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RESEARCH ARTICLE

Growth suppression of colorectal cancer expressing S492R EGFR by monoclonal antibody CH12

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Abstract **Colorectal cancer (CRC) is a common malignant tumor in the digestive tract, and 30%–85% of CRCs express epidermal growth factor receptors (EGFRs). Recently, treatments using cetuximab, also named C225, an anti-EGFR monoclonal antibody, for CRC have been demonstrated to cause an S492R mutation in EGFR. However, little is known about the biological function of S492R EGFR. Therefore, we attempted to elucidate its biological function in CRC cells and explore new treatment strategies for this mutant form. Our study indicated that EGFR and S492R EGFR accelerate the growth of CRC cells *in vitro* and *in vivo* and monoclonal antibody CH12, which specifically recognizes an EGFR tumor-specific epitope, can bind efficiently to S492R EGFR. Furthermore, mAb CH12 showed significantly stronger growth suppression activities and induced a more potent antibody-dependent cellular cytotoxicity effect on CRC cells bearing S492R EGFR than mAb C225. mAb CH12 obviously suppressed the growth of CRC xenografts with S492R EGFR mutations *in vivo*. Thus, mAb CH12 may be a promising therapeutic agent in treating patients with CRC bearing an S492R EGFR mutation.**

Keywords S492R EGFR ectodomain mutation; colorectal cancer; mAb CH12; immunnotherapy

Introduction

Colorectal cancer (CRC) is a major risk to human health. Although the mortality rates of patients with CRC have gradually declined with the improvement of medical standards, CRC remains the third major cancer worldwide, and the mortality rate for CRC ranks second among known malignancies [1–3]. At present, surgical resection and systematic chemotherapy are the main treatment options for CRC. Recurrence and metastasis remain the most intractable problems [4,5]. In recent years, the targeted drugs, such as VEGFR (vascular endothelial growth factor receptor) inhibitors and EGFR inhibitors, and many small-molecule kinase inhibitors that target intracellular signaling pathways are showing excellent promise to enhance the efficacy of chemotherapy drugs on CRC [6–10].

EGFR is a tyrosine kinase receptor related to tumor metastasis, invasion, and angiogenesis [11]. EGFR is overexpressed in numerous epithelial-originated cancer types, including breast carcinoma, lung cancer, glioma, prostate cancer, head and neck squamous cell carcinoma, ovarian cancer, and even CRC [12–15]. At present, two types of EGFR inhibitors (cetuximab and panitumumab) are mainly used to treat CRC [16,17]. Cetuximab is vital to the treatment of CRC without KRAS mutation. Recent studies disclosed that S492R EGFR mutation occurs during cetuximab and panitumumab treatment [18–21]. Newhall *et al*. [21] reported that the EGFR S492R mutation was detected in 16% of patients treated with cetuximab.

However, the biological function of S492R EGFR has never been elucidated, and mutated S492R EGFR cannot efficiently bind to cetuximab and thus may cause the resistance of CRC against cetuximab.

A monoclonal antibody CH12 that is independently developed by our research group can recognize a tumor-specific EGFR epitope exposed in activated EGFR. Our previous studies indicated that mAb CH12 preferentially binds to EGFRvIII or de4 EGFR and effectively inhibits the growth of Huh7-EGFRvIII and SMMC-7721 or U87MG-de4 EGFR xenografts *in vivo* and has a higher growth inhibition rate than cetuximab [22,23].

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Therefore, we proposed that mAb CH12 may efficiently bind to S492R EGFR and inhibit the growth of CRC xenografts expressing S492R EGFR. In this study, we will elucidate the biological function of S492R EGFR and explore the antitumor activities of mAb CH12 in CRC with S492R EGFR.

Materials and methods

Cell culture

The HT-29 and Caco-2 cells were purchased from the Chinese Academy of Sciences (Shanghai, China). Caco-2 was cultured in Dulbecco's Minimum Essential Medium that contained 20% fetal bovine serum (PAA Laboratories), and HT-29 was maintained in McCoy's 5A medium containing 10% fetal bovine serum, 100 U/mL penicillin, and 100 μg/mL streptomycin in a 37 °C environment at 95% air and 5% carbon dioxide atmosphere. A fresh medium was provided every 2 or 3 days. The medium and supplements were purchased from Gibco (Grand Island, NY, USA).

Construction of lentiviral vectors

Lentiviral packaging systems plasmid pWPT-GFP, psPAX2, pMD2.G were provided by Dr. Didier Trono (University of Geneva, Switzerland). Wild-type EGFR plasmid pLWERNL was provided by Steven Wiley (University of Utah). pWPT-EGFR plasmids were constructed in our laboratory [24]. The primers used were as follows: S492R EGFR forward primer: F 5′-TCTGTTTCTTATAATTTTGGTTTTCTGACC-3′, S492R EGFR reverse primer R 5′-CCAAAATTATAA-GAAACAGAGGTGAAAACA-3′, MluI primer 5′-CGACGCGTACGTACTAGTTAATTAA-3′, and BstEII primer 5′-AACGGGTCACCCCGTAGCTCC-3′. A pWPT-EGFR plasmid was used as a template, and a PCR-amplified fragment was obtained after bypass. The PCR products and pWPT-EGFRwt vector were then digested with MluI and BstEII. Finally, we combined the two products with T4 DNA ligase.

Transduction with a lentiviral vector

According to the manufacturer's instructions, the recombinant lentiviruses were prepared by transfecting 293T cells with Lipofectamine™ 2000 (Invitrogen). Human 293T embryonic kidney fibroblasts were cultured in Dulbecco's Minimum Essential Medium that included 10% fetal bovine serum. 5×10^6 cells were seeded at 10 cm dish including 10 mL of media the day before transfection. The next day, cells were transfected with 12 μg of plasmid vector, 9 μg of psPAX2 packaging plasmid, and 3.6 μg of pMD2.G, which encodes the G-protein of the vesicular stomatitis virus (VSV-G) envelope. The viral supernatant was cultured for 72 h at 37 °C and then harvested. HT-29 (2×10^5) and Caco-2 (1×10^6) were transduced with recombinant lentiviruses containing 6 μg/mL Polybrene (Sigma, St. Louis, MO, USA).

