EGF Receptor Tyrosine Kinase Inhibitor Tyrphostin AG1487 Induce Human Tongue Cancer Cells Tca8113 Cell Cycle at G1 Phase and Apoptosis

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Abstract: Objective. This study was to investigate the effects of tyrphostin AG1487 on expression of activated ERK1/2, distribution of cell cycle and apoptosis in Tca8113 cells. **Materials and Methods.** Tca8113 cells were exposed to different concentrations of phosphotyrosine kinase inhibitor tyrphostin AG1487, and then immunocytochemistry, Western blot, flow cytometry, and electron transmission microscopy were employed to investigate the effects of AG1487 on expression of activated ERK1/2, distribution of cell cycle and apoptosis of Tca8113 cells. **Results.** The results of immunocytochemistry showed intensity of activated ERK1/2 reactivity in treated cells was marked decreased. These results were corresponded with the expression of activated ERK1/2 in Western blot: expressions of activated ERK1/2 gradually reduced along with the increasing of concentration of tyrphostin AG1487. Cell cycle kinetic analysis demonstrated that AG1487 induced a delay in cell cycle progression and arrested at G1 phase. Furthermore, AG1487 could induce a marked apoptosis of Tca8113 cells. All these effects were in a dose-dependent pattern. **Conclusion.** EGF receptor tyrosine kinase inhibitor tyrphostin AG1487 can inhibit the expression of activated ERK1/2, induce cell cycle arrest at G1 phase and apoptosis. [Life Science Journal. 2005;2(1):77-84] (ISSN: 1097-8135).

Keywords: Tca8113 cell; EGF receptor; ERK1/2; cell cycle; apoptosis

Abbreviations: EGF, epidermal growth factor; EGFR, epidermal growth factor receptor; MAPK, mitogen-activated kinase; ERK, extracellular signal- regulated kinase

1 Introduction

The EGF receptor belongs to the erbB family of four closely related cell member receptors: EGF receptor (erbB1), erbB2, erbB3 and erbB4. All these receptors are transmembrane glycoproteins that consist of an extracellular ligand-binding domain, a transmembrane domain, and an intracellular domain with tyrosine kinase activity for signal transduction[1-3]. After ligand binding, the tyrosine kinase of the receptor is activated, and initiates a cascade of intracellular events[4]. These events lead to recruitment and phosphorylation of several intracellular substrates, such as extracellular signal-regulated kinase (ERK), which lead to the mitogenic signaling and other cellular activities[1,4]. Many studies have shown EGF receptor is highly expressed in malignent tumors, and is associated with progression of malignant tumors and poor prognostic features. EGF receptor and its downstream signaling pathways are, therefore, becoming the promising antitumor targets for cancer therapy.

A variety of different approaches are currently being used to target the EGF receptor, the most promising strategies include to prevent ligand binding and to inhibit autophosphorylation of EGF receptor. Therapeutic mAbs targeting the extracellular domains of EGF receptor have similar affinity binding to EGF receptor as EGF and TGF-α, compete with these ligands for receptor binding, and block activation of receptor tyrosine kinase induced by EGF and TGF-α[5,6]. Small molecule inhibitors are generally reversible competitors with respect to ATP for binding to the intracellular catalytic domain of the tyrosine kinase[5]. Some studies had shown that small molecule inhibitors not only compete with ATP in the classical mode of action but also induce the formation of inactive, unphosphorylated EGFR dimmers[5,7].

Both *in vitro* and *in vivo* studies have demonstrated that EGF receptor targeted agents could in-

hibit the processes involved in tumor growth and progression, including proliferation, apoptosis, metastasis and angiogenesis[4,5,8]. All these effects are correlated with the blockade of EGF receptor signaling. ERKs, as one of the important signaling molecule that lies downstream of EGF receptor, usually be highly expressed in malignant tumor cells[9]. Some studies demonstrated EGF receptor targeted agents could inhibit the activation of EGF receptor and ERKs in malignant tumor cells[10-12].

For further study the EGF receptor agents effects on malignant tumor cells, we adopted small molecule inhibitor of EGF receptor tyrphostin AG1487 to treat human tongue cancer cells (Tca8113), and then to investigate the effects of AG1487 on expression of activated ERK1/2, distribution of cell cycle and apoptosis in Tca8113 cells.

