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Cetuximab Combined with Vemurafenib Can Significantly Inhibit the Proli- Feration Of Braf V600e Mutant Egfr Wild-Type Colorectal Cancer Cells

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Abstract

Background: Cetuximab (Cetuximab, C225) is relatively limited in improving the survival of patients with advanced colorectal cancer. One possible reason is that some patients with colorectal cancer have high-frequency mutations of BRAF V600E leading to resistance. Vemurafenib is a newly developed inhibitor that specifically blocks the activation of signaling pathways caused by BRAF V600E mutations. Therefore, this study thoroughly studied whether the combined application of C225 and Vemurafenib can inhibit the proliferation of EGFR wild-type, BRAF V600E mutant colorectal cancer cells and the molecular mechanism.

Methods: The effects of different concentrations of C225 and BRAF V600E potent mutant (Vemurafenib) on cell proliferation of colorectal cancer cell lines HT-29 (EGFR wild-type and BRAF V600E mutant) were determined by using CCK-8 and plate cloning assay.Western Blot was used to detect the changes in apoptosis (Caspase-3) and proliferation-related markers (Ki-67, CD31) protein levels of cells treated with C225 alone, Vemurafenib alone or in combination with the two drugs.

Results: The results of CCK-8 clone formation experiment showed that the combined application of C225 and Vemurafenib can significantly reduce cell viability and cell clone formation ability compared with the two single-agent groups. After treatment of cells with the combination of C225 and Vemurafenib, the activity of Caspase-3 was increased, the expression of CD31 and Ki67 was significantly inhibited, the expression of p-EGFR was down-regulated, the total amount of ERK decreased, and the corresponding phosphorylated p-ERK level appeared Elevated.

Conclusion: The combined application of C225 and Vemurafenib can significantly inhibit the proliferation and clone formation of colorectal cancer cell lines. When used in combination, the expression of Caspase-3 was significantly increased, and the expression of Ki-67, CD31 and EGFR phosphor - lation was inhibited. This study provides a theoretical basis for the combined administration of EGFR monoclonal antibody C225 and Vemurafenib for clinically EGFR wild-type and BRAF V600E mutant colon cancer patients, provides preliminary theoretical basis and support for subsequent clinical research.

Keywords: Cancer, Cetuximab, Vemurafenib, BRAF V600E Mutant, Proliferation

Introduction

The global morbidity and mortality of colorectal cancer rank among the top three in malignant tumors. The molecular targeted drug therapy for metastatic colorectal cancer mainly includes two categories: one is Epidermal growth factor receptor (EGFR) as the target, one type takes vascular endothelial growth factor vascular growth factor

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(VEGF) as the target [1, 2]. However, from the perspective of clinical efficacy the combined endothelial application of anti-EGFR monoclonal antibodies on the basis of chemotherapy for advanced colorectal cancer can improve overall survival but it does not achieve the expected results. The first-line treatment is increased by 8 months, and the second-line treatment is only 2 Month [3]. Combined with domestic and foreign data, the reasons may be that there are two factors that limit the survival of patients with colorectal cancer: (1) the efficacy of anti-EGFR in the treatment of colorectal cancer has not reached the expected; (2) patients resist EGFR The monoclonal antibody has acquired resistance, which limits its therapeutic effect.

EGFR is a member of the tyrosine kinase type I receptor subfamily. Members of this family have common characteristics: it is divided into extracellular receptor domain and intracellular ligand binding domain, transmembrane domain and intracellular kinase domain. [4, 5]. Under normal physiological conditions, activated EGFR can promote cell proliferation and differentiation. It is an important receptor molecule on the cell surface. Activated EGFR can regulate cell functions through multiple signaling pathways in the cell: such as activation of MEK-ERK, PI3K-AKT signaling pathway inhibits apoptosis and so on [6]. However, recent studies have found that EGFR is highly expressed in a variety of tumors and plays an important role in the abnormal proliferation of tumors. Therefore, a variety of monoclonal antibodies (such as C225, Panitumumab, Nimotuzumab) have been developed for EGFR, and some small molecule compounds that target to inhibit its activity have been developed such as: Gefitinib, Erlotinib, icotinib, etc. The clinical application of these drugs can significantly prolong the survival period of patients and significantly improve the quality of life of patients [3, 7, 8].

