Epidermal growth factor receptor inhibitor AG1478 affects HepG2 cell proliferation, cell cycle, apoptosis and c-Myc protein expression in a dose-dependent manner

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ABSTRACT
The aim of this study was to observe the effect of AG1478, an inhibitor of the epidermal growth factor receptor, on cell proliferation, cell cycle and apoptosis of human hepatoma HepG2 cells. Cell counting kit-8 assay was employed to examine the survival rates of cultured HepG2 cells after treatments with different concentrations of AG1478 for 24 h. Flow cytometry was performed to determine the effect of AG1478 on cell cycle and apoptosis. Immunohistochemistry was used to measure the expression of c-Myc protein. The survival rates of human hepatoma HepG2 cells after treatments with 5, 10, 20 and 40 \( \mu \text{mol/L} \) of AG1478 for 24 h were 76.0%, 59.6%, 51.2% and 42.1%, respectively. The apoptotic rates after treatments with 5, 10, 20 and 40 \( \mu \text{mol/L} \) of AG1478 were (12.88 \( \pm \) 1.91)%, (23.16 \( \pm \) 2.67)%, (35.36 \( \pm \) 1.95)% and (47.16 \( \pm \) 3.78)%, respectively. HepG2 cells were mainly arrested in the G0/G1 phase after treatment with 10, 20 and 40 \( \mu \text{mol/L} \) of AG1478. The c-Myc protein was highly expressed in HepG2 cells, whereas treatment with 20 \( \mu \text{mol/L} \) AG1478 substantially inhibited its expression. Overall, AG1478 inhibited the proliferation of human hepatoma cells in vitro, arrested the cells in G0/G1 phase, induced apoptosis and reduced the expression of c-Myc protein. These results also indicated that AG1478 blocked the proliferation and induced apoptosis of hepatoma cells in a dose-dependent manner.

Introduction
Primary liver cancer (hepatocellular carcinoma) is one of the mostly detectable cancers, with a total of 630,000 new liver cancer cases and 600,000 deaths all over the world every year. Primary hepatocellular carcinoma is a type of highly malignant cancer with easy recurrence and metastasis, resulting in poor 5-year survival rates in patients [1–3].

Epidermal growth factor receptor (EGFR) is a member of the EGFR family [4–6]. It is the expression product of oncogene c-erbB-1 located on the short arm of human chromosome 7, with a molecular weight of 170–175 kD. EGFR is a transmembrane receptor protein with tyrosine kinase activity. Through binding with its corresponding ligands such as epidermal growth factor (EGF) and transforming growth factor-\( \alpha \) (TGF-\( \alpha \)), EGFR undergoes molecular conformational changes, and its inner membrane domain binds to ATP for autophosphorylation, finally leading to the activation of the tyrosine kinase domain. Phosphorylated tyrosine kinase mediates the transduction of cytoplasmic signals into the nucleus through multiple pathways, such as Ras/Raf/mitogen-activated protein kinase (MAPK) and phosphatidylinositol-3-kinase (PI3K)/protein kinase B (Akt) [7–9]. This process changes cell cycle-related genes, induces cancer cell proliferation and suppresses apoptosis [10,11]. The levels of EGFR in normal tissues were very low, while EGFR was highly expressed in many tumour tissues [12,13]. In our previous study [14], immunohistochemistry was used to detect the expression of EGFR in hepatocellular carcinoma and adjacent tissues. The results showed that the level of EGFR in liver cancer tissues was significantly higher than that in adjacent noncancerous tissues, and was dependent on cancer stages. Jiang et al. [15] reported that EGFR was highly expressed in human hepatoma tissues, and EGF might induce cancer cell proliferation through MAPK extracellular signal-regulated kinase (ERK) pathway. AG1478, an EGFR-specific inhibitor, can suppress this process effectively, suggesting that EGFR can be an ideal target of anti-cancer drugs.