2 Material and Methods

2.1 Tumor cell lines and regents

The cell line of human squamous cell carcinoma of tongue, Tca8113 cells, were obtained from Shanghai Institute of Cell Biology, Chinese Academy of Sciences and cultured in PRMI medium 1640 (GIBCO) containing 10% heat-inactivated FBS, 100 units/ml penicillin G and 100 unites/ml streptomycin at 37℃ in humidified air containing 5% CO_2. Polyclonal anti-EGF receptor antibodies obtained from Boster Biological Technology Co. (Boster, Wuhan, China), anti-total ERK1/2 antibodies and anti-activated ERK1/2 antibody obtained from Sigma Chem. Co. (St. Louis, MO, USA), Tyrphostin AG 1487 obtained from Calbiochem (San Diego, CA, USA).

2.2 Immunocytochemistry

Cells were seeded on coverslips coated with poly-L-lysine placed in six culture plates under the same conditions. On the next day, PRMI medium 1640 was refreshed and tyrphostin AG 1487 was added at indicated concentration (100 nM) to the cultures for 2 h. Then the slides were removed from the culture plates, and cells on coverslips were fixed in 2% paraformaldehyde solution with 0.1% Triton X-100 for 30 min at room temperature. Nonspecific protein reactivity was blocked with 10% goat serum (SABC) in PBS for 10 min. And endogenous peroxidase reactivity was blocked in 3% hydrogen peroxide solution for 10 min. Incubation with primary antibodies were made at room temperature for 1 h at following dilutions: EGF receptor (1∶100), activated ERK1/2 (1∶1000) and total ERK1/2 (1∶1000). After being washed with PBS three times, the coverslips were incubated with goat anit-mouse IgG-HRP or goat anti-rabbit IgG-HRP antibodies (Boster, Wuhan, China) for 1 h at room temperature. Coverslips were visualized using 3-3′-diaminobenzidine as a chromogen for 5 min. Then the specimens were dehydrated and coverslips were mounted on the glass slides using mounting media. Positive and negative controls were included in each staining run.

2.3 Western blot

Western blot was performed as reported previously[13]. Cells were seeded in parallel and under the same conditions. When cells grew to monolayers, PRMI medium was refreshed and phosphotyrosine kinase inhibitor tyrphostin AG1487 was added at the indicated concentrations (0 nM, 50 nM, 100 nM, 200 nM) and incubated for 2 h. Then the cells were scraped into 0.5 ml of cold lysis buffer [50 mM Tris-HCI (pH8.0), 150 mM NaCI, 0.1% SDS, 100 μg/ml PMSF, 1 μg/ml Aprotinin, 1% NP − 40, 0.5% sodium deoxycholate], and protein was extracted. The concentration of protein was determined by Bradford method. Western blots were performed to detect the expression of total ERK1/2 (Sigma 1∶10,000) and activated ERK1/2 (Sigma 1∶10,000).

2.4 Flow cytometry

Tca8113 cells were cultured at same condition and then exposed to indicated concentrations of tyrphostin AG 1487 (0 nM, 50 nM, 100 nM, 200 nM) and treated for 24 h, 48 h and 72 h respectively. Cells were washed with PBS, trypsinized and harvested, and then fixed with pre-cold (−20℃) ethanol for 2 h. The cells were centrifuged for 5 min at 1500 rmp and quickly removed the supernate, washed with PBS and added RNase 50 μl (20 μg/ml) for 10 min at room temperature, then stained with propidium iodide (PI, sigma) 200 μl (5 ng/ml) for 20 min, washed with PBS and resuspended. After filtering cells through 100 μm pore size mesh, cell cycle distribution was analyzed by a flow cytometer (Becton Dickinson FAC Sort).

2.5 Electron transmission microscopy

Tca8113 cells were cultured as above and exposed to different concentrations of tyrphostin AG1487 (0 nM, 50 nM, 100 nM, 200 nM) for 48 h, then washed with PBS, trypsinized and harvested. Cells were fixed with 2% glutaraldehyde solution. Ultrathin Epon plastic sections were prepared and stained with uranyl acetate followed by lead citrate and stabilized for transmission electron microscopy (HITACHI. H-300). Changes of Tca8113 cells after exposing to tyrphostin AG 1487 were clearly identified by its characteristics.

