

# Investigation of combination therapy modes of bevacizumab and paclitaxel for NSCLC *in vivo*

Jian Wang<sup>1</sup>, Hua Shen<sup>1</sup>, Qian Jiang<sup>2</sup> and Yongqian Shu<sup>1\*</sup>

<sup>1</sup>Department of Oncology, First Affiliated Hospital of Nanjing Medical University, Nanjing, Jiangsu Province, China

<sup>2</sup>Department of Clinical Nephrology, People Hospital of Yixing, Wuxi, Jiangsu Province, China

**Abstract:** To explore the optimized scheduling and mode of administration of bevacizumab combined with paclitaxel *in vivo*. Human lung cancer A549 cells was subcutaneously injected in to 40 nude mice, which were stochastically divided into five groups: group A, physiological saline as control group, group B, paclitaxel 10mg/kg/d, i.v.bolus, q5d×4; group C, paclitaxel (10mg/kg/d) 24 hours prior to bevacizumab (5mg/kg/d), i.v.bolus, q5d×4; group D, paclitaxel (10mg/kg/d) 24 hours after to bevacizumab (5mg/kg/d), i.v. bolus, q5d×4; group E, paclitaxel (10mg/kg/d) 24 hours later following bevacizumab (1.25mg/kg/d), i.v. bolus, q5d×4. The tumor growth and side-effects of each therapy group were investigated. Micro vessel density (MVD), Evans blue (EB) quantitative detection, and EB content were analyzed and the tumor vascular permeability was calculated. Circulating endothelial progenitor cells (CEPs) were marked by CD31/CD133/CD117 positive cells and measured by flow cytometry. Lower tumor volume, lower tumor weight, and higher inhibitory rates of tumor growth were witnessed in combination therapy groups (group C, D and E) in comparison with control group (P<0.05). The tumor growth inhibitory rates in groups B, C, D and E were 7.44%, 55.43%, 66.22%, and 75.79%, respectively. The side-effects in combined therapy group were tolerated. Compared with the control group, MVD in all treatment groups were decreased significantly (P<0.05). Furthermore, MVD in combined therapy groups were decreased than single therapy (P<0.05). The EB content of tumor tissues in combined therapy groups was significantly upregulated in comparison with the control group (P<0.01). The changes of CEPs in combined therapy groups were notably higher than that in control group. Bevacizumab has synergetic inhibitory effect with paclitaxel against lung aden carcinoma A549 cell xenografts in mice by inhibiting angiogenesis of the tumor. Different modes of administration of bevacizumab with paclitaxel showed various anti-tumor and anti-angiogenesis effectiveness, in which bevacizumab prior to paclitaxel was better than paclitaxel prior to bevacizumab and consecutive administration of bevacizumab was better than administration of bevacizumab at intervals.

**Keywords:** Non-small cell lung cancer (NSCLC); paclitaxel; bevacizumab; anti- angiogenesis therapy; circulating endothelial progenitor cells (CEPs).

## INTRODUCTION

Lung cancer is the most common cancer in terms of both incidence and mortality all over the world (Jemal *et al.*, 2011). It is a heterogenous disease with two major subtypes: non-small cell lung cancer (NSCLC), which accounts for 85% and small cell lung cancer (SCLC), which accounts for 15%. Despite the improvement of innovative therapies over the last decade, the five-year survival of NSCLC is still low (~<20%) (Gadgeel SM *et al.*, 2012). Since most newly diagnosed patients present distant metastases, chemotherapy is the main treatment strategy. However, chemotherapy can-not achieve satisfactory effectiveness due to drug resistance of cancer cells that is either intrinsic or obtained after an initial round of treatment in many cases. Recently, more and more studies showed that the combined administration of bevacizumab in chemotherapy upon the treatment of selected NSCLC patients has significantly benefited in survival (Sandler *et al.*, 2006). The purpose of this study is to explore the optimized scheduling and mode of

administration of bevacizumab combined with paclitaxel *in vivo*.

## MATERIALS AND METHODS

### *Cell culture*

Human lung cancer cells A549 were all cultured in RPMI-1640 medium supplemented with 10% fetal calf serum (Gibco BRL, Grand Island, NY) in a humidified ambience containing 5% CO<sub>2</sub> at 37°C. Logarithmic growth phase cells were chosen for following experiments.

