that the relative area occupied by positive nuclei decreased following treatment of xenograft With DDP (Fig. 4). The effect was greater for DDP+Indo than DDP alone. Where the PCNA index was much lower following treatment with DDP+Indo than that of control tumors or DDP tumors .

Indomethacin enhanced autophagy in a mouse model of human lung cancer

TEM analyses, which are considered the gold standard for investigations of autophagy . To investigate whether the combination therapy could increase autophagy, we performed TEM analyses and western blotting after treatments of SPC-A1 cells with DDP or DDP+Indo for 3 weeks . Typical double membrane structure vesicle containing portions of the cytoplasm was found in DDP group and combined therapy group (Fig. 5). The late autophagy vacuoles (AVd) contained partially degraded cytoplasmic material.Compared to the control group, Indo combined DDP group showed increased numbers of autophagosomes. These data together demonstrated Indomethacin enhanced autophagy in a mouse model of human lung cancer

Western blotting analyses (Fig. 6) indicatd increased expression of LC3Ⅱ protein either exposed to DDP or DDP+indo. The expression levels of p62 protein (the degradation substrate of autophagy) were decreased, while those of Beclin1 increased. Both decreased p62 levels and increased Beclin1 levels correlated with enhanced autophagy

Discussion

Indomethacin, a potent NSAID, is reported to have antiproliferative and pro-apoptotic effects on many cancer cells [16]. In the present study, our results not only demonstrated that indomethacin combined with cisplatin (DDP) can slow down the growth of lung cancer but also demonstrated the effects of indomethacin on autophagy during the chemotherapy. It also showed the inhibitory effect of indomethacin on murine lung cancer growth in vivo and its possible mechanisms. In conclusion, indomethacin could be an effective chemosensitizer.

Autophagy is a biochemical catabolic process that can delivers cytoplasmic materials or organelles into lysosomes for degradation, which is also a progress of nutrient recycling[17], it plays an important role in promoting apoptosis in NSCLC, and many studies have proved that autophagy is a self-protective cellular mechanism, which can provides energy through the degradation and recycling of cytoplasmic contents[18]. Indeed, autophagy functions as a double-edged sword in the process of carcinogenesis. On the one hand, it is always restrained by many proteins, such as LC3-II, PI3K, and p53, which may prevent protein over degradation in many stressed cancer cells [19]. On the other hand, autophagy can be activated continuously so that it can cause autophagic programmed cell death or apoptosis [20]. Our results showed that indomethacin enhanced autophagy in lung cancer SPC-A-1 cells because the expression levels of LC3-II protein was significantly up-regulated after treatment with indomethacin, and the transmission electron microscope results also show that compared with the control group and DDP group, the autophagy bodies increased significantly when treated with indomethacin.

Currently, many studies on indomethacin mostly focused on signaling pathway to regulate autophagy and inducing apoptosis of cell tumor [21–22]. It is well-known that mTOR is a downstream effector of AKT, and it plays an important role in the regulation of autophagy [23]. Furthermore, a previous study has demonstrated that NSAID can sensitize CD44-overexpressing cancer cells to Hsp90 inhibitor through

autophagy activation [24], Liang et al [25] study also proved that autophagy inhibition potentiates the anti-angiogenic property of multikinase inhibitor anlotinib through JAK2/STAT3/VEGFA signaling in NSCLC cells. Recently, many other researches have reported that the autophagy-related proteins' expression were rely on NF-κB p65 Ser536 phosphorylation [26]. In our study, we can find that in the treatment of lung cancer, Indomethacin combined with DDP can trigger autophagy-associated cell death and further improve chemotherapy efficacy, and it proved that indomethacin can increase the sensitivity of cisplatin to chemotherapy drugs through enhance autophagy, and this is the specific mechanism of indomethacin for chemosensitivity enhancement.

