Adenomatous polyposis coli determines sensitivity to the EGFR tyrosine kinase inhibitor gefitinib in colorectal cancer cells

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Abstract. Epidermal growth factor receptor tyrosine kinase inhibitors (EGFR-TKIs) have been used to treat colorectal cancer (CRC). However, resistance to EGFR-TKIs presents a great challenge for the treatment of CRC, and the mechanisms of resistance are poorly understood. The adenomatous polyposis coli (APC) protein has been known to contribute to the carcinogenesis of CRC. However, its role in the sensitivity of CRC cells to gefitinib has not been investigated. Human CRC HCT-116 (wild-type APC) and HT-29 (mutant APC) cells were used to investigate the effect of APC on the sensitivity of CRC cells to gefitinib. The MTT assay was used to measure cell viability after exposure to gefitinib. Cell apoptosis, migration and invasion were determined by flow cytometry, wound healing assay and Transwell assay, respectively. Knockdown and overexpression of APC were performed, and activation of the EGFR and its downstream pathway was determined. Gefinitib inhibited viability, promoted apoptosis, and reduced the migration of HCT-116 and HT-29 cells. HT-29 cells exhibited increased sensitivity to gefinitib when compared to HCT-116 cells. Knockdown of APC expression increased the sensitivity of HCT-16 cells to gefitinib, accompanied by downregulation of pEGFR, p-AKT and pERK1/2. In contrast, overexpression of APC decreased the sensitivity of HT-29 cells to gefitinib, accompanied by upregulation of pEGFR, p-AKT and pERK1/2. APC plays an important role in the sensitivity of CRC cells to gefitinib. APC may represent a potential therapeutic target for the treatment of CRC.

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Introduction

Colorectal cancer (CRC) is one of the leading causes of cancer-related morbidity and mortality worldwide. Although surgery is the most effective treatment for advanced CRC, recurrence frequently occurs within a few years after surgery. In addition to surgical treatment, chemotherapy has been widely used to improve the survival rate, but the existing chemotherapy regimens such as oxaliplatin or irinotecan plus 5-fluorouracil have not achieved satisfactory results (1,2). It is well known that several signaling pathways such as the epidermal growth factor receptor (EGFR) pathway are involved in the carcinogenesis and progression of CRC (3,4). Developing novel chemotherapeutic strategies targeting these signaling pathways is critical for reducing the carcinogenic progression of CRC.

EGFR is a transmembrane tyrosine kinase receptor that is thought to control cell growth, differentiation and survival (5). Two main intracellular pathways activated by the EGFR are the mitogen-activated protein kinase (MAPK) pathway and the phosphatidylinositol 3-kinase (PI3K)-protein kinase B (AKT) pathway, which are involved in cell proliferation, migration, differentiation and apoptosis (3,6). The EGFR and its downstream signaling pathways are frequently overexpressed in CRC, and are associated with a high risk of metastasis and poor prognosis of CRC (3,7). Antitumor agents targeting the EGFR such as gefitinib have been used for the treatment of CRC (8). Gefitinib (Iressa, ZD-1839), an EGFR-tyrosine kinase inhibitor, binds at the ATP site on the EGFR, and thus blocks its downstream signaling pathway that is involved in the proliferation and survival of cancer cells (9,10). Gefitinib has been used for the treatment of non-small cell lung cancer, and improves the survival rate of patients with lung cancer with acceptable toxicity (11-13). Gefitinib has also been reported to reduce tumor regression in patients with metastatic CRC (14). However, a randomized phase II study demonstrated that gefitinib is inactive as a single agent in patients with previously treated CRC (15). In addition, adding gefitinib into the chemotherapy regime using capecitabine (16), raltitrexed (17) or 5-fluorouracil, folinic acid and irinotecan (18) does not improve the efficacy in patients with CRC. These studies suggest that CRC cells may exhibit resistance to gefitinib.

The adenomatous polyposis coli (*APC*) gene encodes a 310-kDa tumor-suppressor protein that plays a role in cell growth, differentiation, migration, adhesion and apoptosis (19). Mutations in the *APC* gene have been found in more than 80% of sporadic CRCs and are responsible for hereditary forms of CRC (19,20). The best-known function of the APC protein is as a scaffolding protein in a protein complex, including GSK-3β, axin and APC, that regulates the cellular level of β-catenin in the Wnt signaling pathway (21). Mutations in the APC genes lead to aberrant activation of β-catenin, which causes transcriptional activation of several target genes of the Wnt signaling pathway such as cyclin D1 and c-Myc, thus, promoting cell proliferation and differentiation (19,20). In addition, the Wnt/β-catenin and EGFR signaling pathways can crosstalk in cancer (22). However, it remains unclear whether changes in APC can regulate EGFR signaling in CRC cells and alter their sensitivity to gefitinib.