Western blot analysis

We collected cell lysates and centrifuged them for 10 min at 13 000 r/min. The supernatant was transferred to a clean tube, and the proteins were quantified with a BCA Kit (Pierce, Rockford, IL, USA). The proteins (40 μg) were separated on 10% SDS–PAGE gels and transferred to nitrocellulose membranes (Millipore Billerica, MA, USA) [25], which were blocked with 5% skim milk. The membranes were incubated overnight at 4 °C with primary antibodies. The following antibodies were used: mAb 7F4 (developed by our laboratory) [26], anti-phospho-EGFR (Tyr1068) (Abcam, Cambridge, UK), and anti-GAPDH (Kang-Chen Bio-tech, Shanghai, China). The anti-Akt, anti–phospho-Akt (Thr308), anti-ERK1, anti–phospho-ERK, and anti–Bcl-2 antibodies were purchased from Santa Cruz Biotechnology (Santa Cruz, CA, USA). The anti-STAT3 and anti-phospho-STAT3, anti–phospho-Akt (Ser473), anti-cyclin D1, were obtained from Cell Signaling Technology (Danvers, MA, USA). For the testing of the immune complexes, the membrane was incubated with horseradish peroxidase-conjugated goat anti-mouse antibody (Shanghai Raygene Biotechnology, Shanghai, China) for 1 h at room temperature. Then, the membranes were exposed for the enhancement of chemiluminescence reagents (Pierce, Thermo Scientific, Rockford, IL, USA).

FACS analysis

Fluorescence-activated cell sorting analysis was performed according to the methods described by a previous research [27]. Cultured parental and transfected CRC cell lines (1×10^6 cells) were collected by centrifugation and incubated with 20 μg/mL primary antibody or phosphate-buffered saline negative control in PBS that contained 1% newborn calf serum for 45 min at 4 °C. The cells were washed with cold PBS containing 1% NCS and then incubated for 45 min at 4 °C with FITC-conjugated goat anti-human antibody at 1:50 dilution (Kang-Chen Bio-tech, Shanghai, China) in the dark. After two subsequent washes, at least 10 000 cells for each sample were analyzed by FACS cytometry (Beckman Coulter Epics Altra, Miami, FL, USA) and WinMDI 2.9 software.

In vitro cell proliferation assay

4×10^3 cells per well were seeded in 96-well plates in triplicate. After 24 h, the cells were placed in a complete medium of C225 or CH12 with final concentrations of 20, 40, 80, and 160 μg/mL. After the cells were cultured for 72 h at 37 °C, cell proliferation was detected with CCK-8 assay kit (Dojindo Laboratories, Rockville, MD, USA). 10 μL of CCK-8 solution was added to 100 μL of culture media, and then the optical density was measured at 450 nm. The experiment was repeated three times. Percentage inhibition was calculated by the following formula: [1 − (number of viable cells in well treated with antibody/number of viable cells in well treated with the isotype control antibody)] × 100%.

Antibody-dependent cell-mediated cytotoxicity assay

ADCC was conducted with Promega's Cyto Tox 96® non-radioactive cytotoxicity assay kit (Promega, Madison, WI, USA) according to the manufacturer's instructions. Colorectal carcinoma cells HT-29, HT-29-S492R EGFR, Caco-2, and Caco-2-S492R EGFR were used as target cells at a density of 1×10^4 cells per well. Peripheral blood mononuclear cells (PBMCs) were added to the target cells to serve as effector cells, and the ratio between the effector and target cells was 20:1. Various concentrations of the C225 or CH12 antibody (0.001–10 μg/mL) were added in 96-well microtiter plates. The assay was performed in triplicate.

Tumor formation

5×10^5 tumor cells were inoculated subcutaneously to 4–6-week-old female BALB/c nude mice for the tumor growth assay. After 18 days of tumor cell inoculation, the mice were euthanized, and the tumor volumes were measured. The weight of subcutaneous tumors in each group was recorded.

In vivo antitumor effects

HT-29, HT-29-EGFR, and HT-29-S492R EGFR cells (1×10^6) were subcutaneously injected into the right flank of 4–6-week-old female BALB/c nude mice. When the average tumor volume reached approximately 100 mm^3, the mice were randomly divided into three groups ($n = 6$) and injected with the following three injections: (1) vehicle (sterile PBS), (2) 25 mg/kg mAb C225 dissolved in PBS, or (3) 25 mg/kg mAb CH12 dissolved in PBS [22]. The mice received intraperitoneal injections three times per week for 2 weeks. Vernier caliper was used to measure tumor volume every other day. The tumor volumes were calculated by using the following formula: (length × $width^2$) × 0.5. After the experiment, the mice were killed by cervical dislocation, and the tumors were resected and weighed. The data were expressed in terms of percentage inhibition of tumor growth. Animal experiments were conducted following the agreement approved by the Shanghai Medical Experimental Animal Care Commission.

Immunohistochemical analysis

Anti-Ki-67 (Santa Cruz Biotechnology) was used for the evaluation of angiogenesis and cell proliferation in formalin-fixed paraffin-embedded tumor tissues. After deparaffinization and rehydration, the tissue sections were incubated with 3% hydrogen peroxide in methanol for the elimination of endogenous peroxidase. The sections were blocked for 30 min with 1% BSA and incubated primary antibody at 4 °C overnight. Then, the sections were washed with PBS and incubated for 30 min with an HRP-conjugated secondary antibody. Finally, the products and counterstain in hematoxylin were visualized by using a diaminobenzidine staining kit (TIANGEN Biotech, Beijing, China). As a measure of proliferation, the Ki-67 labeling index was determined as the ratio of labeled nuclei to total nuclei in high power fields (200 ×) [22].

