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ORIGINAL RESEARCH

Investigation of the efficacy of a bevacizumab-cetuximab-cisplatin regimen in treating head and neck squamous cell carcinoma in mice

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Abstract The efficacy of bevacizumab plus cetuximab-based chemotherapy remains unclear in head and neck squamous cell carcinoma (HNSCC). The aim of this study was to investigate the anticancer efficacy of a bevacizumab-cetuximab-cisplatin triple-agent combination in treating mouse HNSCC. SCC-VII tumor-bearing C3H mice were treated with bevacizumab, cetuximab and cisplatin either alone or in various combinations. In vivo results showed that the largest delay in tumor growth and the maximum increase in survival were not in the triple-agent combination therapy, but in the bevacizumab plus cisplatin therapy. TUNEL assay showed that the apoptosis indices in bevacizumab plus cisplatin group and a triple-agent combination group were 31.6±12.0% and 6.9±1.3%, respectively. Western blot showed that down-regulation of Bcl-2 and up-regulation of cleaved caspase-3 contributed to the anticancer effect of a triple-agent combination and bevacizumab plus cisplatin. Moreover, the maximum level of cleaved caspase-3 expression was not found in the triple-agent combination group but in the bevacizumab plus cisplatin group. Bevacizumab plus cisplatin therapy is better than bevacizumab-cetuximab-cisplatin triple therapy in the mouse HNSCC model. Our findings suggest that the bevacizumab-cetuximab-cisplatin regimen may not be suitable for treating HNSCC.

Keywords Bevacizumab · Cetuximab · Cisplatin · Chemotherapy · Head and neck squamous cell carcinoma (HNSCC) · Anticancer activity

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Introduction

Multiple cellular pathways are involved in the growth and dissemination of tumors. The two key pathways are the vascular endothelial growth factor (VEGF) pathway and the epidermal growth factor receptor (EGFR) pathway. Evidence has shown that the VEGF and EGFR pathways are closely related [1]. VEGF signaling is up-regulated by EGFR expression, and VEGF overexpression promotes EGFR resistance. Therefore, in theory, a strategy that inhibits both pathways may improve anticancer efficacy [2].

Scientists have developed some inhibitors specific to VEGF and EGFR pathways. Bevacizumab (Avastin®) and cetuximab (Erbitux®) are the two main target drugs. Bevacizumab is a humanized monoclonal antibody. It binds to and inhibits VEGF biological function [3]. Cetuximab is a recombinant chimeric human-murine immunoglobulin G1 antibody. It binds to the extracellular domain of EGFR and promotes receptor internalization and degradation without receptor phosphorylation and activation [4, 5]. Both bevacizumab and cetuximab have been used with standard chemotherapy in treating certain cancers including lung cancer [3], colon cancer [6], and advanced head and neck squamous cell carcinoma (HNSCC) [7].

Bevacizumab in combination with paclitaxel can yield a remarkable inhibition of HNSCC tumor xenografts [8]. In a phase I study, Seiwert et al. showed that bevacizumab at a dose of 10 mg/m^2 every 2 weeks with decreased chemo-radiotherapy (fluorouracil, hydroxyurea, and radiation) has an antitumor activity in poor-prognosis head and neck cancer patients [9]. In addition, in a phase II clinical study, Merlano et al. showed that the combination of cetuximab, cisplatin, fluorouracil, and radiotherapy yielded a very high proportion of complete responses (71%) in locally advanced head and neck cancer [10]. In another preclinical study, Prichard et al. showed that bevacizumab combined with cetuximab and doxorubicin dramatically delayed an orthotopic model of anaplastic thyroid carcinoma growth in athymic nude mice [11]. Bozec et al. also reported that bevacizumab combined with another EGFR tyrosine kinase inhibitor, erlotinib, and irradiation could significantly decrease the tumor mass and reduced the number of metastatic nodes [12].