In the present study, we aimed to investigate the effect of APC on the sensitivity of CRC cells to gefitinib, using human HCT-116 (wild-type APC) and HT-29 (mutant APC) CRC cells. We found that HT-29 cells exhibited enhanced sensitivity to gefitinib when compared to HCT-116 cells. Knockdown of APC expression increased the sensitivity of HCT-116 cells to gefitinib, whereas overexpression of APC decreased the sensitivity of HT-29 cells to gefitinib. Our data suggest that inhibiting APC may represent a potential novel therapeutic method for increasing gefitinib sensitivity in the treatment of CRC.

Materials and methods

Cell culture. The human colorectal cancer cells, HCT-116 containing wild-type APC, HT-29/APC (APC inducible) containing mutant APC, and HT-29/βGal (β-galactosidase inducible), were purchased from the Type Culture Collection of the Chinese Academy of Sciences (Shanghai, China). Cells were cultured in RPMI-1640 medium supplemented with 10% fetal bovine serum (FBS), 100 U/ml penicillin, and 100 mg/ml streptomycin in a humidified atmosphere in a 37˚C, 5% CO_2 incubator.

RNAi and cell transfection. The APC-specific siRNA sequence was 5'-GATCCATACACATTCAAACACTTATTCAAGAG ATAAGTGTTTGAATGTGTATGGA-3', and was cloned into the pSilencer 4.1-CMV neo Vector. Scramble siRNA was used as a negative control. HCT-116 cells were transfected with the pSilencer 4.1-CMV neo Vector containing either APC-specific shRNA or the scramble control, using Lipofectamine (Invitrogen). Forty-eight hours after transfection, cells were used for the following experiments. To test the effect of overexpression of APC on the sensitivity of HT-29 cells to gefitinib, HT-29/APC and HT-29/βGal cells were cultured in the presence of 0, 50 or 100 μM $ZnCl_2$ for 24 h. Cells were then used for the following experiments.

Cell viability assay. MTT assay was used to measure cell viability following exposure to gefitinib. Cells were seeded in 96-well plates at a density of $5x10^3$ cells/well, and allowed to grow in RPMI-1640 medium for 24 h. Gefitinib was added at final concentrations of 0-100 μM. After 0, 24, 48 and 72 h, cells were incubated with 5 mg/ml MTT for 4 h, and subsequently solubilized in DMSO (100 μl/well). The absorbance at 570 nm was then measured using an ELISA reader. Experiments were repeated at least three times.

RNA extraction and reverse transcription-polymerase chain reaction (RT-PCR). Total RNA was isolated from the HCT-116 and HT-29 cells using TRIzol reagent (Gibco-Invitrogen, Carlsbad, CA, USA). RNA was reverse transcribed into complementary DNA using a reverse transcription kit (Takara, Dalian, China) according to the manufacturer's protocol. PCR was performed in a mixture containing 2 μl cDNA, 2 mM $MgCl_2$, 0.03 U/l *Taq* DNA polymerase, 0.4 mmol/l dNTP, and 0.5 μM primers at a final volume of 25 μl. Primers used for amplification of APC were 5'-CTGCAGCTTCATATGATC-3' (sense) and 5'-AGAGTCTTTGTCATTGCAT-3' (antisense). β-actin was used as an internal control. The reaction condition was as follow: 94˚C for 2 min; 35 cycles of 94˚C for 30 sec, 50˚C for 30 sec, and 72˚C for 1 min; and 72˚C for 10 min. The mRNA expression of APC was normalized to the expression of β-actin. The relative expression of APC was calculated using the $2^{-\Delta\Delta CT}$ method.

Western blot analysis. Cells were homogenized on ice in lysis buffer. The lysates were centrifuged at 12,000 rpm for 30 min at 4˚C. Protein concentrations were determined using the BCA method. Proteins were separated by electrophoresis in 10% SDS-PAGE, and transferred onto polyvinylidene fluoride membranes by electroblotting. Membranes were incubated with primary antibodies against APC, EGFR, ERK, p-ERK, AKT and p-AKT (dilution 1:1,000) at 4˚C overnight. β-actin was used as a loading control. Membranes were then incubated with horseradish peroxidase-linked goat anti-rabbit secondary antibodies (dilution 1:2,500) at room temperature for 2 h. All antibodies were purchased from Santa Cruz Biotechnology (Santa Cruz, CA, USA). Bands were visualized using a chemiluminescence detection system.

Flow cytometry. Flow cytometric analysis was performed on a FACSCalibur (Becton-Dickinson). For determination of the cell cycle, 500 μl of cell culture was incubated with 30 μg/ml propidium iodide (PI) for 30 min at 37˚C before analysis. For detection of apoptotic cells, cells were harvested, washed twice with phosphate-buffered saline (PBS), and then incubated for 30 min at 37˚C with a solution of fluorescence isothiocyanate (FITC)-conjugated Annexin V (2.5 μg/ml) and PI (5 μg/ml) (Bio-Sea, China) before analysis for apoptosis.

