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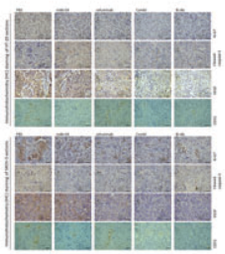

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RESEARCH PAPER

A human IgG-like bispecific antibody co-targeting epidermal growth factor receptor and the vascular endothelial growth factor receptor 2 for enhanced antitumor activity

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ABSRTRACT

Both Epidermal Growth Factor Receptor (EGFR) and the Vascular Endothelial Growth Factor Receptor 2 (VEGFR2) play critical roles in tumorigenesis. We hypothesized co-targeting EGFR and VEGFR2 using a bispecific antibody might have significant therapeutic potential. Here,we designed and produced a human IgG-like bispecific antibody (Bi-Ab) based on the variable regions of cetuximab (an anti-EGFR antibody) and mAb-04 (an anti-VEGFR2 antibody developed in our lab) . The Bi-Ab was found to inhibit the proliferation, survival and invasion of cancer cells via ablating phosphorylation of receptor and downstream signaling. In vivo efficacy was demonstrated against established HT-29 and SKOV-3 xenografts grown in nude mice. Studies revealed our Bi-Ab was able to restrain xenografted tumor growth and prolong survival of mice through inhibiting cell proliferation,promoting apoptosis and anti-angiogenesis. In contrast to cetuximab or mAb-04 alone, our Bi-Ab exhibits enhanced antitumor activity and has equal or slightly superior activity to their combination (Combi). It shows promise as a therapeutic agent, especially for use against tumors EGFR and/or VEGFR2 over-expressing malignancies.

Abbreviations: EGFR, epidermal growth factor receptor; VEGFR2, vascular endothelial growth factor receptor 2; NSCLC, non-small cell lung cancers; VEGF, vascular endothelial growth factor; MAPK, mitogen-activated protein kinase; AKT, protein kinase B; JNK, c-Jun N-terminal kinase;; CHO, chinese Hamster Ovary; NO, nitric oxide; PCR, Polymerase Chain Reaction; SPR, surface plasmon resonance

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Introduction

Epidermal Growth Factor Receptor (EGFR) is the cell-surface receptor for some extracellular protein ligands, such as epidermal growth factor family.[1] It has been involved in various malignancies such as non-small cell lung cancers (NSCLC) and colon cancers.[2-5] EGFR is activated through interaction with its specific ligands, then undergoes a transition from an inactive monomeric form to an active homodimer.[6] Moreover, EGFR may pair with another member of the ErbB receptor family to create an activated heterodimer. The dimerization irritates its intrinsic intracellular protein-tyrosine kinase activity, which promotes autophosphorylation of several tyrosine (Y) residues in the C-terminal domain of EGFR, including Y992, Y1045, Y1068, Y1148 and Y1173.[7] The downstream signaling proteins trigger several signal transduction cascades, principally the mitogen-activated protein kinase (MAPK), c-Jun N-terminal kinase (JNK) and protein kinase B (AKT) pathways.[8] Additionally, activation of EGFR improves angiogenesis by enhancing vascular endothelial growth factor (VEGF) levels and increasing cellular levels of nitric oxide (NO).[9]

Vascular Endothelial Growth Factor Receptor 2 (VEGFR2) is a member of VEGF receptor family, which regulate the formation of blood and lymphatic vessel.[10] VEGFR2 is expressed by endothelial cells and various cancers including breast cancer and NSCLC.[11-12] As the principal receptor leading angiogenesis in tumoral tissue, VEGFR2 has potent tyrosine kinase activity and is primarily associated with the induction of angiogenesis mediated by VEGF.[13] VEGF-induced homodimerization of VEGFR2 leads to a strong autophosphorylation of VEGFR2 on tyrosine residues.[14] Once autophosphorylated, VEGFR2 activates MAP-kinase and DNA synthesis, leading to pathological angiogenesis.[15] Other VEGFR2 dependent pathways reported include PI3K-PKB-AKT focal adhesion kinase, Src kinase, Rho family of GTPases, and other multifunctional docking proteins and adaptors.[16] Therefore, VEGFR2 could be used as a target for tumor therapy.[17-20]

Cetuximab, a monoclonal antibody targeting EGFR, is used to treat a lot of cancers. Unfortunately, majority of patients acquire resistance in the end.[21] The EGFR-independent activation of the AKT and MAPK pathway (such as VEGFR2-inducing) may be one of the resistance mechanisms.[22] Although, blocking VEGF/VEGFR2 with antibody such as the ramucirumab (a VEGFR2 antibody) is a strategy for tumor therapy,

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EGF/EGFR-induced VEGF production may limit the effect of this strategy.[23]

Based on the literature we hypothesized co-targeting EGFR and VEGFR2 may be beneficial in minimizing the activation of MAPK and AKT signaling and inhibition of angiogenesis for the treatment of EGFR related cancers. Previously, we reported a human anti-VEGFR2 antibody (mAb-04), which inhibits the activation of VEGFR2.[24] In this report, we designed and produced a bispecific antibody (Bi-Ab) with the variable regions from 2 different antibodies. The Bi-Ab is expected to demonstrate superior antitumor activity to the parental antibody and could therefore be applied to EGFR- and VEGFR2-overexpressing malignancies.

Results

Bi-Ab targets EGFR and VEGFR2

We generated Bi-Ab from the DNA sequences of anti-EGFR/anti-VEGFR2 taFv and the human IgG1 Fc fragment. The presence of hinge region in the Fc resulted in the production of stable dimmers (Fig. 1A-B) that showed stability at 37°C for 15 d (Fig. 1C). SDS-PAGE analysis of Bi-Ab under non-reducing conditions yielded a protein band of ~150kDa (similar to cetuximab and mAb-04). Under reducing conditions, Bi-Ab yielded a single protein band of ~78kDa with expected mobility. As controls, cetuximab and mAb-04 gave 2 major bands: the IgG light chain (~25kDa) and the IgG heavy chain (~50kDa). Results of SPR analysis showed that the Bi-Ab can bind both EGFR and VEGFR2 simultaneously. (Fig. 1D)

SPR analysis was employed to measure the binding affinities of Bi-Ab to EGFR and VEGFR2 (Fig. 2A-B). The data indicated that the affinity of Bi-Ab to EGFR/VEGFR2 is similar to mAb-04 and cetuximab (Table 1). The binding affinities of cetuximab, mAb-04 and Bi-Ab to HT-29 or SKOV-3 cells were investigated with flow cytometer. All the 3 antibodies demonstrated relatively high binding affinity to HT-29 and SKOV-3 cells (Fig. 2C-D). The binding levels of 10 μg/ml Bi-Ab with HT-29 / SKOV-3 cells were 46.5 / 40.6 %, that of mAb-04 and cetuximab were 33.5 / 27.1% and 61.4 / 64.0%, respectively.

