



Effects of cetuximab combined with afatinib on the expression of KDR and AQP1 in lung cancer

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ABSTRACT. In this study, we examined the effect of cetuximab (epidermal growth factor receptor monoclonal antibody) combined with afatinib (epidermal growth factor receptor and human epidermal growth factor receptor 2 tyrosine kinase irreversible inhibitor) on the apoptosis of A549 cells and on kinase domain receptor (KDR) and aquaporin 1 (AQP1) expression in A549 cells. A549 cells were cultured in RPMI-1640 and then divided into 4 groups: control group, 1-nM cetuximab group, 25- μ M afatinib group, and 1-nM cetuximab + 25- μ M afatinib group. After incubation for 48 h, the cell inhibition rate, cell cycle distribution, and invasive ability of A549 cells before and after treatment were examined using MTT, flow cytometry, and transwell assays, respectively. Gene and protein expression levels of KDR and AQP1 were detected by reverse transcription-polymerase chain reaction and western blot analysis. Cetuximab and afatinib significantly inhibited A549 cell growth. Their combination produced greater growth inhibition ($P < 0.01$). Cetuximab and afatinib both induced the apoptosis of A549 cells, and their combination produced a higher apoptosis rate ($P < 0.01$). Compared with monotherapy, cetuximab in combination with afatinib induced G1 phase arrest and downregulated the gene and protein

expression of KDR and AQP1 ($P < 0.05$). Cetuximab in combination with afatinib synergistically inhibited the growth and migration of cells and downregulated the gene and protein expression of KDR and AQP1, indicating that a combination of cetuximab and afatinib is a potential strategy for lung cancer therapy.

Key words: Apoptosis; Aquaporin-1; Cetuximab; Kinase domain receptor; Afatinib

INTRODUCTION

Lung cancer is a human malignancy with a high incidence and a fatality rate that ranks first in malignant tumors. Invasion and metastasis of lung cancer are leading causes of death and failure in treatment. Angiogenesis plays an extremely important role in the invasion and metastasis of lung cancer (Donnem et al., 2011; Kim et al., 2011). Recent studies have shown that the expression of aquaporin-1 (AQP1) and vascular endothelial growth factor II/kinase domain receptor (KDR) was elevated in many tumors and vascular endothelial cells, which plays a key role in promoting angiogenesis and tumor cell proliferation (Jiang, 2009; Gershtein et al., 2010; Waldner et al., 2010; Wang and Owler, 2011). In this study, lung adenocarcinoma A549 cells were examined to observe the effects of cetuximab, epidermal growth factor receptor (EGFR) monoclonal antibody, and afatinib, an EGFR and HER2 tyrosine kinase irreversible inhibitor, on the proliferation and apoptosis of A549 cells and on the expression of kinase insert domain receptor (KDR) and AQP1 *in vivo* when used alone and in combination, in order to investigate the synergistic effect between these treatments and their mechanism to provide experimental evidence for the clinical treatment of lung cancer.

MATERIAL AND METHODS

Reagents and instruments

The lung adenocarcinoma cell line A549 was obtained from the Shanghai Cell Institute of the Chinese Academy of Sciences. RPMI 1640 medium, fetal bovine serum, and trypsin were purchased from Gibco Company (Grand Island, NY, USA). SPB was purchased from Merck, (Kenilworth, NJ, USA). Afatinib was from Boehringer Ingelheim (Ingelheim, Germany). Cetuximab was from Merck. Penicillin was obtained from Beijing Chemical Factory (Beijing, China). Streptomycin was from PeproTech (Rocky Hill, NJ, USA). The 5% tetrazolium blue solution (MTT solution) was from Shanghai Sino-American Biotechnology Company (Shanghai, China). The Coulter XL flow cytometer was obtained from Beckman Coulter (Brea, CA, USA). The 550 microplate reader was purchased from Bio-Rad Inc. (Hercules, CA, USA). The CKX4 biological inverted microscope was obtained from Olympus (Tokyo, Japan).

Methods

Cell culture and grouping

A549 cells were incubated with RPMI1640 medium containing 10% fetal bovine serum in a humidified incubator with 5% CO₂ at 37°C. Cells were seeded at a density of 1 x 10⁶/100-mL flask

and conventionally cultured for 24 h. Drug treatment was performed as follows: control group [1 mL phosphate-buffered saline (PBS) solution], 1-nM cetuximab group; 10- μ M afatinib group; 1-nM cetuximab + 10- μ M afatinib group. The drug concentrations were determined based on previous studies (Wang et al., 2009; Chen et al., 2010).