TUNEL assay

After deparaffinization and rehydration, the tumor tissue sections were then incubated with proteinase K with a concentration of 20 μg/mL for 20 min at 37 °C. The sections were washed with PBS several times and then incubated for 1 h with the TUNEL assay buffer (Beyotime Biotechnology, Nanjing, China) at 37 °C in the dark. Then, the slides were rinsed with PBS three times and visualized under a Zeiss LSM confocal microscope (Carl Zeiss, Jena, Germany). TUNEL positive cells were counted under 200 × magnification. The apoptotic cell number: the total cell number in each field served as the apoptotic index [22].

Statistical analysis

Data are expressed as mean ± standard deviation (SD) and were analyzed by the Student's *t*-test. $P < 0.05$ was statistically significant.

Results

Established EGFR and S492R EGFR-overexpressing cell lines

The EGFR and S492R EGFR-overexpressing cell lines were established by infecting HT-29 and Caco-2 cells with lentiviruses carrying the EGFR and S492R EGFR (HT-29-

EGFR, HT-29-S492R EGFR, Caco-2-EGFR, and Caco-2-S492R EGFR) and with empty vectors (HT-29-GFP and Caco-2-GFP). The expression of EGFR and S492R EGFR in the established cell lines was confirmed by Western blot (Fig. 1A and 1B). The results indicated that HT-29 and Caco-2 had endogeneous EGFR expression.

EGFR or S492R EGFR promotes the proliferation of CRC cells *in vitro* and *in vivo*

The effect of S492R EGFR on the proliferation of the CRC cells was examined through CCK-8 assay. The results indicated that the number of HT-29-EGFR and HT-29-S492R EGFR cells was approximately 1.59- and 1.53-fold of the number of HT-29 cells, and the number of Caco-2-EGFR and Caco-2-S492R EGFR cells was 1.63- and 1.68-fold of the number of Caco-2 cells after 7 days of culture (Fig. 1C and 1D). To investigate the growth-promoting-capacity of S492R EGFR expression *in vivo*, HT-29-GFP, HT-29-EGFR, and HT-29-S492R EGFR cells were inoculated in 4–6-week-old female BALB/c nude mice ($n = 6$). After 18 days of inoculation, the median tumor volume of HT-29-EGFR and HT-29-S492R EGFR xenografts were 1.36- and 1.31-fold of that of the HT-29 xenografts ($P < 0.05$; Fig. 1E). Although EGFR and S492R EGFR can promote the growth of CRC cells or xenografts, they showed no obvious difference.

Binding specificity of antibodies to cell lines

To determine the binding specificity of mAbs CH12 and C225 (cetuximab), we performed FACS assay on the HT-29, HT-29-S492R EGFR, Caco-2, and Caco-2-S492R EGFR cells. We observed that CH12 bound with a higher ratio to HT-29-S492R EGFR and Caco-2-S492R EGFR cells than to HT-29 and Caco-2 cells, whereas C225 bound to the two cell lines at a similar ratio, indicating the preferential binding of CH12 to S492R EGFR (Fig. 2).

Effect of C225 or CH12 on the viability of colorectal tumor cell lines *in vitro*

To reveal the growth inhibition capacity of mAbs C225 or CH12 on colorectal tumors, different doses of antibodies (20–160 μg/mL) were used to treat the CRC cells. The optimal concentrations of the antibodies used in our research were similar to those in previous *in vitro* studies [28,29]. Our data illustrated that mAb CH12 can inhibit the growth of S492R EGFR overexpressing cell lines in a dose-dependent manner and had a higher growth inhibitory