Given that agents targeting the EGFR or VEGF pathways have demonstrated beneficial effects in these settings, an obvious next step would be to combine EGFR- and VEGF-specific inhibitors for cancer treatment. However, two prospective randomized phase III studies using bevacizumab plus cetuximab-based chemotherapy in treating metastatic colorectal cancer have reported unfavorable results: addition of cetuximab resulted in a worse outcome in both studies [13, 14]. Why does the outcome differ from expected results? In this study, we investigate the efficacy of bevacizumab plus cetuximab-based therapy and its underlying mechanism using a mouse HNSCC allograft model.

Materials and methods

Drugs and reagents

Cisplatin was purchased from Qilu Pharmaceutical Co. LTD. (Shandong, China). Bevacizumab was obtained from Genetech (San Francisco, CA, USA). Cetuximab was produced by Imclone Systems (New York, NY, USA). Dulbecco's Modified Eagle's Medium (DMEM) and fetal bovine serum were purchased from Invitrogen (Carlsbad, CA). Antibodies were purchased from the following vendors: cleaved caspase-3 antibody, Cell Signaling Technology (Beverly, MA, USA); Bcl-2, Bax and β-actin antibodies, Santa Cruz Biotechnology Inc. (Santa Cruz, CA, USA). An In Situ Cell Death Detection kit was purchased from Promega (Madison, WI, USA).

Cell culture

The murine head and neck squamous carcinoma cell line SCC-VII was maintained in DMEM containing 10% fetal bovine serum in a humidified incubator with 5% CO_2. Cells in midlogarithmic growth (~75% confluence) were used for the following experiments.

Tumor allograft model

To establish the SCC-VII tumor allograft model, 4×10^6 SCC-VII cells were subcutaneously (s.c.) injected into the right flank of 6-week-old C3H female mice. Tumors were allowed to reach 120–160 mm^3 in size, and mice were randomized into eight groups of five animals each. Mice were treated with cisplatin in a dose of 8 mg/kg of body weight, cetuximab at a dose of 1 mg/kg of body weight by intraperitoneal (i.p.) injection once a week, and bevacizumab at a dose of 15 mg/kg of body weight by intratumoral injection twice a week. The animals were treated for 3 weeks. Tumors were measured twice a week with a digital caliper. Tumor volume was calculated using the following formula: $1/2\times\text{length}\times\text{width}^2$ [15]. Mouse weight was monitored for evaluating the toxicity of drugs. Tumors reaching 2000 mm^3 were considered a lethal burden for the mice. Ten mice per group from two independent experiments were used to generate the survival curve. All procedures involving animals were approved by Peking University's Institutional Animal Care Committee. All possible measures were taken to minimize any pain or discomfort.

Detecting apoptosis by TUNEL assay

The terminal deoxynucleotidyl transferase dUTP nick end labeling (TUNEL) assay was performed according to the manufacturer's instructions. The sections were counterstained with 4′,6-diamidino-2-phenylindole (DAPI) and recorded using an Olympus dynamic positioning (DP) controller. Apoptotic cells were expressed as the mean of the four highest areas identified within a 400-fold field. The apoptosis index was determined by counting TUNEL-

positive cells per total number of DAPI-positive cells in four high-power fields, avoiding necrotic tumor areas [16, 17].

Western blot analyses of molecules involved in apoptosis

Proteins were prepared from SCC-VII tumors. Mice with SCC-VII tumor allografts were treated briefly with corresponding regimens as described above. Twenty-four hours post-treatment, tumor tissue samples from each group were ground quickly in liquid nitrogen, homogenized in RIPA lysis buffer with protease inhibitors on ice. Homogenates were centrifuged at 12,000×g for 30 min at 4°C, and supernatants were retained. Protein concentration was determined using the bicinchoninic acid (BCA) protein assay, and 40 μg of protein were loaded for each sample. Proteins were separated on SDS-polyacrylamide gel and transferred to polyvinylidene difluoride membrane. The membranes were blocked in 5% non-fat dry milk for 1 h, and probed with the following antibodies against Bcl-2, Bax, cleaved caspase-3, and β-actin separately at 4°C overnight. After incubation with peroxidase-linked secondary antibodies, immunoreactive proteins were visualized by ECL reagent (Applygen Technology Inc. Beijing, China).

