Combined effects of indomethacin and oxaliplatin on lymph node metastasis related factors in human lung cancer xenografts in nude mice

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Abstract: To investigate the combined effects of indomethacin and oxaliplatin on expressions of epidermal growth factor receptor (EGFR), E-cadherin (E-cad), intercellular adhesion molecule-1 (ICAM-1) and CD44v6 related to lymph node metastasis of human lung cancer cell lines. Human lung adenocarcinoma A549 cells were inoculated subcutaneously into the left armpit of nude mice to establish human lung cancer xenografts. The mice were randomly divided into control group, indomethacin group, oxaliplatin group and combination therapy group, which were treated with sterile distilled water, indomethacin, oxaliplatin and indomethacin combined with oxaliplatin, respectively. After 42 days, the mice were sacrificed. The immunohistochemistry and reverse transcription polymerase chain reaction were used to detect the expressions of EGFR, E-cad, ICAM-1 and CD44v6 in tumor tissues. Compared to control group, the protein and mRNA expressions of EGFR, ICAM-1 and CD44v6 in the indomethacin, oxaliplatin, and combination therapy groups were significantly reduced (P<0.05) and the protein and mRNA expressions of E-cad expression were significantly increased (P<0.05). Compared to indomethacin group and oxaliplatin group, the protein and mRNA expressions of EGFR, ICAM-1 and CD44v6 in combination therapy groups were significantly reduced (P<0.05), and the protein and mRNA expressions of E-cad expression were significantly increased (P<0.05). There was no significant difference between indomethacin and oxaliplatin groups. Indomethacin and oxaliplatin have synergistic effect on expressions of lymph node metastasis related factors in lung cancer cell lines.

Keywords: Lung cancer; Cyclooxygenase-2 inhibitor; epidermal growth factor receptor; CD44v6; intercellular adhesion molecule-1; E-cadherin.

INTRODUCTION

In recent years, the mortality of lung cancer has been increasing yearly. Invasion and metastasis are the leading causes of a poor prognosis and short survival in lung cancer. As such, anticancer drugs that target tumor metastasis-related factors are highly desirable for the treatment of patients with advanced lung cancer (Hirsh, 2014). Previous studies have shown that cyclooxygenase-2 (Cox-2) is involved in the occurrence and development of tumors; up regulation of Cox-2 is associated with tumor cell proliferation, transformation, angiogenesis, invasion, and metastasis (Koki and Masferrer, 2002). In the recent years, Cox-2 inhibitors have been recognized as a potential therapy for non-small cell lung cancer. Cox-2 inhibitors can inhibit the proliferation, and promote the apoptosis, of tumor cells (Nadda *et al.*, 2013; Cho *et al.*, 2009; Qiu *et al.*, 2012), inhibit tumor angiogenesis (Nadda *et al.*, 2013; Nadda *et al.*, 2012), and weaken tumor-mediated immune inhibition (Nadda *et al.*, 2013; Lee *et al.*, 2009). However, the effect of Cox-2 inhibitors on cancer lymph node metastasis and the mechanisms through which this might occur, has not been widely studied. This study investigated the combined effects of non-specific Cox-2 inhibitor indomethacin and oxaliplatin on expressions of epidermal growth factor receptor (EGFR), E-cadherin (E-cad), intercellular adhesion molecule-1 (ICAM-1) and CD44v6 related to lymph node metastasis of human lung adenocarcinoma A549 cells. The purpose was to provide a reference for further apllication of indomethacin and oxaliplatin in treatment of human lung cancer.

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MATERIALS AND METHODS

Cell culture

The human lung adenocarcinoma cell line, A549, was a gift from the Medical Department of Peking University. Fetal bovine serum (FBS) was obtained from Thermo Fisher Biochemical Products Co., Ltd. (Beijing, China); trypsin and disposable flasks were purchased from Hyclone (USA); and F12k culture medium was purchased from Macgene Technology Co., Ltd. (Beijing, China). Cells were cultivated in F12k medium at 37°C and 5% CO_2.

A single cell suspension of A549 cells, in the logarithmic phase of growth, was prepared and diluted with serum-free F12k medium for cell counting using a hemocytometer. A cell suspension with 1×10^7/ml was prepared and after skin disinfection, 0.2 ml of this was injected subcutaneously into the left armpit of nude mice using a 1 ml syringe. Mice were observed daily for any changes in their behavior, weight, appetite, or defecation.