3 Results

3.1 Expression of EGFR

The results of immunocytochemical staining demonstrated EGF receptor protein of Tca8113 cells was mainly in the cytoplasm and on the plasma membrane. There was no significant difference about expression of EGF receptor in before and after treated cells (Figure 1a, b). Unfortunately, we did not detect the expression of activated EGF receptor. But in a few of studies shown AG1478 and other EGFR inhibitors could inhibit EGFR phosphorylation without reduced expression of EGFR protein[2,14].

3.2 Expression of activated ERK1/2 and total ERK

To study the effects of tyrosine kinase inhibitor tyrphostin AG1487 on EGF receptor signaling of Tca8113 cells, we adopted immunocyotchemistry and Western blot methods to detect the expression of total ERK1/2 and activated ERK1/2. The results of immunocytochemistry showed that there was no significant difference in intensity of total ERK1/2 reactivity in Tca8113 cells before and after treated with AG1487 (100 nM) (Figure 1c, d). As for activated ERK1/2, it was marked decreased of Intensity of reactivity in nucleus and made nucleus to be a shadow (Figure 1e, f). These results were corresponded with the expression of total ERK1/2 and activated ERK1/2 in Western blot (Figure 2): Along with the increasing of concentration of tyrphostin AG-1487, expression of activated ERK1/2 was reduced or abolished in Western blot. But there was no significant difference in expression of total ERK1/2.

3.3 Distribution of cell cycle after treatment with AG1487

In order to investigate the effects of AG1487 on cell cycle in Tca8113 cells, we employed different concentrations of AG1487 to treat the cells for different time (0 h, 24 h, 48 h, 72 h). The results suggested that the percentage of cells in G1 phase was correlated with the concentration of tyrphostin AG1487 and treating time. Along with the increasing of concentration of tyrphostin AG1487 and treating time, the percentage of cells in G1 phase also increased (Figure 3). When exposing Tca8113 cells to 100 nM AG1487 for 72 h, the percentage of cells in G1 phase reached to maximum (77.57%, Table 1).

3.4 Effect of AG1487 on apoptosis of Tca8113 cells

Some studies demonstrated EGF receptor targeted agents could induce tumor cells apoptosis *in vivo* or *in vitro*, and this effect was correlated with inhibition of EGF receptor signaling pathway. In the present study, effect of tyrphostin AG1487 on apoptosis of Tca8113 cells was also studied. The results showed EGF receptor tyrosine kinase AG1487 could induce apoptosis of Tca8113 cells, and the effect was correlated to the concentration of tyrphostin AG1487. Along with the increase of concentrations of AG1487, the number of apoptotic cells gradually increased (Figure 4a, b). Comparing with untreated cells, the number of apoptotic cells was nearly 7 fold in Tca8113 cells treated with high concentration of AG1487 (200 nM) (Figure 5).

Table 1. Distribution of cell cycles of Tca8113 cells exposed to different concentrations of AG1487 (nM) and treated for different times

Time	Cell cycle (%)											
	G1				S				G2			
(hour)	0 (nM)	50 (nM)	100 (nM)	200 (nM)	0 (nM)	50 (nM)	100 (nM)	200 (nM)	0 (nM)	50 (nM)	100 (nM)	200 (nM)
24	32.35	34.65	38.81	47.70	54.89	50.78	49.17	45.64	12.76	14.57	12.02	6.67
48	32.15	45.50	60.67	74.04	55.20	48.34	33.48	21.07	11.90	6.16	5.85	4.89
72	32.35	75.64	77.57	54.04	54.85	18.06	17.19	38.05	10.90	6.30	5.23	7.91