Bi-Ab inhibits phosphorylation of EGFR and VEGFR2 and down-regulates PI3K/AKT and MAPK signaling

To analyze the synergistic blocking of both EGFR and VEGFR2 through the antibodies (Combi or Bi-Ab) in PI3K/AKT and MAPK signaling pathway, we examined the phosphorylation of EGFR, VEGFR2, AKT and Erk1/2 (Fig. 3A) in HT-29 and SKOV-3 cells treated with the antibodies. We first examined the effect of the antibodies on tyrosine phosphorylation of EGFR and VEGFR2 activated by EGF and VEGF. The phosphorylation of EGFR and VEGFR2 were blocked by cetuximab and mAb-04 respectively. Further, both EGFR and VEGFR2 were blocked by Bi-Ab or Combi. Either cetuximab or mAb-04 inhibited the phosphorylation of AKT and Erk1/2 incompletely in the presence of ligands (EGF and VEGF). However, the Bi-Ab and the Combi significantly inhibited the phosphorylation of AKT and Erk1/2 (Fig. 3B-C). These results suggested that co-targeting EGFR and VEGFR2 with Bi-Ab has superior

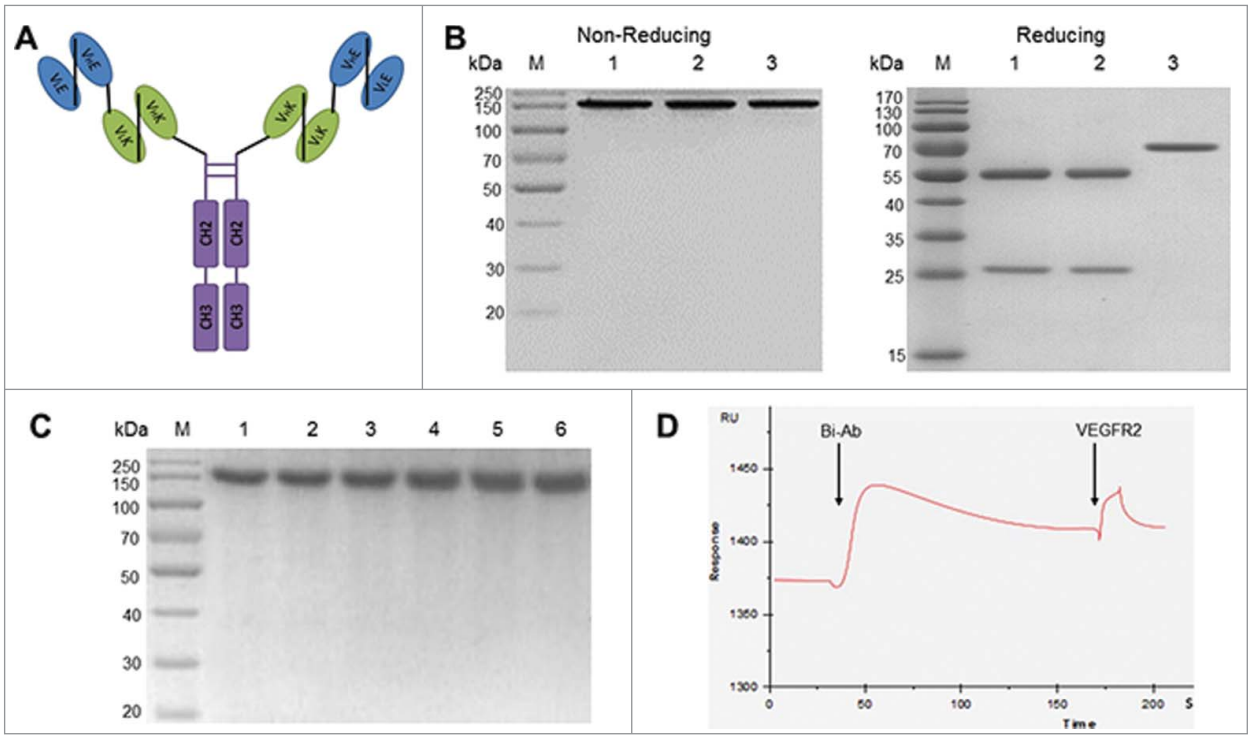

Figure 1. The structure, stability and bispecificity of Bi-Ab. Structure of the anti-EGFR/VEGFR2 bispecific antibody (A) V_LE, variable light region of cetuximab; V_HE, variable heavy region of cetuximab; V_LK, variable light region of mAb-04; V_HK, variable heavy region of mAb-04. SDS-PAGE analysis of the purified Bi-Ab (B) Lane M, marker; lane 1, cetuximab; lane 2, mAb-04; lane 3, Bi-Ab. SDS-PAGE was used to analyze the thermostability of Bi-Ab (C) Lane M, marker; lane 1–6, sample incubated for 0, 3, 6, 9, 12 and 15 d at 37°C. The surface plasmon resonance spectroscopy analysis (D) VEGFR2 was injected after Bi-Ab were flowed over the EGFR-immobilized sensor chip. RU, resonance units.

Table 1 SPR analysis of the binding affinities of antibodies to EGFR or VEGFR2.

Binding	k_a ($M^{-1}s^{-1}$)	k_d (s^{-1})	K_D (M)	Chi^2
Bi-Ab-EGFR	2.22×10^{6}	3.21×10^{-3}	1.45×10^{-9}	0.856
Bi-Ab-VEGFR2	3.51×10^{6}	8.32×10^{-3}	2.37×10^{-9}	1.721
cetuximab-EGFR	1.83×10^{6}	1.35×10^{-3}	7.38×10^{-10}	0.538
mAb-04-VEGFR2	1.91×10^{6}	2.71×10^{-3}	1.42×10^{-9}	1.204

Chi^2, correlation fit between the calculated and experimental data. The data on sensorgrams of mAb-04-VEGFR2 and cetuximab-EGFR are not shown.

antitumor activity on co-expression high levels of EGFR and VEGFR2 in cancer cells.

Bi-Ab effectively inhibits proliferation and invasion of cancer cell, and preserves antibody-dependent cell-mediated cytotoxicity (ADCC) activity

MTT assay was used to analyze the effect of Bi-Ab on HT-29 and SKOV-3 cells proliferation. The results showed that, Bi-Ab treatment effectively inhibited the proliferation of HT-29 and SKOV-3 cells with EGF and VEGF stimulated in dose-dependent manner *in vitro* (Fig. 4A-B). Notably, although Combi treatment showed enhanced inhibition of HT-29 and SKOV-3 proliferation compared with cetuximab or mAb-04 treatment alone, all the other treatments showed less potent than Bi-Ab, especially at high antibody concentrations (over 6nM for HT-29, over 125nM for SKOV-3). When stimulated with EGFR/VEGFR2, inhibition levels (%) of Bi-Ab on HT-29 / SKOV-3 was about 70 / 53 at most, that of mAb-04, cetuximab and Combi were 16 / 18, 37 / 27 and 44 / 39, respectively.

The effect of Bi-Ab on HT-29 and SKOV-3 cells invasion was analyzed by Transwell assay. The invasion was significantly reduced with the different antibodies, and the Bi-Ab demonstrated high inhibitory potential on HT-29 and SKOV-3 invasion than cetuximab and mAb-04 alone or Combi.(Fig. 4C-D).