Experimental animals

A total of 25 male BALB/c nude mice (aged 4-5 weeks, weighing 20-24g) were purchased from Beijing Huafukang Biological Technology Co., Ltd. (Beijing, China). The experimental animal certificate number was: SCXY (Jing)-2009-0004. The animals were maintained in individual ventilated cages (IVC) and given sterile food and purified water. This study was strictly performed according to recommendations in the Guide for the Care and Use of Laboratory Animals of the National Institutes of Health. The animal used protocol has been reviewed and approved by the Institutional Animal Care and Use Committee (IACUC) in Hebei Medical University.

Nude mice grouping and processing

Vernier calipers were used to measure the length and width (x, y) of palpable tumors. Tumor volume (V) was approximated using the formula $V=xy^2/2$. When the average diameter of the tumor xenografts reached 4mm, the mouse with the smallest tumor nodule was excluded. The remaining mice were randomly divided into four groups: The control group; the indomethacin group; the oxaliplatin group and the indomethacin and oxaliplatin combination therapy group. There were six mice in each group. The oxaliplatin group was injected intraperitoneally with 10mg/kg oxaliplatin (Shenzhen Neptunus Pharmaceutical Co., Ltd. (Shenzhen, China), approval number: Zhunzi H20031048) once a week and given sterile distilled water orally every day. The indomethacin group was administered 2.5mg/kg indomethacin orally (Hebei Yongfeng Pharmaceutical Co., Ltd. (Shijiazhuang, China), approval number: Zhunzi H3020834) every day and given an intraperitoneal injection of saline solution once a week. The combination therapy group was treated as described for the single medication treatment groups, although no water or saline was given. The control group received sterile distilled water daily and saline weekly. After 42 days, the mice were sacrificed, the tumor tissues were removed and part was fixed in 4% paraformaldehyde solution and the remaining part was frozen in liquid nitrogen for RNA extraction and analysis by reverse transcription polymerase chain reaction (RT-PCR). The fixed tissue was prepared for immunohistochemical analysis by paraffin embedding and sectioning.

Immunohistochemistry

The streptavidin-peroxidase (SP) conjugate method was used for immunohistochemical analysis according to the reported method (Wang *et al.*, 2000). Sections were dew axed and transferred to water using graded ethanols, and antigens were retrieved using heat. Primary antibodies for EGFR (mouse anti-human polyclonal antibody diluted 1:100), ICAM-1 (rabbit anti-human polyclonal antibody diluted 1:200), E-cad (mouse anti-human polyclonal antibody diluted 1:200) was purchased from Bioword Company (Nanjing, China). The CD44v6 primary antibody (mouse anti-human monoclonal antibody diluted 1:300) and secondary antibodies were purchased from Beijing Zhongshan Company (Beijing, China). Primary and secondary antibodies were added drop-wise prior to 3,3-diaminobenzidine and hematoxylin counterstaining. The sections were observed and photographed using BX43 optical microscope (Olympus Corp., Tokyo, Japan).

Judgment of staining performance and quantitative analysis

According to the reported method (Zhang *et al.*, 2014), EGFR positive staining was located in the cytoplasm and/or cell membrane, which resulted in the cytoplasm, perinucleus and/or cell membrane appearing yellow. E-cad positive staining was located in the cell membrane, which resulted in the cell membrane appearing yellow. ICAM-1 positive staining was located in the cytoplasm and/or cell membrane, which resulted in the cytoplasm, perinucleus, and/or cell membrane appearing yellow. CD44v6 positive staining was located in the cell membrane, which resulted in the cell membrane appearing yellow. Tissue sections were fully observed under low magnification prior to observation using a 200× objective. The Beihang true color pathological image analysis system was used to calculate the average integrated optical density values from ten views that were randomly captured.

RT-PCR

RNA from 50mg tissue was extracted in an ice bath homogenizer using 1ml Trizol (Beijing Trans Gen Biotech Inc, Beijing, China), followed by chloroform and isopropanol addition and centrifugation. The purity and integrity of the extracted RNA was tested using 1% agarose gel electrophoresis and a 756 UV spectrophotometer.