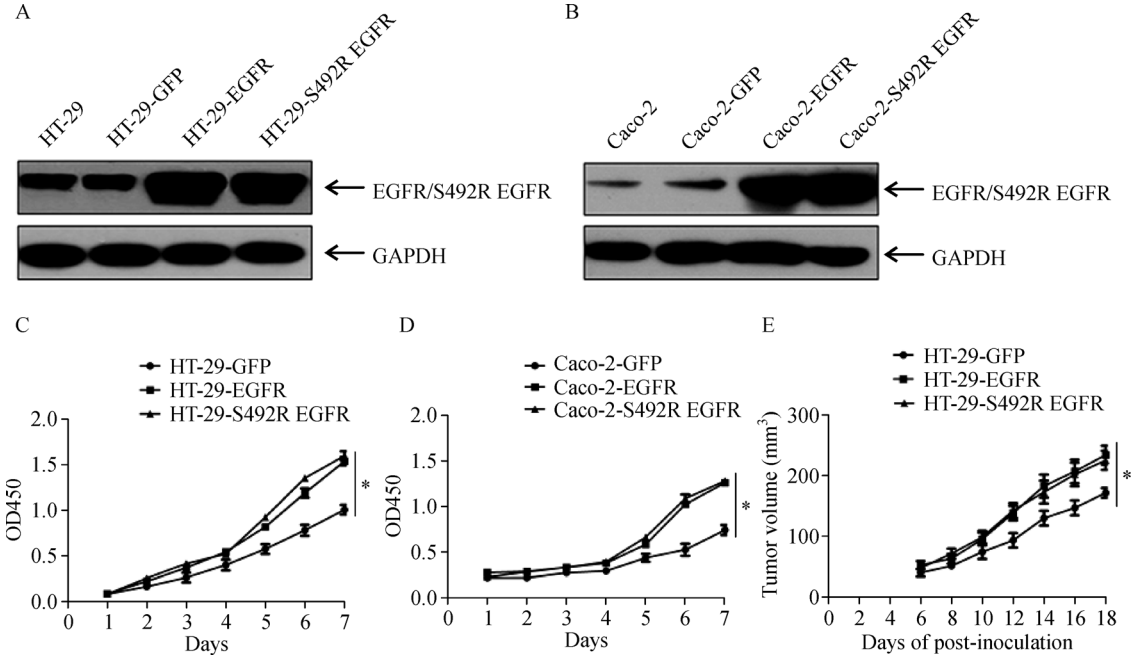

Fig. 1 S492R EGFR expression in the established cell lines and S492R EGFR promoted tumorigenicity in colorectal carcinoma. (A and B) Cell extracts from S492R EGFR-transfected cells were subjected to Western blot analysis. The blot was incubated with a monoclonal anti-EGFR antibody 7F4 (approximately 170 kDa) by using monoclonal antibody 7F4; GAPDH was used as a loading control. (C and D) *In vitro* cell growth of control and transfected colorectal carcinoma cell lines stably expressing EGFR and S492R EGFR by the CCK-8 Assay Kit. (E) S492R EGFR promoting the proliferation of colorectal carcinoma cell *in vivo*. HT-29, HT-29-EGFR, and HT-29-S492R EGFR cells (5×10^5) were injected into 4–6-week-old female BALB/c nude mice ($n = 6$), respectively. Six days later, the tumor volume was measured every other day. Data are expressed as mean tumor volume ± SD.

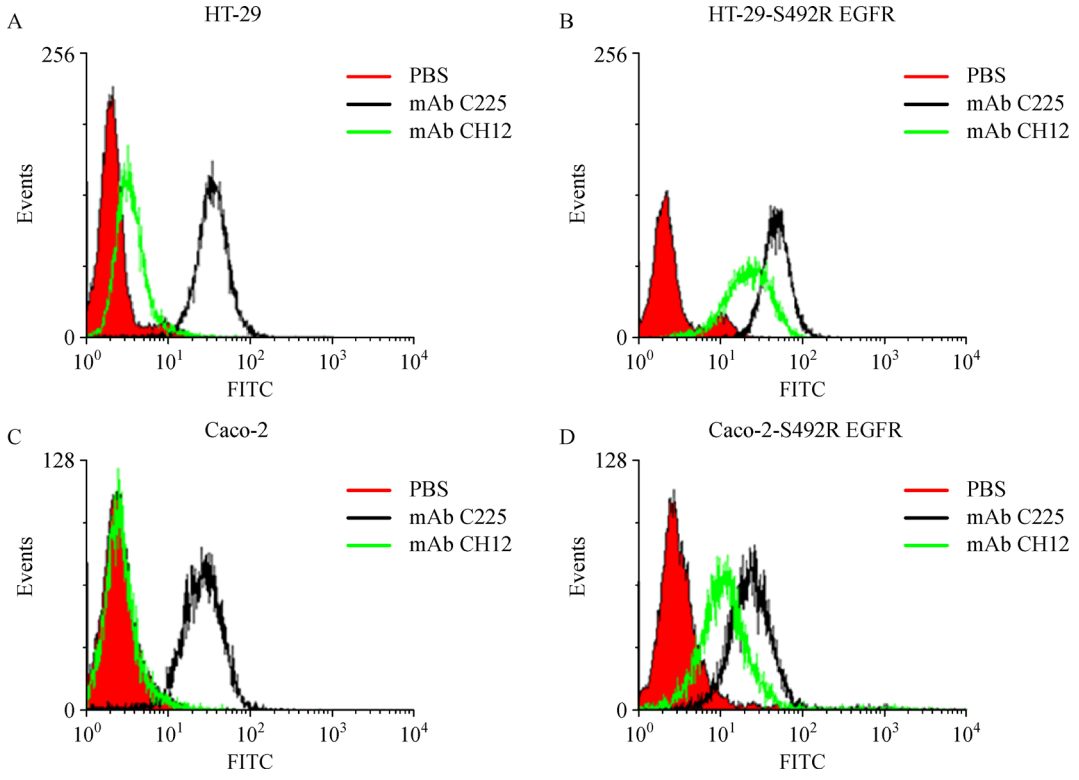

Fig. 2 FACS analysis of parental and transfected colorectal carcinoma cell lines stably expressing S492R EGFR. Cells were incubated with C225 (black line) and CH12 (green line) followed by FITC-conjugated goat anti-human IgG antibody. The negative control (PBS) fluorescence is plotted on each panel (red line).

effect than C225 on both cell lines with S492R EGFR expression (Fig. 3B and 3E). However, the C225-mediated inhibition of tumor cell proliferation was stronger than that medicated by CH12 in HT-29 and Caco-2 cell lines (Fig. 3A and 3D). Furthermore, cell proliferation assay demonstrated that mAb CH12 effectively suppresses the growth of CRC cells bearing S492R EGFR relative to the control group (Fig. 3C and 3F).

ADCC induced by CH12 or cetuximab against colorectal carcinoma cell lines

To verify whether antibodies can induce specific cleavage of target cells, we performed ADCC assays on the parental and S492R EGFR-transfected colorectal carcinoma cell lines by using different concentrations C225 or CH12 (0.001–10 μg/mL) at an effector: target cell ratio of 20:1. As shown in Fig. 4, both mAbs CH12 and C225 elicit dose-dependent specific cytotoxicity on HT-29-S492R EGFR and Caco-2-S492R EGFR cells. However, the ADCC effect of CH12 on these two cell lines with S492R EGFR expression is higher than that of C225. By contrast, ADCC activities of C225 on parental colorectal carcinoma cell lines appeared to be stronger than those of CH12. As for the isotype control antibody, no cytotoxicity effect was observed (Fig. 4).

mAb CH12 treatment inhibits colorectal xenograft tumor growth with S492R EGFR expression *in vivo*