Additionally, Bi-Ab showed comparable or slightly lower ADCC activity than cetuximab, however it was significantly

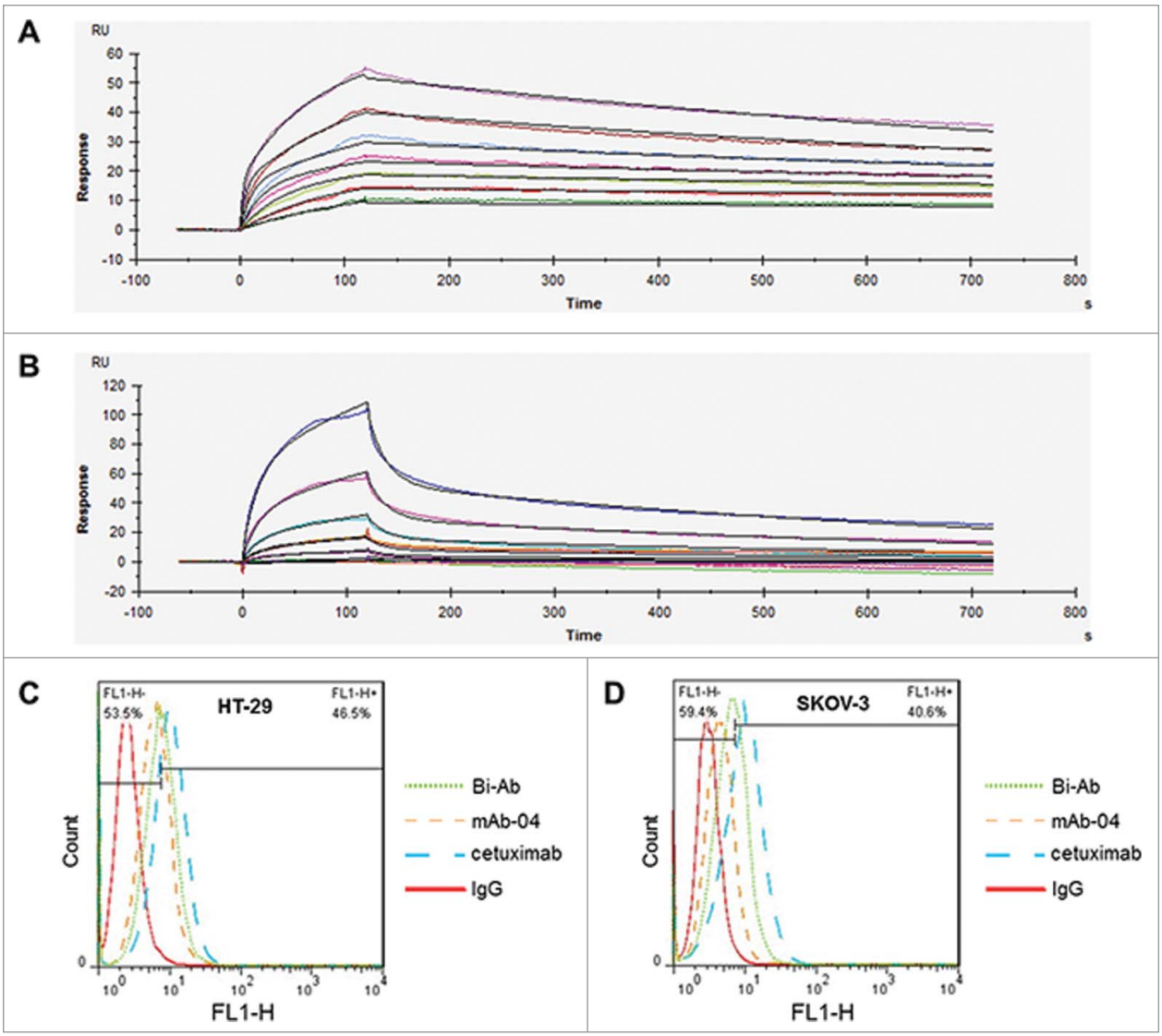

Figure 2. The binding of Bi-Ab to recombinant EGFR/VEGFR2 ectodomains and membrane-associated EGFR/VEGFR2. Surface plasmon resonance spectroscopy was used to analysis the binding kinetics of Bi-Ab to recombinant EGFR and VEGFR2 ectodomains ((A) and B). The equilibrium dissociation rate constant (K_D) of Bi-Ab to EGFR or VEGFR2 were 1.45×10^{-9}M and 2.37×10^{-9}M respectively. Flow cytometry was used to investigate the binding of Bi-Ab to membrane-associated EGFR and VEGFR2 ((C)- F). The histogram overlay showed that Bi-Ab binds to HT-29 and SKOV-3 cells line with a relatively high binding levels.