Cetuximab (Cetuximab, C225) has relatively limited efficacy in improving the survival of patients with advanced colorectal cancer. One possible reason is that some colorectal cancer patients have high-frequency mutations in BRAF V600E, which makes anti-EGFR monoclonal antibodies to EGFR The inhibitory effect of C225 cannot be smoothly transmitted to the downstream through the EGFR-RAS-RAF-MAPK signaling pathway, and cannot inhibit cell proliferation and growth through this signaling pathway. The inhibitory effect of C225 on other signaling pathways downstream of EGFR may lead to the RAS-RAF-MAPK signaling pathway Negative feedback expression of the key molecules of the drug increases, leading to drug resistance [9-11]. Vemurafenib is a newly developed inhibitor that specifically blocks the activation of the signaling pathway caused by the BRAF V600E mutation and has a strong inhibitory effect on BRAF and its downstream signaling pathway molecules [12, 13]. Therefore, we hypothesized that the combined application of C225 and Vemurafenib can have a combined inhibitory effect on EGFR wild-type and BRAF V600E mutant colorectal cancer cell lines. This experiment will verify this conjecture through in vivo and vitro experiments and conduct a preliminary discussion of the relevant mechanisms of signal pathways.

Materials and Methods

Cell culture

The colorectal cancer cell line HT-29 belongs to BRAF V600 mutant and EGFR wild-type cells and was purchased from the Shanghai Cell Bank of the Chinese Academy of Sciences. The cells were cultured with 10% fetal bovine serum, 1% penicillin and streptomycin, DMEM medium, inoculated in a cell culture flask placed in an incubator under the same conditions for overnight culture, and passaged according to 1:3, 37°C, 5% CO2 incubator.

CCK-8 method to detect the proliferation activity of colorectal cancer cell lines

The cells in the exponential proliferation phase were collected, trypsin-zed, inoculated into 96-well plates according to (1×10^3 cells/well), cultured overnight in an incubator, and C225 (25μg/ml, 50μg/ml, 100μg/ml, 200μg/ml, 400μg/ml) and Vemurafenib (2.5μg/ml, 5.0μg/ml, 10μg/ml, 20μg/ml, 40μg/ml) HT-29 cells alone or in combination, after treatment for different time add 10μl/ Incubate the CCK-8 mother liquor in the wells in a 37℃, 5% CO2 incubator for 2 hours. Use a microplate reader to continuously detect the absorbance at a 450nm at different time points set 3 multiple wells for each well and drawn a growth curve based on the detection data.

Clone formation experiment

Digest the cells with trypsin, centrifuge (1000 rpm, 5 minutes) and discard the supernatant. Resuspend the cells with complete cell culture medium, inoculate them into 6-well plates, put them in the incubator and replace with new culture medium every 2-3 days. After about 10-14 days a clonal cell cluster is formed, the culture medium is discarded to culture the tissue cells, the cells are washed with PBS buffer for a total of 3 times and an appropriate amount of 4% paraformaldehyde is

added to fix the cells for 30 minutes. Wash the cells with PBS 3 times, add 2ml of 0.1% crystal violet solution for staining and then wash with PBS 3 times after staining. Collect cell pictures, take cell clusters ≥50 cells as the number of clones, and count the numbers.