MTT assay

A549 cells were seeded on 96-well plates at a density of 5×10^4 cells/well. Grouping was performed as described above. Each group used 3 parallel wells; after 48 h incubation at 37°C with 5% CO₂, the supernatant was removed. The cells were rinsed with saline once and 20 μ L 5 mg/mL MTT was added into each well. After another 4-h incubation, the supernatant was discarded, and the cells were incubated with 200 μ L 10% sodium dodecyl sulfate overnight. After shocking for 15 min, the absorbance value at 570 nm was detected using an automatic microplate reader. Inhibition rate (%) = (A control - A experiment) / (A control - A blank) x 100%.

Flow cytometry to evaluate cell progression

Cells in each group were collected and washed twice with cold PBS; the cell concentration was adjusted to 1×10^6 /L; cells were fixed in 70% cold ethanol (4°C) for 24 h. After washing, the cells were incubated with Tris-HCL buffer (pH 7.4), containing 10 μ g/mL RNA enzyme for 30 min. Next, 50 μ g/mL propidium iodide was used to stain the DNA. DNA distribution was determined by flow cytometry over 1 h, and the percentage of cells in each cycle was calculated.

Transwell chamber invasion assay

A polycarbonate membrane filter was capped with Matrigel (150 μ g/well). In the polymerized lower chamber, 10% fetal bovine serum was added as conditioned medium, while 100 μ L A549 cell suspension (total number of cells: 3×10^5 /L) was added to the upper chamber. After incubation for 24 h, non-invasive tumor cells in the upper chamber were carefully wiped off with a wet cotton swab, and then the membrane filter was fixed in 95% ethanol for 5 min, gently rinsed by PBS 3 times, and stained with hematoxylin and eosin. After natural drying, the upper polycarbonate membrane was carefully removed using a scalpel by slicing along the edge and fixed on glass slides with resin glue (inner side facing up) for sheet-sealing. Invasive cells in the upper, lower, left, right, and middle fields were counted under a high-power microscope and the average was calculated. Three parallel chambers were used for each group.

Reverse transcription-polymerase chain reaction (RT-PCR)

The cell suspension in each group was centrifuged at 800 *g* for 5 min (rotor radius: 16 cm). The cells were collected, placed in a 1-mL homogenizer, and ground with 1 mL TRIzol reagent on ice. Total RNA was extracted using TRIzol reagent, according to the manufacturer instructions (Takara, Shiga, Japan). RNA content was determined using a UV spectrophotometer. Using total RNA as a template and Oligo (dT) as the primer, mRNA was reverse-transcribed into cDNA using a 2-step RT-PCR kit (Takara). The reaction parameters were as follows: 30°C, 2°C for 10 min, 99°C for 30 min, and 5°C for 5 min, 5 min. The cDNA obtained was stored at -20°C. The

cDNA was used as a template for amplification, and primer sequences are shown in Table 1. The reaction included 0.5 μ L upstream primer and 0.5 μ L downstream primer in a 40- μ L total volume. Amplification parameters were as follows: 94°C for 2 min, followed by 94°C for 30 s, 55°C for 30 s, and 72°C for 30 s for 45 cycles, and then 72°C for 5 min. PCR products were separated by agarose electrophoresis and analyzed using an FR200 image analysis system.

Table 1. Primer sequences.

Genes	Primer sequence (5'-3')	Product length (bp)	Temperature (°C)
β -actin	Upstream: AGAGG GAAAT CGTGC GTGAC Downstream: ACATC TGCTG GAAGG TGGAC	224	55
AQP1	Upstream: GGCCACGACCCCTTTGTCTTCAT Downstream: TCCCACAGCCAAGTGTAGTCGATAG	515	55
KDR	Upstream: GCTCAAGACAGGAAGACCAAGAA Downstream: TTCCCAATACTTGTCTGTCTGAT	408	55