Wound healing assay. Cells were seeded at a density of $2.5x10^5$ cells/ml in 6-well plates. Cells were cultured until reaching full confluency. A cell monolayer was carefully scratched across the diameter of the wells using a sterile 1-ml pipette tip. The cells were further cultured for 48 h, and the wound closure was examined under a microscope. Experiments were repeated three times.

Transwell assay. Cell invasion assays were performed using Transwell membrane filter inserts (Corning Costar Corp., Corning, NY USA). The upper surface of the Transwell membrane was coated with Matrigel™ Basement Membrane

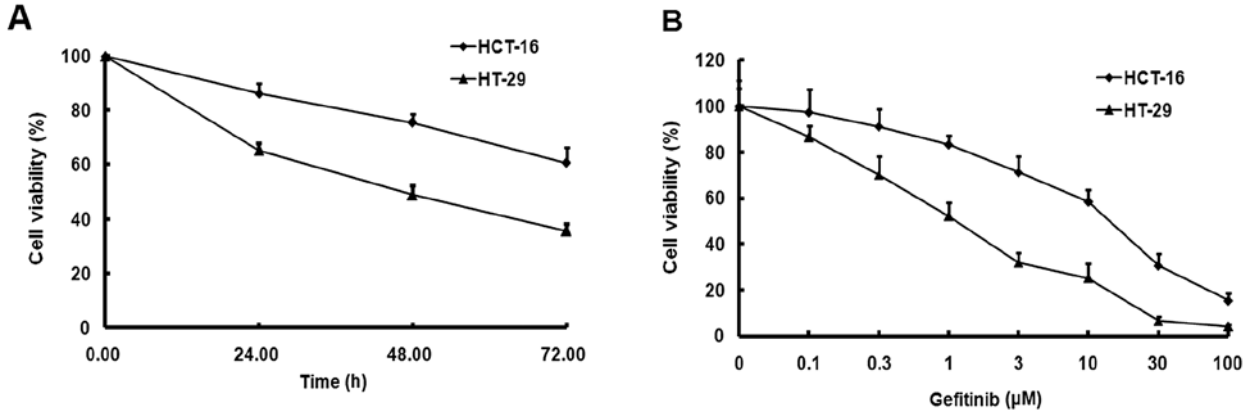

Figure 1. Increased gefitinib sensitivity in HT-29 cells. (A) The time-dependent and (B) dose-dependent effects of gefitinib on the survival of HCT-116 and HT-29 cells were determined. Cells were seeded in 96-well plates at a density of $5x10^3$ cells/well for 24 h. (A) The survival rate of HCT-116 and HT-29 cells after 1 μM gefitinib treatment for 0, 24, 48 and 72 h. (B) The survival rate of HCT-116 and HT-29 cells after treatment with different concentrations of gefitinib (0-100 μM) for 48 h; n=9 for each data point.

Matrix (BD Biosciences, San Jose, CA, USA) for 4 h. The membrane was placed in the upper compartment of 24-well culture plates. Cells in serum-free medium were harvested and seeded at a density of $2.5x10^5$ cells/ml onto Transwell inserts. The lower compartment was filled with cell culture medium containing 20% FBS. Cells were allowed to migrate for 24 h at 37˚C. The Matrix was erased and the inserts were washed with PBS for 3 times. The chamber was then fixed in 95% methanol for 20 min. Cells on the upper surface of the membrane were removed by wiping with a cotton swap. Membranes were stained in 0.1% hexamethyl pararosaniline for another 20 min. The membrane was then mounted onto a glass slide. Cell invasion was determined by counting the number of invasive cells. Ten random fields per membrane (x200) were counted for each assay, and three separated assays were performed.

Statistical analyses. Statistical analyses were performed using SPSS 13.0. All values are presented as the mean and standard deviation. The Student's t-test was used to compare differences between two groups. A probability value <0.05 was considered to indicate a statistically significant result.

Results

HCT-116 and HT-29 cells exhibit differential sensitivity to gefitinib. We investigated the effect of APC on the sensitivity of human HCT-116 (wild-type APC) and HT-29 (mutant APC) CRC cells to gefitinib. Cells were treated with various concentrations of gefitinib (0-100 μM) for 0-72 h, and cell viability was examined using MTT assay. Gefitinib inhibited the cell viability of the HCT-116 and HT-29 cells in a time-dependent manner (Fig. 1A). At 24, 48 and 72 h after gefitinib treatment, the survival rate of HCT-116 cells was significantly higher than that of HT-29 cells (P<0.05). The survival rates of both cells decreased in a dose-dependent manner. The IC_{50} value for gefitinib in HCT-116 cells was 15.64±1.48 μM, which was ~15-fold higher than that of the HT-29 cells (1.19±0.04 μM) (Fig. 1B).