Western blot detection

Collect the cells, add the lysate to refill, and after the cells are fully lysed, collect the cell lysate in a centrifuge tube, centrifuge at 4°C, 12000 rpm for 15 minutes, collect the total cell protein, and then use the BCA method to detect the protein concentration. Add 60μg of sample to each well use 10% polyacrylamide gel for protein electrophoresis, set the electrophoresis voltage to 90V and 120V constant voltage, after completion wet transfer to PVDF membrane dilute the membrane in PBS The 5% skimmed milk powder was sealed at room temperature for 2 hours. Incubate with the primary antibody overnight at 4°C, use β-actin as the internal reference protein add a 1: 2,000 peroxidase-labeled secondary antibody incubate at room temperature for 2 hours, wash the membrane with TBST buffer after the reaction 5min for 3 times, the strip is placed in a dark room an appropriate amount of HRP substrate exposure solution is dropped on the surface of the strip for development and the gel imager collects pictures and performs grayscale analysis.

Data analysis

SPSS19.0 software was used to analyze all the data. Quantitative data were expressed as mean ± standard error (x ± s). The comparison between the two groups was performed by t test. The count data was tested by $\chi 2$. The expression difference of $p<0.05$ was statistically significant.

Results

Cetuximab (Cetuximab, C225) and Vemurafenib (Vemurafenib) combined application on the activity of colorectal cancer cells

As the first monoclonal antibody targeting EGFR, C225 is relatively limited in improving the survival of patients with advanced colorectal cancer. One possible reason is that some colorectal cancer patients have high-frequency mutations in BRAF V600E, which makes anti-EGFR monoclonal antibodies against EGFR. The inhibitory effect of EGFR-RAS-RAF-MAPK cannot be smoothly transmitted to the downstream through the EGFR-RAS-RAF-MAPK signaling pathway and cannot inhibit cell proliferation and growth through this signaling pathway. Vemurafenib is a potent BRAF inhibitor against BRAF V600E mutation and has a strong inhibitory effect on BRAF and its downstream signaling pathway molecules. Therefore, we hypothesized that the combined application of C225 and Vemurafenib can have a synergistic inhibitory effect on colorectal cancer with wild-type EGFR and BRAF V600E mutation. Therefore, the EGFR wild-type and BRAF V600E mutant colorectal cancer cell line HT-29 was used for the test. First use different concentrations of C225 (25μg/ml, 50μg/ml, 100μg/ml, 200μg/ml, 400μg/ml) and Vemurafenib (2.5μg/ml, 5.0μg/ml, 10μg/ml, 20μg/ml, 40μg/ ml) Treat HT-29 cells alone or in combination and use CCK-8 to detect cell viability. The results are shown in Figure 1: Compared with the two single-drug groups, combined use of 50ug/ml or 100ug/ml C225 and 5ug/ml or 10ug /ml Vemurafenib can significantly reduce cell activity ($P<0.05$). Therefore, in subsequent experiments cells were treated with this concentration to conduct other functional experiments.

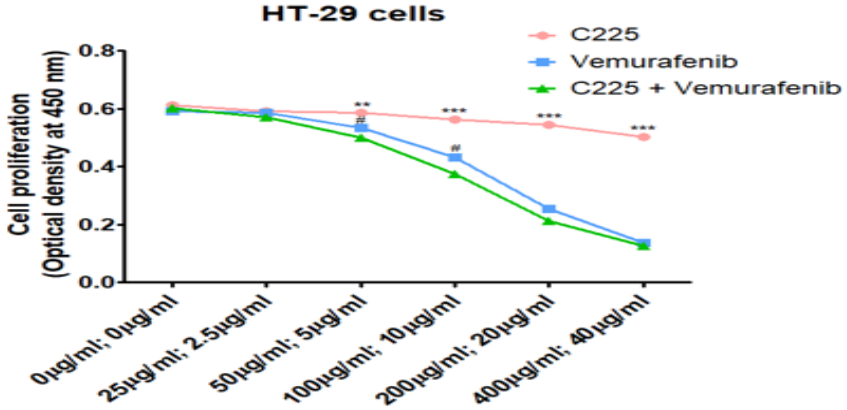

Figure 1. **CCK-8 detects the effect of C225, Vemurafenib and C225+Vemurafenib combined application on colon cancer HT-29 cell viability, #P<0.05, ** P<0.01, ***P<0.001**