There are a variety of EGFR-targeted drugs that are being used clinically at present. These drugs are mainly classified into two groups: monoclonal antibodies and...
small molecule inhibitors. Monoclonal antibodies compete with physiological ligands for binding to EGFR and thus inhibit the ligand-activated tyrosine kinase activity of EGFR, resulting in anti-tumour effects. Small molecule inhibitors are usually referred to as molecules that bind to ATP-binding sites of the intracellular segment of EGFR to block the EGFR signalling pathway [16]. AG1478 is a synthetic small molecule that is a protein tyrosine kinase inhibitor specific for EGFR [17–19]. Through competitive binding to ATP-binding sites to inhibit protein tyrosine kinase activity, AG1478 blocks EGFR-mediated signalling pathway, inhibits cell proliferation of several tumours and induces apoptosis [20]. This study was designed to investigate the effects of AG1478 on hepatoma cell proliferation, cell cycle and apoptosis.

Materials and methods

Reagents

Dulbecco’s modified Eagle’s medium (DMEM), fetal calf serum and 0.25% trypsin were products of HyClone (USA). AG1478, dimethyl sulfoxide and Hoechst33258 were purchased from Sigma-Aldrich (Germany). Cell counting kit-8 (CCK-8) was purchased from Dojindo Molecular Technologies, Inc. (Japan). Propidium iodide (PI), cell cycle and apoptosis analysis kit, and annexin V/PI double staining kit were purchased from Invitrogen (USA). AG1478 was dissolved in DMEM to reach a concentration of 1 mmol/L, and then aliquoted for storage at –20°C. The antibodies against c-Myc, β-actin, histone H1 and goat anti-mouse IgG-HRP (Cat# sc-2005) were purchased from Santa Cruz Biotechnology, Inc. (Santa Cruz, USA). The ECL system was from Pierce Biotechnology (Rockford, IL, USA).

Cell line and cell culture

Human hepatoma HepG2 cells provided by the General Surgery Key Laboratory of our hospital were cultured in DMEM (Dulbecco’s Modified Eagle Medium) supplemented with 10% fetal calf serum. The cells were kept at 37°C in a 10% CO2/90% air atmosphere.

Cell morphology observation

HepG2 cells were treated with vehicle control (dimethyl sulfoxide; 0.006%, vol/vol) or AG1478 (20 μmol/L) for 48 h. Cell morphology was observed under a light microscope (Olympus, Japan). Meanwhile, the cells were stained with 10 μg/mL of Hoechst33258 at 37°C for 30 min. Then the stained cells were observed under a fluorescence microscope (Nikon, Japan).

Cell proliferation assay

HepG2 cells were digested using trypsin, diluted and seeded into 96-well microplates (5000 cells/well, 200 μL) in triplicate for both the control group and the experimental group. The experimental group contained four subgroups with 5, 10, 20 and 40 μmol/L AG1478 being added to the culture medium, respectively. After 24 h treatment, 10 μL of CCK-8 was added for further incubation for 3 h. A 96-well microplate reader was used to measure the absorbance at 450 nm (A450), using empty wells as blanks. Every assay was performed in triplicate at least. The cell survival rate was calculated by the formula of (average A450 of each experimental group/average A450 of the control group) × 100 (%).

Flow cytometry

To detect apoptosis, HepG2 cells were harvested from the plates after 24 h treatment with 0, 5, 10, 20 and 40 μmol/L of AG1478. After being digested by ethylenediaminetetraacetic acid (EDTA)-free trypsin to obtain single cell suspension, cells were centrifuged at 2000 r/min for 5 min. The supernatant was discarded after centrifugation, and the cells were washed twice in precooled phosphate-buffered saline (PBS). The binding buffer, Annexin V-fluorescein isothiocyanate (FITC) and PI were added sequentially according to instructions of the Annexin V-FITC/PI kit manufacturer. Flow cytometry was performed after incubation at 4°C for 30 min in darkness. Cells (1 × 10^6) were counted each time, using 488 nm as excitation wavelength. Cell Quest analysis software was used to calculate the apoptotic rate. Normal living cells were insensitive to Annexin V and PI staining (Annexin-V - PI-), and were distributed in the lower left area of the flow cytometric diagram. Early apoptotic cells were sensitive to Annexin V but insensitive to PI staining (Annexin-V+PI-), and were located in the upper left area. Late apoptotic cells or dead cells were sensitive to both Annexin V and PI staining (Annexin V+PI+), and were usually found in the upper right area [21].