Isolated RNA was subjected to reverse transcription and the resulting cDNA was stored at -20°C for the future use. For the PCR reactions, the following primers (synthesized by Shanghai Biological Engineering Company, Shanghai, China) were used: GAPDH upstream primer 5'-TGAAC-GGGAAGCTCACTGG-3' and downstream primer 5'-GCTTCACCACCTTCTTGATGTC-3'; EGFR upstream primer 5'-CGCAAAGTGTGTAACGGAAT-AG-3' and downstream primer 5'-CCAGAGGAGGAGTATGTGT-GAA-3'; E-cad upstream primer 5'-TTCACA-GCAGAA-CTAACACACG-3' and downstream primer 5'-TTGGG-TTGGGTCGTTGTA-3'; ICAM-1 upstream primer 5'-TTGTCATCATCACTGTGGTAGC-3' and downstream primer 5'-GGCTTGTGTGTTCGGTTTC-3'; and CD44v6 upstream primer 5'-GGAGCCAAATGAAGAAAATGA-A-3' and downstream primer 5'-TGAAATGGTGCTGG-AGATAAAA-3'. Reactions were performed in a volume of 20µl. The relative quantitative (RQ) values of the expressed target genes were used for statistical analyses.

STATISTICAL ANALYSIS

Data were expressed as the mean ± the standard deviation (SD). Inter group comparisons were estimated using ANOVA analysis, and $P<0.05$ was considered statistically significant. All the analyses were analyzed using SPSS 16.0 software.

RESULTS

Tumorigenicity and general conditions

In 25 nude mice with inoculation, 24 mice successfully developed palpable tumors, with success rate of 96%. The tumors formed 5-11 days post-inoculation. No mouse died during the experimental period.

Protein expression

An image analysis system was used to integrate the optical density values from sections immunohistochemically stained for EGFR, E-cad, ICAM-1 and CD44v6 (table 1, figs. 1, 2). ANOVA showed that, compared with the control group, EGFR, CD44v6, and ICAM-1 protein expression was significantly reduced in the indomethacin, oxaliplatin, and combination therapy groups, while E-cad protein expression was significantly increased ($P<0.05$). EGFR, CD44v6, and ICAM-1 protein expression were significantly reduced in the combination therapy group compared with the monotherapy groups, as was E-cad protein expression significantly increased ($P<0.05$). No significant differences in EGFR, CD44v6, ICAM-1, or E-cad protein expression between the oxaliplatin and the indomethacin treated groups were detected ($P>0.05$).

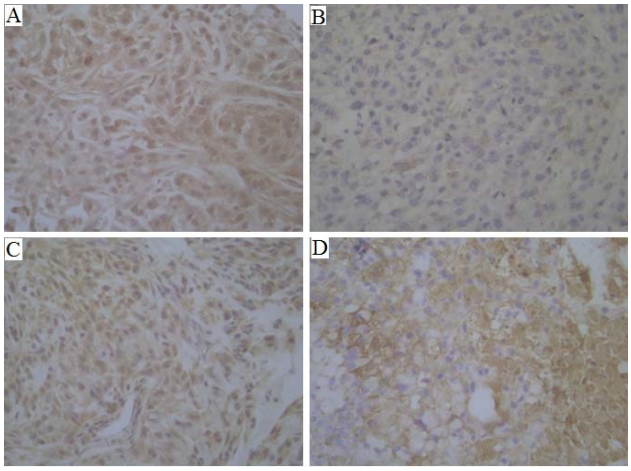

Fig. 1: Protein expressions in control group. A: EGFR; B: E-cad; C: ICAM-1; D: CD44v6.

mRNA expression

Fluorescent quantitative RT-PCR was performed to test the expression of *EGFR*, *E-cad*, *ICAM-1* and *CD44v6* mRNA (table 2). ANOVA showed that the *EGFR*, *CD44v6* and *ICAM-1* mRNA expression in the indomethacin, oxaliplatin, and combination therapy groups were significantly reduced, while *E-cad* mRNA expression was significantly increased ($P<0.05$). The expression of *EGFR*, *CD44v6*, and *ICAM-1* mRNA in the combination therapy group was significantly less than in either of the monotherapy groups, conversely *E-cad* mRNA expression was significantly greater in the combination therapy group ($P<0.05$). There was no significant difference in *EGFR*, *CD44v6*, *ICAM-1*, and *E-cad* mRNA expression between the oxaliplatin and indomethacin groups ($P>0.05$).