### *In vivo tumor xenograft model*

40 BALB/C nude mice with a body weight of approximately 20g were bought and raised in standard vinyl cages with air filter tops in a filtered laminar air flow room at 25°C on a 12h light/dark cycle; Water and food were autoclaved and supplied. For tumor establishment, human lung cancer A549 cells were injected subcutaneously in a volume of 0.1mL into the flank of mice. After inoculation, tumor-bearing mice were divided stochastically into 5 treatment groups (8 mice per group) and treatment initiated when the xenograft solid

\*Corresponding author: e-mail: shuyongqian1999@126.com

tumors grew to a volume of about 50-75mm<sup>3</sup>. Each mouse was treated with different drugs as below: group A, physiological saline as control group, group B, paclitaxel 10mg/kg/d, i.v. bolus, q5d×4; group C, paclitaxel (10mg/kg/d) 24 hours prior to bevacizumab (5mg/kg/d), i.v. bolus, q5d×4; group D, paclitaxel (10mg/kg/d) 24 hours after to bevacizumab (5mg/kg/d), i.v. bolus, q5d×4; group E, paclitaxel (10mg/kg/d) 24 hours later following bevacizumab (1.25mg/kg/d), i.v. bolus, q5d×4. After xenograft transplantation, mice bearing tumors were continuously observed and tumor sizes were measured once every 3 days. Evans Blue (20mg/kg) was injected into the four nude mice, which were randomly selected from each group after four cycles over a week. 1 ml peripheral blood was collected from every nude mice. Tumor weight, length (a), width (b) were measured and tumor volume (TV) and growth inhibition ratio (GIR) were calculated.

$$TV = \frac{1}{2} \times a \times b^2$$

#### Tumor vascular permeability evaluation

Evans Blue (20mg/kg) was injected into the four nude mice, which were randomly selected from each group. 3h later, nude mice were euthanized. And tumor tissues were collected for further analysis. Take 0.1g tumor tissues into 2ml formamide solution soak, 56°C water bath for 24h. Then the leaching agent's OD values were measured by the spectrophotometry and the EB content were calculated through the standard curve regression equation.

The animal tests have been approved by the ethics committees of Nanjing Medical University, Nanjing, China.

#### Circulating endothelial progenitor cells (CEPs) detection

Circulating endothelial progenitor cells (CEPs) were marked by CD31/CD133/CD117 positive cells and measured by flow cytometry. Briefly, 1ml of anti coagulated whole blood was collected from each mouse into 1ml lymphocyte stratified fluid to collect the mononuclear cells. Then, added Fc receptor blockers and incubated for 15 min. Hence, the cells were incubated for 30 min with the following combination of anti-mouse monoclonal antibodies: 1μg of anti-CD133 conjugated with phycoerythrin (PE) (eBioscience), 0.06μg of anti-CD117 conjugated with allophycocyanin (APC) (eBioscience) and 0.5μg of anti-CD31 conjugated with APC (eBioscience). After incubation, the cells were washed with phosphate-buffered-saline (PBS) and analyzed using a FACSCalibur flow cytometer and FACSDiva Software (Becton Dickinson).

## STATISTICAL ANALYSIS

Numerical data were shown as mean ± SD. The difference between means was analyzed with Student's t test. All statistical analyses were performed using SPSS17.0 software. For all tests, the significance level for statistical analysis was set at P<0.05.