Western blotting

After RT-PCR, the sample was treated with lysate and protease inhibitors and then centrifuged at 1500 rpm for 30 min at 4°C. The supernatant was collected, and protein content was determined using the Coomassie brilliant blue method. The sample was separated on a 5% stacking gel at a constant voltage of 40 V for 1 h, and then on a 10% separating gel at a constant voltage of 60 V for 3.5 h. Bands were transferred to a membrane at a constant voltage of 14 V for 14 h and sealed for 2 h on a shaker at 37°C. The membrane was washed 3 times for 10 min each. KDR primary polyclonal antibody (1:1000), AQP1 polyclonal antibody (1:1000), or anti-mouse β -actin (1:5000) was added to the membranes, following which they were incubated at 4°C overnight. The following day, goat anti-rabbit secondary antibody (1: 700) was added and the samples were incubated at 37°C in a shaker for 1.5 h. This was followed by washing the membranes with Tris-buffered saline containing Tween 20 3 times for 5 min each time and then washing with Tris-buffered saline 3 times for 5 min each time. The nitrocellulose membrane was treated with luminescent liquid (Pierce, A, B each 100 mL) for 5 min, covered with plastic wrap, and exposed in the cassette for 5 min. Developing, washing, post-fixing observation, and Quantity One image analysis (Bio-Rad) were performed. The gray values of target bands were divided by β -actin gray values to analyze the results.

Statistical analysis

The SPSS13.0 statistical software was used for analysis (SPSS, Inc., Chicago, IL, USA). Measurement data are reported as means \pm standard deviation; continuous variables between 2 groups were compared using the *t* test, while continuous variables among groups were compared using analysis of variance and covariance correction. Two-sided test level $\alpha = 0.05$.

RESULTS

Morphological observation

A549 cells were observed under an optical microscope and showed vigorous cell growth, dense distribution, mostly polygonal cells, and the convergence of cell trend. The polygonal cells had plumb cell bodies, 2-3 flat and long processes, and large, oval, and multi-center nuclei (Figure 1).



Figure 1. A549 morphology, as observed under the light microscope (200X).

OD₄₉₀ values of A549 cells in each group after treatment

The inhibition rate in the cetuximab monotherapy group was $30.6 \pm 1.2\%$. The inhibition rate in the afatinib monotherapy group was $29.8 \pm 1.4\%$. The inhibition rate in the 1 nM cetuximab + 25 μM afatinib group was $61.4 \pm 2.2\%$, indicating that the treatments had a synergistic effect (Table 2).

Table 2. OD₄₉₀ values of A549 cells in each group after treatment (means \pm SD, N = 9).

Group	OD ₄₉₀	Inhibition rate (%)
Control	0.57 ± 0.06	
Cetuximab	$0.38 \pm 0.08^{*\Delta}$	$30.6 \pm 1.2^{*\Delta}$
Afatinib	$0.39 \pm 0.05^{*\Delta}$	$29.8 \pm 1.4^{*\Delta}$
Combination	$0.27 \pm 0.06^{**}$	$61.4 \pm 2.2^{**}$

Compared with control group, *P < 0.05; compared with combination group, Δ P < 0.05; compared with control group, **P < 0.01.

Cell cycle progression, as evaluated by flow cytometry

As shown in Table 3, cells in G1 phase increased significantly in the cetuximab group, afatinib group, and combination group, compared with the control group. The largest numbers of cells in G1 phase were observed in the combination group, while the cells in S phase correspondingly decreased, with statistically significant differences (P < 0.05). No significant changes were observed for M phase, indicating better efficacy of 1 nM cetuximab + 25 μM afatinib than monotherapy.

Changes in A549 cell invasion *in vitro*

Figure 2 shows that a larger number of A549 cells passed through the membranes in control group. Penetrating cells significantly decreased in the cetuximab and afatinib groups. The

lowest number of penetrating cells was observed in the combination group. The results showed that 1 nM cetuximab + 25 μ M afatinib more effectively reduced the invasiveness of A549 cells.

Table 3. Effect on A549 cell cycle in each group (means \pm SD, N = 3).

Group	G0/G1	S	G2/M
Control	40.9 \pm 1.3	48.2 \pm 2.4	8.2 \pm 0.8
Cetuximab	58.1 \pm 3.5* Δ	35.4 \pm 2.1* Δ	7.9 \pm 1.3
Afatinib	58.4 \pm 2.7* Δ	35.2 \pm 2.3* Δ	7.8 \pm 2.0
Combination	68.8 \pm 4.2*	22.35 \pm 1.2*	10.0 \pm 1.6

Compared with control group, *P < 0.05; compared with combination group, Δ P < 0.05.

