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Original Article

Effects of aspirin on non-small cell lung cancer cells via regulation of angiogenesis factors

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Abstract: Aspirin is a non-steroidal anti-inflammatory drug with many pharmacological properties. We aim to investigate the effects of aspirin on the viability, migration and angiogenesis process of non-small cell lung cancer cells. MTT assay was employed to measure the viability of human non-small cell lung cancer cell line A549 cells treated with different concentrations of aspirin. The scratch wound experiment was applied for the migration of A549 cells. The anticancer activity of aspirin was then evaluated in a mouse model of A549 xenografts, to evaluate the role of antiangiogenesis of aspirin in A549 xenograft tissues, CD34 immunohistochemical staining was employed to analyze the mean vascular density. The in vivo anti-tumor effect of aspirin was compared with carboplatin, and the combination effect of aspirin and carboplatin in vivo was also detected. Western blot was used to analyze expression of related protein levels. After A549 cells were treated with aspirin, the viability and migration of the cells were inhibited significantly. The levels of angiogenic factors including vascular endothelial growth factor, vascular endothelial growth factor receptor 2, and matrix metalloproteinase-2 were also decreased. We concluded that aspirin might be associated with tumor angiogenesis and growth in non-small cell lung cancer, and could be considered as a potential combination regimen choice for cancer chemotherapy.

Keywords: Aspirin, non-small cell lung cancer, angiogenesis, carboplatin

Introduction

Lung cancer is one of the leading cause of cancer-related death worldwide characterized by uncontrolled cell growth in tissues of the lung with approximately 1.4 million deaths worldwide annually [1, 2]. Most primary lung cancers started in the lung are carcinomas that derive from epithelial cells. There are two main primary types of lung cancer known as non-small cell lung cancer (NSCLC) (representing 80-85% of cases) and small cell lung cancer (SCLC) (representing 15-20%) [3]. If left untreated the primary lung carcinomas can spread beyond the lung focal by process of metastasis and invasion into nearby tissues/organs or other parts of the body.

The role for nonsteroidal anti-inflammatory drugs (NSAIDs) in preventing lung carcinogenesis is suggested by animal models, in which aspirin and other NSAIDs inhibited the formation of chemically-induced lung tumors [4-7]. Cyclooxygenase 2 (COX-2) has been implicated to involve in several process of carcinogenesis including apoptosis resistance, angiogenesis, decreased host immunity, and enhanced invasion and metastasis, and thus COX-2 is become one of the critical novel targets being studied for lung cancer therapy and chemoprevention [8].

Although the tumor-suppressive effects of NSAIDs were attributed to their ability to act as COX-2 inhibitors, some effects of these agents cannot be explained by inhibition of COX-2 [9]. Acetylsalicylic acid (aspirin), with characteristic of NSAIDs, has been considered to prevent colorectal adenocarcinoma growth through the mechanism of anti-inflammation, e.g. the inhibi-

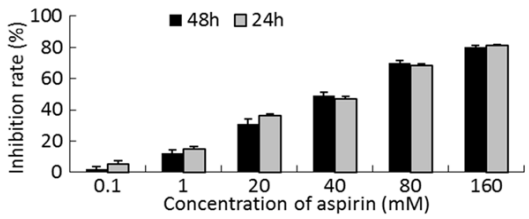

Figure 1. Aspirin induced cell death of A549 cells. The cells were treated with different concentrations of aspirin for 48 h, and then percentage of cell survival was determined using the MTT assay and denoted as a percentage of untreated controls at the concurrent time point. The bars indicate mean ± S.D. (n=3).

tion of COX-2 and NF-kappaB, and it was reported that regular aspirin use may be inversely associated with lung cancer in women, although regular aspirin use in men is not previously proved to associate with the decreased risk of lung cancer [4, 10-12]. And there is also limiting factor for aspirin use characterized as gastrointestinal toxicity, particularly gastrointestinal bleeding [13]. However, considering both its benefits and risks of COX-2 inhibition, there is still great concern regarding the potential use of aspirin and other COX-2-specific inhibitors in combination with other anti-cancer therapeutics. In this study, we examined the effects of aspirin on the growth of A549 human non-small cell lung cancer cells, and the combination effects of aspirin with carboplatin were also detected.