Statistical analyses

The product-limit method, with Kaplan-Meier curves, was used to summarize the distribution of survival time, and a logrank test was used to compare with survival curves among these groups. Tumor size was expressed as mean± standard deviation (SD) of two independent experiments and compared using the analysis of variance between groups (ANOVA) test. $P<0.05$ was considered statistically significant.

Results

Bevacizumab and cisplatin combination therapy was better than a bevacizumab-cetuximab-cisplatin triple-agent combination therapy

We evaluated the effect of a bevacizumab-cetuximab-cisplatin triple-agent combination on SCC-VII tumors propagated in the right flank of C3H mice. A triple-agent combination therapy significantly delayed tumor growth compared with single-agent treatments ($P<0.05$). Bevacizumab alone and cetuximab alone did not, in fact, affect tumor growth rate compared with control (both $P>0.05$). Cisplatin alone produced slight delayed tumor growth compared with control ($P>0.05$, Fig. 1a). In comparison with double-agent treatments, a triple-agent combination therapy yielded the similar result in tumor growth as cetuximab plus cisplatin therapy. Interestingly, the maximum delay in tumor growth was found in a bevacizumab and cisplatin combination therapy. There was significant difference in tumor size between the bevacizumab and cisplatin combination and the triple-agent combination therapy ($P<0.05$). But bevacizumab plus cetuximab did not affect tumor growth rate compared with control (both $P>0.05$). In addition, bevacizumab plus cisplatin and cetuximab plus cisplatin produced a statistically significant decrease in tumor size compared with single-agent treatments and untreated control ($P<0.05$, Fig. 1b).

The Kaplan-Meier curve showed that a triple-agent combination therapy significantly increased the survival time compared with bevacizumab alone ($P<0.01$), cisplatin alone ($P<0.01$), and control ($P<0.01$) (Fig. 1c). A cetuximab and cisplatin combination produced a survival result similar to that of a triple-agent combination therapy. Interestingly, the maximum increase in survival was found in the bevacizumab and cisplatin combination group: 45 days post-treatment, 70% of the mice were alive in a bevacizumab and cisplatin combination group, whereas only 30% of the mice were alive in a cetuximab plus cisplatin group, and 20% of the mice were alive in a triple-agent combination group ($P<0.05$, Fig. 1d).

More apoptotic cells were found in tumors treated with bevacizumab and cisplatin combination therapy via TUNEL assay

Animals with SCC-VII-bearing allografts were administered cisplatin, bevacizumab plus cisplatin, cetuximab plus cisplatin, or bevacizumab plus cetuximab plus cisplatin for 24 h. Tumor samples were removed and subjected to histological examination and apoptosis TUNEL assay analyses. Quantitative analyses showed that the apoptosis indices for untreated control, bevacizumab plus cisplatin, cetuximab plus cisplatin and a triple-agent combination were 1.2±0.6%, 31.6±12%, 6.9±1.3%, and 9.7±2.8%, respectively. There was a significant difference between a bevacizumab plus cisplatin therapy versus control, a cetuximab plus cisplatin therapy, and a triple-agent combination therapy ($P<0.05$ for all comparisons; Fig. 2), while no significant difference was found between cetuximab-cisplatin and a triple-agent combination therapy ($P>0.05$; Fig. 2).

Down-regulation of Bcl-2 and up-regulation of cleaved caspase-3 expression contributed to tumor cell apoptosis

Western blot analysis showed that Bcl-2 was clearly down-regulated in tumors treated with cetuximab plus cisplatin, and completely inhibited with bevacizumab plus cisplatin