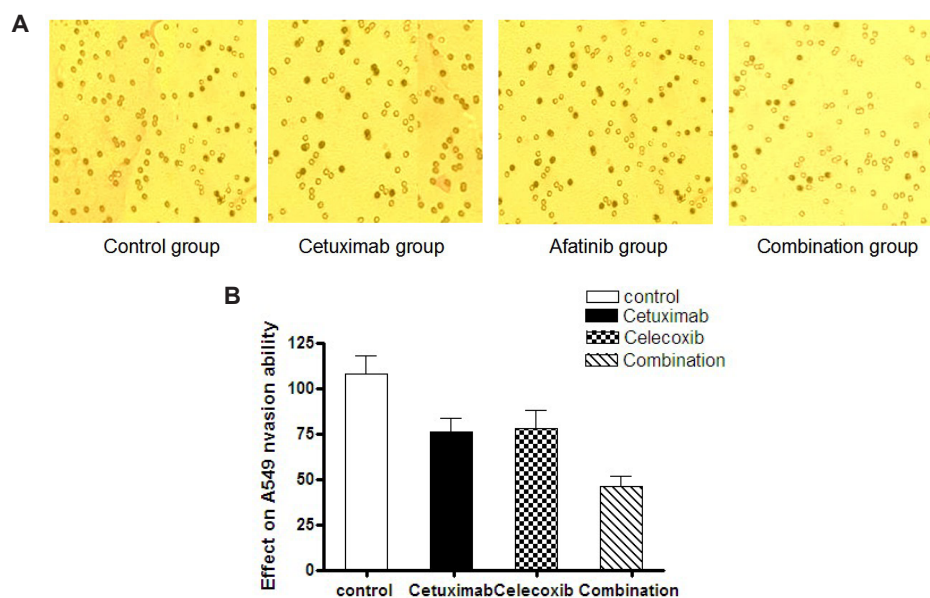


Figure 2. Changes in the invasion ability of A549 cells in each group.

AQP1 and KDR mRNA expression

After corresponding treatment, AQP1 and KDR mRNA expression levels in the combination group decreased compared with the cetuximab group and afatinib group; the difference was statistically significant (P < 0.05). Expression levels were significantly lower than those in the control group were, and the differences were statistically significant (P < 0.05), as shown in Figure 3.

AQP1 and KDR protein expression

After corresponding treatment, AQP1 and KDR protein expression levels in the combination group decreased compared with the cetuximab and afatinib groups; the difference was statistically significant (P < 0.05). Expression levels were significantly lower than those in the control group were, and the differences were statistically significant (P < 0.05), as shown in Figure 4.