Conclusion

In summary, our findings demonstrated that indomethacin can sensitizes human lung cancer cells to DDP through enhance autophagy, and this also explains why indomethacin combined with DDP can inhibits the tumor growth of murine SPC-A-1 lung cancer in vivo. What's more, the results and the mechanistic investigation also need to be further studies in vitro. We hope that indomethacin could be regarded as a novel combined chemotherapy agent against lung cancer in the future.

Abbreviations

DDP
cisplatin; NSAID:non-steroidal anti-inflammatory drugs;
NSCLC
non-small cell lung cancer.

Declarations

Acknowledgements

The study was supported by Key Laboratory for Modern Medicine and Technology of Shandong Province, Institute of Basic Medicine, Shandong.

Authors' contributions

YYZ, the corresponding author, designed and conducted this study and was a

major contributor in writing the manuscript. DL TY WZ and YL consulted literatures and took part in the writing of manuscript. NS, ZY, ZG, ZQ and ZX performed the experiment conduction and data analysis. All authors read and approved the final manuscript.

Funding

This research was supported by the following funds：National Natural Science Foundation of China (81403150)；Natural Science Foundation of Shandong Province of China (ZR2014HL064)；Developmental

Project of Traditional Chinese Medical Sciences and Technology of Shandong Province (2015-325); Innovation Project of Shandong Academy of Medical Sciences□2019□□Key R & D project of Shandong Province (Major Key Technologies) (2016ZDJSO7A15).

Availability of data and materials

The datasets used and/or analysed during the current study are available from

the corresponding author on reasonable request.

Consent for publication

Not applicable.

Competing interests

The authors declare that they have no competing interests.

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Figures

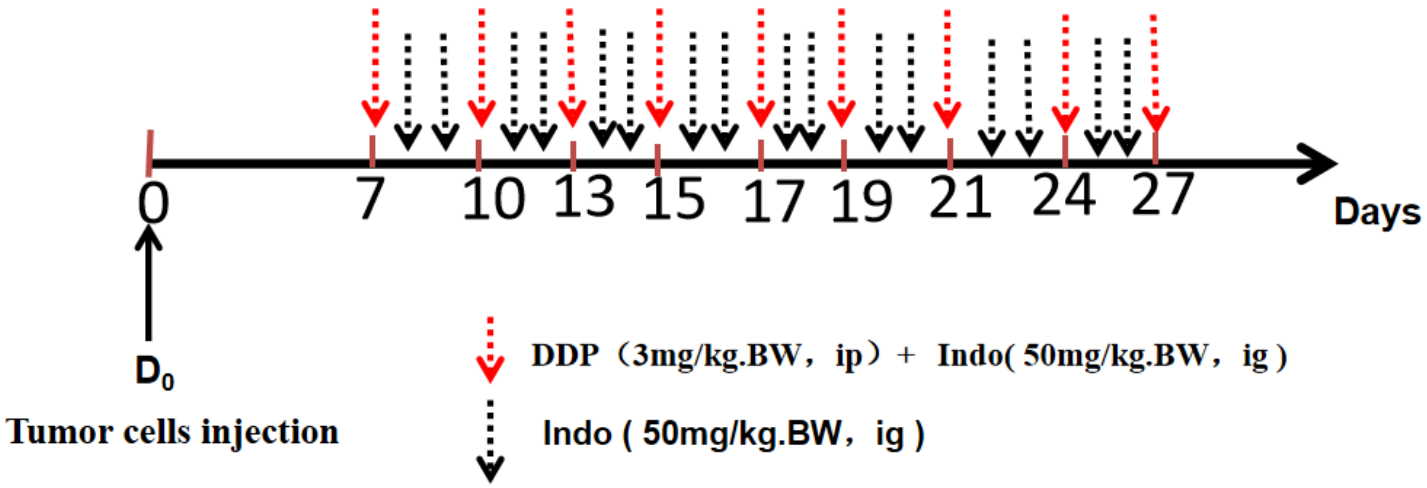

Figure 1

Treatment scheme of SPC-A1 xenograft. After injection of SPC-A1 cells(1x106)into right flank of male nude mice 1 weeks, the mice were divided into three groups and treatments were innitiated according to the schedule shown in the figure.