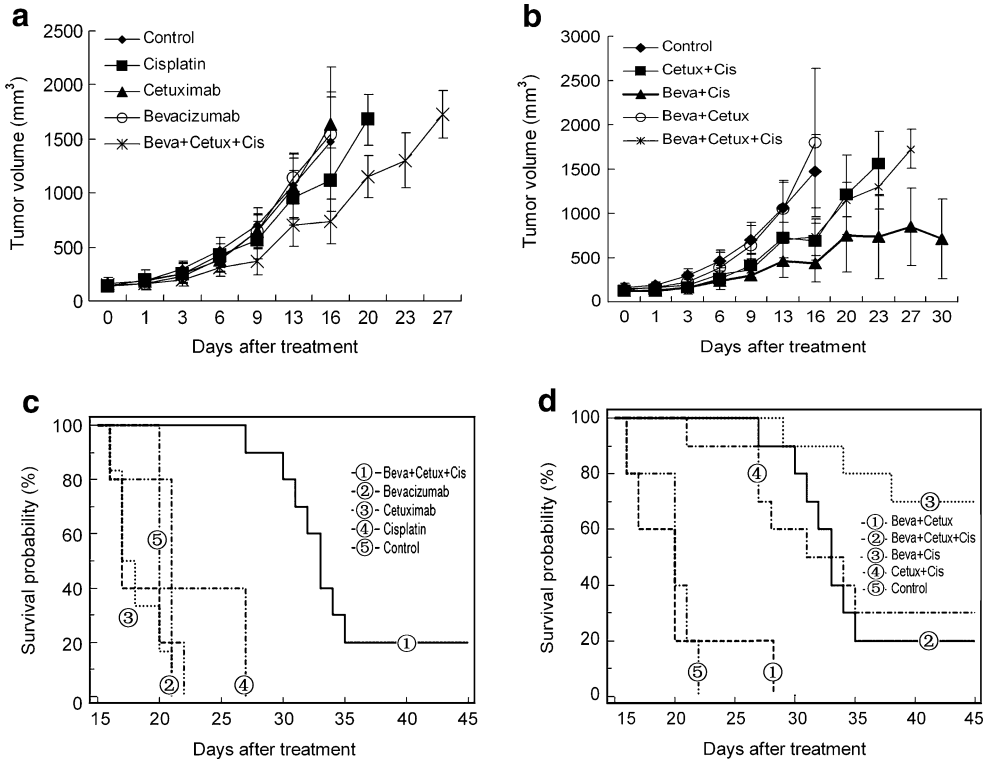

Fig. 1 Tumor growth and survival curve of SCC-VII tumor allografts in C3H mice treated with various combinations of bevacizumab, cetuximab and cisplatin. SCC-VII tumor-bearing mice were established and treated as described in the "Materials and methods" section. Each data point represents the mean volume±standard deviation. Ten mice/group from two independent experiments were monitored for survival. Inhibition of tumor growth was described as a bevacizumab-cetuximab-cisplatin triple-agent combination versus a single-agent therapy (**a**) or versus a double-agent therapy in tumor growth (**b**). Increase in survival was shown in a triple-agent combination versus the single-agent therapies (**c**) or versus the double-agent therapies (**d**), respectively. The bevacizumab plus cisplatin therapy was the best strategy, significantly delaying SCC-VII tumor growth and significantly prolonging survival time compared with other groups ($P<0.05$)

treatment and a triple-agent combination treatment. Bax was only slightly up-regulated in bevacizumab alone-treated tumors and bevacizumab plus cisplatin-treated tumors. Furthermore, it was clearly up-regulated in the other treatments. The maximum level of Bax expression was found in tumors treated with bevacizumab plus cetuximab and in a triple-agent combination. All treatments enhanced cleaved caspase-3 expression as compared with untreated control tumors. Its relative levels (from low to high) were untreated control, bevacizumab alone, cetuximab alone, bevacizumab plus cetuximab, cetuximab plus cisplatin, a triple-agent combination, cisplatin alone, and bevacizumab plus cisplatin. The maximum level of cleaved caspase-3 expression was found in tumor treated with bevacizumab plus cisplatin (Fig. 3).

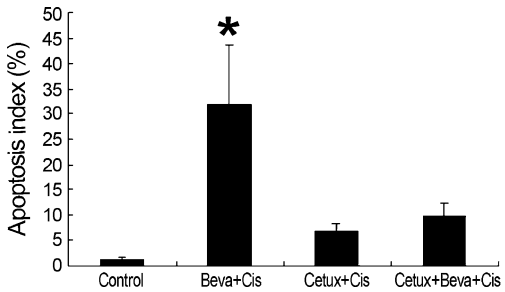

Fig. 2 TUNEL assay analyses. Apoptosis indices showed that a markedly higher frequency of apoptotic tumor cells was found in tumors from animals treated with bevacizumab plus cisplatin compared with untreated control, and animals treated with cetuximab plus cisplatin or a triple-agent combination. *$P<0.05$