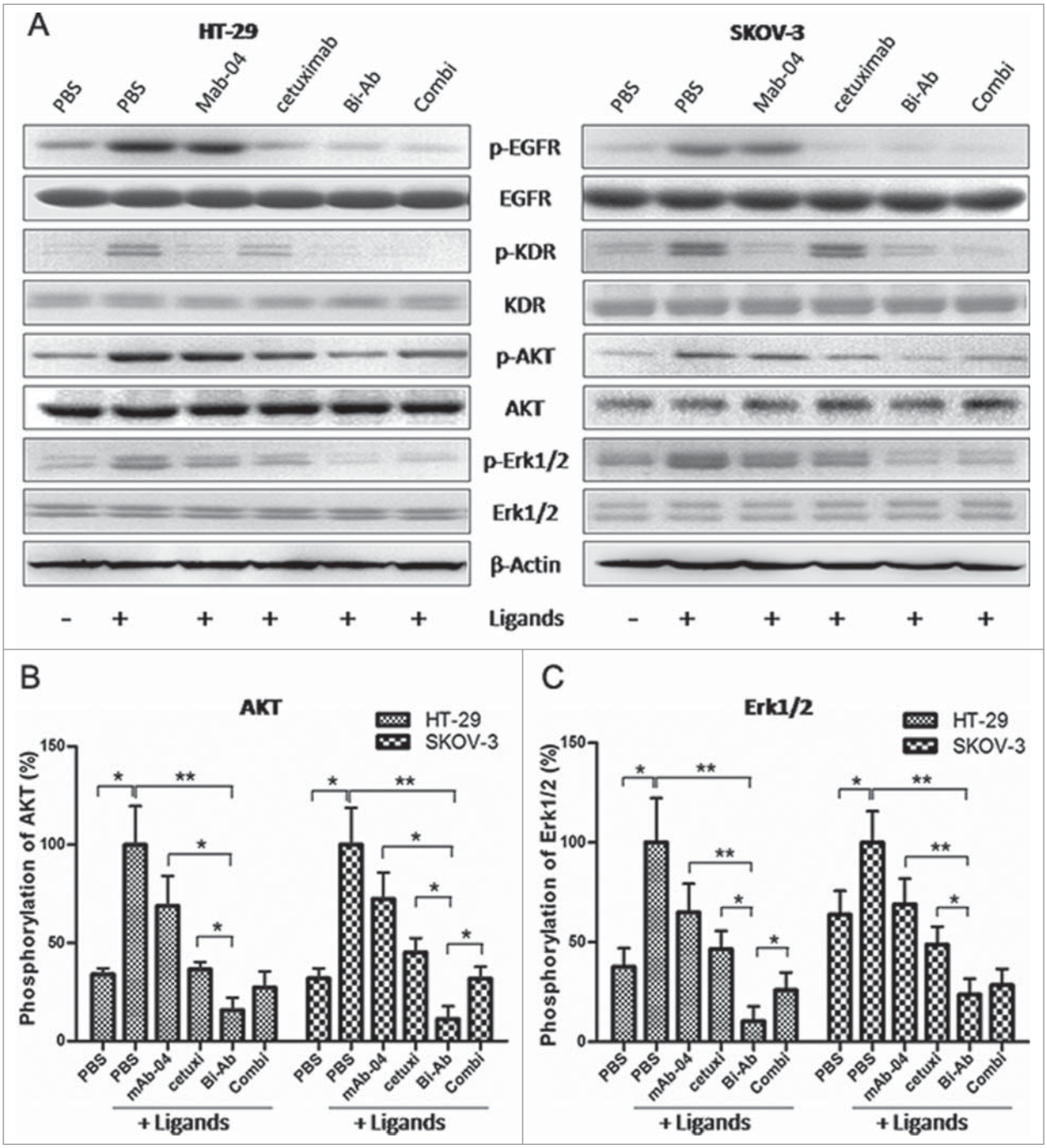

Figure 3. Bi-Ab inhibits phosphorylation of EGFR and VEGFR2 and down-regulates AKT and MAPK signaling. (A) Western blot to show the inhibition on phosphorylation of EGFR, VEGFR2, AKT and Erk1/2 in HT-29 and SKOV-3 cells after antibodies treatment. ((B) and C) Phosphorylation level of AKT and Erk1/2 in HT-29 and SKOV-3 cells. The data presented as the mean ± SD, are from a representative experiment, n = 3. $^{*}P < 0.05$; $^{**}P < 0.01$.

higher than that of mAb-04, all the treatment conditions were less potent than that of Combi (Fig. 4E). These data suggest that Bi-Ab remains effective in killing EGFR- and/or VEGFR2-overexpressing tumor cells through ADCC *in vivo*.

Bi-Ab induces cancer cell apoptosis, inhibits endothelial tube formation

Apoptosis assay was carried out to assess the ability of Bi-Ab to stimulate apoptosis in HT-29 and SKOV-3 cells. Annexin V-FITC/PI Vybrant apoptosis assay kit was used. The Bi-Ab demonstrated a more potent apoptotic stimulating potential than cetuximab, mAb-04 and Combi in SKOV-3 cells *in vitro*. However, HT-29 cells were insensitive to the treatment of all antibodies (Fig. 5A, C).

Since EGFR signaling and VEGFR2 signaling have been demonstrated to enhance angiogenesis,[10,25] the tube formation assay was carried out to investigate the anti-angiogenic potential of Bi-Ab, as against cetuximab or mAb-04 on tube formation by HUVEC cells. Similar to the Combi, Bi-Ab demonstrated relatively more potent restraining effect on tube formation by HUVEC cells compared to mAb-04 or cetuximab (Fig. 5B, D).

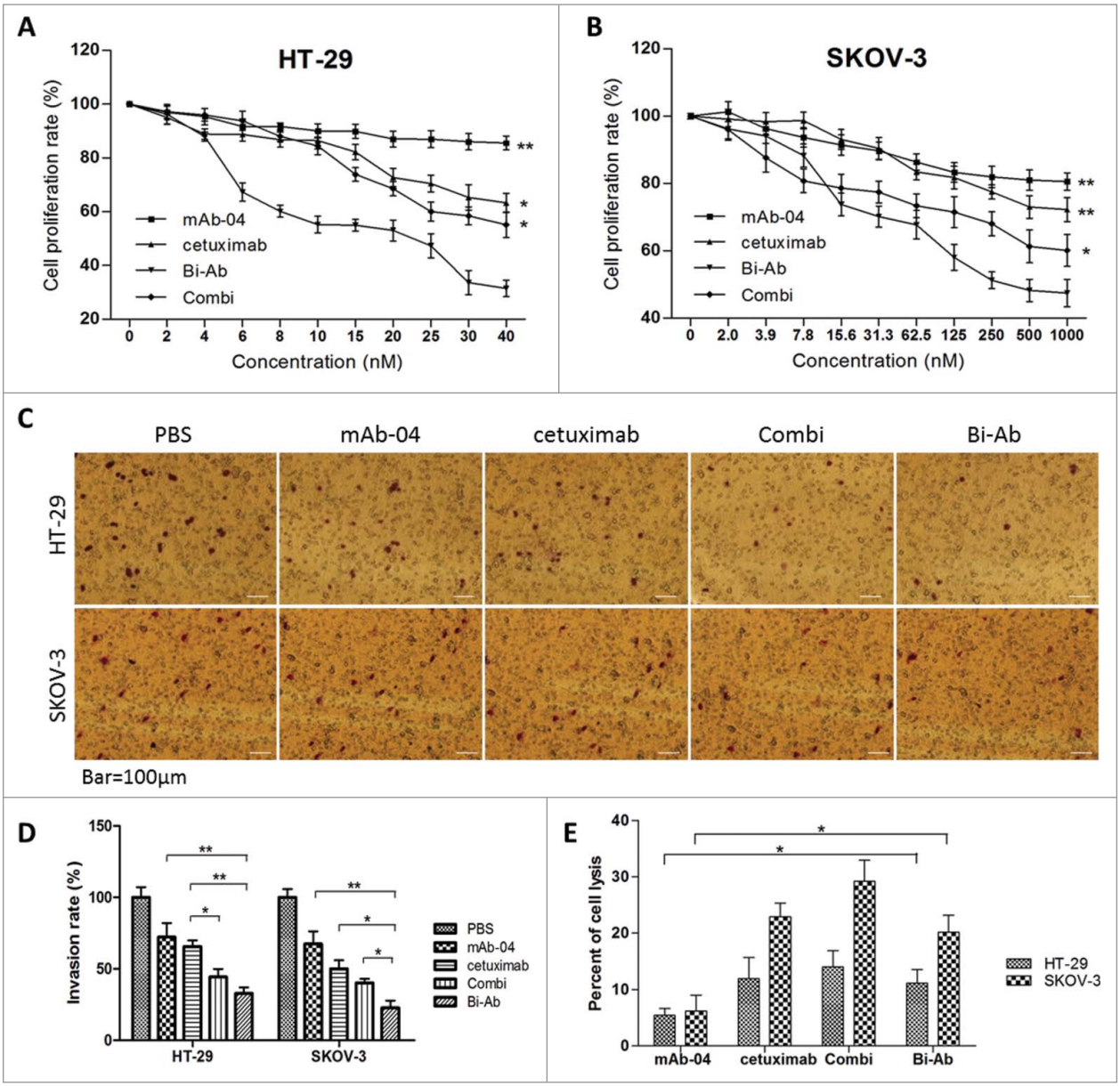

Figure 4. Bi-Ab showed the most effective inhibition of proliferation on HT-29 and SKOV-3 cells compared to mAb-04, cetuximab or Combi with EGF and VEGF stimulated ((**A**)**and B**). Three independent experiments were performed in triplicate, the means ± SD of triplicate experiment are shown, $^{*}P < 0.05$; $^{**}P < 0.01$ versus treatment with Bi-Ab treatment. Photomicrographs of transwell invasion assay indicated that Bi-Ab could effectively inhibit the invasion of HT-29 and SKOV-3 cells induced by EGF and VEGF ((**C**)**and D**). Quantitative analysis of the transwell invasion assay showing that Bi-Ab treatment significantly increased the inhibition of HT-29 and SKOV-3 cells invasion when compared to mAb-04 and cetuximab. The data presented as the mean ± SD, are from a representative experiment, 5 independent experiments were performed in triplicate, $^{*}P < 0.05$; $^{**}P < 0.01$. Percent ADCC of the antibodies on HT-29 and SKOV-3 (E). The data presented as the mean ± SD, each antibody was tested in triplicate, the assays were repeated once, n = 3, $^{*}P < 0.05$.