To determine the cell cycle distribution, HepG2 cells were harvested after 24 h treatment with 0, 5, 10, 20 and 40 μmol/L of AG1478 followed by trypsin digestion for cell collection. Cells were washed by precooled PBS twice and centrifuged at 2000 r/min for 5 min. The supernatant and cell debris were discarded after centrifugation, and precooled 70% ethanol was then added to fix cells at 4°C overnight. After centrifugation and washing, 600 μL PI and RNase inhibitor were added for 30 min staining at room temperature in dark. The cell density was adjusted to 1 × 10^6/mL by filtering through a # 300 sieve. Cell cycle analysis was performed using flow cytometry by measuring cellular DNA content. The experiments were performed in triplicate.
**Immunohistochemistry assay**

Immunohistochemistry was performed using the streptavidin-peroxidase method. The cells were fixed and washed by PBS, and then incubated in 3% H₂O₂ for 10 min at room temperature. After washing with PBS, samples were blocked by goat normal serum for 30 min at room temperature. The serum was discarded before adding 1:100 diluted anti-c-Myc monoclonal antibody (primary antibody) into a humidified chamber, followed by incubation at 4 °C overnight. Biotin-labelled secondary antibody was then dripped onto the cells at 37 °C over a period of 30 min in a humidified chamber before washing with PBS. Samples were incubated in peroxidase-labelled streptavidin for 30 min at room temperature before washing with PBS. Chromogenic reagent 3,3’-diaminobenzidine was then used to develop the immunostains, and the slides were rinsed with water thoroughly before being restained by hematoxylin. Conventional dehydration, transparent treatment and mounting procedures were carried out. Light microscopy was used for observation. PBS was employed instead of primary antibody as control.

**Immunoblotting assays**

Total proteins were harvested from cells, separated in 10% SDS/PAGE gels, and then subjected to immunoblot analyses. The membrane was incubated with primary antibodies against c-Myc (1:500), β-actin (1:5000) and histone H1 (1:5000) and then with the secondary antibody of the goat anti-mouse IgG-HRP. The bound antibodies were detected using the ECL system. The immunoblot experiments were repeated more than three times. The mean normalized optical density (OD) of the protein bands relative to the OD of the β-actin band from the same conditions was calculated.

**Statistical analysis**

SPSS 17.0 statistical package for Windows was used for data processing. Results were presented as means with standard error (± SEM), and statistical differences between the control and experimental groups were evaluated using t test. P < 0.05 was considered statistically significant.

**Results and discussion**

**AG1478 causes morphological changes in HepG2 cells**

To observe the effect of AG1478 on the morphology of HepG2 cells, microscopy was used. Under an inverted microscope, HepG2 cells in the control group were growing adherently with fusiform or polygonal shapes. The cells contained abundant cytoplasm and underwent vigorous growth, with the neighbouring cells converging into a cell layer (Figure 1A). After adding AG1478 into the medium, the cultured HepG2 cells showed blurred edges. Black particles were visualized in the nucleus without clear gaps between particles. In addition, more and more cells detached and floated in the medium (Figure 1B). To further support this, we also used Hoechst 33258 staining to evaluate the phenotypic characteristics of AG1478-treated cells. The apoptotic nuclei with obvious morphological alterations were observed and dose-dependently (Figures 1C,D), showing that AG1478 may induce HepG2 cell apoptosis. These data suggested that AG1478 caused morphological changes in HepG2 cells.

**AG1478 exerts inhibitory effect on HepG2 cell proliferation in a dose-dependent manner**

To investigate the effect of AG1478 on the proliferation of HepG2 cells, HepG2 cells were treated with 5, 10, 20 or 40 μmol/mL AG1478 for 24 h (Table 1). The survival rate of HepG2 cells after 24 h treatment with different concentrations of AG1478 decreased with the increase of drug concentrations in a dose-dependent manner (P < 0.05 for all doses). These data demonstrated that
AG1478 exerted inhibitory effect on HepG2 cell proliferation in a dose-dependent manner.