To determine the antitumor effect of CH12 or C225 *in vivo*, we treated the mice bearing HT-29, HT-29-EGFR, and HT-29-S492R EGFR xenografts with vehicle and 25 mg/kg C225 or 25 mg/kg CH12 per day intraperitoneally three times per week for 2 weeks. All the mice were well tolerated during the study period, with no obvious sign of toxicity and had stable body weight. After 28 days of tumor inoculation, as shown in Fig. 5A–5D, we found that C225 displayed significantly higher growth suppression effect on HT-29 xenograft than CH12 ($P < 0.001$), with a median inhibitory ratio of 41.20% and 14.42%, respectively. C225 also displayed a significantly higher growth suppression effect on HT-29-EGFR xenograft than CH12 ($P < 0.001$), with a median inhibitory ratio of 60.24% and 25.05%, respectively (Fig. 5I–5L). By contrast, CH12 can significantly inhibit the growth of HT-29-S492R EGFR xenograft model with an inhibitory ratio of 39.68% (CH12 vs. PBS, $P < 0.001$), whereas C225 displayed a minimal growth-suppression effect with an inhibitory ratio of 8.25% (C225 vs. PBS, $P > 0.05$) (Fig. 5E–5H).

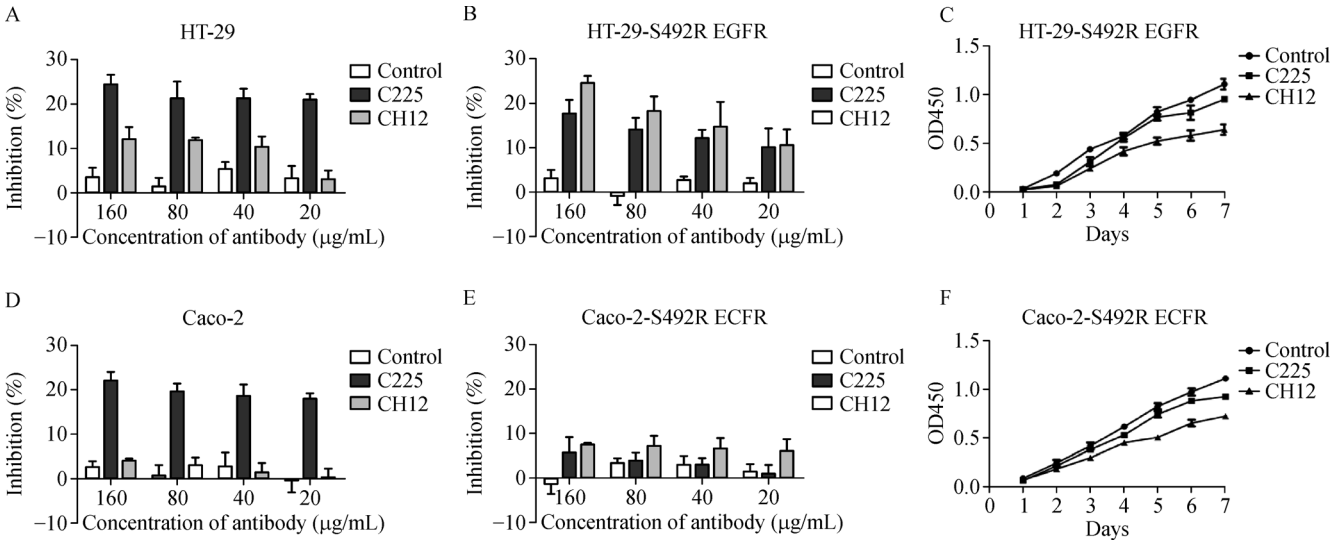

Fig. 3 *In vitro* growth suppression effects of cetuximab (C225) or CH12 on parental or S492R-EGFR-transfected human colorectal carcinoma cell lines. (A, B, D, E) Each cell line was treated with cetuximab or CH12 at concentration ranging from 20, 40, 80, 160 μg/mL for 72 h. Data are expressed as the mean percentage inhibition of cell growth ± SD. (C and F) *In vitro* cell proliferation assay after 80 μg/mL of antibody therapy in S492R EGFR over-expressed colorectal carcinoma cell lines. Results were presented as three independent experiments.