Bi-Ab shows potent antitumor effect in HT-29 and SKOV-3 xenograft models

Balb/C nude mice xenografted with HT-29 and SKOV-3 tumors were treated with antibodies. PBS-treated tumors grew rapidly, whereas tumors were inhibited in different extent with the different antibodies (Fig. 6A-B). Compared with PBS, mAb-04 or cetuximab treatment, Bi-Ab treatment significantly inhibited the growth of HT-29 or SKOV-3 tumors xenografts.

The survival rates of HT-29 and SKOV-3 tumor-bearing mice were compared following the 5 different treatment regimens (Fig. 6C-D). Median survival times and terminal survival rate of HT-29/SKOV-3 tumor-bearing mice for the 5 different groups are shown in Table 2. These studies showed that the Bi-Ab treatment did not only demonstrate better inhibition of tumor growth but also prolonged median survival of xenograft-bearing animals.

Effect of Bi-Ab on proliferation, apoptosis and angiogenesis in vivo

To further investigate the anti-tumor mechanisms of Bi-Ab *in vivo*, the proliferation, apoptosis and angiogenesis in the tissue sections of the tumors were immunohistochemically analyzed. Cell proliferation and apoptosis were evaluated with Ki-67 and cleaved caspase-3 staining methods respectively. In addition,

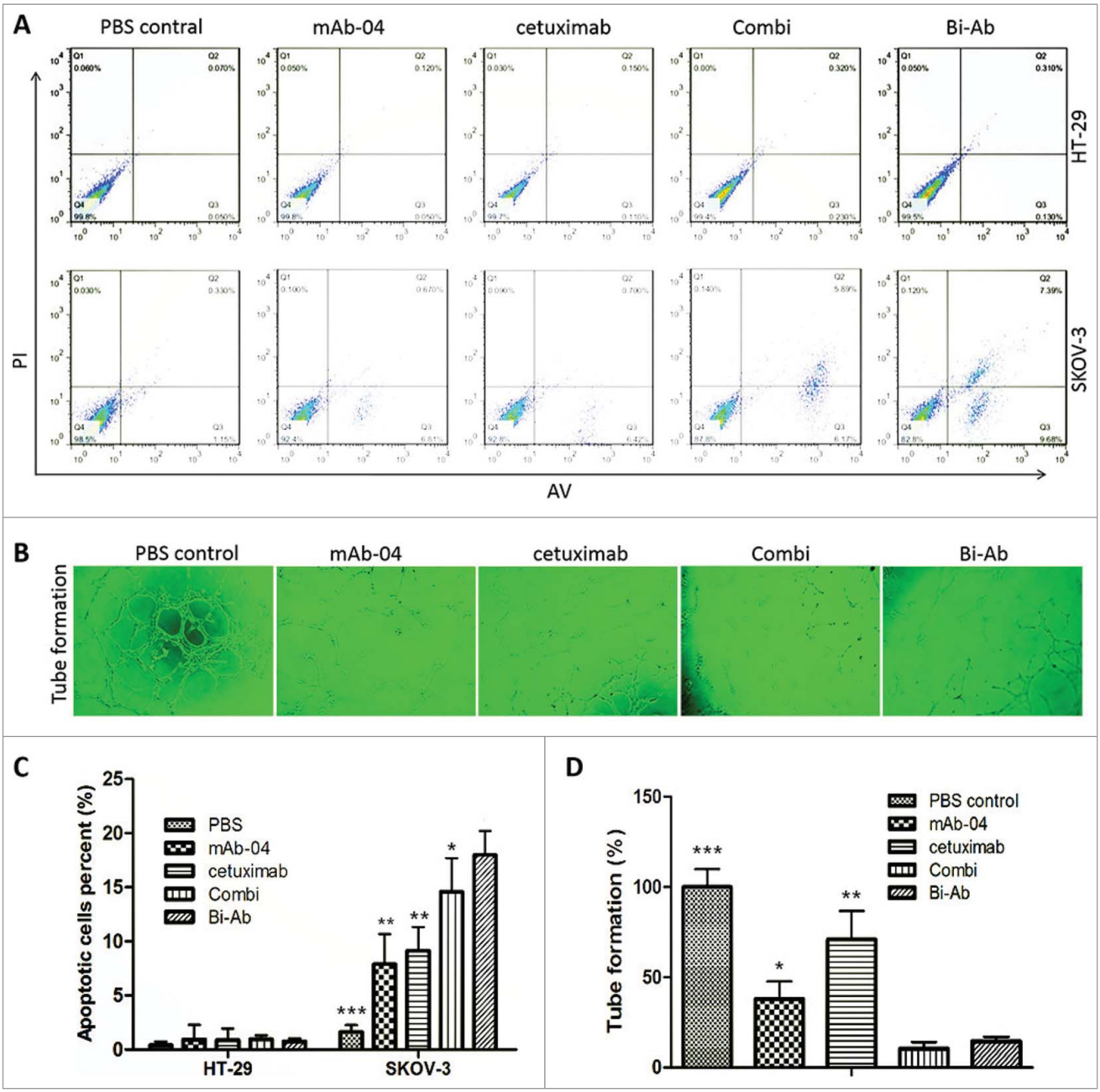

Figure 5. The apoptosis was analyzed by flow cytometry, endothelial tube formation was performed using HUVECs tube formation assay. ((A)and C) Bi-Ab treatment increased apoptosis in SKOV-3 cells than cetuximab and mAb-04 alone or Combi, but not HT-29. (B) HUVEC tube-like photomicrographs showing the significant effects of Bi-Ab on HUVECs tube formation. (D) Similar to the Combi, Bi-Ab demonstrated relatively more potent restraining effect on tube formation by HUVEC cells compared to mAb-04 or cetuximab. Three independent experiments were performed in triplicate, the means ± SD of triplicate experiment are shown ($^{*}P < 0.05$; $^{**}P < 0.01$ vs. Bi-Ab treatment).

anti-VEGF and CD31 antibodies were used to evaluate tumor angiogenesis (Fig. 7A-B). These results are consistent with the *in vitro* studies. Bi-Ab and the Combi, significantly reduced the percentage of Ki-67-positive cells compared to cetuximab or mAb-04 alone (Fig. 7C). Contrary to the cell line study, the results of cleaved caspase-3 staining demonstrated that Bi-Ab treatment induced significant apoptosis in HT-29 and SKOV-3 tumors (Fig. 7D). The VEGF and CD31 staining showed that, treatment with Bi-Ab significantly reduce blood vessel density compared with mAb-04 or cetuximab treatment (Fig. 7A-B and E).

