Synergistic effects of adina rubella hance and taxol

The synergistic effects of Adina rubella Hance combination with taxol on Lewis Pulmonary Carcinoma bearing in Mice

Running title: synergistic effects of Adina rubella hance and taxol

Highlights

1. The synergistic effects of Adina rubella Hance combination with taxol were found.
2. Their combination synergisticly inhibited tumor growth.
3. Their combination synergisticly inhibited angiogenesis by reducing the level of VEGF.

Abstract:

Objective: The purpose of the study was to assess the effects of Adina rubella Hance combination with taxol on Lewis pulmonary carcinoma bearing mice and explore the possible mechanism.

Methods: Based on the established Lewis pulmonary carcinoma bearing mice model, the effects ofAdina rubella Hance combination with taxol on mice survival rate and tumor growth were assessed. The level of microvessel density (MVD) and VEGF were measured by immunohistochemistry. Results: Compared with control group, taxol alone treatment and Adina rubella Hance combination with taxol treatments all showed significant effects on increasing the mice survival rate ($P < 0.05$), inhibiting the tumor growth ($P < 0.05$) and reducing the level of VEGF and MVD ($P < 0.05$).

Compared with the taxol alone treatment, the inhibiton effects of tumor growth was more obvious in Adina rubella Hance combination with taxol treatment and presented a dose-dependent manner on Adina rubella Hance.

Furthermore, the level of VEGF and MVD were more significantly decreased in treatment of Adina rubella Hance combination with taxol. These indicated that Adina rubella Hance combination with taxol might involved in the development of lung cancer through inhibiting the angiogenesis.

Conclusion: The results indicate that Adina rubella Hance combination with taxol synergisticly exhibit the effects of antitumor and anti-angiogenesis via reducing the level of VEGF on Lewis pulmonary carcinoma. These will provide valuable evidence for the clinical application of combined therapy of Adina rubella Hance and with taxol.

Keywords: Adina rubella Hance; taxol; Lewis Pulmonary Carcinoma; MVD; VEGF

1. Introduction

Lung cancer is the leading cause of cancer-related death in the wordwide and more than 70% of deaths are attributed to tumor metastasis [1]. Lung tumor metastasis are largely dependent on development of new blood vessels (neovascularization), thus inhibition of tumor-induced angiogenesis should prevent the growth of tumors and reduce development of metastases [2]. A number of recent preclinical studies have sparked interest in the concept of exploiting conventional chemotherapeutic drugs for anti-angiogenics. However, conventional chemotherapy schemes for the treatment of cancer mostly produce a limited improvement, associated with considerable side-effects and acquired drug resistance [3]. Recently, herbs in combinations with anticancer drugs have been found to be capable of resensitizing the chemoresistance and enhance therapeutic effect, especially

in cancer chemotherapy [4]. Therefore, exploration for the effective combination therapy that herbs in combinations with anti-angiogenics drugs is of great significance.

Taxol (paclitaxel) is a cancer chemotherapeutic agent that is commonly used as first line therapy for many common malignancies [5, 6] . Recently, a number of studies showed that taxol has antiangiogenic activity that could be ascribed to the inhibition of either tubule formation or cell migration, and to an antiproliferative effect towards activated endothelial cells [7, 8]. However, serious side effects such as allergies, neutropenia and neurotoxicity were observed during the clinical application of paclitaxel [9, 10], as well as the limited source and expensive price all hinder the extensive clinical application of taxol. Recently, the combination therapy of taxol with traditional Chinese medicine showed some advantages. For instance, a synergistic effect was found between taxol and baicalein. Their combined treatment resulted in significantly higher apoptsis rate than that alone [11]. The clinical study found that gemcitabine combination taxol treatment for non-small cell lung cancer (NSCLC) significantly increased the curative effects, patients life quality and reduced the side effects [12].