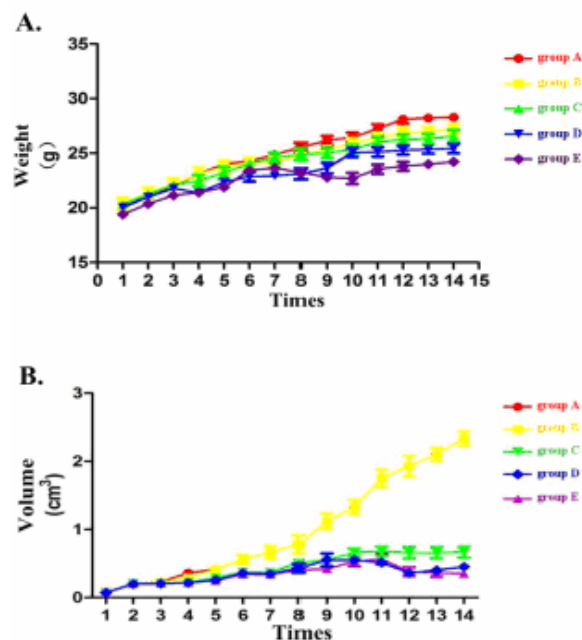
## RESULTS

### Establishment of A549 xenograft nude mice model

We established human lung cancer A549 xenograft nude mice models successfully. Subcutaneous nodules could be observed after subcutaneously transplantation of human lung cancer A549 cells into 40 nude mice. Nude mice's weight and tumor volume showed no significant differences before treatment (table 1).

### Paclitaxel in combination with bevacizumab exhibits synergistic effect to inhibit A549 tumor growth in vivo

Nude mice's weight in each group showed no significant differences before and after treatment (P>0.05) (fig.1A). Growth curve of tumor volume about nude mice in each group after treatment was shown in fig. 1B.



**Fig. 1:** A, weight changes of nude mice in each group; B, changes of tumor volume.

### Tumor weight and growth inhibition ratio

Tumor weight and growth inhibition ratio of each group were showed in table 2. The tumor weight and size in PBS control group were 2.05±0.47g and 1.928±0.429 cm<sup>3</sup>. The tumor weight of B, C, D, E control group were 1.90±0.69g, 0.91±0.21g, 0.69±0.18g and 0.50±0.24g, respectively. The tumor size of B, C, D, E control group were 1.456±0.545cm<sup>3</sup>, 0.648±0.263cm<sup>3</sup>, 0.362±0.120

cm<sup>3</sup>, and 0.380±0.179cm<sup>3</sup>, respectively. The differences between group B, C, D, E and group A had statistical significance (P<0.05). The combined therapy showed lower tumor volume, lower tumor weight and higher tumor growth inhibition rates than control group (P<0.05), in which group E show the best anti-tumor effect (P<0.05 vs. C group) and group E was better than group C (P<0.05), group D was better than group C (P<0.05).

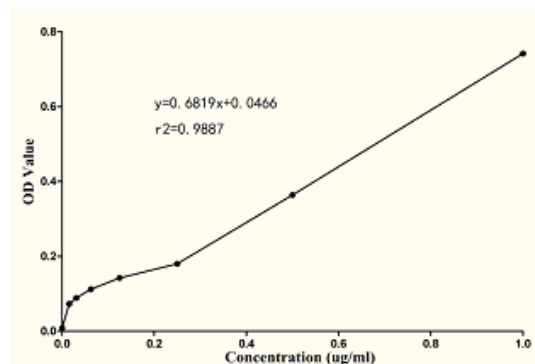


Fig. 2: Standard curve of EB.

### MVD

MVD of each group was showed in table 3. The MVD of PBS control group was 38.750±5.909. In-group B, C, D and E, the MVD were 27.250±3.403, 20.250±1.708, 12.750±2.217 and 6.750±3.202, respectively. In comparison with the control group, MVD in treatment group decreased evidently (P<0.05), and in combined therapy was lower than the single therapy (P<0.05). MVD in-group D was lower than which in-group C (P=0.002), and in-group E was lower than that in-group C (P<0.0001).

Table 1: Nude mice's weight and tumor volume before treatment

Group	No.	Weight (g)	Tumor volume (mm <sup>3</sup> )
A	8	20.2±0.9	0.069±0.017
B	8	20.4±1.5	0.069±0.015
C	8	20.3±1.0	0.072±0.019
D	8	20.0±0.8	0.070±0.021
E	8	19.4±0.9	0.072±0.019

### Tumor vascular permeability

1. Optical density (OD) value of different concentrations and standard curve of EB Optical density value of different concentrations of EB and standard curve of EB were showed in table 4 and fig. 2.

2. The changes of tumor vascular permeability after treatment As it can be seen in table 5, the EB content of tumor tissues in group A, B, C, D, E were 2.19±0.62μg/g, 2.77±0.41μg/g, 4.38±0.82μg/g, 5.97±1.14μg/g and 7.64±1.19μg/g, respectively. The EB content in combined therapy groups was notably higher in comparison with the

control group (P<0.01), and in Group C was significantly lower than Group E (P=0.01), but was not significantly different than Group D (P=0.06) (table5).