Discussion

EGFR has been involved in various malignancies such as NSCLC, colon, breast, head and neck, and pancreatic cancers. VEGFR2 is over-expressed in various cancers such as breast cancer and non-small cell lung cancer. It has been reported that inhibition of EGFR or VEGFR2 signaling restrains the proliferation of ovarian tumors.[23,26] In addition, the combination of EGFR and VEGFR2 antagonists enhanced anti-tumor effect, which suggests EGFR and VEGFR2 co-targeted therapy has therapeutic potential in colon tumors.[27-28] In this article, we designed a bispecific antibody based on a known EGFR antibody and our

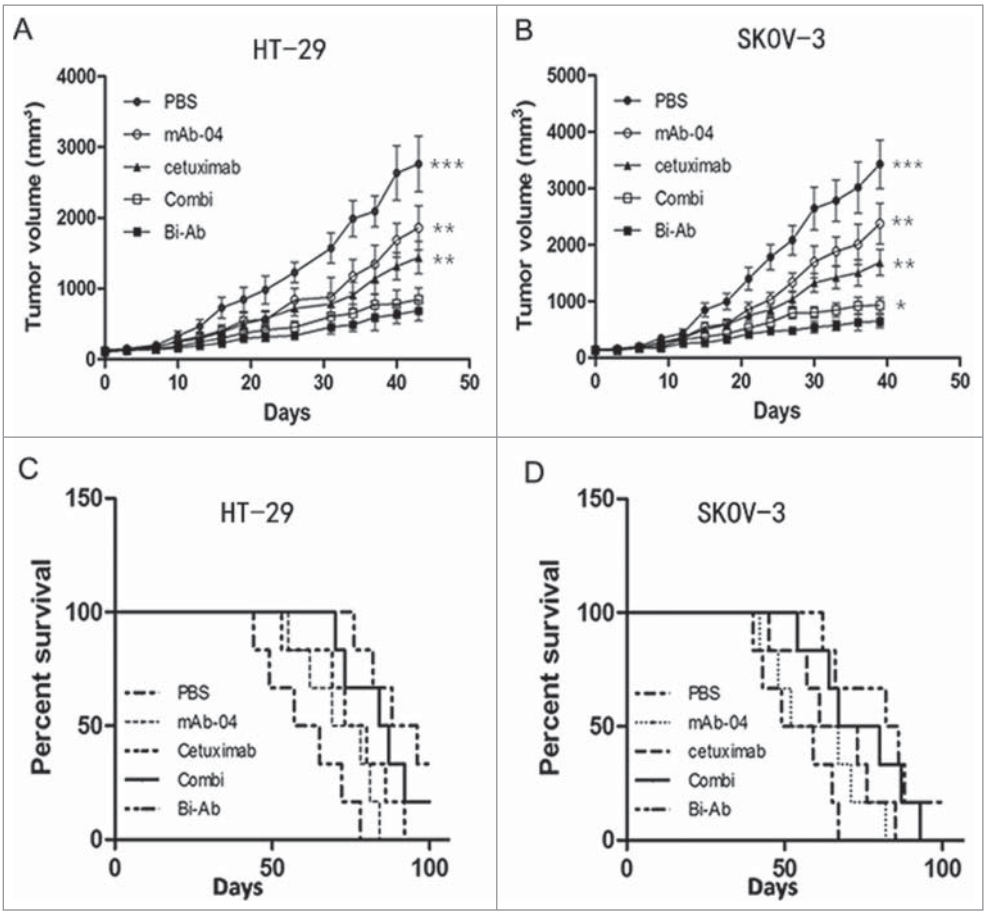

Figure 6. The Bi-Ab shows potent antitumor effect on HT-29 and SKOV-3 tumor xenografts in nude mice. ((A) and B) Bi-Ab suppressed tumor growth, tumor diameter was measured with a vernier caliper ($^{*}P < 0.05$; $^{**}P < 0.01$; $^{***}P < 0.005$ versus treatment with Bi-Ab). The survival rates of HT-29 and SKOV-3 tumor-bearing mice ((C)and D). The median survival and terminal survival rate were shown in Table 2.

VEGFR2 antibody. Its efficacy was evaluated both in vitro assays and nude mice tumor xenograft models, indicating co-targeting EGFR and VEGFR2 may be of clinical relevance in treating EGFR and/or VEGFR2 overexpressing malignant.

The functional affinity of Bi-Ab to EGFR or VEGFR2 were measured by SPR. The result showed that there were disparity between the level of signal in the SPR experiments and the predicted Rmax based on the molecular weights of the ligand and analyte. This may be explained by the inactivation of ligands in immobilization and the steric inhibition of binding for all the ligands and analytes are biomacromolecules. Further investigation will be needed in future.

It has been reported that, both EGFR and VEGFR2 contribute to the activation of MAPK and AKT signaling in cancer cells and angiogenesis in tumors.[8,16] Activation of MAPK and AKT pathways mediated by mutations or amplification of cell surface receptors is a frequent event in human cancers, which leads to molecular alterations in genes encoding key components of the pathways.[29–31] These intracellular signaling pathways regulate proliferation, motility, differentiation and survival of cancer cells. The PI3K and MAPK pathways interact in multiple ways that limit the activity of targeting the MAPK or the PI3K pathways.[32–33] In cancer cells which express both EGFR and VEGFR2, MAPK and AKT signals are regulated by both EGFR and VEGFR2. Hence, simultaneous targeting to both receptors would yield an enhanced inhibition in the signal transduction. We found that both Bi-Ab and Combi treatment inhibited phosphorylation of AKT and Erk1/2 more thoroughly compared with cetuximab or mAb-04 treatment alone (Fig. 3). Interestingly, the effect of co-targeting EGFR- and VEGFR2-overexpressing cancer cells with Bi-Ab has slightly superior activity to the Combi, which is presumably due to the enhanced inhibition of AKT and MAPK signal pathway and ligand-induced EGFR phosphorylation in HT-29 cells and SKOV-3 cells (Fig. 3). Nonetheless, intensive understanding will be further needed through investigating whether heterodimer exists between EGFR and VEGFR2 and whether it contributed to this result via steric to antibodies (cetuximab and mAb-04).

ADCC, an important mechanism of action for antitumor antibodies, is mediated by Fc fragment. ADCC assay demonstrates that the Fc mediated cytotoxicity activity of Bi-Ab was significantly higher than mAb-04 and slightly lower than cetuximab. This phenomenon may be explained by the same Fc fragment in antibodies and the different binding affinity of antibodies to cancer cells (Fig. 2C-D).

Angiogenesis is a critical step in tumor progression, providing nutrients and oxygen for the proliferation and metastasis of the tumor cells. VEGF and its receptors (VEGFR1, VEGFR2 and Neuropilin1) are key regulators of angiogenesis and have been the key targets for anti-angiogenesis therapy in recent

Table 2 Median survival and 100-day survival rate (%).

Drug	Median survival (d) (HT-29)	Survival at 100d (%) (HT-29)	Median survival (d) (SKOV-3)	Survival at 100d (%) (SKOV-3)
PBS	61**	0	54**	0
mAb-04	73.5*	0	59.5**	0
cetuximab	76.5*	0	67*	0
Combi	85.5	16.7	73.5*	0
Bi-Ab	92	33.3	84	16.7

*$P < 0.05$
**$P < 0.02$, versus Bi-Ab treatment, analyzed by log rank tests.

times.[34] In this study, targeting VEGFR2 was used to suppress angiogenesis. Surprisingly, co-targeting EGFR and VEGFR2 was more effective to suppress angiogenesis than VEGFR2-targeting alone, which may be due to the decrease in the production of VEGF by EGFR-inhibition (Fig. 7). Although, VEGFR2 is the main receptor of VEGF in angiogenesis, VEGF also induces angiogenesis through VEGFR1 and Neuropilin1.[35–36] As such, targeting EGFR decreases VEGF production, resulting in angiogenesis suppression from synergistic VEGFR2-targeting.[9]

Our current data showed that none of the treatments induced apoptosis in HT-29 cells (Fig. 5A and C). The failure of Bi-Ab on stimulating apoptosis of HT-29 is partly due to coexpression of multiple EGFR family members such as EGFR, HER2 and HER4, especially HER2.[37] HER2 stimulate S-phase proliferation and inhibit apoptosis by increasing the expression of Wilms' Tumor 1 protein in cancer cells.[38] Degradation of HER2 induces apoptosis in HER2-overexpressing cancer cells.[39-40]

Our data suggest that Bi-Ab has anti-angiogenic effect *in vivo*. Antiangiogenic therapy lessened leakage of plasma proteins from microvessels in the tumor tissue, and reduced vascular density and blood flow in total tumor. This may contribute to the initiation of tumor cell apoptosis.[41] It has also been reported that, anti-VEGFR2 antibody was able to promote apoptosis *in vivo*.[42] These could account for the reason why the Bi-Ab falied in promoting apoptosis in HT-29 cells *in vitro*, but stimulated apoptosis in HT-29 tumor xenograft (Figs. 5 and 7).