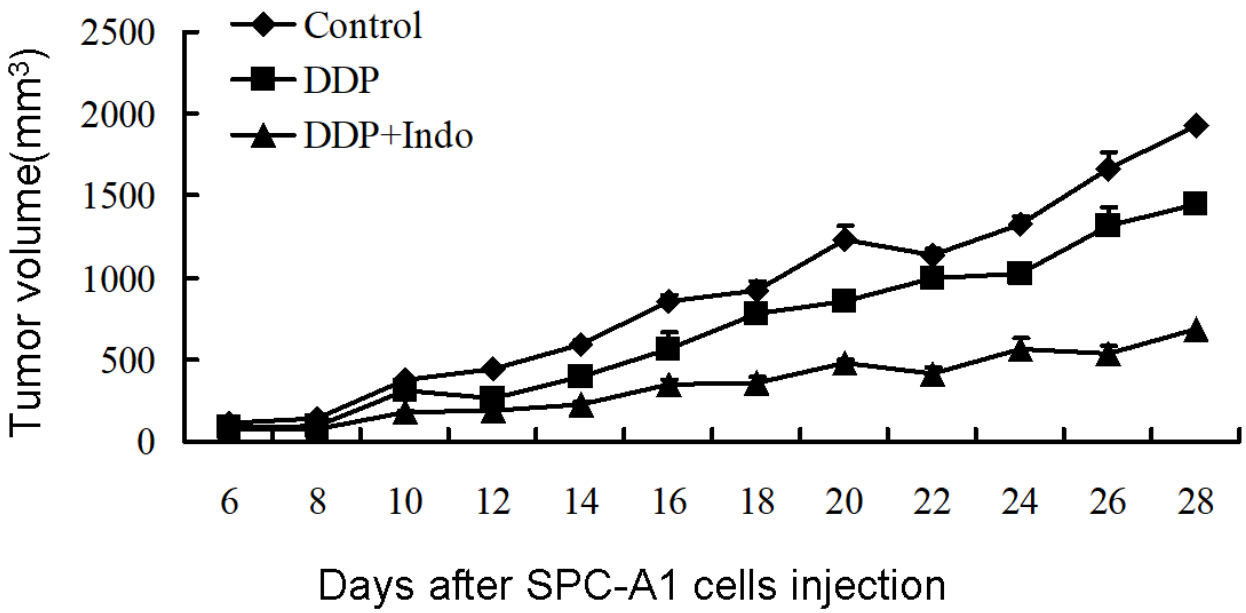

Figure 2

Growth curves of SPC-A1 xenografts after treatment with DDP or Indo following DDP chemotherapy. Control animals were given vehicle solution. Indo(50 mg per kg per day, for 3 weeks) given between courses of DDP increased the growth delay of SPC-A1 tumors and the effects seemed additive to those from chemotherapy Points, means for at least 10 tumors; bars, SE