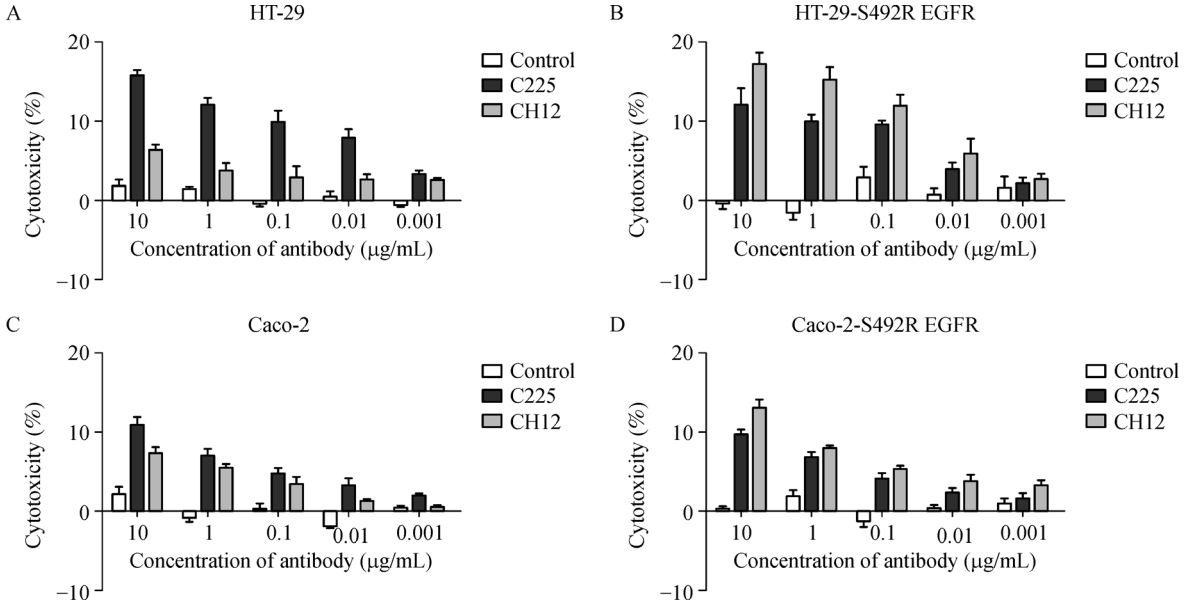

Fig. 4 Antibody-dependent cellular cytotoxicity mediated by mAb CH12 or C225 on HT-29 (A), HT-29-S492R EGFR (B), Caco-2 (C), and Caco-2-S492R EGFR (D) cells. C225 or CH12-mediated ADCC activity with PBMCs from healthy donors at an effector: target cell ratio of 20:1. Antibody concentrations ranged from 0.001 to 10 μg/mL. Rituximab was used as an antibody control. Data are presented as mean percentage ± SD of cytotoxicity of triplicate determinations. Results are representative of three separate experiments.

CH12 treatment reduces S492R EGFR autophosphorylation and downregulates cyclin D1 and Bcl-2 expression in HT-29-S492R EGFR CRC tumors

To elucidate the possible molecular events of CH12 in the treatment of tumors, some key signaling molecules in the EGFR pathway were analyzed by Western blot. Given that S492R EGFR promotes the proliferation and inhibits the apoptosis of CRC cells, we detected the phosphorylation status of EGFR and AKT, which are the vital downstream

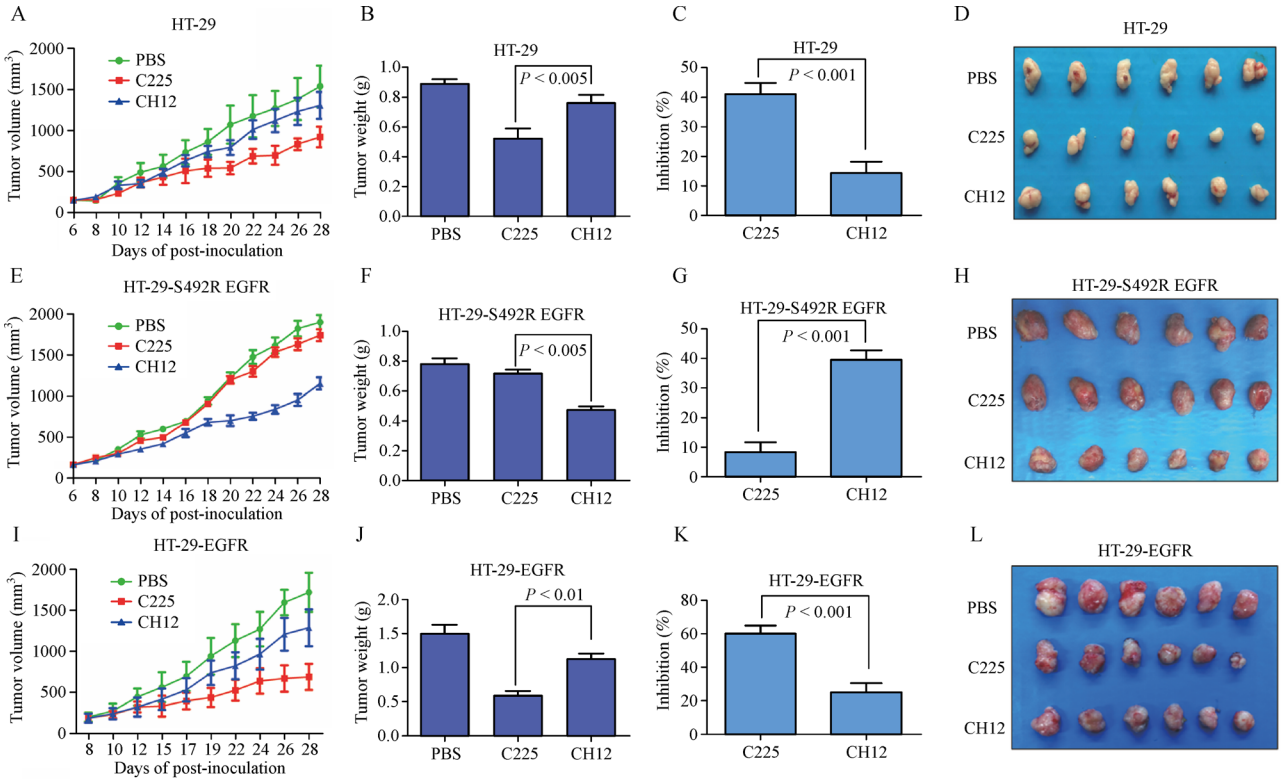

Fig. 5 Antitumor effects of C225 and CH12 on colon cancer xenografts. HT-29, HT-29-EGFR, and HT-29-S492R EGFR cells (1×10^6) were subcutaneously injected into 4–6-week-old female BALB/c nude mice. When the tumors had reached a mean tumor volume of 100 mm^3, the mice were randomly divided into three groups and treated with PBS, C225, or CH12. (A, E, I) Tumor growth curves. (B, F, J) Average weight of the isolated tumor tissues in each group. (C, G, K) Data are expressed as the percentage inhibition of tumor growth ($P < 0.05$). (D, H, L) Photos of tumor body of three groups treated with PBS, C225, or CH12.

signal transductors of EGFR and the expression levels of the anti-apoptotic protein Bcl-2 and the cyclin D1 which is involved in regulating the G1/S transition. As shown in Fig. 6, compared with the PBS or C225 therapy group, the p-EGFR, p-Akt, p-ERK, and p-STAT3 levels were dramatically downregulated in the CH12 therapy group. In our previous study, CH12 treatment reduced the expression of Bcl-2 and cyclin D1 [22]. Therefore, we also determined Bcl-2 and cyclin D1 expression in the CH12 or C225-treated tumors in this study. We found that Bcl-2 and cyclin D1 were more noticeably downregulated in the CH12 treatment group compared with the C225 treatment group (Fig. 6).