Materials and methods

Cell lines and animals

The human non-small cell lung cancer cell line A549 was purchased from American Type Culture Collection (Manassas, Virginia, USA) and cultured in RPMI-1640 medium (Gibco) supplemented with 10% heat-inactivated fetal bovine serum (Gemini Bio-Products, USA), penicillin-streptomycin (100 IU/ml-100 μg/ml) and 10 mM Hepes buffer at 37°C in a humid atmosphere (5% CO_2 -95% air).

Balb/c-nu mice (female, 6 weeks of age) were purchased from the Animal Centre of China Academy of Medical Sciences (Beijing, China) and kept under pathogen-free conditions. The research protocol was in accordance with the institutional guidelines of Animal Care and Use Committee at Qingdao University Medical College and the Institutional Animal Care and Use Committee specifically approved this study.

Cell proliferation assay

A549 cells (5×10^3 per well) seeded in 96-well plates were exposed to increasing concentrations of aspirin (0.1-160 mM) for the indicated time. The medium was then removed and the wells were washed with PBS. The 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) assay was performed by adding 20 μl of MTT (5 mg/ml, Sigma, USA) for 4 h. Then 150 μL DMSO was added to dissolve the formazan precipitate before absorbance was measured at 570 nm using a THERMO^{max} microplate reader (Molecular Devices, USA). Triplicate experiments were performed.

Wound scratch assay

Cells were seeded into 6-well culture dishes at 3.0×10^5 cells/well. After cells attaching on the dishes and reaching about 80% confluence, a scratch about 1 mm width was made through the culture dish with a sterile plastic 200 μl micropipette tip to generate one homogeneous wound, then the peeled off cells were washed twice with PBS. Cells were further incubated without or with 20 mM, 40 mM and 80 mM aspirin for 12 and 24 h. The wound widths were measured under microscope using an ocular grid at each time point. Images were taken using a microscope at 100 magnification (Olympus IX51, Japan). Cell migration = initial wound width (1 mm) -12 or 24 h wound width. The migration was represented as percentage of untreated control as 100%. The experiments were repeated at least three times.

Western blot

Cells (3.0×10^5) seeded in 6-well plates were treated with with 20 mM, 40 mM and 80 mM aspirin for 48 h. Then cells were harvested and cell lysates (30 μg of protein per lane) were fractionated by 10% SDS-PAGE. After electrophoresis, proteins were electro-transferred onto PVDF membranes and then detected using dilutions of primary antibodies including anti-VEGF (sc-152, Santa Cruz, USA), anti-phospho-VEGF receptor 2 (19A10, Cell Signaling Technology, USA), anti-MMP-2 (sc-10736, Santa Cruz, USA) and anti-β-actin (#ab6276, Abcam, Cambridge, MA, USA) which