Adina rubella hance, as traditional Chinese medicine, has exhibited potenitial an-titumor activity in kinds of digestive tract tumors [13-15]. To date, few studies have been focused on the antitumor effects of Adina rubella Hance combination with paclitaxel on angiogenics. It has been reported that agiogenesis is tightly regulated by pro-angiogenic and anti-endothelial growth factors. VEGF is one of the most essential pro-angiogenic growth factors, and play a critical role in the angiogenic process [16, 17].

Microvessel density (MVD) is accepted as a standard indicator of angiogenesis and VEGF expression is strictly correlated with MVD [18, 19]. Therefore, based on the Lewis lung carcinoma mice model, the present study was designed to evaluate the antitumor effects of Adina rubella Hance combination with taxol and further efficacy on angiogenesis via assessing the level of VEGF and MVD.

2. Methods and materials

2. 1 Materials

The healthy male/female C57BL/6 mice (6–8 week age) weighing between 18 g and 23 g were purchased from Vital River Laboratory Animal Technology Companyï¼ˆBeijingï¼‰, and housed under pathogen-free conditions. Lewis lung carcinoma cell line was obtained from immunology ResearchInstitution ofChina Medical University.

Adina rubella Hance was supplied by China Medical University. Taxol(30 mg/5 ml) was supplied by Sichuan Sunnyhope Pharmaceutical Company. Immunohistochemical kit (Boster, Wuhan).

2. 2 The preparation of taxol solution (1mg/ml)

Taxol solution (1 mg/ml) was made from total of 25 ml normal saline added totaxol injection (30 mg/5 ml) and for standby.

2. 3 Establishment oflewis pulmonary carcinoma xenograft model

Lewis lung carcinoma cells were diluted to 110/ml with normal saline and injected intraperitoneally to 6 C57BL/6 mice. When obvious abdomen bulge was observed, about 3-5ml ascites was extracted and diluted with normal saline, then centrifuged at 5, 000 g for 5 min. The cellular precipitation was

washed twice with distilled water and harvested. Viability of the cells was determined with trypan blue exclusion method and always found to be 95% or more viable. After that, the cells were adjusted to a concentration of 2×10^{7} /ml, and 0. 2 ml cell suspensions were injected subcutaneously into thearmpit of each mouse (totally 200). After injection for 48h, the mice were randomized into the following five groups (40 mice per group)ï¼šcontrol group(equal volume of normal saline once a day)ï¼Œtaxol group (10 mg/kg)ï¼Œ taxol + Adina rubella Hance (1 mg/kg) groupï¼Œtaxol + Adina rubella Hance (3 mg/kg) groupï¼Œtaxol + Adina rubella Hance (5 mg/kg) group. Taxol was through tail intravenous injection and adina rubella hance was through intraperitoneal injection. The total injection volume was 0. 2 ml. All treatments lasted for 15 days and when a mouse died during the experiment period, the number of living days was recorded and calculated for the survival rate.

2. 4 The inhibition rate of tumor

After consecutive injection for 15 days, the remaining mice in all groups were executed by cervical dislocation and their metastatic tumors in the armpit were fully excised followed by calculating the tumorinhibition rate: inhibitive rate of tumor (%) = (1- average tumor weight in treated group/average tumor weight in control) × 100%.

2. 5 Detection of microvessel density (MVD)

Tumor tissues were fixed immediately in 10% buffered formalin phosphate and embedded in paraffin. Five-μm sections of each specimen were stained according to the manufacture kit. MVDwas assessed by immunohistochemical analysis with antibodies to the endothelial marker

CD31 (anti-CD31: 1: 100, Dako, Japan) and determined according to the method ofWeidner [20]. Briefly, the immunostained sections were initially screened at low magnifications (40× and 100×) to identify hot spots, which are the areas of highest neovascularization. Within the hot spot area, the yellow brown stained microvessels were counted in a single high-power (400×) field, and the average vessel count in 5 hot spots was considered the value of MVD. All counts were performed by three investigators in a blinded manner. Microvessel counts were compared between the observers and discrepant results were reassessed. The consensus was used as the final score for analysis. In order to avoid interference with the count, larger blood vessels with thicker smooth muscle bundle on pipe wall around or lumen > 8 red blood cell area of blood vessels were not included to count.

