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STUDY ON TARGETING OF LUNG CANCER WITH POLYPEPTIDE RGD

Jieke Tang[1], Zhao Zhong[1*#], Fangchao Chen[2], Jinyong Liu[2*]
[1] *Department of Anesthesiology, First Affiliated Hospital of Jinan University, Guangzhou, China*
[2] *Guangzhou First People's Hospital, the Second Affiliated Hospital of South China University of Technology, Guangzhou, China*
[*]*Corresponding E-mail: liujinyong87@126.com*
[#]*These authors contributed equally to this work and are Co-first-author of this article.*

Abstract: In this study, we aimed to study the selectivity of therapeutic targets in cancer chemotherapy through a new targeting peptide. Peptide specifically binding to human lung adenocarcinoma was identified through a NCI-H1299 subcutaneous xenograft model, results showed that RGD was concentrated mainly in the tumor while less accumulation of fluorescence in the tumor was found in control group animals. Ability of RGD to target human lung cancer tissue in vitro was investigated through tissue microarray, results show that a 7-fold higher detection rate of $[RGD]^{FITC}$ was achieved compared with the control group, indicating the potential of RGD as carriers for targeted cancer therapy drug. In conclusion, RGD is specifically targeted to human lung adenocarcinoma xenografted and had high specifically and affinity with lung cancer tissues, it might be an efficient method to increase the selectivity of therapeutic targets in cancer chemotherapy.
Keywords: Lung Cancer, Polypeptide, arginine-glycine-aspartic acid.

1. INTRODUCTION

Lung cancer is one of the most common cancers, it is estimated that 4292,000 new cancer cases and 2814,000 cancer deaths would occur in China in 2015, with lung cancer being the most common incident cancer and the leading cause of cancer death [1]. The 5-year survival rate is 52.2% for cases detected when the disease is still localized, 24.3% for patients with regional disease, and 3.6% for patients with distant stage disease [2].

Currently the three main treatment are chemotherapy, surgery and radiation therapy. Of them, chemotherapy is still the most important method for comprehensive cancer treatment[3]. However, chemotherapy is limited by the side effects, including damage to the kidney, neurotoxicity, hearing, and bone marrow suppression [4,5]. In view of these limitations, a major challenge in chemotherapy is to achieve the specific delivery of drugs to tumor tissue instead of normal tissues.

The integrins are a family of cell-surface glycoproteins and play an important role in cells and extracellular matrix (ECM) combining, cell adherens, complement binding and phagocytosis. Integrin is a dimmer consisting of two subunits, α and β peptide. α chain has 16 subtypes and β chain has 8 subtypes. Different subtypes of integrin have different combining abilities with ECM [6]. Integrin function is central to inflammation, immunity, and tumor progression, previous studies have confirmed that integrins and tumor metastasis are closely related.

The arginine-glycine-aspartic acid (RGD) sequence was discovered as cell attachment site in fibronectin some 20 years ago, then were reported in other extracellular matrix (ECM) proteins as well[7].Many studies show that polypeptide containing drug to the tumor site may be a more effective method in cancer chemotherapy as a targeting ligand[8]. We hypothesized that RGD in combination with chemotherapy drugs would lead to their preferential uptake by non-small cell lung carcinoma cell after i.v. administration, in a NCI-H1299 lung cancer model. To test the targeting of lung cancer with polypeptide rgd, tissue distribution of rgd was analyzed in an animal model and tissue specificity was tested by tissue microarray.

2. METHODS

2.1. Cells, animals and materials

Cell Culture. The human non-small cell lung carcinoma cell line (NCI-H1299) was purchased from the Type Culture Collection of the Chinese Academy of Sciences, Shanghai, China. Fetal calf serum (FCS) and DMEM medium were purchased from GiBCO (USA).The NCI-H1299 cell line were cultured in a DMEM medium supplemented with 10% fetal calf serum.