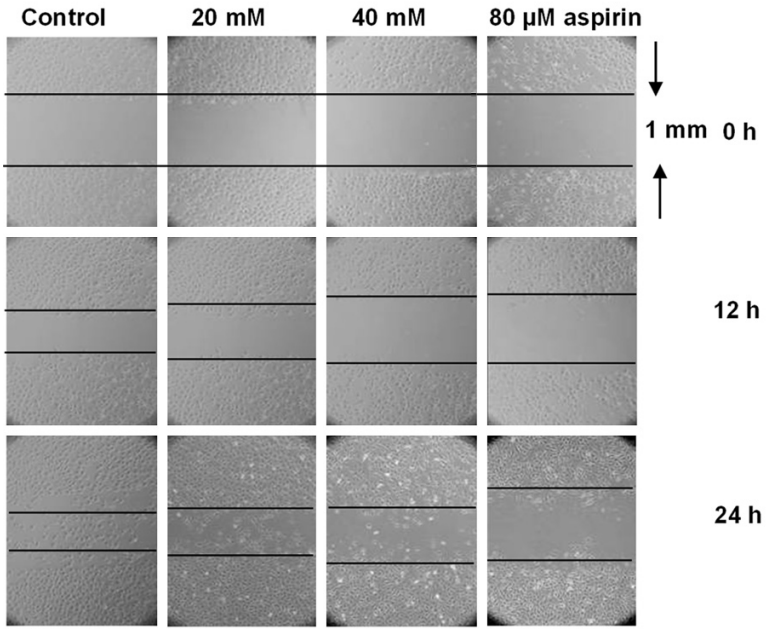

Figure 2. Inhibition of A549 cell migration by different concentrations of aspirin. Scratch assay was performed by plating cells in 6-well culture dish. After cells were allowed to attach and reach 80% confluence, a scratch (1 mm) was made through culture dish with a sterile plastic 200 μl micropipette tip to generate one homogeneous wound. Cells were further incubated without or with aspirin for 12 h and 24 h, respectively. The wound widths were measured under microscope using an ocular grid (magnification, × 100).

was used as loading control. The primary antibodies were washed in 0.05% Tween-20/PBS and then incubated with appropriate horseradish peroxidase-conjugated secondary antibodies. The bound antibodies were visualized using an enhanced chemiluminescence reagent (Millipore, USA), and quantified by densitometry using a ChemiDoc XRS + image analyzer (Bio-Rad, USA). Triplicate experiments were performed.

Inhibition of tumor growth in xenograft mouse model

The *in vivo* efficacy of aspirin was assessed using A549 xenograft mouse model. The model was established by subcutaneously inoculating A549 cells (8×10^6 per mouse) into the armpit of nude mouse. After about 7 days when the tumor volume reached about 0.1 cm^3, mice were randomly divided into 6 groups (n=6): vehicle control, carboplatin (35 mg/kg biweekly ip) group, aspirin (150 mg/kg/day by p.o. gavage) group, and aspirin (150 mg/kg/day orally) + carboplatin (35 mg/kg biweekly ip) group. Both drugs and vehicle were given for 3 weeks totally.

Immunohistochemical staining for micro vessel density (MVD)

CD34 is considered to be a marker of capillary endothelial cells, the inhibitory effect of aspirin on angiogenesis in A549 xenograft tissues was evaluated by detection of CD34 immunohistochemical staining. Specifically, the formalin-fixed paraffin-embedded tumor tissues were cut into slices of 4 μm thick, then deparaffinized, and dehydrated by decreasing concentrations of ethanol solutions. The sections were microwaved twice for 15 min at 600 W in 10 mM citrate buffer for antigen retrieval, then endogenous peroxidase activity was blocked by incubation with 3% hydrogen peroxide in methanol for 5-10 min. Non-specific binding was blocked by incubating with 5% bovine serum albumin (BSA) at room temperature for 20 min. After incubation with anti-CD34 antibody (BA0532, Boster, China) at 4°C, the sections were washed and treated with biotinylated anti-immunoglobulin, washed, reacted with avidin-conjugated horseradish peroxidase H complex, and incubated in diaminobenzidine and hydrogen peroxide, rinsed in distilled water, counterstained with hematoxylin, and mounted. The vascular density was calculated by counting CD-34 positive blood micro vessels in the composite images of each slide for the six animals in each group.

Statistical analysis

Data were all described as mean ± S.D. Statistical significance was analyzed by one-way analysis of variance (ANOVA) followed by Dunnett's multiple range tests using the SPSS/Win13.0 software (SPSS, Inc., Chicago, Illinois, USA). $P < 0.05$ was considered as statistically significant.