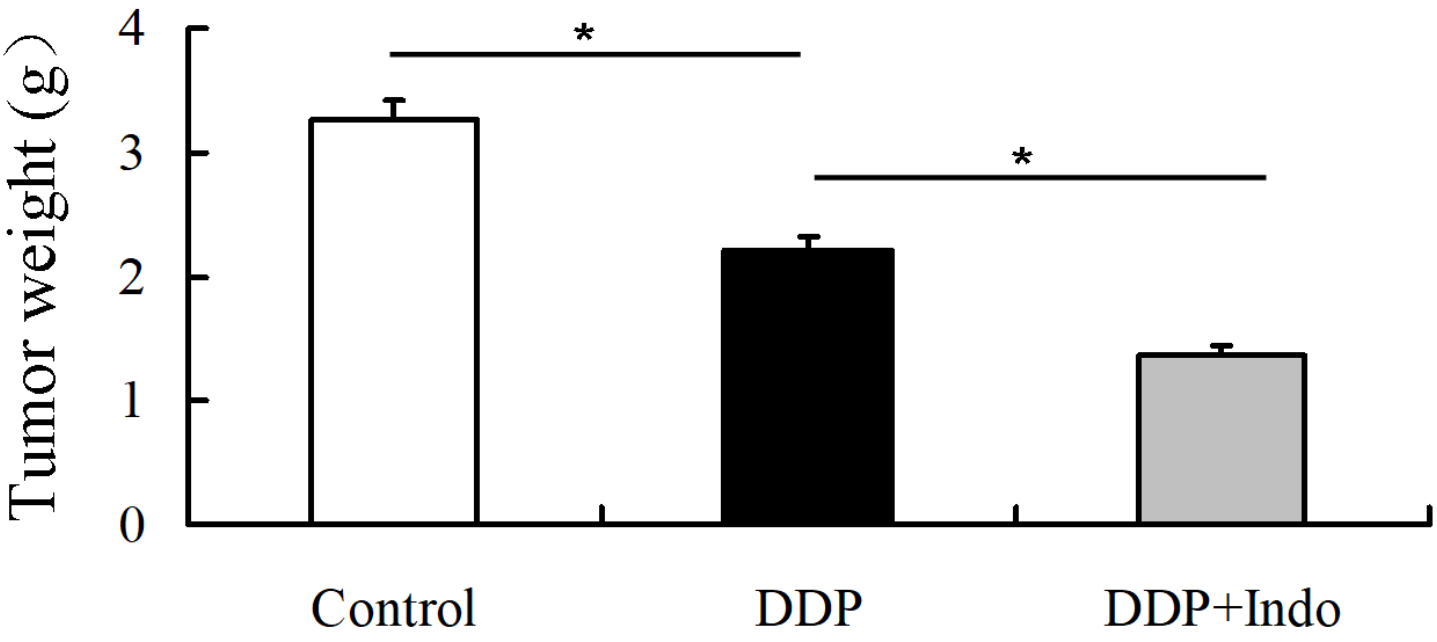

Figure 3

Tumor weight of SPC-A1 xenografts at 28 days after injection. The tumor weight decreased significantly in combined group. (N=5;*p<0.05)Results are expressed as means ± SEM (n=5). ∗P<0.05