### The changes of CEPs in nude mice

The single therapy was not significantly different than control group (P>0.05) and combined therapy groups was significantly higher compared with the control group (P<0.05). CEPs showed no significant differences among the there combined therapy groups (P>0.05). (table 6).

## DISCUSSION

The growth of solid neoplasms is a complicated process and always accompanied by neovascularization (Folkman 1971; Rafii *et al.*, 2008). Tumor angiogenesis is regulated by tumor microenvironment which is composed of tumor cells, cancer stem cells, stromal cells, endothelial cells, bone marrow-derived cells (Gomes *et al.*, 2013). Tumor cells secreted cytokines and growth factors to break the balance of blood-vessel growth factor and angiogenesis factor, trigger a series of cascade reaction, and eventually lead to a large amount of new angiogenesis with disordered and abnormal structure and function (Sakurai *et al.*, 2011).

Antiangiogenic therapy has become increasingly important for cancer therapy, and bevacizumab is the first anti-angiogenic drug approved by U.S. FDA. The mechanism of anti-tumor activity of bevacizumab is most likely due to its anti-angiogenesis effect through binding and neutralization of secreted VEGF (Wang *et al.*, 2004). Therefore it is rational to combine bevacizumab with chemotherapy or radiotherapy to enhance anti-tumor effectiveness. Bevacizumab alone can-not improve PFS and OS existing in patients with advanced cancer. Shaked *et al.* found that certain chemotherapy drugs, e.g., paclitaxel, can rapidly induce proangiogenic bone marrow-derived circulating endothelial progenitor (CEP) mobilization and subsequent tumor homing. While other drugs, e.g., gemcitabine, do not exert similar characteristics (Shaked *et al.*, 2004). Only those chemotherapy drugs which significantly increase CEPs combining with anti-angiogenic drugs combination can bring a better anti-tumor effect (Bertolini *et al.*, 2011). So we speculated that CEPs was invovled in bevacizumab's antiangiogenic activity in many solid malignancies when combined with cytotoxic chemotherapy.

None-small cell lung caner (NSCLC) is the leading cause of death worldwide and its incidence is still increasing. Median survival of metastatic NSCLC is about 8~10 months even if treated with conventional chemotherapy. (Shan Y *et al.*, 2015). This study established a non-small cell lung cancer xenografted nude mice model successfully. Pharmacodynamics experiments showed that bevacizumab has synergetic inhibitory effect with paclitaxel against lung adencarcinoma A549 cell

**Table 2:** Growth inhibition ratio of each group

Group	No.	Tumor volume (cm <sup>3</sup> )	Tumor weight (g)	Growth inhibition ratio (%)
A	8	1.928±0.429	2.05±0.47	/
B	8	1.456±0.545	1.90±0.69	7.44%±2.12
C	8	0.648±0.263	0.91±0.21	55.43%±5.01
D	8	0.362±0.120	0.69±0.18	66.22%±4.42
E	8	0.380±0.179	0.50±0.24	75.79%±2.11

xenografts in mice by inhibiting tumor angiogenesis. Different modes of administration of bevacizumab with paclitaxel showed different anti-tumor and anti-angiogenesis effect, in which bevacizumab prior to was better than paclitaxel prior to bevacizumab and consecutive administration of bevacizumab was better than interval administration of bevacizumab.

**Table 3:** Analysis of MVD in each group

Group	No.	MVD
A	4	38.750±5.909
B	4	27.250±3.403*
C	4	20.250±1.708**
D	4	12.750±2.217**
E	4	6.750±3.202**

\* P<0.05, \*\*P<0.01

The inhibitory effects of bevacizumab combined with paclitaxel on tumor blood vessels were consistent with that of in pharmacodynamics experiment. MVD and EB in different modes of administration of bevacizumab with paclitaxel were both more decreased more than those in the paclitaxel single therapy. Similarly, MVD and EB in consecutive administration of bevacizumab decreased obviously than administration of bevacizumab at intervals. There was no significantly statistical differences between group C (paclitaxel (10mg/kg/d) 24 hours prior to bevacizumab (5mg/kg/d)) and group D (paclitaxel (10mg/kg/d) 24 hours after to bevacizumab (5mg/kg/d)) due to small sample sizes. However, the result showed a trend that MVD and EB in-group C was lower than group D. These results indicated that different modes of administration of bevacizumab with paclitaxel affected the quantity and quality of tumor blood vessels.