Effect of Cetuximab (Cetuximab, C225) and Vemurafenib (Vemurafenib) on the colonization ability of colorectal cancer cells

In order to further examine the molecular mechanism of C225 and Vemurafenib inhibiting the activity of colorectal cancer cells we further used a plate clone formation experiment to detect the effect of the combination on cell proliferation. First HT-29 was inoculated into a 6-well plate and then 50 μg/ml was added C225, 5μg/ml Vemurafenib and the combination group (50ug/ml C225 and 5ug/ml Vemurafenib) were cultured for 10 days, then fixed and stained, and the number of colonies formed was counted under the microscope. The experimental results are shown in Figure 2: Compared with the blank control (Control), C225 treatment has no significant effect on the cloning ability of HT-29 cells. Vemurafenib treatment can significantly inhibit the ability of cell clone formation. After the combination treatment, compared with the two single-agent groups, the cell clone formation ability was significantly reduced (vs C225, P<0.01 vs Vemurafenib P<0.05). We infer that the combined application may have an effect on the long-term proliferation of cells.

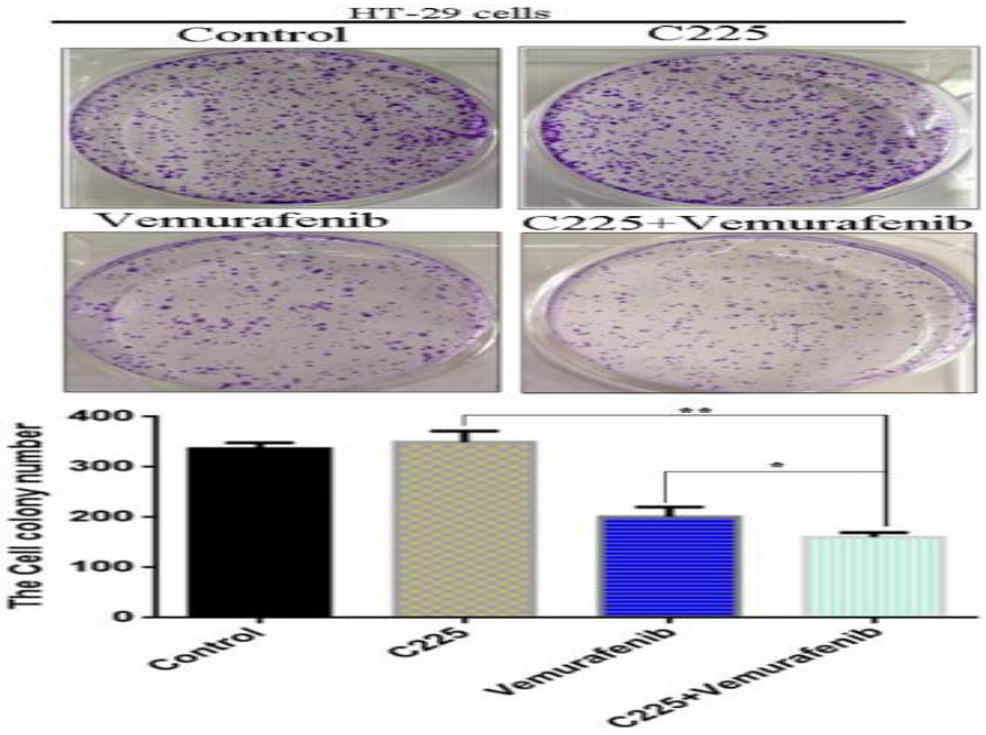

Figure 2. **The clone formation experiment detects the effect of C225, Vemurafenib and C225+Vemurafenib combined application on the clone formation ability of HT-29 cells, *P<0.05, ** P<0.01**

Effect of Cetuximab (Cetuximab, C225) and Vemurafenib (Vemurafenib) on key signaling molecules in colorectal cancer cells