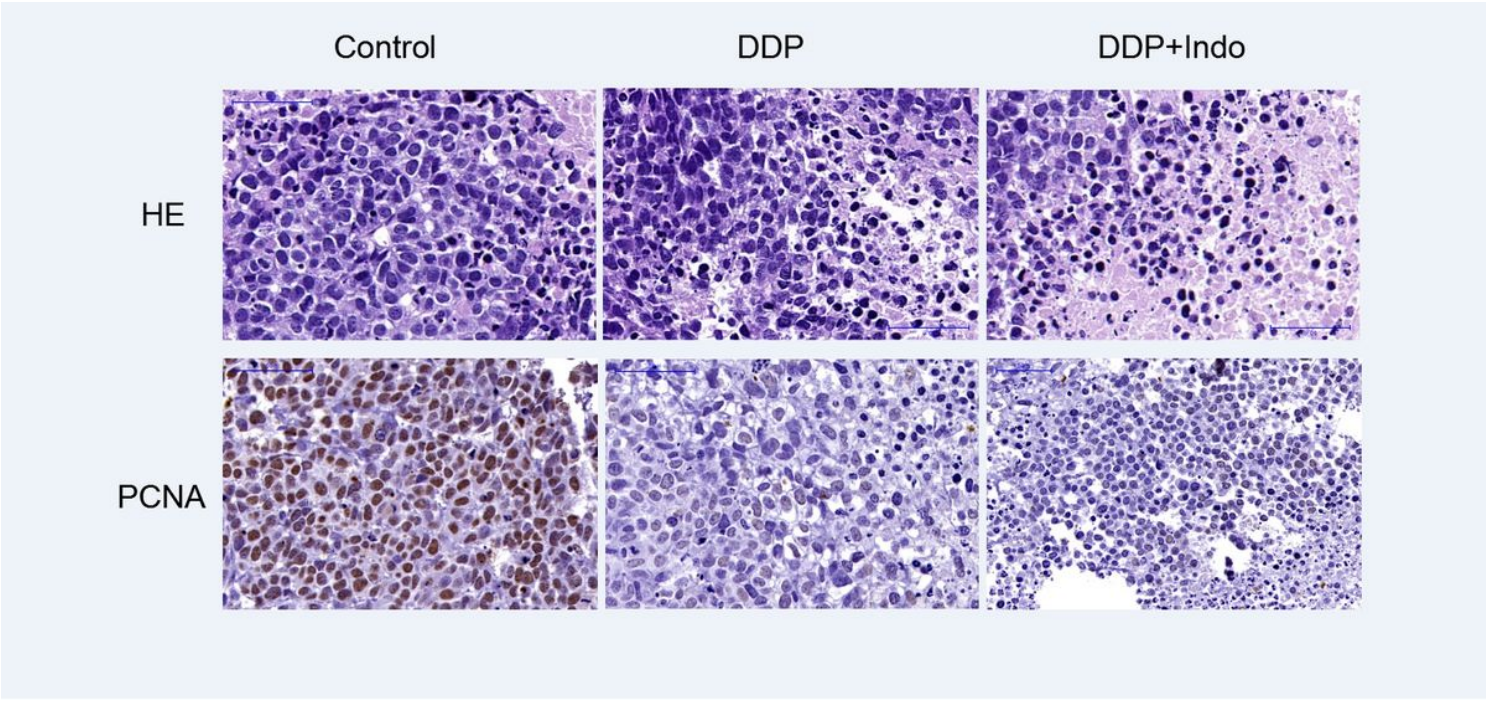

Figure 4

Effects of DDP+Indo on histology and on cell proliferation (as indicated by PCNA immunostaining) in SPC-A1 xenografts.DDP Used Alone or DDP Chemotherapy with Indo.