2. 6 Immunohistochemical detection VEGF

Tumor tissues were fixed immediately in 10% buffered formalin phosphate and embedded in paraffin. Five-μm sections of each specimen were stained according to the manufacture kit. VEGF was assessed by immunohistochemical analysis with

anti-VEGF antibody diluted for 1: 50 (Santa Cruz Biotechnology, Santa Cruz, CA, USA). VEGF staining was considered positive if unequivocal yellow brown staining was seen in the tumor cell cytoplasm, and the immunoreactivity was scored semiquantitatively as the intensity of the immunoreactive reaction and positive percent of tumor cells [3]. The intensity of the immunoreactive reaction were graded as 0, no immunoreactivity; 1, weak intensity; 2, moderate intensity; 3, strong intensity. Positive percent were graded as 0 to 3 score (1, < 10%; 2, 10%–50%; 3,≥ 50%). After multiplying the scores of

intensity and positive percent, the positive degree was determined as following: 0 as negative (-), 1 to 3 as weak expression (+), 4 to 6 as moderate expression (++) and 7 to 9 as strong expression (+++).

2. 7 Statistical analysis

All statistical analyses were performed using SPSS 17. 0 software package, measurement data between groups were subjected to statistical analysis by ANOVA, enumeration data were assessed with chi-square and Fisher tests. The statistical significance was set at a value of $P < 0.05$ or $P < 0.01$.

3. Results

3. 1 The effects of Adina rubella Hance cooperate with taxol on mice survival rate

As the results showed in Table 1, all of the mice were sacrificed or died within 15 days of treatments. The cumulative survival rate in control groupï¼Œtaxol groupï¼Œ taxol + Adina rubella Hance (1 mg/kg) groupï¼Œtaxol + Adina rubella Hance (3 mg/kg) groupï¼Œtaxol + Adina rubella Hance (5 mg/kg) group was 60ï¼…, 70ï¼…, 77. 5ï¼…, 80ï¼… and 82. 5ï¼…respectively. A significant difference between control group andalltreatment groups was observed, while no significant difference was observed between treatment groups.

3. 2 The effects of Adina rubella Hance cooperate with taxol on tumor

Compared with control group (Table 2), the tumor weight was significant reduced in all treatment groups ($P < 0.05$). Meanwhile, the inhibitory rate of tumor in the combination group was significantly higher than that of the taxol alone group and that presented a dose-dependent manner. Moreover, a significant reduction of tumor in Adina rubella Hance (5 mg/kg) combination

withtaxol was showed compared with taxol alone group. These results suggested that combined therapy with Adina rubella Hance and taxol resulted in a tumor inhibition.

3. 3 Effect of Adina rubella Hance cooperate with taxol on expression of MVD in tumor of Lewis Pulmonary Carcinoma-bearing mice

Compared with the control group (Figure 1), MVD value was obviously decreased in all treatment groups ($P < 0.05$). In addition, compared with taxol alone group, taxol + Adina rubella Hance (3 mg/kg) group and taxol + Adina rubella Hance (5mg/kg) group both showed a significant reduction of MVD value. While a reduction was observed in taxol + Adina rubella Hance (1 mg/kg) group, but the difference did not reach statistical significance ($P > 0.05$)

3. 4 Effect of Adina rubella Hance cooperate with taxol on expression of VEGF in tumor of Lewis Pulmonary Carcinoma-bearing mice

The positive expression of VEGF in all treatment groups was significantly lower ($P < 0.05$) than in control group. Furthermore, compared with the taxol alone group, thepositive expression of VEGF in combination group presented an inverse correlation. Namely, as the increasing of Adina rubella Hance, the positive expression of VEGF was becoming lower. A significant difference was found in Adina rubella Hance (3 mg/kg) group and taxol + Adina rubella Hance (5mg/kg) group, and especially in Adina rubella Hance (5mg/kg) group, the positive expression of VEGF was most lowest (Table 3).