Gefitinib induces increased apoptosis in HT-29 cells when compared with the HCT-116 cells. We further examined the effect of gefitinib on the apoptosis of HCT-16 and H-29 cells, using flow cytometry. Gefitinib treatment increased apoptosis of both cell lines in a dose-dependent manner (Fig. 2A). Gefitinib (10 μM) significantly induced increased apoptosis in HT-29 cells when compared with that in HCT-116 cells (P<0.05; Fig. 2A). Exposure of HCT-116 cells to gefitinib resulted in an increase in the percentage of G0/G1-phase cells in a dose-dependent manner. Gefitinib-treated HT-29 cells were arrested in the S phase of the cell cycle in a dose-dependent manner (Fig. 2B).

Gefitinib-induced inhibition of cell migration is greater in HT-29 cells than that in HCT-116 cells. We investigated the effect of gefitinib on the cell mobility of HCT-116 and HT-29 cells using a monolayer wound-healing assay. Gefitinib inhibited the cell migration of both HCT-116 and HT-29 cells in a dose-dependent manner. Following gefitinib treatment for 48 h, HCT-116 cells migrated more slowly than HT-29 cells (Fig. 3A). Gefitinib-induced inhibition of cell migration was further confirmed by Transwell migration assay (Fig. 3B). After gefitinib treatment for 48 h, the number of HCT-116 cells that migrated into the lower chamber was significantly higher when compared with that of HT-29 cells (P<0.05; Fig. 3B).

Knockdown of APC increases the sensitivity of HCT-116 cells to gefitinib. We further tested whether APC contributes to the sensitivity of HCT-116 cells to gefitinib using APC siRNA to knock down endogenously expressed APC. RT-PCR results showed that APC siRNA decreased the expression of APC in HCT-116 cells (Fig. 4A). The APC siRNA-induced inhibition of APC expression was further confirmed by western blot analysis (Fig. 4B). We then examined the sensitivity of HCT-116 cells to gefitinib after reducing APC expression. Following gefitinib treatment, the survival rate of HCT-116 cells treated with APC siRNA was significantly reduced when compared with cells treated with scramble siRNA (P<0.05; Fig. 4C), suggesting that knockdown of APC increased the sensitivity of HCT-116 cells to gefitinib.

Enhanced expression of APC decreases the sensitivity of HT-29 cells to gefitinib. We further investigated whether