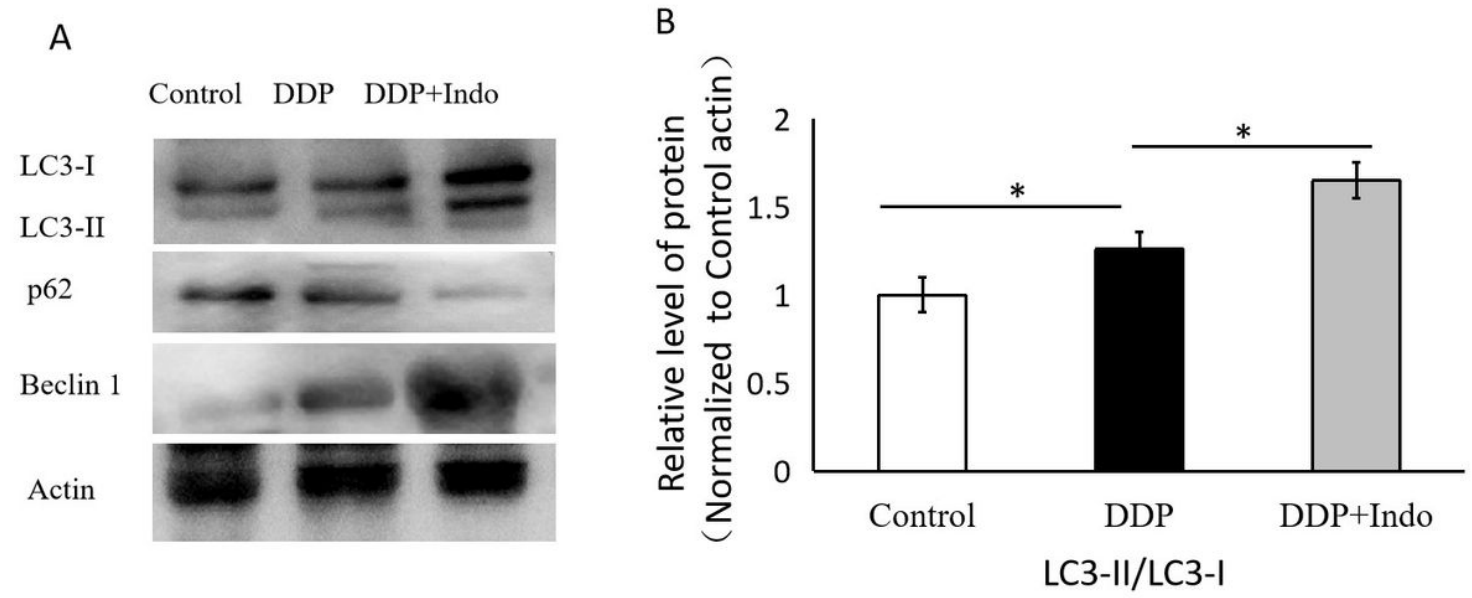

Figure 5

The expression of autophagy related protein LC3-II and P62 . A.Representative western blotting of LC3,p62 and Beclin 1 in SPC-A1 xenografts.B.Relative expression levels of the ratio LC3-II/LC-I (Gray value) from 3 replicate experiments are quantitated and subjected to statistical analysis.

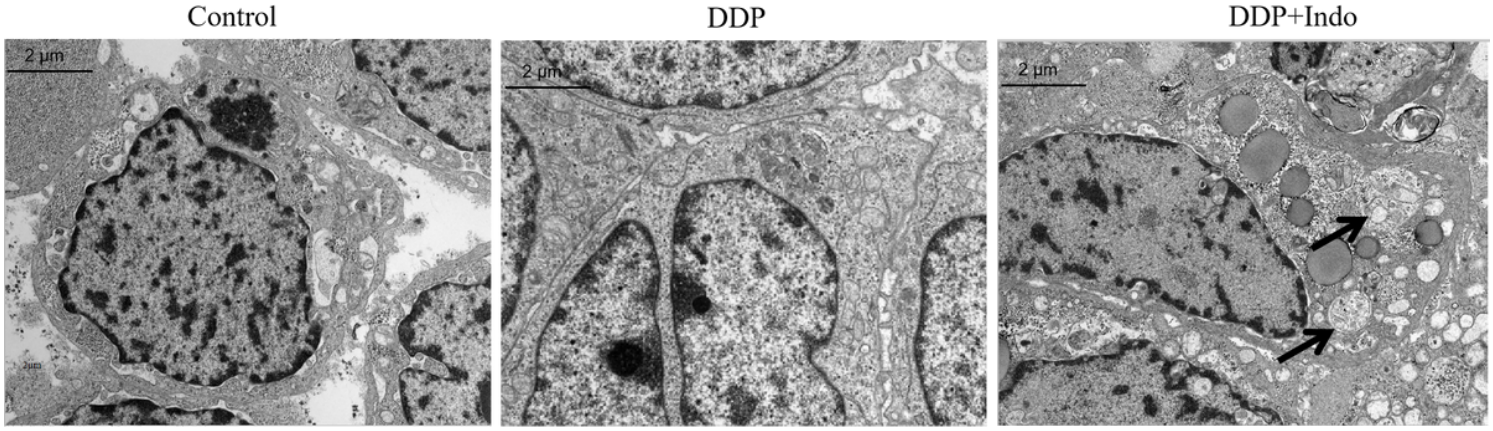

Figure 6

Indomethacin+DDP promoted autophage in SPC-A1 xenografts.in a mouse model of human lung cancer. Representative TEM images for autophagesomes in threee groups. Arrows show antophagosomes.