**All phases of the apoptosis of HepG2 cells were enhanced by AG1478 in a dose-dependent manner**

To study the influence of AG1478 on the apoptosis of HepG2 cells, flow cytometry and Annexin V/PI double staining were employed to count the number of apoptotic cells. After treatment with 0, 5, 10, 20 or 40 μmol/mL AG1478, the apoptotic rates of HepG2 cells in the experimental groups were (12.88 ± 1.91)%, (23.16 ± 2.67)%, (35.36 ± 1.95)% and (47.16 ± 3.78)%, respectively, which were significantly higher than that in the control group [(5.44 ± 0.95)%] (P < 0.05). In addition, the ratios of the apoptotic cells in the late phase of the death process were also significantly increased in the experimental groups compared with that in the control group (P < 0.05) (Table 2). These data showed that all phases of the apoptosis of HepG2 cells were enhanced by AG1478 in a dose-dependent manner.

**AG1478 increases the percentage of G0/G1-phase HepG2 cells in a dose-dependent manner**

To test the impact of AG1478 on HepG2 cell cycle, HepG2 cells were treated with 0, 5, 10, 20 or 40 μmol/mL AG1478 for 24 h and analyzed using flow cytometry (Figure 2A). The results showed that the number of S-phase cells decreased gradually, whereas the number of G1-phase cells increased. In addition, the percentage of G1-phase HepG2 cells was significantly increased in the groups treated with 10, 20 or 40 μmol/mL AG1478, whereas that of S-phase cells was decreased in a dose-dependent manner (P < 0.05). However, there were no

Figure 2. AG1478 induces cell cycle arrest (A) and apoptosis (B) in HepG2 cells.
Note: (A) Flow cytometric analysis of HepG2 cells treated with 0, 5, 10, 20 or 40 μmol/mL AG1478 for 48 h, followed by staining with PI. (B) Flow cytometric analysis with Annexin V/PI double staining of HepG2 cells treated with 0, 5, 10, 20, or 40 μmol/mL AG1478 for 24 h.

### Table 1. Survival rates of HepG2 cells after 24 h treatment with different concentrations of AG1478.

| AG1478 (μmol/L) | Survival rate (%) |
|-----------------|-------------------|
| 0               | 100               |
| 5               | 76.0*             |
| 10              | 59.6*             |
| 20              | 51.2*             |
| 40              | 42.1*             |

Note: *, P < 0.05 compared with the 0 dose group. N = 3.
significant changes in the percentage of G2-phase cells in any of the experimental groups compared with the control \( (P > 0.05) \) (Table 3). These data suggested that AG1478 increased the percentage of G0/G1-phase HepG2 cells in a dose-dependent manner.

**AG1478 treatment reduces expression of c-Myc protein in HepG2 cells**

To detect the expression of c-Myc protein in HepG2 cells, immunohistochemistry assay was performed. The microscopic images showed that c-Myc protein was mainly localized in the cytoplasm of HepG2 cells, being positively stained as brown granules (Figure 3(A)). After treatment with AG1478 (20 μmol/L), the number of brown granules in HepG2 cells was reduced and the colour became lighter, indicating that the expression level of c-Myc had declined (Figure 3(B)). These results indicated that AG1478 treatment reduced the expression of c-Myc protein in HepG2 cells.

Furthermore, AG1478–treated HepG2 cells were harvested and separated into nuclear and cytoplasmic fractions. Equivalent amounts of each fraction were analyzed by immunoblotting with anti-c-Myc. Histone H1 and actin were used as the internal controls for nuclear proteins and cytoplasmic proteins, respectively (Figure 3(C)). The results showed that c-Myc protein was mainly expressed in cell cytoplasm.

To further investigate the effects of AG1478 on HepG2 cells, the total proteins were harvested and immunoblot analyses were performed. As indicated in Figure 4(A,B), the levels of c-Myc proteins were significantly reduced. In this experiment, β-actin was used as the control.

### Comparative analysis

In this study, we found that the number of cultured HepG2 cells was reduced significantly after being treated with different concentrations of AG1478. With the increase of drug concentrations, the morphology of HepG2 cells was changed from the original polygonal shape to fusiform shape. In addition, the detached cells accumulated and became floating in the medium. CCK-8 assay and flow cytometry were used to study the effect of AG1478 on HepG2 cell proliferation and cell cycle, respectively. The results showed that AG1478 effectively inhibited the proliferation of HepG2 cells in a dose-dependent manner, with the survival rate of cultured cells being gradually reduced with the increase of drug concentrations. Flow cytometric analysis indicated that AG1478 induced apoptosis in HepG2 cells with the effect being stronger as the concentration increased. Apoptotic cell counts by flow cytometry after Annexin V/PI double staining suggested that the apoptotic rates in all