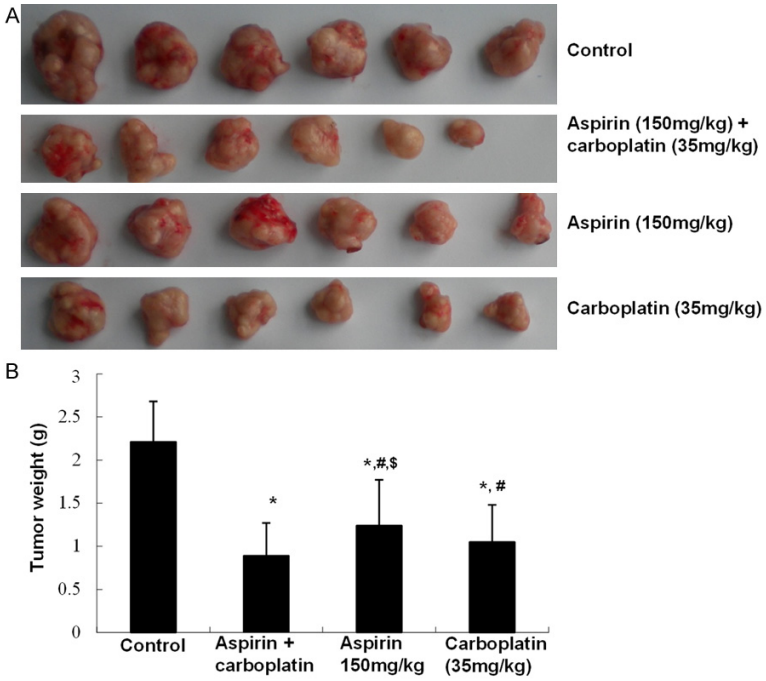

Figure 3. Effects of aspirin and carboplatin on the growth of A549 xenografts bearing in mice. The mice were administrated with aspirin and carboplatin for three weeks. A. Tumor measurement was made after mice were sacrificed. B. Tumor weights were measured after tumor tissues were isolated. The bars indicate means ± S.D. $^{*}P < 0.05$ *vs.* control group; $^{\#}P < 0.05$ vs. aspirin + carboplatin group; and $^{\$}P < 0.05$ *vs.* carboplatin group.

Table 1. The count of mean vascular density in xenograft tissues of each group

Group	Control	Aspirin	Carboplatin	Aspirin + carboplatin
MVD	12.1±2.4	7.6±2.9$^{*,\#}$	6.8±3.4$^{*,\$,\#}$	5.1±3.2*

*, *P < 0.05* vs control group; $^{\$}$, P > 0.05 vs aspirin group; $^{\#}$, P < 0.05 vs aspirin + carboplatin group.

Results

Inhibition of cell proliferation

A549 cells were exposed to different concentrations of aspirin and then detected the proliferation ability using MTT assay. Our results showed that aspirin significantly inhibited A549 cell proliferation. Aspirin had a dose- dependent anti-proliferative effect on A549 cells in the range of 0.5-160 mM (0.1 mM-24 h, P > 0.05; 1-160 mM-24 h, P < 0.01; 0.1 mM, P < 0.05; 1-160 mM, P < 0.01 *vs.* vehicle controls). As shown in **Figure 1**, the inhibition rates of 0.5-160 mM of aspirin varied from 2.1% to a maximum of 81.5% after 24 h and 48 h of exposure. Although the inhibition rate of 48 h treatment was a little higher than 24 h treatment group, we did not observed statistical significance at each concentration between 24 h and 48 h treatment group (P > 0.05 24 h *vs.* 48 h at each concentration of aspirin).

Decrease of A549 cell migration by aspirin

The migration ability of A549 cells was measured in the presence of aspirin using a wound scratch assay. Our results showed continuous cell migration in the control group for up to 24 h, the confluent monolayer region gradually migrated into the cell-free 'scratch' region (**Figure 2**). The distances of cell migration were significantly reduced in the presence of aspirin (**Figure 2**), the percentage of inhibition of 80 mM aspirin was 9.8%, 38.3%, and 83.5% respectively after a 24 h exposure. (**Figure 2**, P < 0.05 *vs.* spontaneous migration distances in control group).

Inhibition of tumor growth in vivo by aspirin treatment

The anticancer activity of aspirin and its anticancer synergy effect with carboplatin was evaluated in A549 xenografts in mice after 3 weeks of the treatments respectively. As shown in **Figure 3**, 150 mg/kg of aspirin delayed the growth of A549 xenografts by 43.9%, however the inhibition rate of aspirin was lower than carboplatin (35 mg/kg) at 52.5% (P < 0.05 vs. control group; P < 0.05 vs. carboplatin group). And in Aspirin 150 mg/kg + carboplatin (35 mg/kg) combination group, the inhibition rate was elevated to 64.2% (P < 0.05 vs. control group; P < 0.05 vs. aspirin group and carboplatin group).