After EGFR is activated it can be smoothly transmitted to the downstream through the EGFR, RAS, RAF and MAPK signaling pathway to promote cell proliferation and growth. The inhibitory effect of C225 on other signaling pathways downstream of EGFR may lead to negative feedback expression of key molecules in the RAS-RAF-MAPK signaling pathway increase. Based on the above research results and the currently known mechanism of action of EGFR monoclonal antibody C225 and BRAF V600E mutation potent inhibitor Vemurafenib we first used 50μg/ml C225, 5μg/ml Vemurafenib and the combination group (50ug/ml C225 and 5ug /ml Vemurafenib) treatment of HT-29 cells, Westen-blot test results showed that compared with the Control group the combination of drugs (50ug/ml C225 and 5ug/ml Vemurafenib) treated cells increased Cleaved caspase-3 expressions but there was no significant effect on the expression of MEK and phosphorylated p-MEK, and significantly reduces the expression of vascular endothelial cell marker CD31. At the same time the expression of the nuclear protein Ki-67 which is related to cell proliferation is significantly reduced and down-regulated. The expression of p-EGFR and the total amount of ERK decreased but the corresponding phosphorylated p-ERK level increased (image 3).

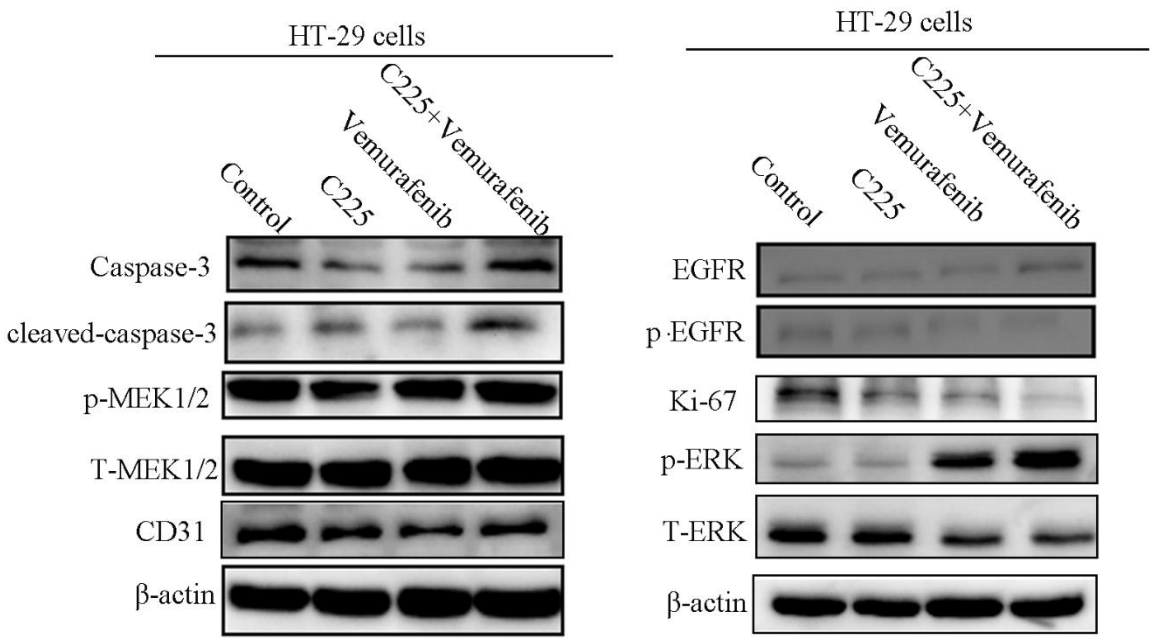

Figure 3. **Western blot detection of the expression changes of key molecules after C225, Vemurafenib and C225+Vemurafenib combined treatment of HT-29 cells**