Animal. Animal care and handling were performed with the approval of the Animal Ethics Committee at the Institute of Medical Plant Development, Chinese Academy of Medical Science. Male and female BALB/c nu/nu nude mice (4–5 weeks old and weighing 15–25 g) were obtained from Guang Dong Research Center of Laboratory Animals. Male and female KM mice were purchased from Guang Dong Research Center of Laboratory Animals. All mices were housed in laminar-flow cabinets under specific pathogen-free conditions and had free access to water and standard animal diet.

Model of Human Non-small Cell Lung Cancer in Nude Mice. Human lung cancer cell line NCI-H1299 digested with 0.25% trypsin containing 0.01% EDTA, centrifugal and serum-free DMEM medium was added and resuspended. 1×10^6 NCI-H1299 cells were injected subcutaneously in the flank of mice. Solid tumors were formed from the inoculated human lung cancer cells (NCI-H1299) with sizes at about 10 mm×10 mm×5 mm.

Peptide. Peptide RGD were synthesized and labeled with fluorescein isothiocyanate (FITC) by Beijing Scilight-peptide Ltd, all peptides are >95% pure as determined by a reverse-phase HPLC.

2.2 Peptide distribution after i.v. injection, in a tumor-bearing nude mice model

Human non-small cell lung tumor was induced in ten female nude mice as described above, when tumor volume reached 500 mm^3, the mice were randomly divided into 2 groups (each n=10): RGD group and control group. Peptide RGD was labelled with fluorescein isothiocyanate (FITC) and given to mice by intravenous injection at the dose of 10um.kg^{-1}, suspended in 0.15 mL PBS. Furthermore, pure FITC (purchased from Sigma-Aldrich, USA) was used as a control. Ten minute post injection, all mice were anesthetized with diethyl ether, perfused through the left ventricle at a perfusion pressure of approximately 120 mmHg [9]. Perfusion was initiated by flushing with 25 ml of 0.9% saline solution , followed immediately by 4% w/v paraformaldehyde, then their heart, liver, spleen, lung, kidney, brain and tumor were removed and made into frozen sections, fluorescence intensity were visualized by Leica Fluorescence Microscope System .

2.3 In vitro targeting study use a tissue microarray

To validate that RGD is specifically targeted to human lung cancer tissue in vitro, the human lung cancer tissue microarray (Fanpu Biotech, Inc, China) was used. The microarray includes 70 cases of human lung cancer tissue with different types, two cases of normal tissue and three cases of pneumonia tissue. After deparaffinized with different concentrations of ethanol, the microarray was treated in citrate buffer (pH 6.0, 95℃) for antigen retrieval. The microarray was then washed and incubated in blocking buffer (10% BSA), followed by incubation overnight at 4℃ with $[RGD]^{FITC}$ or pure FITC. Fluorescence of the tissue microarray was scanned by GenePix 4000B Microarray Scanner System (Molecular Devices, USA).

2.4 Statistical analysis

Statistical analysis was performed using SPSS17.0 software.

3. RESULTS

3.1 Peptide distribution in NCI-H1299 tumor-bearing nude mice model

Peptide specifically binding to human lung adenocarcinoma was identified through a NCI-H1299 subcutaneous xenograft model. Peptide RGD was labeled with FITC (green) and given to mice by intravenous injection. As a control, pure FITC was used. All mice were perfused with 0.9% saline solution and 4% w/v paraformaldehyde, then tissues were made into frozen sections, fluorescence intensity was visualized by Leica Fluorescence Microscope System. As seen, RGD was concentrated mainly in the tumor, showing bright green fluorescence, heart, liver, and kidney can also be found have greater accumulation of fluorescence compared with spleen, lung, and brain (Fig. 1 I). As a control, pure FITC was used. Fig. 1 II

reveals that pure FITC without peptide attached was concentrated mainly in the heart, liver and kidney. Moreover, less accumulation of fluorescence in the tumor was found in control group animals. Tumor image in RGD group revealed a greater level of fluorescence compared with the very low level in control group (Fig. 2). Results indicate that peptide RGD is specifically targeted to human lung adenocarcinoma cell (NCI-H1299).