Besides, we found that CEPs in combined therapy groups was notably higher in comparison with the control group. However, CEPs showed no significant differences among combined therapy groups.

Researchers found that acute CEP mobilization was mediated, at least in part, by systemic induction of SDF-1 $\alpha$  and could be prevented by various procedures such as treatment with anti-VEGFR2 blocking antibodies or by paclitaxel treatment in CEP-deficient Id-mutant mice and both of which resulted in enhanced anti-tumor effects mediated by paclitaxel (Shaked *et al.*, 2004). SDF-1 is one of the chemokine endothelial progenitor cells (epcs),

and it can recruit endothelial progenitor cells to the angiogenesis (Du *et al.*, 2008). SDF-1 receptor signaling blockage suspended the progenitor cell homing, while injecting SDF-1 to target organs increased progenitor cell homing (Weis *et al.*, 2011). However, researchers also found that SDF-1 could not enhance the recruitment of bone marrow-derived cells when VEGF was missing. When SDF-1 receptor was blocked, bone marrow-derived cells in the target organs were still reduced even in the presence of high level of VEGF. In our study, we found that anti-tumor and anti-angiogenesis effect in-group C (paclitaxel (10mg/kg/d) 24 hours prior to bevacizumab (5mg/kg/d)) was better than in group D (paclitaxel (10mg/kg/d) 24 hours after to bevacizumab (5mg/kg/d)). We speculated that it might be because bevacizumab blocked VEGF signals, and paclitaxel induced the decreased expression of the CEPs. However, we only selected a point in time to test the number of CEPs. Further studies on different time point will be necessary to confirm our findings. Angiogenesis is a long-term and complicated process (Kubota, 2012; Yan *et al.*, 2015).

Many researches showed that bevacizumab transiently normalized the dysfunctional vasculature and improved tumor oxygenation which is a rapid and instantaneous process and a vascular normalization time window could be accompanied (Myers *et al.*, 2010; Arjaans *et al.*, 2013). Consecutive administration of bevacizumab could prolong the time window to achieve a better anti-tumor effectiveness.

In conclusion, in this present study, we showed that bevacizumab has synergetic inhibitory effect with paclitaxel against lung adenocarcinoma A549 cell xenografts in mice by inhibiting angiogenesis of the tumor. Different modes of administration of bevacizumab with paclitaxel showed different anti-tumor and anti-angiogenesis effect.

Further studies on different time point and large samples will be necessary and are of great interests.

## CONCLUSION

This study showed Bevacizumab has synergetic inhibitory effect with paclitaxel against lung adenocarcinoma A549 cell xenografts in mice by inhibiting angiogenesis of the tumor. Different modes of administration of bevacizumab

**Table 4:** Optical density (OD) value of different concentrations of EB

Concentrations ( $\mu\text{g/ml}$ )	1	0.5	0.25	0.125	0.0625	0.0313	0.0156	0
OD	0.742	0.364	0.180	0.142	0.112	0.089	0.073	0.008

**Table 5:** The EB content of tumor tissues in each group\*\*P < 0.01

Group	A	B	C	D	E
EB ( $\mu\text{g/g}$ )	2.19 $\pm$ 0.62	2.77 $\pm$ 0.41	4.38 $\pm$ 0.82**	5.97 $\pm$ 1.14**	7.64 $\pm$ 1.19**

\*\*P &lt; 0.01

**Table 6:** CEPs in five different therapy groups

Group	A	B	C	D	E
CEPs (%)	8.208 $\pm$ 2.075	7.639 $\pm$ 2.397	4.102 $\pm$ 1.066	3.389 $\pm$ 1.286	3.026 $\pm$ 1.086

with paclitaxel showed various anti-tumor and anti-angiogenesis effectiveness, in which bevacizumab prior to paclitaxel was better than paclitaxel prior to bevacizumab and consecutive administration of bevacizumab was better than administration of bevacizumab at intervals.

