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Original Article

Effect of angiogenesis inhibitor SU6668 in combination with 5-Fu on liver metastasis from transplantation tumors of human colorectal cancer in nude mice

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Abstract: This study was to investigate inhibiting effect of angiogenesis inhibitor SU6668 in combination with 5-Fu on liver metastasis from human colon cancer. Results showed that metastasis rates in SU6668+5-Fu group, SU6668 group, 5-Fu group decreased obviously ($P<0.01$). Compared with 5-Fu group and control group, microvessel density significantly decreased in SU6668+5-Fu group and SU6668 group ($P<0.05$). Vascular endothelial growth factor and base fibroblast growth factor reduced obviously in SU6668+5-Fu group, SU6668 group and 5-Fu group compared with control group, and there were significant differences among SU6668+5-Fu group, SU6668 group and 5-Fu group ($P<0.05$). Thus, SU6668 can inhibit liver metastasis from colorectal cancer through anti-angiogenesis, and it would have a synergistic effect in combination with 5-Fu. Therefore, SU6668 combined with 5-Fu could be considered as a safe and effective antitumor strategy.

Keywords: Neovascularization, pathologic, 5-Fu, colon neoplasm, liver metastasis, mice, inbred BALB C

Introduction

Colorectal cancer is a common and highly malignant gastrointestinal cancer, and can be treated by surgery with chemoradiotherapy as auxiliary treatment. However, the outcome is not ideal, and the postoperative liver metastases occur easily to cause relapse [1]. Data indicated that about 20% to 40% of the patients had liver metastases when clinically diagnosed; the incidence of liver metastasis following radical resection of colorectal cancer was still up to 40% to 50%, while liver metastasis occurred in more than 50% of the colorectal cancer-associated mortality [2]. Inhibition of liver metastasis was proved to significantly improve the therapeutic outcomes of colorectal cancer, as well as patients' survival and life quality. In clinic, the hepatectomy method was often used to cure liver metastasis from colorectal cancer. However, only 10% to 20% patients with liver metastasis from colorectal cancer are suitable for direct hepatic resection so far [3]. The growth, metastasis and relapse of colorectal cancer are angiogenesis-dependent. Therefore, antiangiogenic cancer therapy of angiogenesis inhibitors for colorectal cancer can be adopted to induce apoptosis in cancer cells, making the tumors remain in a dormant state, so that the growth, metastasis and relapse of colorectal cancer can be effectively inhibited [4]. In the present study, on an animal model of liver metastasis from colorectal cancer established by splenectomy, immunochemical tests were performed to detect the expressions of microvessel density (MVD), vascular endothelial growth factor (VEGF) and base fibroblast growth factor (bFGF) protein in liver metastasis from colorectal cancer, as so to investigate the efficacy of angiogenesis inhibitor SU6668 in the treatment of liver metastasis from colorectal cancer.

Materials and methods

Cell lines and culture

Human colorectal cancer cell line HT-29 were kindly provided by Dr. Jia Xiaoqing from Qilu Hospital of Shandong University and grown in RPMI Medium 1640 containing 10% fetal calf

serum (Gibico, USA); the culture contained 100 U/ml of penicillin and streptomycin each and was monolayer cultured in a saturated humidity incubator at 37°C and 5% CO_2. The cultured cell line was digested by 0.25% trypsin and underwent digestion and passage, before entering the exponential growth phase. The experimental cells were stained by using 0.4% trypan blue, with a dye exclusion rate >95%.

Experimental animals

BALB C, nu/nu female nude mice (5-6 weeks old and weighted 19 to 22 g) were purchased from the Institute of Laboratory Animal Sciences, CAMS&PUMC (Animal quality certificate no: Beijing 017) and reared under SPF conditions.

Major reagent

SU6668 was a product from Sugen, Inc. (U.S.). Polyclonal rabbit anti-mouse VEGF antibody, polyclonal bFGF antibody, monoclonal CD34 antibody, SABC kit and DAB chromogenic kit were all purchased from Wuhan Boster Biological Technology, Ltd.

Establishment and randomization of the models

Liver metastases from colorectal cancer models were established according to the splenectomy method proposed by Huang et al. [5]. Such animal models were well mimicking clinical signs of liver metastasis following radical operation of colorectal cancer, without concomitant induction of spleen metastasis during the formation of liver metastasis [6]. 48 nude mice were randomized into 4 groups by using a random number table; each group contained 12 mice. 0.1 ml of HT-29 cell suspension (containing 1×10^6 cells) were injected into the spleen of each nude mouse and the injections were finished within 1 min. 1 week after inoculation, the 4 groups of mice received once daily intraperitoneal injections of saline (control group), fluorouracil (30 mg/kg, 5-Fu group), SU6668 (200 mg/kg, SU6668 group) and 5-Fu combined with SU6668 (5-Fu 30 mg/kg, SU6668 200 mg/kg, 5-Fu+SU6668 group), respectively, for a total course of six weeks.