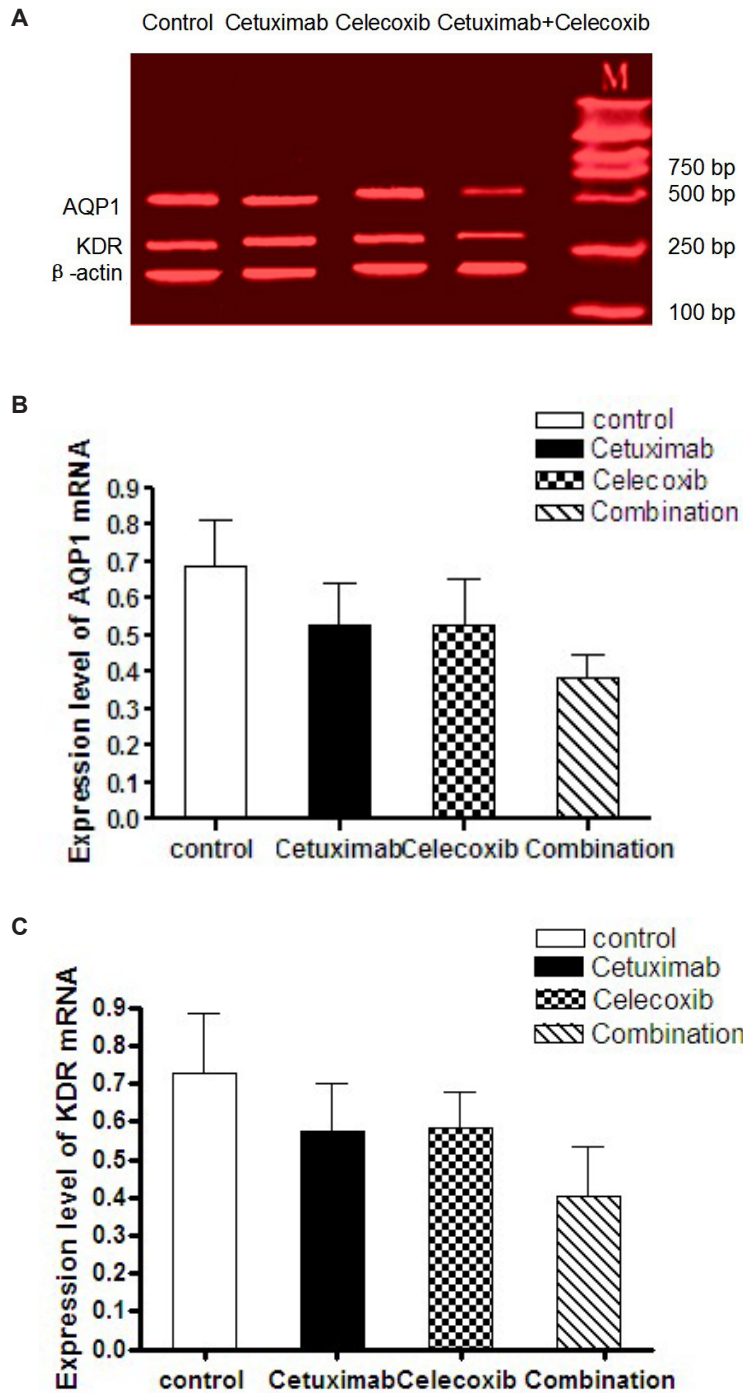


Figure 3. Changes in *AQP1* and *KDR* mRNA levels in each group of cells.