Inhibition of vascular density in xenograft tissues

The mean vascular density in xenograft tissues was analyzed to evaluate the effect of aspirin

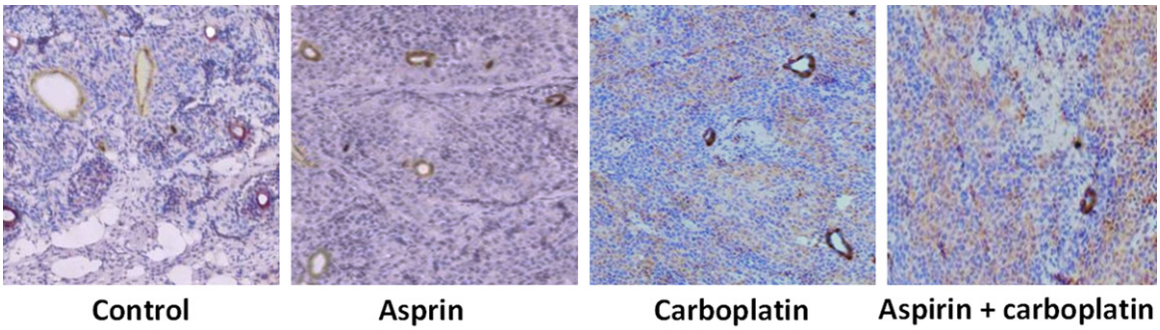

Figure 4. Immunohistochemical staining of CD34 expression in cancer tissues. The number and size of blood vessel in the tumor tissues were demonstrated by CD34 staining.