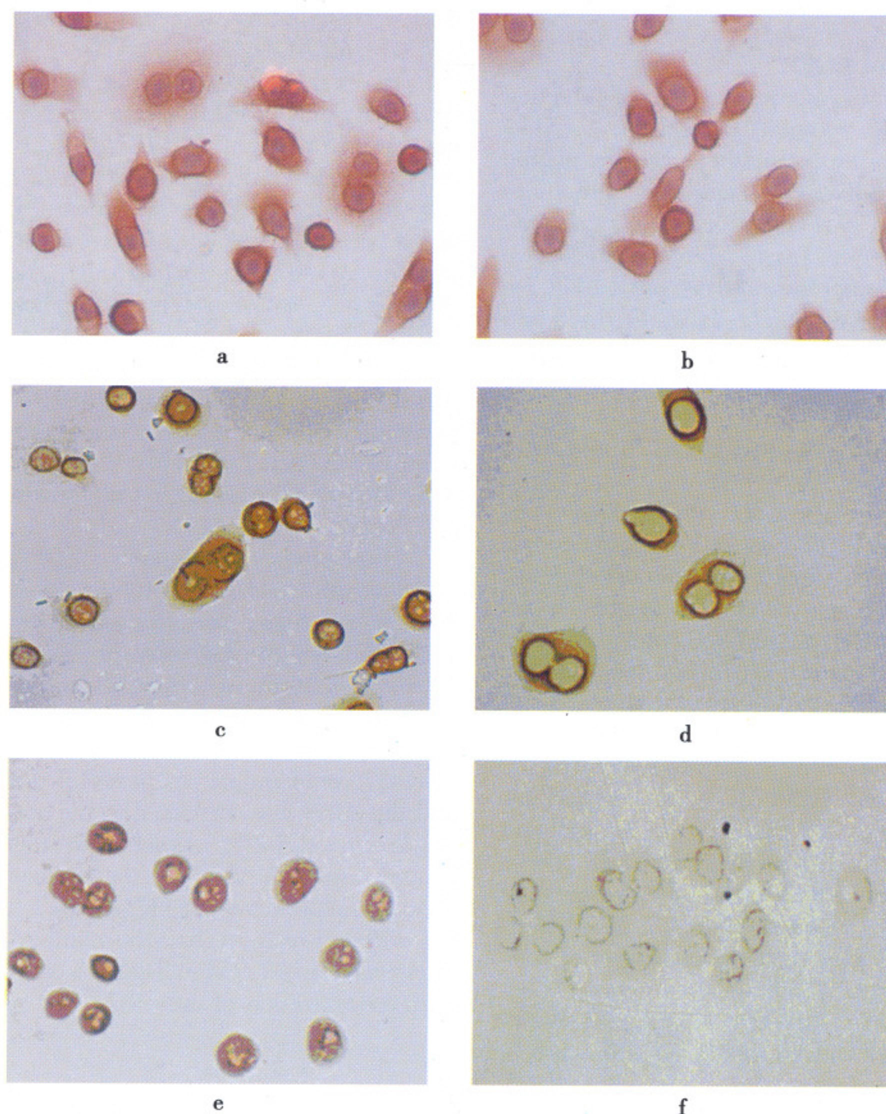

Figure 1. Immunochemistry for EGF receptor, total ERK1/2 and activated ERK1/2 in Tca8113 cells treated or untreated with AG1487 (×400). a. EGF receptor immunochemical staining in untreated cells. b. EGF receptor immunochemical staining in treated cells. c. Total ERK1/2 immunochemical staining in untreated cells. d. Total ERK1/2 immunochemical staining in treated cells. e. Activated ERK1/2 immunochemical staining in untreated cells. f. Activated ERK1/2 immunochemical staining in treated cells.

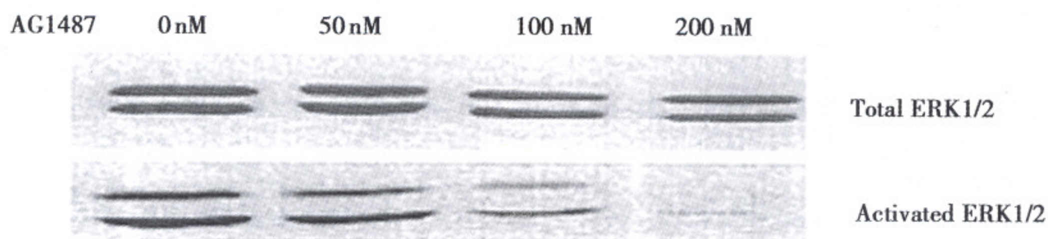

Figure 2. Expression of total ERK1/2 and activated ERK1/2 in Tca8113 cells treated or untreated with AG1487. Tca8113 cells pretreated with 0, 50, 100 and 200 nM AG1487 in an equal amount of DMSO (final concentration 0.5%) for 2 h at 37℃. Cell lysates were prepared and Western blot analyses were performed with antibodies to total ERK1/2 and activated ERK1/2.