Discussion

It seems the bevacizumab plus cetuximab-based chemotherapy could be a promising approach for cancer treatment. However, the unexpected outcomes indicate the complexity of underlying mechanisms. Tol et al. compared capecitabine and oxaliplatin chemotherapy plus bevacizumab with or without cetuximab in 755 colorectal cancer patients [13, 18] and found that outcome (progression-free survival) was significantly worse in patients who received cetuximab. A similar result was reported by Cohen et al. using the EGFR tyrosine kinase inhibitor erlotinib com-

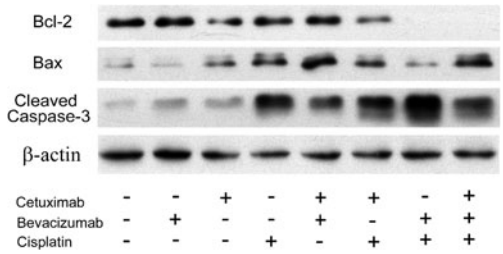

Fig. 3 Western blot analyses. Total tumor lysates were resolved by SDS-PAGE followed by immunoblot analyses to detect the Bcl-2, Bax, and cleaved caspase-3 expression. The results showed that down-regulation of Bcl-2 and up-regulation of cleaved caspase-3 expression contributed to apoptosis caused by bevacizumab-cetuximab-cisplatin and bevacizumab plus cisplatin treatments. The maximum level of cleaved caspase-3 expression was found in the tumor treated with bevacizumab plus cisplatin, which was consistent with tumor growth and survival results. β-actin served as a loading control

bined with bevacizumab to treat patients with recurrent or metastatic HNSCC. They found that although patients tolerated the combination of erlotinib and bevacizumab well, only a few patients seemed to derive a sustained benefit and complete responses [19]. Punt and Tol provided some explanations: 1) enhancement of adverse effect of treatment; 2) the signaling pathway interactions caused by the different drug combinations; and 3) attenuation of the effect of oxaliplatin owing to high serum protein levels induced by the administration of two monoclonal antibodies (bavacizumab and cetuximab) [20]. These may contribute to the detrimental results. Owing to the complicated mechanism of bevacizumab plus cetuximab-based chemotherapy, in this study we explored the underlying mechanism from a different perspective, apoptosis. Apoptosis-related proteins, including Bcl-2 and caspase-3, were chosen as targets.

Normally, human tumors spontaneously arise in an immunocompetent system. In this study, we used a host with an immunocompetent system to investigate the anticancer effect of bevacizumab plus cetuximab plus cisplatin. This would provide a close approximation of the cancer treatment process in human and reduce any bias owing to the use of an immunodeficient mouse model. Mouse HNSCC SCC-VII tumor is the ideal immunocompetent syngeneic preclinical model for approximating human squamous cell carcinoma. It is derived from spontaneous oral squamous cell carcinoma in C3H mouse [21–23].

Before this study, we had confirmed that bevacizumab alone had significant inhibitory effect on murine hemangioendothelioma cell line (EOMA) growth in mice via intratumoral injection [24]. In addition, we also confirmed that cetuximab alone had a growth-inhibitory effect on murine squamous carcinoma cell line SCC-VII in vitro, and its growth-inhibitory effect was significantly enhanced when combined with cisplatin (data not shown). Based on our data and previous studies, we adapted intratumoral injection of bevacizumab and intraperitoneal injection of cetuximab and cisplatin to investigate the anticancer efficacy of a bevacizumab-cetuximab-cisplatin strategy on SCC-VII-bearing mice. We found that bevacizumab plus cisplatin and cetuximab plus cisplatin produced a statistically significant reduction in SCC-VII tumor growth and an increase in survival of SCC-VII tumor-bearing mice. These results indicated that either bevacizumab or cetuximab with cisplatin were highly effective against SCC-VII-established HNSCC tumors. Both bevacizumab and cetuximab exerted their own biological functions in this case. The results were consistent with those obtained in previous studies about bevacizumab- or cetuximab-based chemotherapies in the treatment of colon, lung, breast and pancreatic carcinomas [25, 26].