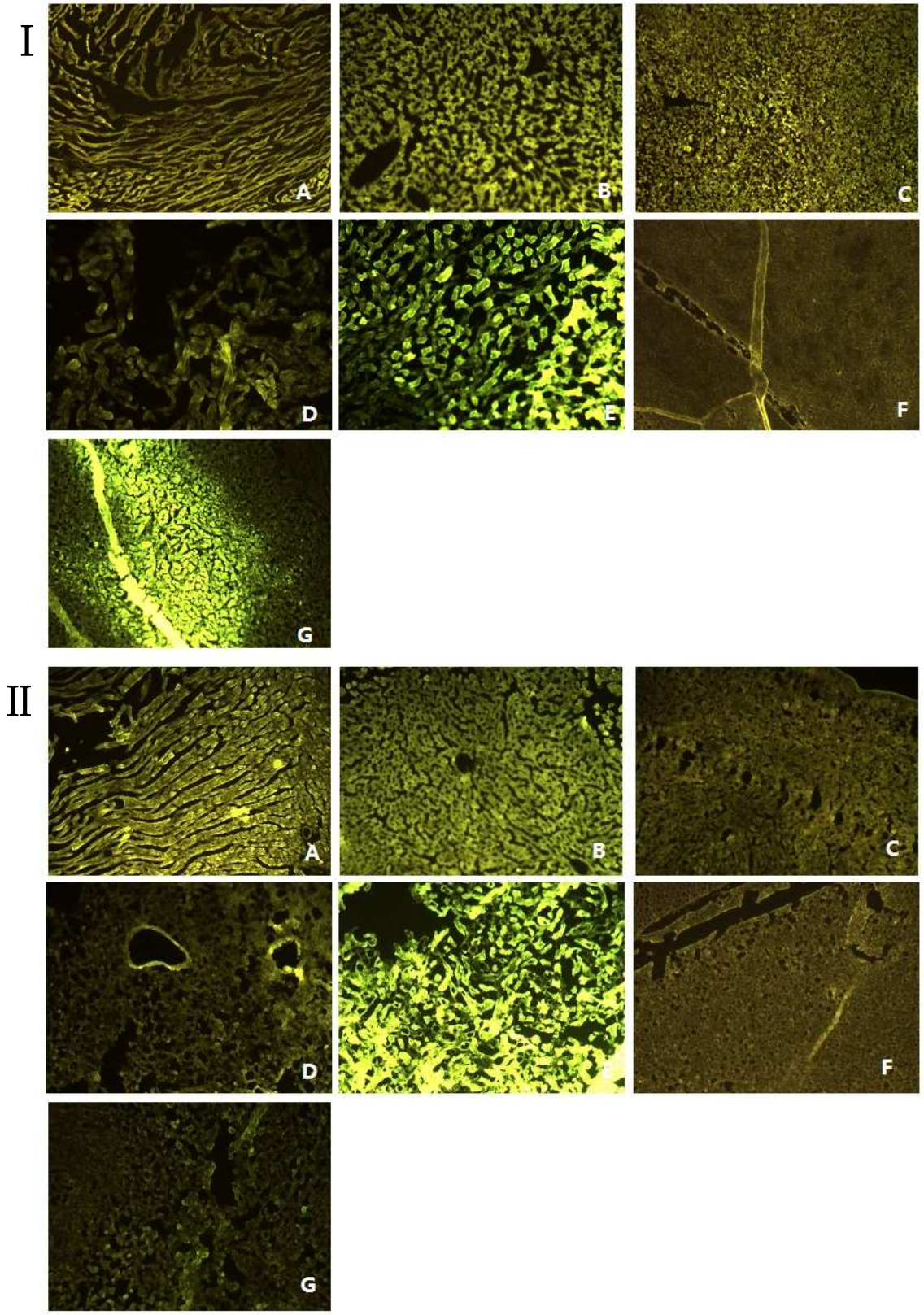

Fig. 1 (I). $[RGD]^{FITC}$ organ distribution in NCI-H1299 human lung adenocarcinoma bearing nude mice,10 min after receiving an i.v. injection via the tail vein. The (A) heart, (B) liver, (C)

spleen, (D) lung, (E) kidney, (F) brain and (G) tumor were removed and made into frozen sections, fluorescence accumulation was visualized by Leica Fluorescence Microscope System. (II) . As a control, pure FITC was used. 10 min after receiving an i.v. injection, The (A) heart, (B) liver, (C) spleen, (D) lung, (E) kidney, (F) brain and (G) tumor were removed and made into frozen sections, fluorescence accumulation was visualized by Leica Fluorescence Microscope System.