## REFERENCES

- Arjaans M, Oude Munnink TH, Oosting SF, Terwisscha van Scheltinga AG, Gietema JA, Garbacik ET, Timmer-Bosscha H, Lub-de Hooge MN, Schröder CP and de Vries EG (2013). Bevacizumab-induced normalization of blood vessels in tumors hampers antibody uptake. *Cancer Res.*, **73**(11): 3347-3355.
- Bertolini F, Mancuso P and Shaked Y (2011). Circulating endothelial cells as biomarkers for patients receiving bevacizumab. *Lancet Oncol.*, **12**(3): 217-218.
- Du R1, Lu KV, Petritsch C, Liu P, Ganss R, Passequé E, Song H, Vandenberg S, Johnson RS, Werb Z and Bergers G (2008). HIF1 $\alpha$  induces the recruitment of bone marrow-derived vascular modulatory cells to regulate tumor angiogenesis and invasion. *Cancer Cell.*, **13**(3): 206-220.
- Folkman J (1971). Tumor angiogenesis: Therapeutic implications. *N. Engl. J. Med.*, **285**(21): 1182-1186.
- Gadgeel SM, Ramalingam SS and Kalemkerian GP (2012). Treatment of lung cancer. *Radiol. Clin. North Am.*, **50**(5): 961-974.
- Gomes FG1, Nedel F, Alves AM, Nör JE and Tarquinio SB (2013). Tumor angiogenesis and lymphangiogenesis: Tumor/endothelial crosstalk and cellular/micro environmental signaling mechanisms. *Life Sci.*, **92**(2): 101-117.
- Jemal A, Bray F, Center MM, Ferlay J, Ward E and Forman D (2011). Global cancer statistics. *CA. Cancer J. Clin.*, **61**(2): 69-90.
- Kubota Y (2012). Tumor angiogenesis and anti-angiogenic therapy. *Keio. J. Med.*, **61**(2): 47-56.
- Myers AL, Williams RF, Ng CY, Hartwich JE and Davidoff AM (2010). Bevacizumab-induced tumor vessel remodeling in rhabdomyosarcoma xenografts increases the effectiveness of adjuvant ionizing radiation. *J. Pediatr. Surg.*, **45**(6): 1080-1085.
- Rafii S and Lyden D (2008). Cancer. A few to flip the angiogenic switch. *Science.*, **319**(5860):163-4.
- Sakurai T and Kudo M (2011). Signaling pathways governing tumor angiogenesis. *Oncology*, **81**(Suppl 1): 24-29.
- Sandler A, Gray R, Perry MC, Brahmer J, Schiller JH, Dowlati A, Lilenbaum R and Johnson DH (2006). Paclitaxel-carboplatin alone or with bevacizumab for non-small-cell lung cancer. *N. Engl. J. Med.*, **355**(24): 2542-2550.
- Shaked Y, Henke E, Roodhart JM, Mancuso P, Langenberg MH, Colleoni M, Daenen LG, Man S, Xu P, Emmenegger U, Tang T, Zhu Z, Witte L, Strieter RM, Bertolini F, Voest EE, Benezra R and Kerbel RS (2008). Rapid chemotherapy-induced acute endothelial progenitor cell mobilization: Implications for antiangiogenic drugs as chemo sensitizing agents. *Cancer Cell*, **14**(3): 263-273.
- Shan Y, Li S.(2015).Expression of Cripto-1 gene protein and Activin-A in human lung adenocarcinoma tissue. *Pak. J. Pharm. Sci.*, **28**(2 Suppl): 739-43.
- Wang Y, Fei D, Vanderlaan M and Song A (2004). Biological activity of bevacizumab, a humanized anti-VEGF antibody *in vitro*. *Angiogenesis*, **7**(4): 335-345.
- Weis SM and Chesh DA (2011). Tumor angiogenesis: Molecular pathways and therapeutic targets. *Nat. Med.*, **17**(11): 1359-1370.
- Yan Y, Wang T, Cao J, Wang M and Li F(2015). Clinical research on intravitreal injection of bevacizumab in the treatment of macula lutea and retinal edema of ocular fundus disease. *Pak. J. Pharm. Sci.*, **28**(4 Suppl): 1481-1484.