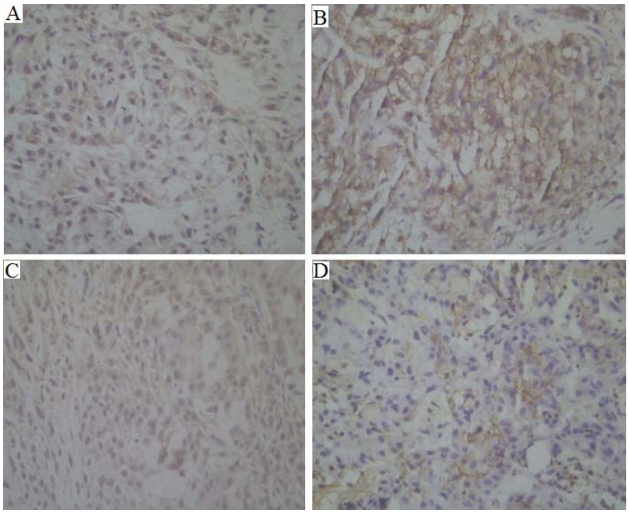

Fig. 2: Protein expressions in combination therapy group. A: EGFR; B: E-cad; C: ICAM-1; D: CD44v6.

DISCUSSION

Alteration in the adhesion properties of tumor cells closely relates to their metastastic capacity, and involves a variety of biological factors and molecular mechanisms. Recently, the role of the cell adhesion molecule family in tumor invasion and metastasis had attracted much attention.

E-cad is a calcium-dependent cell adhesion molecule. The adhesion function of E-cad depends upon the intracellular E-cad-catenin structure. Any factor, which alters the expression and/or integrity of E-cad-catenin structure, will therefore destroy the intercellular connections, thus decreasing intercellular adhesion and promoting tumor cell shedding from the original lesion, and enabling the formation of distant metastases (Zhou *et al.,* 2010). Some studies have shown that EGFR can destroy the E-cad-catenin structure and weaken adhesion between tumor cells, thereby promoting tumor cell shedding from their original location and the formation of distant metastases (Masuelli *et al.,* 2012).

The results of this study indicate that, compared with the control group, the expression of EGFR protein and mRNA is significantly lower in the indomethacin and

Table 1: EGFR, E-cad, ICAM-1 and CD44v6 protein expressions among different experimental groups ($\bar{x}\pm s$)

Group	N	EGFR	E-cad	ICAM-1	CD44v6
Control	6	159.25±5.486$^{\#}$	100.10±3.376$^{\#}$	163.11±4.807$^{\#}$	154.73±4.439$^{\#}$
Indomethacin	6	129.46±5.976$^{*\#}$	118.80±7.768$^{*\#}$	137.85±5.194$^{*\#}$	131.33±8.024$^{*\#}$
Oxaliplatin	6	132.85±2.376$^{\#}$	114.40±9.287$^{\#}$	141.91±4.052$^{\#}$	135.63±7.716$^{\#}$
Combination therapy	6	108.03±5.486*	139.50±8.139*	119.43±6.156*	111.98±2.392*

Table 2: EGFR, E-cad, ICAM-1 and CD44v6 mRNA expressions among different experimental groups ($\bar{x}\pm s$)

Group	N	EGFR	E-cad	ICAM-1	CD44v6
Control	6	0.902±0.043$^{\#}$	0.733±0.204$^{\#}$	0.948±0.031$^{\#}$	0.972±0.020$^{\#}$
Indomethacin	6	0.747±0.043$^{*\#}$	0.809±0.029$^{*\#}$	0.766±0.054$^{*\#}$	0.771±0.031$^{*\#}$
Oxaliplatin	6	0.741±0.043$^{\#}$	0.824±0.300$^{\#}$	0.768±0.075$^{\#}$	0.770±0.029$^{\#}$
Combination therapy	6	0.687±0.015*	0.941±0.136*	0.677±0.035*	0.654±0.024*

Note: $^{*}P<0.05$ vs control group, $^{\#}P<0.05$ vs combination group

combination therapy treated groups (P<0.05), while the levels of E-cad protein and mRNA was significantly higher in these groups (P<0.05). This suggests that indomethacin and the combination of the two drugs could inhibit EGFR expression and promotes the E-cad expression, thus stabilizing the E-cad-catenin complex intracellular structure, enabling normal membrane and extra cellular E-cad expression and tight intercellular connections between the tumor cells, which inhibit tumor cell distant metastases. The expression of EGFR and E-cad were not significantly different between the indomethacin and oxaliplatin groups. A comparison between the combination therapy group and the single drug application groups showed that the expression of EGFR protein and EGFR mRNA were significantly reduced (P<0.05), and the E-cad protein and mRNA levels were significantly increased in the combination therapy group (P<0.05), indicating that the combined administration had a synergistic effect.

