Translational Oncology
www.transonc.com

Volume 7 Number 2 April 2014 pp. 221–229 221

Antiangiogenic Therapy Using Sunitinib Combined with Rapamycin Retards Tumor Growth But Promotes Metastasis[1]

Tao Yin*, Sisi He*, Tinghong Ye*, Guobo Shen*, Yang Wan* and Yongsheng Wang*,†

*State Key Laboratory of Biotherapy, West China School of Medicine, West China Hospital, Sichuan University, Chengdu, People's Republic of China; †Department of Thoracic Oncology, Cancer Center, West China Hospital, Sichuan University, Chengdu, People's Republic of China

Abstract

BACKGROUND: This study investigated the synergistic effect of sunitinib and rapamycin on tumor growth and metastasis in murine breast cancer model. *METHODS:* The synergistic antitumor effect of sunitinib and rapamycin on tumor growth and metastasis was investigated. Myeloid-derived suppressor cells (MDSCs) in spleens and lungs were assessed. Tumor hypoxia, vessel density and micrometastasis were evaluated. Versican, indoleamine 2,3-dioxygenase (IDO), arginase 1, interleukin-6 (IL-6), IL-10, and transforming growth factor β (TGF-β) in the lungs and tumors were examined. IL-6 and TGF-β in the blood were evaluated. *RESULTS:* Synergism between sunitinib and rapamycin on tumor growth was observed. Sunitinib plus rapamycin reduced splenomegaly, MDSCs in spleens and lungs, and microvessel density in tumor microenvironment, while exacerbated hypoxia and promoted cancer lung metastasis. Sunitinib plus rapamycin markedly induced versican, IDO, arginase 1, IL-6, and TGF-β expression in the lungs, whereas it reduced IDO and IL-10 expression in the primary tumor tissues. IL-6 levels in the circulation were increased after rapamycin and combination therapies. *CONCLUSIONS:* The combination of sunitinib plus rapamycin reduced the tumor growth but promoted tumor metastasis. This study warrants that further mTOR inhibition treatment should be closely watched in clinical setting, especially combined with antiangiogenic therapy.

Translational Oncology (2014) 7, 221–229

Introduction

Angiogenesis is essential for tumor growth and progression [1]. Antiangiogenic therapies have been demonstrated effective on the suppression of tumor growth [2]. Paradoxically, antiangiogenic strategies can also induce local and distant metastasis [3]. Reduced oxygen supply leads to the stabilization and activation of the transcription factor hypoxia-induced factor 1 (HIF-1) [4]. Hypoxia and the expression of HIF-1 are correlated with cancer metastasis and unfavorable prognosis [5]. Through activation of the Twist, hypoxia induces epithelial-to-mesenchymal transition [6], which was associated with cancer metastasis [7]. Sunitinib is one type of multitargeted tyrosine kinase inhibitor, which targets several receptor tyrosine kinases, including vascular endothelial growth factor receptor (VEGFR) (VEGFR-1, VEGFR-2, and VEGFR-3), PDGFR (PDGFR-α and PDGFR-β), and stem cell factor receptor (KIT) [8]. Sunitinib monotherapy has activity in advanced breast cancers [9]. Sunitinib has also been demonstrated to be effective in combination with chemotherapy in preclinical models [10]. However, sunitinib therapy can induce intratumoral hypoxia, which enriches cancer stem cells [11].

The mammalian target of rapamycin (mTOR) promotes cell growth, proliferation, and survival in response to nutrient signals and a variety of cytokines. mTOR also plays a vital role in the regulation of cancer cell growth and progression [12]. mTOR promotes cancer cell migration and invasion [13]. mTOR has been demonstrated to impact

Address all correspondence to: Yongsheng Wang, Department of Thoracic Oncology, Cancer Center, State Key Laboratory of Biotherapy, West China Hospital, Sichuan University, Chengdu 610041, People's Republic of China. E-mail: yintao03073@163.com

[1] This work was supported by National Major Project (2011ZX09302-001-01). The authors have declared that they have no competing interests.
Received 5 July 2013; Revised 9 November 2013; Accepted 2 January 2014

1936-5233/14 http://dx.doi.org/10.1016/j.tranon.2014.02.007

angiogenesis. The phosphatidylinositide 3-kinases (PI3K)/Akt signaling pathway is the downstream of VEGF and promotes endothelial cell survival [14]. In the hind limb ischemia, Akt is critical for ischemia and VEGF-induced angiogenesis [15]. Endothelial cells in the tumor microenvironment have chronic Akt activation, and the sustained Akt activation induces the formation of abnormal microvessels, which mimic the effects of VEGF-A–induced angiogenesis [16]. Treatment of cultured cells with rapamycin decreased activation of Akt [17]. Rapamycin can inhibit pathologic angiogenesis through the inhibition of endothelial Akt signaling [16] and VEGF production [18]. Then, mTOR has been considered as a promising target for cancer therapy [19].

mTOR regulates the expression of HIF-1α expression [20]. We then hypothesized that rapamycin could suppress antiangiogenic therapy–induced cancer metastasis. In addition, there is no study investigating the synergism between antiangiogenic therapy and rapamycin on breast tumor model. In our present study, we demonstrate the synergistic effect of rapamycin and sunitinib on tumor regression. However, the hypothesized therapeutic effect of sunitinib combined with rapamycin on lung metastasis was not observed, and, unexpectedly, we found that the combination promoted the lung metastasis of cancer cells.