Growth of abdominal tumors

Neither diet nor drinking restriction was imposed on mice after inoculation. The mice remained usual activity, without any obvious changes in physical appearance. Body weights, spiritual status and dietary status were observed daily. On Week 7, all the nude mice were sacrificed by dislocating their vertebrates, for observing the metastasis of abdominal tumors; tumor nodules on the liver surface were counted by naked eyes while those in liver section were counted under light microscope. The livers of nude mice were removed and fixed with 10% neutral formalin to prepare paraffin-embedded tissue sections (4 μm); a total of 6 coronal sections were prepared and each section was separated by 0.4 cm; by using the largest coronal section as the center, microscopic counting was performed. Nodules that appeared on different sections were counted as 1 nodule. The sum of gross and microscopic counting was taken as the final number of liver metastatic nodules; a negative test referred to as no metastatic nodule revealed under gross or microscopic observation.

Judgment criteria of immunohistochemical findings

The expressions of MVD, VEGF and bFGF proteins in the liver metastasis tissues were detected by using the SABC method. For VEGF and bFGF staining: 100 cells were counted under high power field, if the number of positive cells (brown cytoplasm) positive cells <5%, the results was considered negative; otherwise (≥5%), the result was considered positive. The average gray scale and pixel area of VEGF in the tumor tissues were determined by using an IBSA 2.5 automatic image analysis system (KONTRON, Germany) and converted into positive unit (PU) according to formula; the PU values were used to quantize the VEGF and bFGF expressions. MVD counting was conducted according to the following method: 5 areas with the highest density of blood vessels in the tumors were localized at first under 100 × light microscope; afterwards, MVD was counted under 200× microscope; any yellow-stained cells or cell clusters was calculated as one MVD value, even if without demonstration of a tubular structure [7].

Statistical analysis

Pre-experimental weight, changes in weight after experiment, VEGF and bFGF expressions and MVD counts were tested by One-way ANOVA; while the rates of liver metastasis

Table 1. Liver metastasis rates and number of metastatic nodules in various groups

Groups	Cases (n)	Liver metastasis rate (%)	Number of metastatic nodules (*n*)		
			1≤*n*<10	10≤*n*<20	*n*≥20
Control	12	100	0	4	8
5-Fu	12	75	1	3	5
SU6668	12	41.7	1	1	3
Combined	12	25	1	1	1

among different groups and the proportionalities of nude mice with different number of metastatic nodules were both compared by using χ^2 test; all the data were processed by using SPSS10.0 statistical software.

Results

Changes in the living conditions and body weights of mice

All the 48 mice survived after the operation. On Day 3 after operation, weight loss was observed in some nude mice in every group; the body weights restored to the pre-operative levels on Day 7. On Day 20 post operation, decreased body weight, weight loss and slow motion were observed in some nude mice in the control group, 2 of these mice exhibited the manifestations of malignancy and died on Day 24 and 27, respectively; another mouse had slight ascites. In the other groups, all the nude mice depicted excellent dietary and activity status, while the body weights were slightly increased at the end of experiment. The changes in body weights between the pre-experimental and the post-experimental readings were statistically insignificant (P>0.05).

Liver metastasis in various groups

Most of the nude mice with liver metastasis manifested as multiple gray nodules on the liver surface, with decreased liver volume and hardened liver tissues. In some mice, although no tumor nodule was observed on liver surface, tiny (sesame like) metastatic foci were visible on coronal sections. The healthy liver tissues were brightly red and soft, without any visible nodules under naked eyes or microscope. The liver metastasis rates and number of metastatic nodules in various groups were shown in **Table 1**. The metastasis rates showed a descending order and the inter-group differences were statistical significant (P<0.01); When the number of nodules is larger than 20 in the control group, the difference in the number of liver metastatic nodules among various groups were statistically significant (*P*<0.01).

Expressions of VEGF, bFGF and MVD in liver metastatic tissues

As shown in **Table 2**, both the combined treatment group and the SU6668 group showed significantly decreased MVD (P<0.05) when compared with the 5-Fu group and the control group, respectively; although mice in the 5-Fu group had lower MVD counts than those in the control group (P>0.05). When compared with the control group, the combined treatment group, the SU6668 group and the F-5u group showed significantly decreased VEGF and bFGF levels; and also the inter-group differences among the combined treatment group, the SU6668 group and the F-5u group were statistically significant (*P*<0.05).