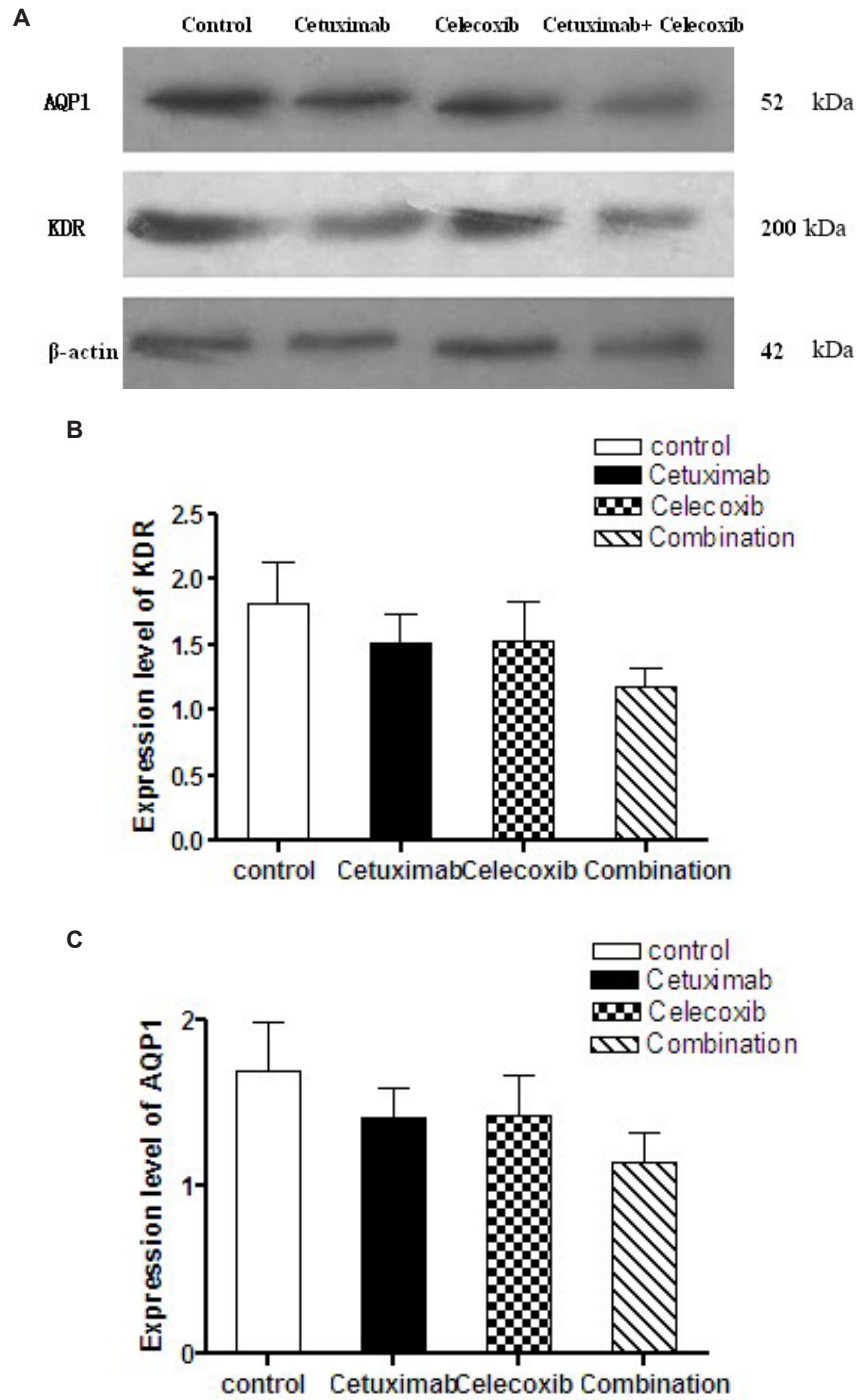


Figure 4. Changes in AQP1 and KDR protein levels in each group of cells.

DISCUSSION

Lung cancer is a common clinical cancer, and its fatality rate ranks first among all types of cancers. It can be divided into small cell lung cancer and non-small cell lung cancer; non-small cell lung cancer accounts for more than 85% of lung cancer. Invasion and metastasis of lung cancer is the main reason for the failure of treatment and death in patients with lung cancer. Tumor angiogenesis plays a vital role in the invasion and metastasis of tumors (Hada and Horiuchi, 2005). Because the continued growth of a tumor must rely on angiogenesis, particularly for tumors >2 mm, limiting angiogenesis would strictly limit tumor growth potential (Siemann et al., 2004; Folkman, 2006).

Recent studies have shown that KDR and AQP1 are closely related to angiogenesis and were expressed not only in endothelial cells but also in tumor cells (Li and Zhang, 2010; Färkkilä et al., 2011). AQP1 is a recently discovered factor involved in tumor angiogenesis and is considered a marker of angiogenesis. Several studies have shown that many tumor tissues, tumor cell lines, and microvascular endothelial cells express high levels of AQP1 (Endo et al., 1999; Bissell, 1999) and that expression level is directly related to the degree of malignancy. A higher degree of malignancy was found to be associated with higher expression of AQP1 (Moon et al., 2003), suggesting that AQP inhibitors or anti-AQP antibody can reduce the expression of AQP1 via lung adenocarcinoma treatment. KDR is also a main regulatory factor in tumor angiogenesis (Pander et al., 2011). Tumor cells secrete vascular endothelial growth factor, which not only affects vascular endothelium KDR in a paracrine manner to promote angiogenesis, but also affects their own KDR in an autocrine manner to directly promote tumor cell growth. Another study found that blocking the expression of KDR induced tumor cell apoptosis. Therefore, inhibition of KDR expression can inhibit tumor angiogenesis and prevent the growth of tumor cells, thereby blocking the growth and metastasis of lung cancer. Thus, KDR can inhibit angiogenesis and tumor growth control and is important for tumor treatment and cancer metastasis prevention.

Recent studies have shown that the cyclooxygenase-2 inhibitor afatinib can inhibit proliferation, induce differentiation, and promote apoptosis in various tumor cells (Xia et al., 2010). Cetuximab is a molecularly targeted cancer drug, which is also widely used in basic and clinical research (Lin et al., 2009; Sun et al., 2012). In this study, the combination of these drugs was applied in A549 cells *in vitro* to explore the effects on KDR and AQP1 expression and the inhibitory effect on tumor cells. We found that in A549 cells, cetuximab and afatinib had some anti-tumor effects *in vitro*, while in combination, they had a significant synergistic effect, which further inhibited cell growth and migration. Monotherapy with cetuximab and afatinib in A549 cells significantly reduced the levels of AQP1 and KDR gene and protein expression. The reduction of AQP1 and KDR gene and protein expression was more pronounced in the combination group. This indicates that reducing the expression of AQP1 and KDR at the gene and protein levels may be a molecular mechanism of synergy between these drugs. We analyzed the specific mechanisms and found that both drugs reduced AQP1 and KDR gene and protein expression by inhibiting cyclooxygenase-2 and exhibited anti-tumor effects to improve the microenvironment. After combination, the apoptosis rate increased ($P < 0.01$); compared with monotherapy, significant G1 phase arrest was observed following combination therapy ($P < 0.01$), and cell invasion was significantly reduced. This study demonstrated that a combination of cetuximab and afatinib had better efficacy in lung cancer treatment, which may be important for clinical treatment.

Conflicts of interest

The authors declare no conflict of interest.

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