4. Discussion

Currently, surgery is the preferred treatment for cancer, but due to be limited to the tumor location, invasion, metastasis and other factors,

chemotherapy has been an alternative treatment for cancer. It has been demonstrated that taxol is an effective drug for lung cancer chemotherapy, but the effect for lung cancer whether treatment with single drug or multidrug therapy (MDT) is still poor [21]. Traditional Chinese medicine with good curative effect and no obvious side effects in combination chemotherapy is the inevitable trend in the prevention and treatment of lung cancer. In the present study, the antitumor effects of Adina rubella Hance combination with taxol were assessed on the Lewis Pulmonary Carcinoma-bearing mice. The results revealed that compared with the taxol alone treatment, the inhibiton effects of tumor growth was more obvious in Adina rubella Hance combination with taxol treatment and presented a dose-dependent manner on Adina rubella Hance. Furthermore, the level of VEGF and MVD were significantly decreased in treatment of Adina rubella Hance combination with taxol. These indicated that Adina rubella Hancecombination withtaxol might involved in the development of lung cancer through inhibiting the angiogenesis.

In this study, a synergistic effect of the two agents was noted in their combined application. The inhibition tumor rate was more significantly in Adina rubella Hancecombination with taxol group and exhibited a dose-dependent manner. It is generally thought that taxol, as a cytotoxic agent, can block cell division in G2/M stage and induce apoptpsis of tumor cells by promoting the polymerization of microtubule and inhibiting their degradation [22, 23]. In addition, it has been reported that the ethylacetat extract from root ofadina rubella hance strongly inhibited tumor cell proliferation[15]. These indicated that Adina rubella Hance combination with taxol could

increase the antitumor effects of taxol. While , the survival rate ofLewis Pulmonary Carcinoma-bearing mice in different treatment groups was significantly higher than that in control group, but significant difference was not observed between taxol alone group and Adina rubella Hance combination with taxol group. These results indicated that Adina rubella Hance combination with taxol could not improve the survival rate of Lewis Pulmonary Carcinoma-bearing mice. Further investigation was needed to explore the proper dose of two agents in order to decrease the side effects of taxol and promote the survival rate.

Angiogenesis is a critical process for the growth, invasion and metastasis of cancer. VEGF is a specific and potent angiogenic factor and contributes to the growth of solid tumors by promoting tumorangiogenesis [24]. VEGF acts directly on the endothelial cells, promoting their proliferation and permeability, and it induces angiogenesis in pathologic and physiologic situation [25]. Therefore, tumor angiogenesis that reflects the growth potential of a tumor could be evaluated by the expression of VEGF [26, 27]. In addition, measurement of tumour microvascular density (MVD) from tumour biopsies is a common method for assessing the efficacy of antiangiogenic drugs [28]. Previous study found that paclitaxel showed an inhibitory effect on tumor angiogenesis in a lung tumor xenograft [29]. Similarly, the results of Adina rubella Hance combination with taxol treatment presented a lower level of MVD and VEGF than taxol alone treatment. Especially in adina rubella hance (5 mg/kg) group, the effects was most significant. Moreover, the loss of VEGF expression in a tumor caused dramatic decreases in vascular density and vascular permeability and

increased tumor cell apoptosis [30]. These results indicated that Adina rubella Hance combination with taxol treatment synergisticly exhibit their anti-angiogenesis effects by inhibiting the level of VEGF.

Conclusion

Adina rubella Hance combination with taxol synergisticly exhibit antitumor and anti-angiogenesis effects on Lewis Pulmonary Carcinoma-bearing mice, which may be by reducing the level of VEGF. These provide evidence for the application of combination therapy of Adina rubella Hance and taxol inlung cancer. However, for the clinical application, the mechanism of Adina rubella Hance combination with taxol involved in the development of lung cancer was still need further research.