Discussion

Metastasis and recurrence are the leading causes of treatment failure of colorectal cancer; the novel anti-angiogenesis therapy provides a new approach for the treatment of colorectal cancer. There are two phases of tumor growth, namely, the avascular stage and the vascular stage When the volume of a solid tumor ≥2 mm, the growth of its central portion requires supply of oxygen and nutrients from the blood vessels; At the same time, the tumor cells start to infiltrate the peripheral tissues and invade the distant tissues via blood and lymphatic vessels, eventually resulted in formation of metastatic foci in the secondary regions [8]. The higher permeability of the neocapillary increases the chance for tumor cells entering the circulation; hence, early inhibition of angiogenesis in primary tumors could prevent tumor cells from entering the circulation and force the tumor cells into dormancy by blocking angiogenesis, so as to inhibit the metastasis.

SU6668 is a small-molecules oxindole receptor tyrosine kinases (RTK) inhibitor targeting at VEGF, bFGF and PDGF (platelet derived growth

Table 2. Expressions of VEGF, bFGF and MVD in liver metastasis liver tissues (n=12, $\bar{x}\pm s$)

Group	MVD	VEGF (PU)	bFGF (PU)
Control	33.84±7.56	18.04±4.20	16.42±4.64
5-Fu	29.65±6.32	14.32±4.33	12.05±3.86
SU6668	18.24±5.06	10.18±2.44	9.66±3.20
Combined	12.22±3.48	7.20±1.98	7.08±2.34

Footnotes: MVD: microvessel density; VEGF: vascular endothelial growth factor; PU: positive unit; bFGF: base fibroblast growth factor.

factor) [9-11]. It could act on multiple targets simultaneously, to block the synthesis and release of vascular endothelial growth factor and exert its anti-tumor effect through its anti-angiogenic properties [12, 13]; however, the product does not directly act on tumor cells [14]. As a novel angiogenesis inhibitor, SU6668 has grasped widely attention and its Phase I clinical trials have proved its safety and non-toxicity [15-18].

The inhibitory efficacy of SU6668 on the growth and metastasis of orthotropic implanted colorectal cancer in nude mice have been proved in a previous study [14]. In the present study, by establishing a nude mice liver metastasis from colorectal cancer mode, it showed that not only both the liver metastasis rates and the number of metastatic nodules reduced in the nude mice after SU6668 treatment, but the expressions of MVD, VEGF and bFGF metastatic tumor tissues also significantly decreased, suggesting that SU6668 exerted the anti-metastasis effect mainly by its anti-angiogenic properties. 5-Fu was able to inhibit, to a certain degree, the formation of new blood vessels by killing tumor cells and reducing VEGF/bFGF secretion, and thus leading to reduce liver metastasis. In various treatment groups, liver metastasis was all markedly inhibited, and such inhibition was particularly more obvious in the 5-Fu+SU6668 group, suggesting that SU6668 had not only strong inhibitory effect against metastasis of colorectal cancer, but also could synergistically acted with conventional chemotherapy drug in combination. Since SU6668 targets at vascular endothelial cells, which are unlikely to mutate due to its higher genetic stability, SU6668 anti-tumor therapy is unlikely to develop drug resistance. Combined therapy of SU6668 and other angiogenesis inhibitors with conventional chemotherapy or radiotherapy will cause an active effect and be a spotlight in the futures studies on its application. Zhang et al. [19] reported a potential improvement in the efficacy of conventional chemotherapy and radiotherapy if combined with SU6668; In patients at tumor regression period following surgical treatment, medication of an angiogenesis inhibitor (e.g. SU6668) has the potential to force the micrometastatic loci into dormancy, so as to inhibit the growth of residual lesion, and even to cure clinical recurrence/distant metastasis caused by small lesions, and thus reaching an unprecedented efficacy for subclinical cancer treatment.

In conclusion, by inhibiting formation of tumor blood vessels, angiogenesis inhibitor SU6668 had strong inhibitory effect against liver metastasis from colorectal cancer in nude mice and could synergistically act with the conventional chemotherapy drug, and thus it could be considered as a new approach for clinical treatment of liver metastasis from colorectal cancer.

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References

[1] van Gijn W, Krijnen P, Lemmens VE, den Dulk M, Putter H and van de Velde CJ. Quality assurance in rectal cancer treatment in the Netherlands: a catch up compared to colon cancer treatment. Eur J Surg Oncol 2010; 36: 340-344.

[2] Cady B and Stone MD. The role of surgical resection of liver metastases in colorectal carcinoma. Semin Oncol 1991; 18: 399-406.

[3] Folprecht G, Grothey A, Alberts S, Raab HR and Kohne CH. Neoadjuvant treatment of unresectable colorectal liver metastases: correlation between tumour response and resection rates. Ann Oncol 2005; 16: 1311-1319.