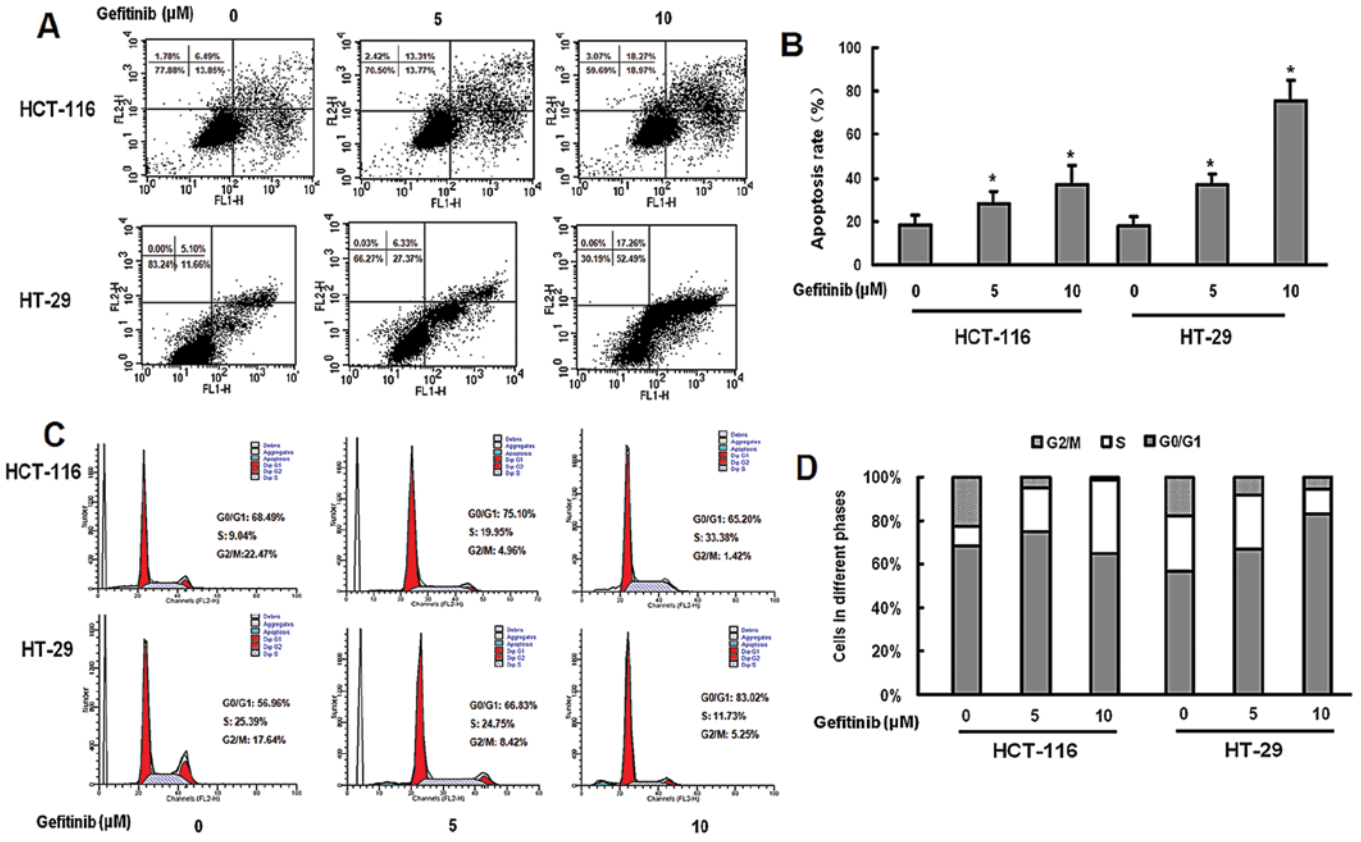

Figure 2. Gefitinib induces apoptosis in HCT-116 and HT-29 cells. (A) Representative flow cytometric analysis of apoptotic HCT-116 and HT-29 cells following treatment with gefitinib (0, 5 and 10 μM) for 48 h. (B) The percentage of apoptotic HCT-116 and HT-29 cells following treatment with gefitinib (0, 5 and 10 μM) for 48 h. Data are represented as mean and standard deviation; n=9. *P<0.05 vs. control. (C) Representative flow cytometry histograms showing cell cycle distribution in HCT-116 and HT-29 cells following treatment with 0, 5 and 10 μM gefitinib for 48 h. (D) Percentage of cells in the different phases of the cell cycle in HCT-116 and HT-29 cells following treatment with 0, 5 and 10 μM gefitinib for 48 h.

enhanced expression of APC in HT-29 cells improves their sensitivity to gefitinib. Western blot results showed that $ZnCl_2$ treatment upregulated the expression of APC in the HT-29/APC cells in a concentration-dependent manner (Fig. 5A). Gefitinib treatment dose-dependently decreased the survival rate of HT-29/βGal cells after exposure to 100 μm $ZnCl_2$. The gefitinib-induced decrease in the survival rate was significantly reduced in HT-29/APC cells compared with that in HT-29/βGal cells (P<0.05; Fig. 5B), suggesting that upregulation of APC inhibited the sensitivity of HT-29 cells to gefitinib.

APC regulates the EGFR and its downstream pathways. We then explored the potential role of APC on the EGFR signaling pathway. Knockdown of APC significantly downregulated the expression of pEGFR, p-AKT and pERK1/2 in HCT-116 cells, whereas overexpression of APC significantly upregulated the expression of pEGFR, p-AKT and pERK1/2 in HT-29 cells (Fig. 6).

Discussion

EGFR kinase inhibitors have been clinically used for the treatment of lung cancer with improved survival (23-25). However, several clinical studies have shown that EGFR kinase inhibitors have not achieved satisfactory outcomes in CRC patients (15-18). The mechanisms underlying CRC resistance to EGFR kinase inhibitors remain unknown. Mutations in APC genes have been associated with CRC (19,20). However, it remains to be determined whether APC is involved in the sensitivity of CRC cells to EGFT kinase inhibitors. In the present study, we investigated the role of APC in the sensitivity of human CRC cells to gefitinib, using HCT-116 cells containing wild-type APC and HT-29 cells containing mutant APC. We found that gefitinib inhibited the viability, promoted apoptosis, and reduced the migration of HCT-116 and HT-29 cells. HT-29 cells exhibited more sensitivity to gefitinib than HCT-116 cells. Furthermore, knockdown of APC expression improved the sensitivity of HCT-16 cells to gefitinib, whereas overexpression of APC decreased the sensitivity of HT-29 cells to gefitinib. The present study suggests that APC is critical for determining the sensitivity of CRC cells to gefitinib.