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**Table 2. Apoptotic rates of HepG2 cells after 24 h treatment with 5, 10, 20 or 40 μmol/mL AG1478.**

| AG1478 (μmol/L) | Total apoptotic rate (%) | Early apoptotic rate (%) | Late apoptotic rate (%) |
|-----------------|--------------------------|--------------------------|------------------------|
| 0               | 5.44 ± 0.95              | 4.18 ± 1.35              | 0.34 ± 0.18            |
| 5               | 12.88 ± 1.91*            | 8.12 ± 1.06**            | 3.86 ± 0.92*           |
| 10              | 23.16 ± 2.67*            | 12.47 ± 1.37*            | 9.60 ± 0.90*           |
| 20              | 35.36 ± 1.95*            | 21.31 ± 2.26*            | 12.9 ± 1.05*           |
| 40              | 47.16 ± 3.78*            | 27.10 ± 2.73*            | 18.13 ± 1.10*          |

Note: Data are means ± SD. *, \( P < 0.01 \) vs control; **, \( P < 0.05 \) vs control. \( N = 3 \).

**Table 3. Cell cycle distribution of HepG2 cells after 24 h treatment with 5, 10, 20 or 40 μmol/mL AG1478.**

| AG1478 (μmol/L) | G0/G1 (%) | S (%) | G2 (%) |
|-----------------|-----------|-------|--------|
| 0               | 52.83 ± 2.02 | 33.90 ± 0.55 | 13.26 ± 2.15 |
| 5               | 54.76 ± 0.55  | 31.26 ± 1.23** | 13.96 ± 1.77 |
| 10              | 59.90 ± 0.90* | 27.66 ± 1.61* | 12.43 ± 1.27 |
| 20              | 64.46 ± 1.96* | 22.53 ± 1.11* | 13.33 ± 2.40 |
| 40              | 69.86 ± 1.19* | 16.36 ± 2.34* | 13.76 ± 3.44 |

Note: Data are means ± SD. *, \( P < 0.01 \) vs control; **, \( P < 0.05 \) vs control. \( N = 3 \).
experimental groups were significantly higher than that in the control group. Furthermore, it was observed that early apoptotic cells began to appear at a low concentration (5 μmol/L), with the apoptotic rates being significantly different between the control group and the experimental groups. Cell cycle analysis showed that the proportion of G1-phase cells was significantly increased, whereas the percentage of S-phase cells was significantly decreased with the increase of AG1478 concentration. These results are consistent with previous reports [22–24], indicating that the main mechanism of action of AG1478 may be through its binding to EGFR, which then inhibits autophosphorylation, blocks EGFR-mediated signalling pathways, hinders cell cycle progression, inhibits cell proliferation and promotes apoptosis.

The c-Myc protein, which has been proved to be an essential factor for cell proliferation, is also an accessory factor for reverse transcription that plays important roles in signal transduction, cell proliferation, differentiation, apoptosis and cell cycle control. The c-Myc encoding gene is an essential early gene for cell proliferation, and the c-Myc protein is a critical factor to control G0-S phase transition. The c-Myc protein is closely related to the MAPK signalling pathway. Wang et al. [25] found that the activation of the MAPK pathway down-regulated the expression of cell cycle-related genes p21 and p16, up-regulated the expression of c-myc, accelerated G1/S phase transition and promoted cell proliferation. Our immunohistochemical data demonstrated that the expression of c-Myc protein in HepG2 cells was significantly decreased after treatment with AG1478 for 24 h, suggesting a close relationship between EGFR and c-Myc expression. This observation indicated that c-Myc might be involved in the EGFR signalling pathway in hepatocellular carcinoma. However, further investigation is needed to elucidate the exact mechanism.

Conclusions

EGFR plays a key role in the occurrence and development of a variety of tumours. Anti-tumour treatment targeting EGFR tyrosine kinase has become a research hotspot for cancer adjuvant therapies in recent years. The present study showed that AG1478, as a tyrosine kinase inhibitor, significantly inhibited the growth of human hepatoma cells, arrested the cell cycle and induced apoptosis.

Disclosure statement

All authors declare no financial competing interests.

All authors declare no non-financial competing interests.

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