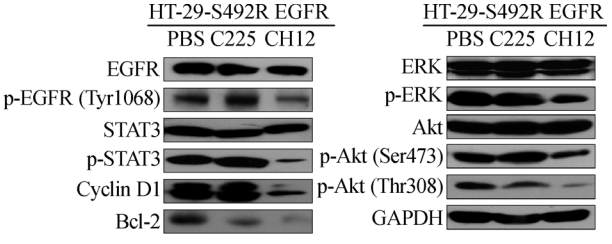

Fig. 6 Mechanisms of antitumor activity following treatment with C225 and CH12. Established HT-29-S492R EGFR xenografts treated with PBS, C225, or CH12 as single agents were excised and prepared by homogenization in cell lysis buffer. Tumor lysates (40 μg) were then subjected to SDS-PAGE and immunoblotted for total EGFR, p-EGFR (Tyr1068), total ERK, p-ERK, total Akt, p-Akt (Ser473), p-Akt (Thr308), total STAT3, p-STAT3, cyclin D1, Bcl-2, as indicated. GAPDH was used as a loading control.

mAb CH12 treatment potently reduced proliferation and induced apoptosis in HT-29-S492R EGFR xenografts

To elucidate the mechanism of CH12 inhibiting tumor growth *in vivo*, we determined the proliferation rates of control mice and treated mice. In the HT-29-S492R EGFR xenograft model, the proliferative index, measured by Ki-67 staining of the CH12-treated tumors, was significantly lower than that of the vehicle control and C225-treated tumors ($P < 0.05$, Fig. 7A and 7B). By contrast, in the HT-29 xenograft model, the proliferative index of the C225-

treated tumors was significantly lower than that of the vehicle control and CH12-treated tumors ($P < 0.05$, Fig. 7A and 7B). Furthermore, TUNEL staining showed that in the HT-29-S492R EGFR xenograft model, the number of apoptotic cells treated with CH12 was significantly higher than that of the control group and the tumors treated with C225 ($P < 0.05$; Fig. 7C and 7D). However, in the HT-29 xenograft model, the apoptotic index of the C225-treated tumors was significantly increased compared with that of the vehicle control and CH12-treated tumors ($P < 0.05$, Fig. 7C and 7D).

Discussion

CRC is a common malignancy in the world with 600 000 cases of death annually worldwide [30]. Surgical resection combined with chemotherapy is the main treatment modality for CRC. Chemotherapy drugs such as 5-fluorouracil combined with oxaliplatin or irinotecan greatly improve the survival of patients with CRC [31–34]. However, owing to the side effects of chemotherapy drugs, facilitating the emergence of molecular-targeted drugs for the treatment of metastatic CRC is extremely urgent. Epidermal growth factor receptor inhibitors cetuximab and panitumumab are the main targeted drugs to treat CRC, and cetuximab has been approved for treating CRC from third-line to first-line as monotherapy or in combination with chemotherapy.

However, the use of cetuximab in the clinical treatment of CRC results in drug resistance owing to KRAS gene mutation or amplification, HER2 gene amplification, and the newly discovered S492R EGFR ectodomain mutation [35]. To the S492R EGFR mutation occurs in the extracellular domain of EGFR, thereby abolishing the binding of EGFR with the cetuximab. The antibody CH12 as that of mAb 806 can recognize the epitope located between animo acids 287 and 302 in the CR1 domain of