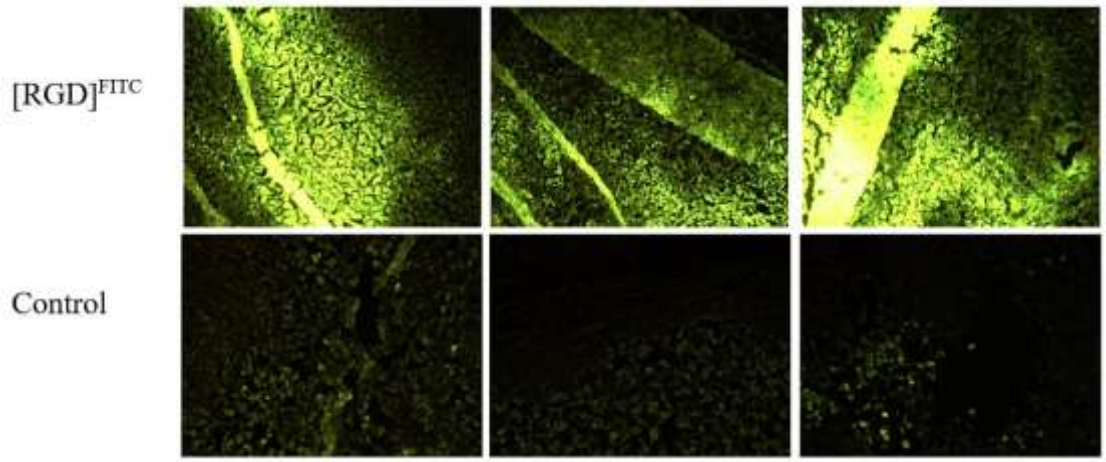

Fig. 2 Fluorescence accumulation in human lung adenocarcinoma subcutaneous xenograft, 10 min after receiving an i.v. injection of $[RGD]^{FITC}$ or FITC.

3.2 In vitro targeting study

To investigate the ability of RGD to target human lung cancer tissue in vitro, tissue microarray was used. After deparaffinized with different concentrations of ethanol, the microarray was treated in citrate buffer for antigen retrieval. Then washed and incubated in blocking buffer (10% BSA), followed by incubation overnight at 4℃ with $[RGD]^{FITC}$ or pure FITC. Fluorescence of the tissue microarray was scanned by GenePix 4000B Microarray Scanner System (Fig. 3). Positive was defined as the fluorescence intensity of lung cancer tissues was twice than the normal and inflammatory tissue. Table. 1 showed that the detection rate of $[RGD]^{FITC}$ was 32.86 %, while the false-positive rate was 0 %. As a control, the detection rate and false-positive rate of pure FITC were 4.29% and 0%. As seen, a 7-fold higher detection rate of $[RGD]^{FITC}$ was achieved compared with the control group, suggesting that RGD had high specifically and affinity with lung cancer tissues.