ICAM-1 is the ligand of lymphocyte function-associated antigen-1 (LFA-1) (Dustin *et al.,* 1986). It has been shown that ICAM-1 promotes the adhesion of tumor cells and lymphocytes, thus enabling them to shed from their original lesions, enter the circulation, evade immune cell targeting, adhere and invade the peripheral lymphoid tissues, and finally form a lymph node metastasis (Basoglu *et al.,* 32007). While soluble ICAM-1, formed by proteolytic cleavage (Mendez *et al.,* 2008), could help free tumor cells evade immune system identification and cytotoxicity, it would be simpler for tumor cells to remain within the lymphatic sinuses and proliferate inside (Zhou *et al.,* 2001).

The results of this study showed that, compared with the control group, the expression of ICAM-1 protein and mRNA in the indomethacin group and the combination therapy group was significantly lower (P<0.05), indicating that indomethacin could inhibit ICAM-1 expression. This could lead to an inhibition of adhesion among tumor cells and lymphocytes, while simultaneously reducing the generation of soluble ICAM-1, which would enhance immune system recognition and destruction of free tumor cells. There were no significant differences in the expression of ICAM-1 protein or mRNA between the indomethacin and oxaliplatin treatment groups (P>0.05). However, the combination therapy group exhibited a significantly lower expression of ICAM-1 protein and mRNA than either of the monotherapy groups (P<0.05), suggesting that the combined administration had a synergistic effect.

CD44v6 belongs to the cell adhesion molecule family. It is up regulated in a variety of tumors, including head and neck cancer, colon cancer, endometrial cancer, ovarian cancer, non-small cell lung cancer, pharyngeal squamous cell carcinoma, thyroid papillary carcinoma, gastric cancer, and esophageal cancer. It has been shown to be closely associated with tumor lymphatic metastasis (Wang *et al.,* 2009; Yamada *et al.,* 2003; Afify *et al.,* 2005; Afify *et al.,* 2001; Afify *et al.,* 2011; Yang *et al.,* 2013; Wu *et al.,* 2013; Liu *et al.,* 2005; Shen *et al.,* 2012). Studies have shown that CD44v6 might promote tumor cell shedding from primary lesions to form tumor thrombus and activate lymphocyte recirculation. CD44v6 can also act as a ligand to promote adhesion between tumor and mesothelial cells, and promote the formation of lymph node metastases (Che *et al.,* 2006; Shi *et al.,* 2013; Riddle *et al.,* 2010). In addition, CD44v6 has also been shown to be associated with the immune escape of tumor cells; tumor cells with high CD44v6 expression can potentially evade recognition and destruction by the host immune system (Lida and Bourguignon, 1997), making it easier remain in the lymph nodes, proliferate, and form lymph node metastases.

The results of this study showed that, compared with the control group, the expression of CD44v6 protein and

mRNA in the indomethacin and combination therapy groups was significantly reduced, indicating that indomethacin could reduce the expression of CD44v6. This would not be conducive for the tumor cells to detach from the primary lesions and adhere to peri-tissue epithelial cells; however, it could improve the recognition and destruction of free tumor cells from by the host immune system. There were no significant differences in the expression of CD44v6 protein and mRNA between the indomethacin and the oxaliplatin group ($P>0.05$). The combination therapy group exhibited a significantly lower level of CD44v6 protein and mRNA expression than either of the monotherapy groups ($P<0.05$), suggesting that the combined administration had synergistic effects.

In conclusion indomethacin can affect the expressions of EGFR, E-cad, ICAM-1 and CD44v6 related to the lymph node metastasis in human lung cancer xenografts in nude mice. The mechanism of this inhibition can entail enhancing adhesion among tumor cells, as well as aiding the host immune system to identify and destroy free tumor cells. The combined indomethacin and oxaliplatin have synergistic effects on the lymph node metastasis related factors.

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