In conclusion, co-targeting EGFR and VEGFR2 with Bi-Ab or Combi has great anti-cancer effect both *in vitro* and *in vivo*. We found that Bi-Ab has equal or slightly enhanced efficacy compared with the Combi. These results indicate that antitumor synergy of combined EGFR and VEGFR2 targeted therapy is more effective than single targeting in inhibiting EGFR- and/or VEGFR2 over-expressing tumors. With lower cost in manufacture and improved efficacy, the Bi-Ab has more potential in targeted therapy for EGFR and/or VEGFR2 over-expressing tumors.

Materials and methods

Construction of Bi-Ab

Employing the reported method, we effectively generated an anti-EGFR/anti-VEGFR2 tandem single-chain variable fragment (taFv) with variable regions of cetuximab targeting EGFR and mAb-04 targeting VEGFR2. The DNA of the taFv was then fused to the Fc fragment of human IgG1 with linker (GGGGS) through overlap PCR. The fusion DNA and pMH3 vector were digested with *NotI* and *XbaI* and the resulting fragments joined using T4 DNA ligase. Nucleotide sequence of the recombinant vector (pMH3-taFv-Fc) was confirmed by Sangon Biotech. (Shanghai, China).

Expression and purification of Bi-Ab

After confirmed, the recombinant expression vector (pMH3-taFv-Fc) was transfected into CHO-s cell line by electroporation. After screening stable transfectants with 1mg/ml G418, Bi-Ab was expressed by CHO-s cells, and purified from the culture supernatants using Protein A affinity purification. Subsequent to purification, samples of Bi-Ab (200mM in PBS) were incubated for 0, 3, 6, 9, 12 and 15days at 37 °C respectively and then analyzed by SDS-PAGE.

Surface plasmon resonance spectroscopy

The functional affinity between Bi-Ab and EGFR or VEGFR2 were analyzed with surface plasmon resonance (SPR) spectroscopy using Biacore system (Biacore X100, GE Healthcare). EGFR/VEGFR2 (ligand) was immobilized on Sensor Chip CM5 (GE Healthcare, BR-1000–12) up to 1000 resonance units. Different concentrations of Bi-Ab or cetuximab/mAb-04 (analyte, 40–0.625nM, fold2- serially diluted) in running buffer (HBS-EP, pH7.4) flowed over the ligand on Sensor Chip CM5. Because the Bi-Ab being bivalent to both EGFR and VEGFR2 (Fig. 1A), association rate constant k_a and dissociation rate constant k_d were determined by Bivalent analyte with the assumption of a 2:1 binding model.

Flow cytometry

After washing twice with phosphate buffered saline (PBS), 5×10^5 HT-29 or SKOV-3 cells were incubated at 4°C for 1 h with 10μg/ml antibodies (mAb-04, cetuximab, Bi-Ab or isotypical IgG) in PBS containing 2% fetal bovine serum (FBS). Cell surface bound antibodies were detected with FITC-conjugated goat anti-human IgG antibody (SANGON, Shanghai, China) by incubation at 4°C for 1 h followed by washing twice with PBS. The cells binding assay was performed with a BD FACS flow cytometer and FlowJo software.

Proliferation assays

HT-29 or SKOV-3 cells were plated on 96-well plates with a final concentration of 2,000 cells per well. After overnight incubation, cells were treated with different concentrations of the antibodies (in media supplemented with 2% FBS containing or not containing 10ng/ml EGF and 10ng/ml VEGF). The plates were then incubated at 37°C with 5% CO_2 for 48 h. The cell proliferation level was detected with MTT assay and the inhibitory rates expressed as percentages of the vehicle control (100%).

Transwell invasion assays

Five$\times10^4$ HT-29 or SKOV-3 cells with or without the antibodies (100nM) were suspended in serum-free medium and added