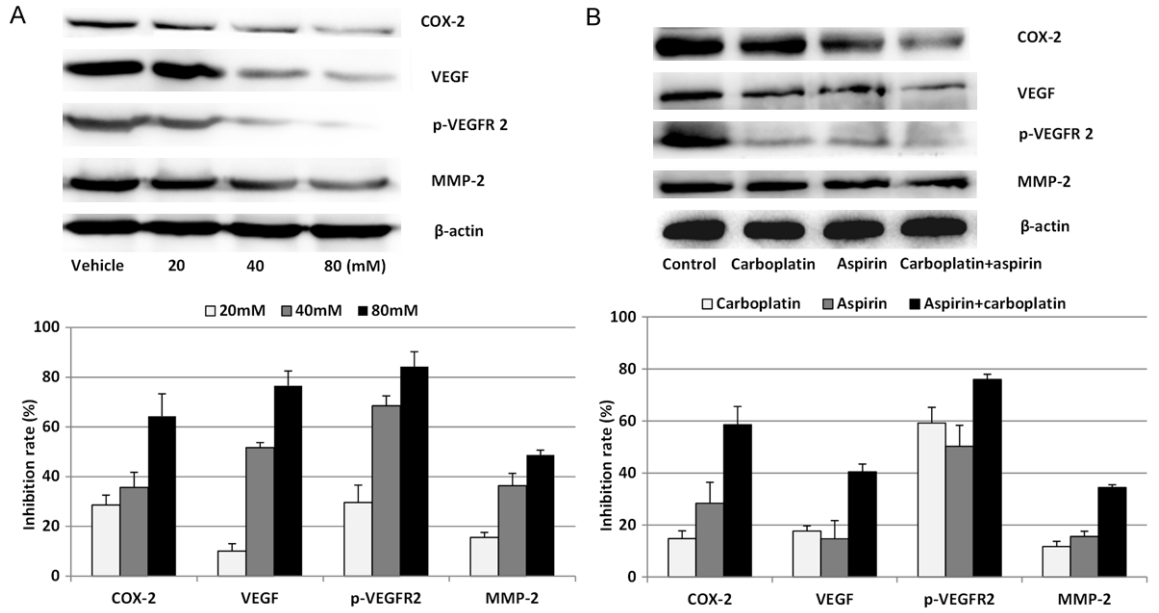

Figure 5. Aspirin decreases expressions of COX-2 and angiogenic molecules in vitro and in vivo as estimated by western blot assay. A. Inhibition of COX-2, VEGF, p-VEGFR2 and MMP-2 expression in A549 cells in vitro by aspirin, cells were treated with 20 mM, 40 mM, and 80 mM aspirin and cell lysates were obtained and the molecules were estimated by western blot analysis. The data are expressed as the relative density of the protein normalized to β-actin. Triplicate experiments with triplicate samples were performed; B. Inhibition of COX-2, VEGF, p-VEGFR2 and MMP-2 expression on in A549 xenografts estimated by western blot analysis, A549 xenografts in nude mice were treated with carboplatin 35 mg/kg, aspirin 150 mg/kg, carboplatin 35 mg/kg + aspirin 150 mg/kg. A549 tumors were collected and the levels of these angiogenic molecules were estimated by Western blot analysis. The data are expressed as the relative density of the protein normalized to β -actin. Triplicate experiments with triplicate samples were performed.

on antiangiogenesis after mice were sacrificed. The number and size of blood vessel in the tumor tissues were demonstrated by CD34 staining. The number of blood vessels was notably decreased in the xenograft tissues in aspirin-treated mice. As shown in **Table 1** and **Figure 4**, the mean vascular density in the untreated control animals was 12.1. The density was then significantly reduced to 7.6, 6.8, and 5.1 after three weeks treatment in aspirin group, carboplatin group, and aspirin + carboplatin group respectively ($P < 0.05$, between drug treated groups and control group; $P > 0.05$ between aspirin group and carboplatin group; $P < 0.05$ between aspirin group/carboplatin group and aspirin + carboplatin group).

Decrease of angiogenic molecules

First we examined the expression of COX-2 in A549 cells and xenografts using western blotting, and then we examined the expressions of several angiogenic molecules, including VEGF, p-VEGFR 2 and MMP-2. The results showed that the COX-2 was inhibited by aspirin treatment in vitro from 26.8% to-70.7% at the rage of 20 mM to 80 mM (**Figure 5A**). As shown in **Figure 5A**, the expression levels of the proteins in A549 cells were significantly reduced by different concentrations of aspirin. At 20 mM, 40 mM and 80 mM of aspirin, the inhibition rate were from 10.6% to 76.9%, for VEGF (**Figure 5A**, 20 mM, $P > 0.05$; 40 and 80 mM, $P < 0.05$ vs. control group); from 29.4% to 83.2%, for p-VEGFR 2 (**Figure 5A**, $P < 0.05$ vs. vehicle control group) and from 16.3% to 49.6 %, for MMP-2 (**Figure 5A**, 20 mM, $P > 0.05$; 40 and 80 mM, $P < 0.05$ vs. vehicle control).

And the in vivo experiment showed that aspirin could also inhibit COX-2 expression (28.8%, $P < 0.05$ *vs.* control, **Figure 5B**), while carboplatin showed a weak down-regulation effect on COX-2 expression (15.1%, $P > 0.05$ *vs.* control). Treatment with aspirin resulted in a significant decrease of VEGF, p-VEGFR 2 and MMP-2 (15.2%, 50.3% and 15.9%, respectively, $P < 0.05$ *vs.* control), carboplatin also decrease VEGF, p-VEGFR 2 and MMP-2 by 17.2%, 59.2% and 11.6%, respectively ($P < 0.05$ *vs.* control). And the percentages of inhibition were up to 43.1%, 75.6% and 35.6% in the aspirin + carboplatin combination group (**Figure 5B**, $P < 0.01$ *vs.* vehicle).