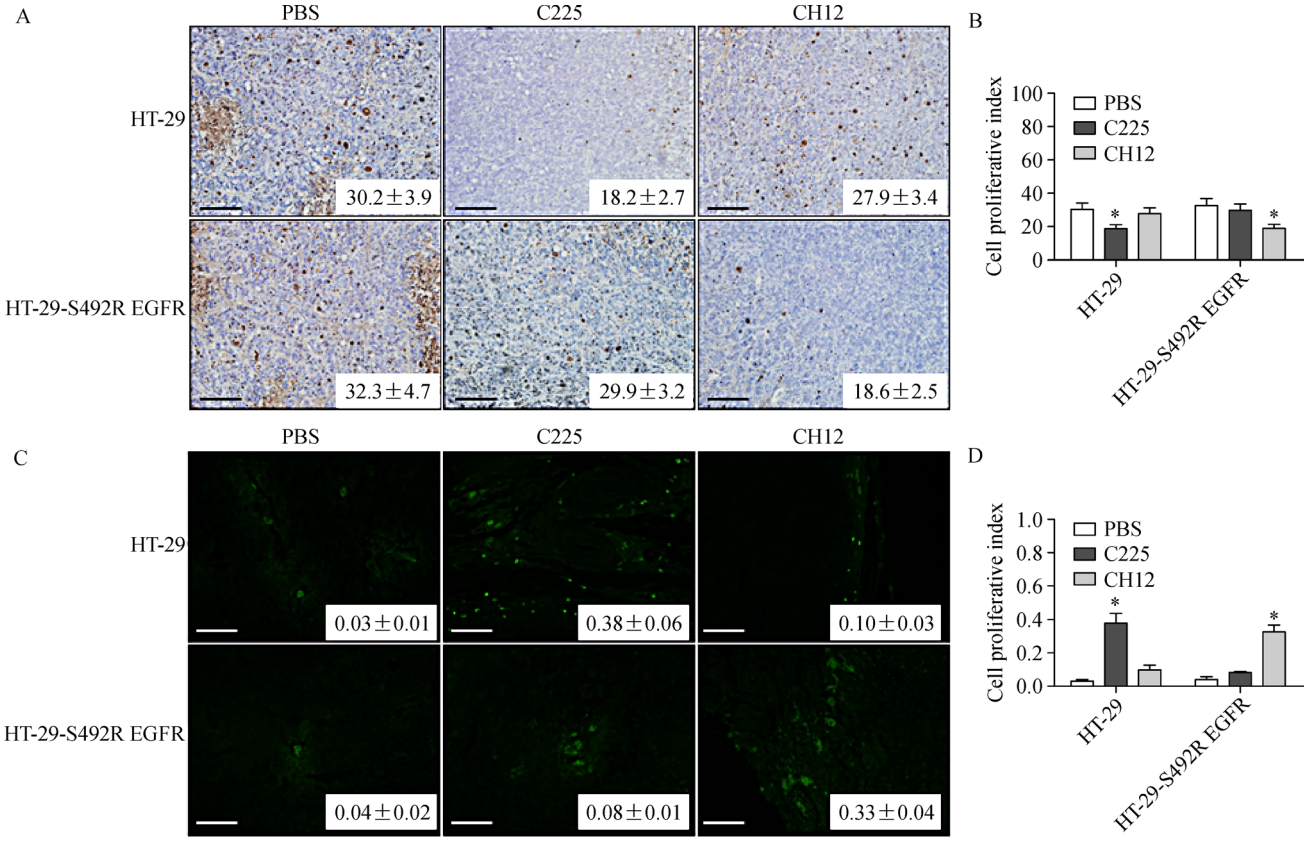

Fig. 7 CH12 reduced proliferation and induced apoptosis in the HT-29-S492R EGFR tumor xenografts. (A) CH12 treatment led to less growth compared with other treatments in HT-29-S492R EGFR xenograft. Tumor sections were stained for Ki-67. The cell proliferative index was assessed as the percentage of total cells that were Ki-67 positive from six randomly selected high power fields (200 ×) in xenografts from six mice of each group. (B) Quantitative analysis of Ki-67 staining. (C) CH12 treatment led to an increase in apoptosis compared with control in HT-29-S492R EGFR xenografts. Apoptotic cells were detected using the TUNEL assay. The apoptotic index was assessed by the ratio of TUNEL-positive cells: total number of cells from six randomly selected high power fields (200 ×) in xenografts from six mice of each group. (D) Quantitative analysis of TUNEL assay. Data are presented as the mean ± SE. *$P < 0.05$ vs. control group.

the EGFR ECD [22]. The epitope is masked in the inactive monomer state or the fully coordinated back-to-back dimer state, which results in the antibody not binding to the resting normal tissue of EGFR. However, conformational exposure to this epitope takes precedence over tumor-specific conditions, including EGFR activity imbalance or ECD mutation [36]. Unfortunately, side effects such as skin rash, diarrhea (20%), and so on, were observed in patients who used panitumumab to treat CRC [37]. Therefore, seeking an alternative monoclonal antibody for S492R EGFR is necessary.

In our research, to avoid the effect of KRAS mutation on CH12 treatment, we chose two colorectal carcinoma cell lines, HT-29 and Caco-2, which carry wild-type KRAS [38]. Our study revealed that both EGFR and S492R EGFR can promote the proliferation of the cancer cells *in vitro* and *in vivo*. As expected, mAb CH12 can selectively bind to the S492R EGFR. Importantly, mAb CH12 can efficiently suppress the growth of the cancer cells with the S492R EGFR, but not the cells without this mutant. Additionally, mAb CH12 can induce obvious ADCC effect on the CRC cells that bear this EGFR mutant. Moreover, mAb CH12 can significantly inhibit the growth of CRC xenografts with S492R EGFR, but not the xenografts without the EGFR mutant.

Further study on the underlying mechanism on the growth inhibition effect indicated that CH12 treatment can decrease proliferation and promote apoptosis. Cyclin D1 participates in the regulation of G1/S transformation and is amplified in CRC [39,40]. Therefore, the decreased proliferation of CRC xenografts after CH12 treatment is at least partially due to the downregulation of cyclin D1 expression. Bcl-2 can regulate migration and invasion of CRC cells [41]. To elucidate the molecular mechanism underlying apoptosis induced by CH12 in CRC xenografts, we examined the expression of anti-apoptotic Bcl-2. CH12 treatment induced downregulation of Bcl-2 expression, which further resulted in hepatoma cells being prone to apoptosis [22]. Therefore, the downregulation of Bcl-2 induced by CH12 should contribute to the apoptosis-promoting effect of CH12 in our study. In our previous research, we found that CH12 therapy can decrease the phosphorylation of EGFR signal pathway in hepatocellular carcinoma xenografts [28]; in the present study, we also observed that CH12 therapy can reduce the phosphorylation of EGFR signal pathway in colorectal carcinoma xenografts, including p-EGFR (Tyr1068), p-Akt(Ser473), p-Akt(Thr308), p-ERK. Intriguingly, we found that CH12 therapy can potently decrease the phosphorylation of signal transducer and activator of transcription 3 (STAT3), which is a member of the STAT family and is vital to tumorigenesis because it regulates cell proliferation, differentiation, and metabolism [42]. We elucidated the inhibitory effect of CH12 on the proliferation and induction of apoptosis in S42R EGFR.

We demonstrated that S492R can promote the growth of CRC cells *in vitro* and *in vivo*. We also demonstrated that monoclonal antibody CH12 exerts a growth-suppression effect on S492R EGFR positive CRC. Thus, CH12 may be a potential novel intervention strategy for patients with CRC bearing S492R EGFR.

Acknowledgements

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Compliance with ethics guidelines

Qiongna Dong, Bizhi Shi, Min Zhou, Huiping Gao, Xiaoying Luo, Zonghai Li, and Hua Jiang declare that they have no conflicts of interest. All procedures followed were in line with the ethical standards of the human laboratory responsibility committee (institutions and countries).

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