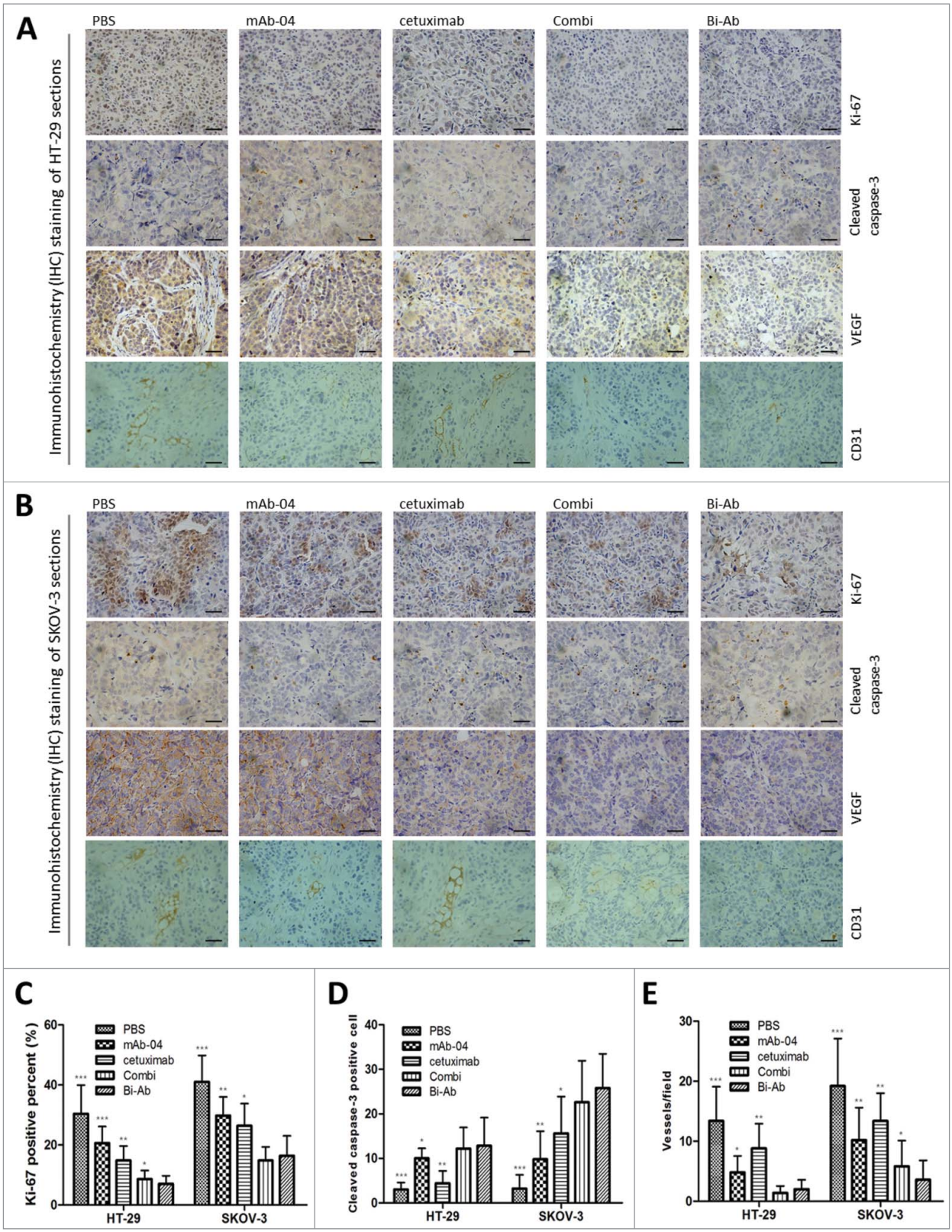

Figure 7. Immunohistochemical analysis was used to measure the effect of Bi-Ab treatment on proliferation, apoptosis and angiogenesis *in vivo*. In HT-29 (A) and SKOV-3 (B) tumors, proliferation and apoptosis were evaluated with Ki-67 and cleaved caspase-3 staining methods respectively, VEGF and CD31 were used to evaluate tumor angiogenesis. (C) Proliferative cells (Ki-67 positive cells) decreased in Bi-Ab-treated HT-29 and SKOV-3 tumors, and it was more effective compared to mAb-04- and cetuximab-treated tumors. (D) Apoptotic cells (cleaved caspase-3 positive cells) increased in Bi-Ab-treated HT-29 and SKOV-3 tumors. (E) The density of CD31-positive blood vessels decreased in Bi-Ab-treated HT-29 and SKOV-3 tumors. The data presented as the mean ± SD, are from a representative experiment, n = 5, $^{*}P < 0.05$; $^{**}P < 0.005$; $^{***}P < 0.0005$ vs. Bi-Ab treatment.

to the upper chamber of 24-well transwell chambers (Millipore, PIEP12R48) coated with 20μl Matrigel (BD Biosciences, 356234) on the bottom. The lower chambers were filled with 600 μl McCoy's 5A (for HT-29 cells) / RPMI-1640 (for SKOV-3 cells) containing 10% FBS, 10ng/ml EGF and VEGF, incubated for 24h (HT2–9) or 12h (SKOV-3). Invaded cells were stained with 1% (w/v) crystal violet and washed thrice with water. Images were then captured with an OLYMPUS inverted microscope at ×100 magnification and cells counted with Image-pro-plus program.

Apoptosis assays

At 70% confluence, HT29 cells and SKOV3 cells were treated with the antibodies (100nM) for 48 hours. They were harvested with trypsin, washed with PBS and centrifuged at 500g for 5 min at 4℃. The apoptotic or necrotic death induced by the antibodies in cells was measured with Annexin V-FITC / PtdIns Vybrant apoptosis assay kit (KeyGEN BioTECH, KGA108). The data of apoptosis was determined with a BD FACS flow cytometer, and FlowJo software was used for the data analysis.

Tube formation assay

Matrigel (BD Biosciences) was coated on a 96-well tissue culture plate and incubated at 37℃ for 1 h to solidify. HUVECs (2×10^4) in 100μl ECM supplemented with 2% (v/v) FBS and 1% (v/v) ECGS containing 1ng EGF and VEGF were added in each well. It was then incubated with antibodies (50nM) for 8h. After incubation, endothelial tube formation was photographed and quantified.[24]

ADCC assay

Target cells (HT-29 or SKOV-3, 2 × 10^4 per well) were incubated with 5μg/ml antibodies at 37°C for 30 minutes in McCoy's 5A (for HT-29 cells) / RPMI-1640 (for SKOV-3 cells) containing 10% FBS and 2mM GlutaMax^{TM}. Subsequently, effector cells (PBMCs, isolated from healthy human donor blood) were added at an effector/target ratio of 50:1. After overnight incubation, lactate dehydrogenase release was measured and then cell-mediated cytotoxicity of the target cells measured as follows: Cytotoxicity (expressed as a percentage) = (experimental lysis - spontaneous lysis) / (maximum lysis - spontaneous lysis) × 100.

Western blotting

At 80% confluence, cells were incubated in serum-free medium overnight, then incubated with the antibodies for 30 min, followed by induction with ligands (10ng/ml EGF and VEGF) for 30 min. Cells were then lysed and harvested with RIPA buffer (Beyotime, P0013C). Proteins were resolved by electrophoresis then transferred onto PVDF membranes.[24] The membranes were blocked and incubated with the following primary mAbs purchased from Cell Signal Technology: rabbit anti-P44/42 MAPK (4695), rabbit anti-Phospho-p44/42 MAPK (4376), rabbit anti-AKT (4691), rabbit anti-Phospho-AKT (4060), rabbit anti-Phospho- VEGFR2 (2478), rabbit anti- VEGFR2 (9698), rabbit anti-EGFR (4267), rabbit anti-Phospho-EGFR (3777), and rabbit anti-â-Actin (4970). Anti-rabbit antibody conjugated to horseradish peroxidase (HRP) and enhanced ECL chemiluminescence reagent (Millipore, WBKLS0500) were used to visualize protein bands. The membranes were then exposed with Bio-Rad detection system.

In vivo tumor xenograft studies

Six-weeks-old nude BALB/c female mice were purchased from the Animal Center of the Yangzhou University. The animal study was done under the protocol approved by the Animal Center of the Yangzhou University. SKOV-3 (5×10^6) or HT-29 (3×10^6) cells were resuspended in 100μl PBS and injected subcutaneously into each nude mouse. Mice were randomly allocated into 6 groups (n = 6 for each treatment group): PBS control, 5mg/kg mAb-04, 5mg/kg cetuximab, Combi (5mg/kg MAb-04 and cetuximab), 5mg/kg Bi-Ab. After the xenograft tumors had reached 50 mm^3, drugs were injected intraperitoneally (I.P.) into each mouse 3 times a week for the duration of the treatment regimen.[43] Tumor diameter was measured with caliper, and the volume calculated with the formula: ð/6 × larger diameter × (smaller diameter).[2] Mice were killed when the tumor load reached 4,000 mm^3, and tumor tissues were harvested.

Immunohistochemistry (IHC) staining and analysis

Xenograft tissues samples (n = 6 for each treatment group) were harvested (from 2.10), fixed in 4% formaldehyde/PBS and embedded in paraffin. Sections were incubated with rabbit anti-cleaved caspase-3 (Cell Signaling Technology, 9661), rabbit anti-Ki-67 (Cell Signaling Technology, 9027), rabbit anti-VEGF (Santa Cruz Biotechnology, sc-152), or rabbit anti-CD31 (Cell Signaling Technology, 3528). The sections were then stained with Peroxidase/DAB and examined in 5 randomly selected areas from each slide at ×100 magnification. Necrotic areas were excluded from analysis.

Statistical analysis

Excel and SPSS 17.0 software were used to analysis the data we got. Results were presented as mean ± SD of the mean (s.e.m.). Student's *t*-test was used to determine the statistical significance, GraphPad Prism 5 software program were used for figures production and log rank test.

Disclosure of Potential Conflicts of Interest

No potential conflicts of interest were disclosed.

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