APC is known to play an important role in the pathogenesis of CRC (20). Mutations in *APC* genes have been found in ~80% of patients with sporadic CRC (19,20). Abnormal production of mutant APC proteins leads to accumulation of β-catenin, which promotes transcriptional activation of many proliferation genes (19,20). The role of APC in CRC is largely attributed to its inhibition of Wnt/β-catenin signaling, which has been found to be associated with resistance to chemotherapy in cancers (26-28). It has been reported that knockdown of the expression of β-catenin increases the sensitivity of lung cancer cells to gefitinib (29). If β-catenin-

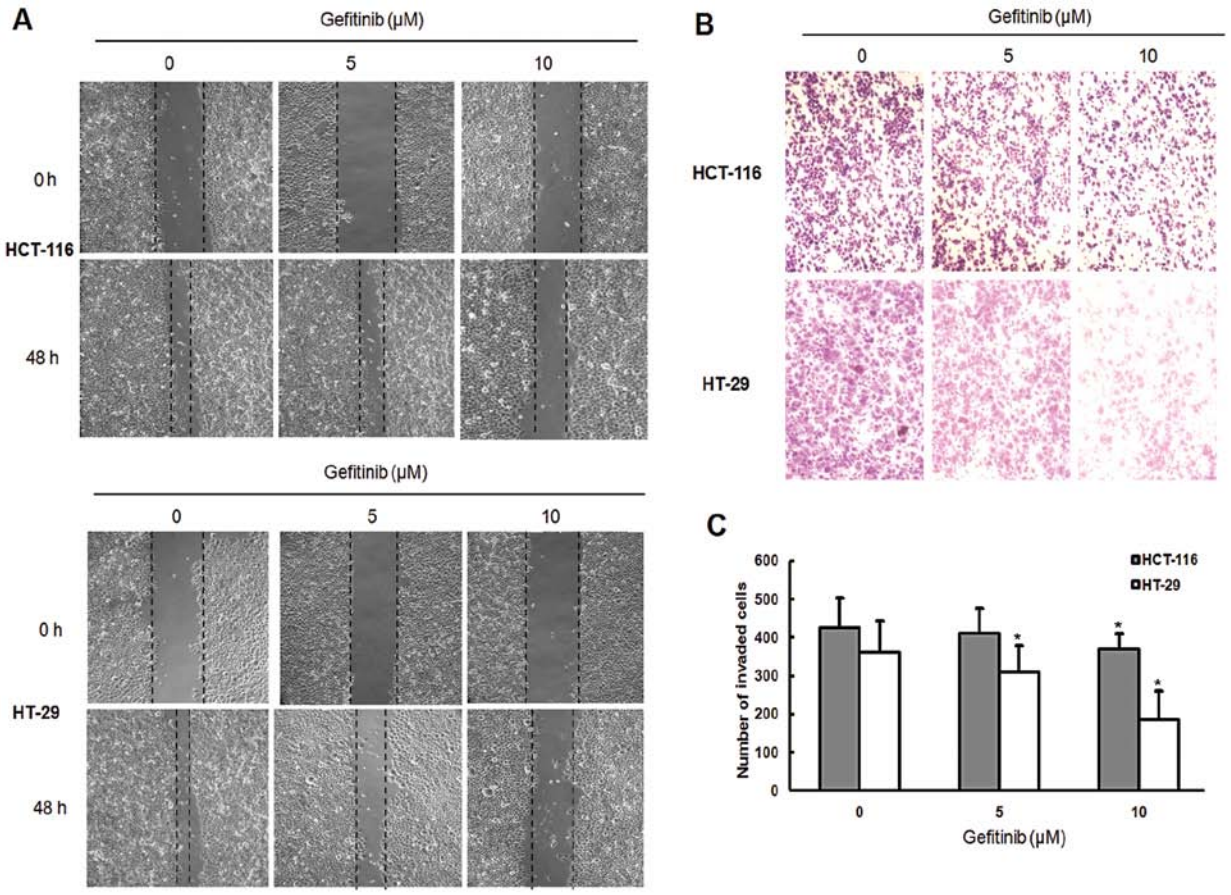

Figure 3. Gefitinib suppresses cell migration and invasion in HCT-116 and HT-29 cells. (A) Wound healing assay was performed to assess the effect of gefitinib on the migration of HCT-116 and HT-29 cells. Gefitinib treatment for 48 h inhibited migration of the cells in a concentration-dependent manner. The broken white line indicates the wound areas without migrating cells. The assay was repeated three times and representative images are shown. (B) Representative images of the invasiveness of HCT-116 and HT-29 cells that migrated through Transwell membranes. Cells were treated with 0, 5 and 10 μM gefitinib for 48 h. (C) Quantification of the Transwell assay by counting the number of invasive cells through the Transwell membranes. Data are mean ± SD; n=9. *P<0.05 vs. control.