[4] Belur LR, Podetz-Pedersen KM, Sorenson BS, Hsu AH, Parker JB, Carlson CS, Saltzman DA, Ramakrishnan S and McIvor RS. Inhibition of

angiogenesis and suppression of colorectal cancer metastatic to the liver using the Sleeping Beauty Transposon System. Mol Cancer 2011; 10: 14.

[5] Huang P, Lou RC, Zhang XD and Zhou XK. Establishment of a model of human colon cell cancer liver metastases in nude mice with the splenectomy and evaluation of liver metastases. Cancer 2000; 19: 879-882.

[6] Higashijima J, Shimada M, Chikakiyo M, Miyatani T, Yoshikawa K, Nishioka M, Iwata T and Kurita N. Effect of splenectomy on antitumor immune system in mice. Anticancer Res 2009; 29: 385-393.

[7] Weidner N. Current pathologic methods for measuring intratumoral microvessel density within breast carcinoma and other solid tumors. Breast Cancer Res Treat 1995; 36: 169-180.

[8] Folkman J and Shing Y. Angiogenesis. J Biol Chem 1992; 267: 10931-10934.

[9] Farace P, Galie M, Merigo F, Daducci A, Calderan L, Nicolato E, Degrassi A, Pesenti E, Sbarbati A and Marzola P. Inhibition of tyrosine kinase receptors by SU6668 promotes abnormal stromal development at the periphery of carcinomas. Br J Cancer 2009; 100: 1575-1580.

[10] Piirsoo A, Kasak L, Kauts ML, Loog M, Tints K, Uusen P, Neuman T and Piirsoo M. Protein kinase inhibitor SU6668 attenuates positive regulation of Gli proteins in cancer and multipotent progenitor cells. Biochim Biophys Acta 2014; 1843: 703-714.

[11] Wang L, Liu Z, Ma D, Piao Y, Guo F, Han Y and Xie X. SU6668 suppresses proliferation of triple negative breast cancer cells through downregulating MTDH expression. Cancer Cell Int 2013; 13: 88.

[12] Heinrich MC, Blanke CD, Druker BJ and Corless CL. Inhibition of KIT tyrosine kinase activity: a novel molecular approach to the treatment of KIT-positive malignancies. J Clin Oncol 2002; 20: 1692-1703.

[13] Laird AD, Vajkoczy P, Shawver LK, Thurnher A, Liang C, Mohammadi M, Schlessinger J, Ullrich A, Hubbard SR, Blake RA, Fong TA, Strawn LM, Sun L, Tang C, Hawtin R, Tang F, Shenoy N, Hirth KP, McMahon G and Cherrington. SU6668 is a potent antiangiogenic a nd antitumor agent that induces regression of established tumors. Cancer Res 2000; 60: 4152-4160.

[14] Zeng QL, Chu ZH, Zhou K and Luo XJ. [Effect of Endostatin and SU6668 combined with 5-FU on human colon cancer xenograft in nude mice]. Zhonghua Wei Chang Wai Ke Za Zhi 2008; 11: 376-378.

[15] Jiang XT, Tao HQ and Zou SC. [Effect of angiogenesis inhibitor SU6668 on the growth and metastasis of gastric cancer in SCID mice]. Zhonghua Wei Chang Wai Ke Za Zhi 2006; 9: 335-337.

[16] Xiong HQ, Herbst R, Faria SC, Scholz C, Davis D, Jackson EF, Madden T, McConkey D, Hicks M, Hess K, Charnsangavej CA and Abbruzzese JL. A phase I surrogate endpoint study of SU6668 in patients with solid tumors. Invest New Drugs 2004; 22: 459-466.

[17] Marzola P, Degrassi A, Calderan L, Farace P, Crescimanno C, Nicolato E, Giusti A, Pesenti E, Terron A, Sbarbati A, Abrams T, Murray L and Osculati F. In vivo assessment of antiangiogenic activity of SU6668 in an experimental colon carcinoma model. Clin Cancer Res 2004; 10: 739-750.

[18] Zhu XD, Sun HC, Xu HX, Kong LQ, Chai ZT, Lu L, Zhang JB, Gao DM, Wang WQ, Zhang W, Zhuang PY, Wu WZ, Wang L and Tang ZY. Antiangiogenic therapy promoted metastasis of hepatocellular carcinoma by suppressing host-derived interleukin-12b in mouse models. Angiogenesis 2013; 16: 809-820.

[19] Zhang GF, Wang YH and Wang Q. Antiangiogenic therapy for gastrointestinal tumors. World Chin J Digestol 2001; 9: 1180-1184.