Based on the data described above, combination of bevacizumab, cetuximab and cisplatin should, in theory, improve anticancer effect in treating SCC-VII tumor. But the maximum delay in tumor growth and the maximum increase in survival were not in mice treated with a triple-agent combination therapy, but in those with treated with bevacizumab plus cisplatin therapy. Our explanation is that cetuximab may attenuate the cooperative effects of bevacizumab and cisplatin in mouse HNSCC allografts. To confirm this suspicion, a histological examination was conducted by TUNEL assay. The apoptosis indices in the bevacizumab plus cisplatin group and a triple-agent combination group were 31.6±12% and 9.7±2.8%, respectively. This could explain why the largest delay in tumor growth and the best survival rate were in the bevacizumab plus cisplatin group.

Western blot results provided evidence concerning the underlying mechanism involved. In the treated tumors, we investigated two molecules from the Bcl-2 family (the anti-apoptosis protein Bcl-2 and the pro-apoptosis protein Bax) along with apoptosis executioner caspase-3 expression. The Bcl-2 family is the best-characterized group of apoptosis-mediating factors [27]. Bcl-2 both directly and indirectly prevents the release of cytochrome *c* from mitochondria and contributes to anti-apoptosis [28]. Bax is a pro-apoptotic protein that resides in the cytosol and translocates to mitochondria upon induction of apoptosis [29, 30]. In the present study, we found that while the Bcl-2 band was completely gone in tumors treated with bevacizumab plus cisplatin and the triple-agent combination, the pro-apoptotic protein Bax was markedly up-regulated in tumors treated with the triple-agent combination. The results suggest that the tumor cells in the bevacizumab plus cisplatin group and the triple-agent combination group were more susceptible to apoptosis than tumor cells in other groups. These data also explain why the two groups

had better anticancer outcomes than other groups. But Bcl-2 expression results cannot explain why the outcomes in the bevacizumab plus cisplatin group were better than those in the triple-agent combination group. Therefore, cleaved caspase-3 was addressed to answer this question. Western blot showed that it was clearly up-regulated by cisplatin and its combinations with other agents. The maximum cleaved caspase-3 expression was in tumors treated with bevacizumab plus cisplatin, and its expression in tumors treated cetuximab plus cisplatin was higher than that in tumors treated the triple-agent combination. These results were consistent with the tumor growth and survival curve results, as well as with the histological examination results. This elucidated why the outcome in the bevacizumab plus cisplatin group was better than that in the triple-agent combination group. Cetuximab may play a role in attenuating the anticancer effect of bevacizumab plus cisplatin.

Cancer cells within tumors may display different phenotypes, and this may make cancer cells sensitive to certain kinds of drugs [31]. Studies have showed that EMMPRIN expression and wild-type BRAF are required for response to bevacizumab therapy in HNSCC xenografts [32] and to panitumumab or cetuximab in metastatic colorectal cancer [33]. In addition, the current classical chemotherapy treatment in head and neck cancer treatment is a doublet with cisplatin and fluorouracil. The effect of bevacizumab-cetuximab-cisplatin-fluorouracil combination therapy for HNSCC tumors with both wild-type BRAF and EMMPRIN phenotypes deserves investigation in the future. This regimen may provide more valuable information for clinical application.

In conclusion, the preliminary results demonstrated that the combination of bevacizumab and cisplatin was highly effective against SCC-VII-established HNSCC tumors. Cetuximab attenuated the cooperative anticancer activity of bevacizumab plus cisplatin. Down-regulation of Bcl-2 and up-regulation of cleaved caspase-3 expression contributed to the tumor cell apoptosis caused by bevacizumab-cetuximab-cisplatin and bevacizumab plus cisplatin treatments. However, the precise mechanism is still unclear. More data will be needed to further evaluate bevacizumab and cetuximab combination-based therapy in the future. Our findings suggest that the bevacizumab-cetuximab-cisplatin regimen may not be suitable for treating HNSCC.

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Conflict of interest statement None declared.

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