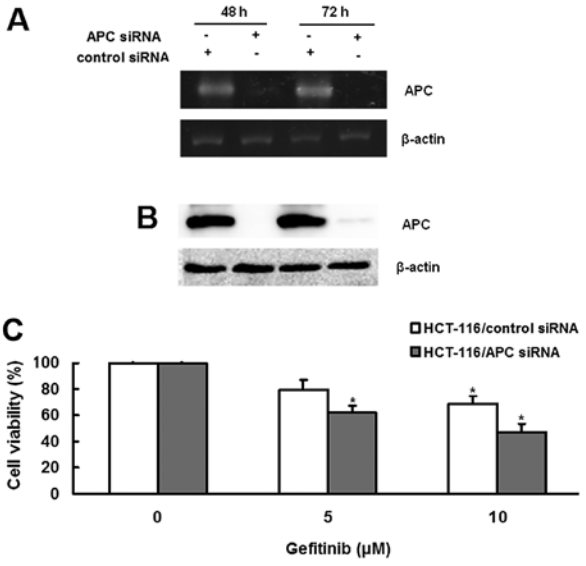

Figure 4. Knockdown of APC increases the sensitivity of HCT-116 cells to gefitinib. (A) Representative RT-PCR and (B) western blot analysis showing the expression of APC in HCT-116 cells treated with APC siRNA or the scramble control for 48 and 72 h. (C) The survival rate of HCT-116 cells transfected with APC siRNA and the scramble control following treatment with 0, 5 and 10 μM gefitinib for 48 h. Cell survival was determined using MTT assay; n=9. *P<0.05 vs. control.

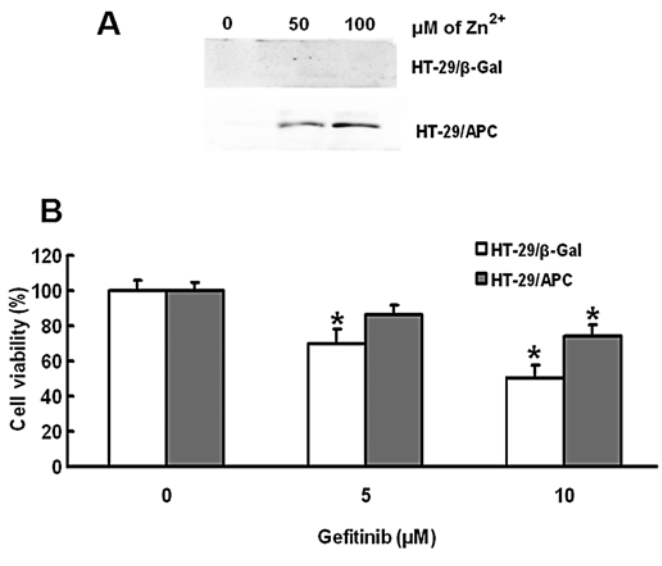

Figure 5. Overexpression of APC decreases the sensitivity of HT-29 cells to gefitinib. (A) Representative western blot analysis showing the expression of APC in HT-29/βGal and HT-29/APC cells treated with 0, 50 and 100 μM $ZnCl_2$ for 24 h. (B) The survival rate of HT-29/βGal and HT-29/APC cells following treatment with 0, 5 and 10 μM gefitinib for 48 h. Cells were pretreated with 100 μM $ZnCl_2$ for 24 h. Cell survival was determined using MTT assay. n=9. *P<0.05 vs. control.