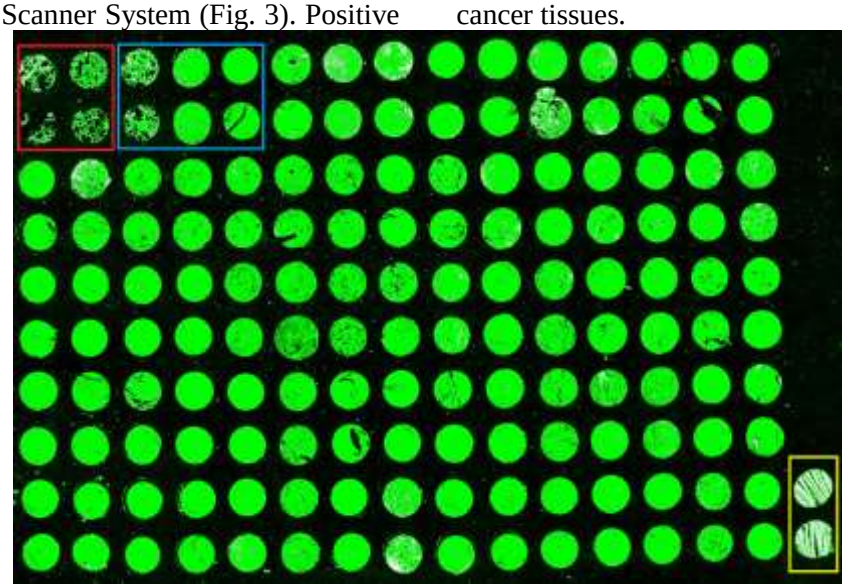

Fig. 3 Tissue microarray was used to investigate the ability of RGD to target human lung cancer tissue in vitro. The microarray includes 70 cases of human lung cancer tissue with different types, two cases of normal tissue (red), three cases of pneumonia tissue (blue) and two marked

points (yellow). Fluorescence of the tissue microarray was scanned by GenePix 4000B Microarray Scanner System.

Table 1 Rate of lung cancer detection when using tissue microarray. Positive was defined as the fluorescence intensity of lung cancer tissues was twice than the normal and inflammatory tissue.

Pathological type	Positive/total
RGD	
Lung squamous cell carcinoma	20/66
Lung adenocarcinoma	14/40
Adenosquamous carcinoma	5/14
Small cell lung cancer	7/14
Lung metastasis	0/6
Total (positive rate)	46/140 (32.86%)
Pneumonia	0/6
Normal	0/4
Total (false-positive rate)	0/10 (0%)
Control	
Lung squamous cell carcinoma	4/66
Lung adenocarcinoma	2/40
Adenosquamous carcinoma	0/14
Small cell lung cancer	0/14
Lung metastasis	0/6
Total	6/140 (4.29%)
Pneumonia	0/6
Normal	0/4
Total (false-positive rate)	0/10 (0%)

3.3 discussion

The arginine-glycine-aspartic acid (RGD) sequence was discovered as cell attachment site in fibronectin some 20 years ago, which may be specifically bound to NCI-H1299 and have potential in targeted cancer therapy. First, Peptide specifically binding to human lung adenocarcinoma was identified through a NCI-H1299 subcutaneous xenograft model. As seen, RGD was concentrated mainly in the tumor while less accumulation of fluorescence in the tumor was found in control group animals. Moreover, heart, liver, and kidneys can also be found have greater accumulation of fluorescence, which may due to the low stability of peptide against proteolysis, leading to a short duration of in vivo activity [10-12]. When Peptide was degraded in vivo, free fluorescein isothiocyanate were distributed, that results fluorescence distribution are visible in various tissues and organs. In addition, the liver plays a major role in drug clearance and kidney is the primary route of drug and/or metabolite excretion, it may have great accumulation of fluorescence in liver and kidney in theory. Follow, ability of RGD to target human lung cancer tissue in vitro was investigated through tissue microarray. While positive was defined as the fluorescence intensity of lung cancer tissues was twice than the normal and inflammatory tissue, results show that a 7-fold higher detection rate of $[RGD]^{FITC}$ was achieved compared with the control group, indicating that RGD is specifically targeted to human lung adenocarcinoma xenografted and had high specifically and affinity with lung cancer tissues.

4. CONCLUSION

The results presented herein reveal that the potential of RGD as carriers for targeted cancer therapy drug. However, there are also some limitations of our study. Our results are lack of the histopathological or morphological examination in this study. Further, other human lung cancer cells were not included in the study. Despite its preliminary character, this study can clearly indicate the validity of a targeted peptide RGD based approach to cancer chemotherapy combining toxicity reduction with selective drug delivery.

5. COMPETING INTERESTS

The authors declare that they have no competing interests.

6. ACKNOWLEDGMENTS

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