Discussion

Aspirin (acetylsalicylic acid) is a non-steroidal anti-inflammatory drug (NSAID) with many pharmacological properties including anti-inflammatory, anti-pyretic, and analgesic. And recent years its anti-cancer and protection effects have drawn more attention of researchers. Previous study has demonstrated that long-term administration of aspirin in humans leads to protection against the development of colorectal cancer as well as other types of cancer cells and it is thought to be a good candidate for chemotherapy because of its selective cytotoxicity and weak mutagenicity [14-18]. Aspirin has been considered to prevent colorectal adenocarcinoma growth through the mechanism of anti-inflammation, also it has been proved that administration of 300 mg or more of aspirin a day for about 5 years reduces the short-term risk of recurrent colorectal adenomas in patients with a history of adenomas or cancer [10, 14, 19].

Most NSCLC patients have advanced disease at diagnosis; 22% have regional lymph node metastases, and 56% have distant metastases in the brain, bone, liver, or adrenal glands [1, 20]. Therefore, understanding and prevention of NSCLC metastasis is an important research for the development of new therapies to improve survival for lung cancer patients. Although it is not sure whether regular aspirin use is inversely associated with lung cancer in human body, here we aimed to measure its anti-cancer ability in human non-small cell lung cancer A549 cell line and in a xenograft mice model, previous researches indicated that the anticancer activity of aspirin may be linked to their ability to inhibit cell proliferation and to induce apoptosis [21-23], in this study we examined its effects on lung cancer cell migration, metastasis and angiogenesis. We found that aspirin could inhibit cell proliferation in a dose-dependent manner (**Figure 1**), and the cell migration ability in vitro was also significantly inhibited (**Figure 2**). Then, the in vivo effects of aspirin were also detected and compared with carboplatin (**Figure 3**). The results suggested that aspirin could be a promising agent for cancer growth though the dose of aspirin used in this study was relatively high than common chemotherapy drugs, and it might involve in the process of metastasis and angiogenesis process.

Many of the molecular changes promote metastatic capability of a tumor cell, enabling it to detach from the primary tumor, invade tissue and enter circulation and lastly colonize and grow in a secondary site. There are a growing number of evidences indicating that the growth and metastasis of malignant tissues largely depends on angiogenesis, and the inhibition of new capillaries and disruption of tumor blood vessels could cause tumor shrinkage and/or tumor cell death [3, 24, 25]. It is believed that angiogenesis in tumor tissues actually starts with cancerous tumor cells releasing growth-related molecules that send signals to vascular endothelial cells [26, 27]. Currently, there are

many different molecules identified as angiogenesis-stimulating factors, such as VEGF, VEGF receptor, EGF receptor and MMPs [28]. VEGF is highly expressed in both NSCLC and SCLC and its expression is associated with poor prognosis in NSCLC, therefore inhibition of VEGF signaling in tumor cells is an important therapeutic target [29]. And among members of the MMP family, the tissue expression and the activity of MMP-2 in the malignant tumors is particularly important for the metastatic spread of cancer cells during the process of type IV collagen degradation which is a major component of the basement membrane [3, 30, 31], the decrease of activity and expression of MMP-2 could be associated with reductions in tumor growth, invasion and metastasis, and improved clinical outcomes. After confirmation of its inhibition effects of cancer cell growth, we examined the effects of aspirin both in vitro and in vivo on the levels of angiogenic factors including vascular endothelial growth factor (VEGF), VEGF receptor 2, and matrix metalloproteinase-2. Our results showed that aspirin could down-regulate VEGF, p-VEGFR 2 and MMP-2 levels in vitro and in vivo, and when combined with carboplatin, the regulation effects were further elevated (**Figure 5**).

Conclusions

In summary, we have demonstrated both in vitro and in vivo that aspirin could inhibit the growth and the activity of human lung carcinoma without significant cytotoxicity, and it could enhance the anti-tumor effects of carboplatin in vivo. These results suggest that aspirin maybe a potential candidate agent for cancer treatment and a promising combination regimen choice for cancer chemotherapy.

Disclosure of conflict of interest

None.

Authors' contribution

Conceived and designed the experiments: Jian Zhang, Xianwen Gu. Performed the experiments: Jian Zhang, Taisheng Chi, Pingping Yao, Yanan Long, Xingguang Wang. Analyzed the data: Jian Zhang, Xianwen Gu. Wrote the paper: Jian Zhang, Linben Gao, Xianwen Gu.

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