Discussion

Vemurafenib (Vemurafenib) is a potent and selective small molecule inhibitor targeting BRAF V600E mutation downstream of the EGFR signaling pathway. It was approved by the US FDA in August 2011 and is currently clinically recommended for treatment with Patients with advanced malignant melanoma with BRAF V600E mutation [12]. Vemurafenib can specifically bind to the BRAF V600E mutation site thereby blocking the signal transduction pathway of cells in the body inducing cell apoptosis and inhibiting tumor growth to disappear [14]. Studies have shown that the clinical use of Vemurafenib to treat patients with BRAF V600E mutations of malignant melanoma is about 50% while the effective rate of Vemurafenib treatment for patients with BRAF V600E mutations in metastatic colorectal cancer is only 5% [13, 15, 16]. In colorectal cancer cells abnormal activation of EGFR downstream pathways has been proven to be a key factor affecting the occurrence of Vemurafenib resistance. The main mechanism is that feedback activation of the EGFR pathway leads to the reactivation of MEK and MAPK signaling pathways, cancer cell proliferation, migration and invasion. The ability is enhanced and the use of EGFR inhibitors can inhibit the activation of MEK and MAPK and induce apoptosis [16, 17]. The combination regimen shows better curative effect than the single medication in terms of curative effect or in reversing resistance [18, 19]. In addition, the adverse reactions of the combination medication did not increase significantly compared with the single medication and the incidence of certain adverse reactions was even lower.

Therefore, in this study we hypothesized that the combined application of C225 and Vemurafenib can have a combined inhibitory effect on EGFR wild-type and BRAF V600E mutant colorectal cancer cell lines. First we used CCK-8 to detect the effect of the drug on the proliferation activity of two colorectal cancer cell lines HT-29 (EGFR wild-type, BRAF V600E mutant). The experimental results show that the combined application of C225 and Vemurafenib can significantly reduce cell activity compared with the two single-drug groups which shows that the combined drug can significantly inhibit cell proliferation. Vemurafenib can also be used in combination with other alkaloids (lycorine) to induce autophagy and apoptosis by targeting MEK [20]. Clinical studies have shown that the combined treatment of EGFR monoclonal antibody (panitumumab) and BRAF V600E inhibitor (Vemurafenib) is well tolerated and can achieve good results in patients with metastatic colorectal cancer with extensive BRAF mutations [21]. C225 can specifically bind to the EGFR-related domains on the surface of cancer cells, competitively block the corresponding ligands, inhibit the proliferation of cancer cells, induce apoptosis of cancer cells and exert anti-tumor effects. The clone formation experiment further proved that C225 and

Vemurafenib can significantly inhibit the growth of colorectal cancer which suggests that the combined drug can inhibit cell proliferation for a long time. Molecular mechanism studies have shown that C225 and Vemurafenib can significantly induce the activation of the apoptosis marker enzyme Caspase-3, the decrease of the expression of the nuclear antigen Ki67 associated with proliferating cells and the decrease of the expression of tumor angiogenesis molecule CD31. Since C225 mainly inhibits the activity of EGFR molecules we also tested the expression of EGFR signaling molecules and important downstream molecules MEK and ERK1/2. The results show that there is no significant effect on the total amount of MEK and phosphorylated p-MEK up-regulating the expression of EGFR, significantly down-regulating the expression of p-EGFR and reducing the total amount of ERK but the corresponding phosphorylated p-ERK level has increased.

In summary, the combined application of C225 and Vemurafenib can significantly inhibit cell proliferation, induce cell apoptosis and cycle arrest, inhibit cell invasion and migration. The molecular mechanism study shows that the combined application can activate the apoptosis molecule Caspase-3 activation, the expression of Ki-67 and CD31 decreased inhibiting the phosphorylation of EGFR and the activation of downstream molecules (MEK and ERK). Therefore, the results of this study prove that C225 combined with Vemurafenib is feasible for the treatment of EGFR wild-type and BRAF V600E mutations in refractory colorectal cancer and the mechanism is explained from the perspective of molecular mechanism which provides theoretical basis and support for clinical application.

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