Materials and Methods

Mice

BALB/c mice (6-8 weeks old) were purchased from Beijing HFK Bioscience Co (Beijing, China) and maintained under pathogen-free conditions in the animal facility with individual ventilation. All animal experiments were carried out according to protocols approved by Sichuan University's Institutional Animal Care and Use Committee.

Cell Lines and Reagents

Murine breast cancer cell lines (4T1) were cultured in the RPMI1640 media supplemented with 10% FBS at 37°C, 5% CO_2 atmosphere. Rapamycin was obtained from Selleck Chemicals (Houston, TX). Sunitinib was purchased from Pfizer company (New York, NY).

Tumor Challenge and Treatment

Syngeneic breast cancers were established by subcutaneous inoculation of 4T1 cells. Briefly, 1×10^6 4T1 cells were injected subcutaneously in the right flank of BALB/c mice. At day 6 after tumor inoculation (tumors reached an average diameter of 5 mm), animals were randomly assigned to four groups and treated with vehicle, rapamycin (4 mg/kg), sunitinib (10 mg/kg), and rapamycin (4 mg/kg) and sunitinib (10 mg/kg), respectively. Sunitinib was administered daily by gavage, and rapamycin was intraperitoneally administered daily. Tumor diameters were measured with a caliper, and tumor volumes were calculated as previously reported [21]. Tumor burden was measured by the tumor volume and the gross wet weight of tumors. Metastatic and disseminated tumors were assessed by gross evaluation and microscopical examination.

Histopathologic Examination and Hypoxia Assessment

At 21-day posttreatment, tumor was harvested, fixed, and embedded in paraffin. Tumor sections were stained with CD31 (Abcam, Cambridge, UK) and counterstained with hematoxylin (Beyotime, Jiangsu, China). Liver and kidney metastases were evaluated on hematoxylin and eosin (H&E)-stained sections. Twenty-one days after treatment, tumor-bearing mice were injected intraperitoneally with the hypoxic cell marker Hypoxyprobe-1 (60 mg/kg; Hypoxyprobe™-1, HPI Inc., Burlington, MA) and killed 90 minutes later. Tumors were excised, and frozen tumor sections were prepared. Tumor sections were stained with fluorescein isothiocyanate–conjugated mouse anti–Hypoxyprobe-1 monoclonal antibody (1:100) at 37°C for 1 hour. The hypoxic tissues were examined under a fluorescence microscope.

Flow Cytometry

At day 21 of posttreatment, spleens were harvested, and erythrocytes were lysed. Spleen cells were centrifuged, washed with phosphate-buffered saline, and then incubated with CD11b-peridinin chlorophyll protein(PerCP)-Cy5.5 Gr-1–phycoerythrin (PE) antibodies (BD Pharmingen, San Diego, CA) for 30 minutes at 4°C. Single-cell suspension of lung cells of tumor-bearing mouse was prepared and then stained with CD11b-PerCP-Cy5.5, Gr-1–PE antibodies (BD Pharmingen) for 30 minutes at 4°C. For flow cytometry analysis, cells were acquired with FACSCalibur flow cytometer and analyzed with CellQuest software (BD Biosciences, San Jose, CA).

Reverse Transcription–Polymerase Chain Reaction

Total RNA was isolated from tumor tissues using an RNA isolation kit (Axygen, Union City, CA, AP-MN-MS-RNA-50) and reverse transcribed (Takara Bio Inc., Otsu, Japan, RR047A) following the manufacturer's protocols. Polymerase chain reaction (PCR) was performed on a CFX 96 real-time PCR thermocycler (Bio-Rad Laboratories, Hercules, CA) using specific primers and SYBR Premix Ex Taq II (Takara Bio Inc., Otsu, Japan, RR820A). Primer pairs are as follows: mouse 18S RNA, forward—CGCCGCTAGAGGTGAAATTCT and reverse—CGAACCTCCGACTTTCGTTCT; mouse interleukin-10 (IL-10), forward—ACCTGCTCCACTGCCTTGCT and reverse—GGTTGCCAAGCCTTATCGGA; mouse IL-6, forward—GATGGATGCTACCAAACTGGAT and reverse—CCAGGTAGCTATGGTACTCCAGA; mouse arginase 1, forward—GCTGTCTTCCCAAGAGTTGGG and reverse—ATGGAAGAGACCTTCAGCTAC; mouse indoleamine 2,3-dioxygenase (IDO), forward—TGGGACATTCCTTCAGTGGC and reverse—TCTCGAAGCTGCCCGTTCT; mouse transforming growth factor β (TGF-β), forward—CTCCCGTGGCTTCTAGTGC and reverse—GCCTTAGTTTGGACAGGATCTG. Data from the real-time PCR reactions were analyzed using CFX Manager Software 2.1 (Bio-Rad Laboratories). Relative changes of mRNA expression were analyzed with the $2-\triangle\triangle C_t$ method, with 18S RNA serving as an internal reference. These standardized data were used to calculate fold changes in gene expression. All real-time PCR amplifications were performed in triplicate.

ELISA

ELISA assay was performed on serum samples taken 21 days post-therapy to determine levels of IL-6 and TGF-β protein in the circulation. Briefly, 96-well microtiter plates (MultiSciences, Hang zhou, China, Catalog No. EK2812; EK2062) were coated with serum from tumor-bearing mouse for 2 hours at 37°C. For TGF-β, serum was acidified with 1 N HCl and then neutralized with 1 N NaOH. Biotinylated secondary antibody was then added to the plates for 1 hour at 37°C. Finally, streptavidin conjugated to HRP