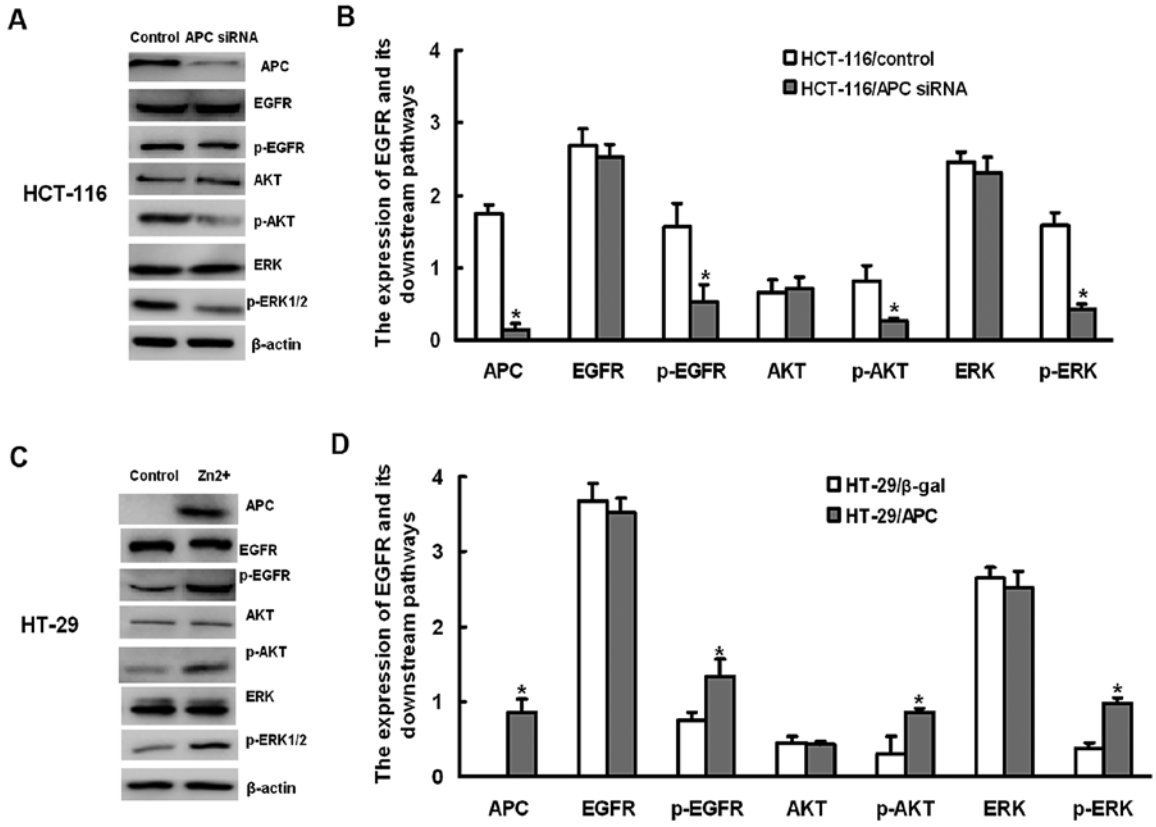

Figure 6. Effect of altering APC expression on the activity of the EGFR and its downstream pathways. Representative western blot analysis shows the expression of EGFR and its downstream AKT and ERK1/2 signaling in (A) HCT-116 cells transfected with APC siRNA and scramble control and in (C) HT-29/βGal and HT-29/APC cells treated with 100 μM $ZnCl_2$ for 24 h. (B and D) Quantitative protein expression levels of APC, EGFR and its downstream signaling normalized to β-actin. Data are presented as means ± standard deviation; n=3. *P<0.05 vs. control.

mediated resistance to gefitinib occurs in CRC, we would expect that the knockdown of the expression of APC, which leads to upregulation of β-catenin, may result in CRC resistance to gefitinib. However, in the present study, we found that the knockdown of APC expression increased the sensitivity of HCT-16 cells to gefitinib. Our results suggest that APC may mediate gefitinib resistance in CRC, at least in part, via a mechanism different from lung cancer. It has been reported that EGFR mutations are associated with the sensitivity of non-small cell lung cancer to gefitinib (23-25). The incidence of EGFR mutations in lung cancer varies in different regions, and has been reported to be 15% in Korea (30), 28% in Greece (31) and 45% in Japan (32). However, the incidence of EGFR mutations in CRC is ~0.34-12% depending on different regions (33,34), which is much lower than that in lung cancer. The low incidence of EGFR mutations in CRC suggests that CRC may exhibit a different mechanism underlying gefitinib resistance compared with lung cancer. Our findings that APC mediated gefitinib resistance suggest that APC may represent a novel mechanism underlying gefitinib resistance.

Gefitinib resistance presents a great challenge for its clinical application for the treatment of cancer. The mechanisms underlying gefitinib resistance in cancer cells remain poorly understood. Altered EGFR and its downstream signaling pathways are likely to contribute to gefitinib resistance. It has been reported that inhibiting sialylation of EGFR increases the sensitivity of CRC cells to gefitinib (35). In addition, mutations in cancer cells with reduced phosphorylation levels of EGFR exhibit enhanced sensitivity to gefitinib (29,36). In agreement with these reports, we found that the phosphorylation level of EGFR was increased in CRC cells with low expression of APC, accompanied with more sensitivity to gefitinib. Furthermore, we found that downregulation of APC increased the activity of the EGFR and its downstream AKT and ERK1/2 signaling pathways. Conversely, overexpression of APC reduced the activity of the EGRF and its downstream pathways. However, the mechanisms underlying APC-mediated regulation of EGFR signaling remain unclear. It has been reported that overexpression of β-catenin confers lung cancer cells resistance to gefitinib (29). Our present finding that the knockdown of APC expression, which should upregulate the expression of β-catenin, increased the sensitivity of HCT-116 cells to gefitinib suggests that APC may not regulate the EGFR signaling via the Wnt/β-catenin. APC has also been reported to regulate cytoskeletal proteins such as F-action (19), which can bind to the EGFR (37), suggesting that APC may regulate EGFR via F-action. Further studies are required to demonstrate the signaling pathway that is involved in APC-mediated regulation of EGFR signaling.

In summary, we found that APC plays an important role in the sensitivity of CRC cells to gefitinib. Our finding that the inhibition of APC increased the sensitivity of CRC cells to gefitinib suggests that APC may represent a potential therapeutic target for the treatment of CRC. Further animal studies

and clinical trials are required to verify the efficacy of the inhibition of APC in the treatment of CRC.

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