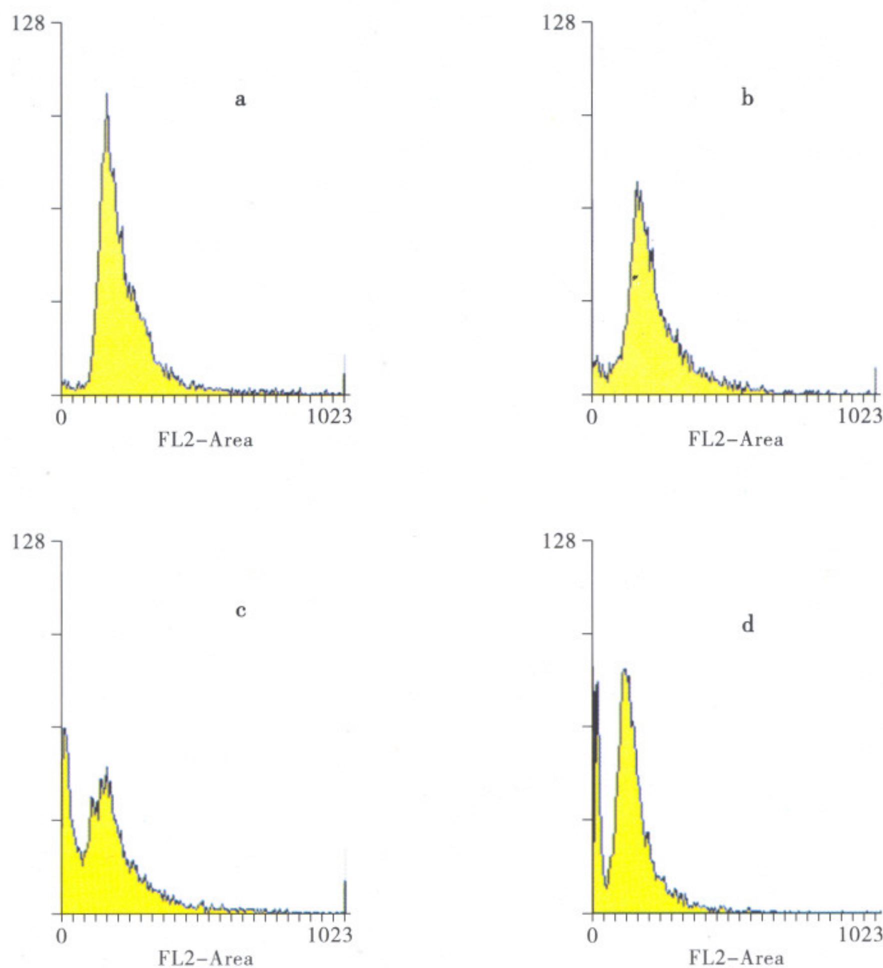

Figure 3. Effects of AG1487 on cell cycle distribution in Tca8113 cells. Tca8113 cells were cultured with PRMI medium 1640 and exposing to different concentrations (0 nM, 50 nM, 100 nM, 200 nM) of AG1487 and for different time (24 h, 48 h, 72 h), and cells were then harvested and stained with propidium iodide as described under Materials and Methods. Their cell cycle distribution was analyzed by flow cytometer. a. Cell cycle distribution in Tca8113 cells untreated with AG1487. b. Cell cycle distribution in Tca8113 cells treated with AG1487 in concentration 50 nM for 24 h. c. Cell cycle distribution in Tca8113 cells treated with AG1487 in concentration 100nM for 48 h. d. Cell cycle distribution in Tca8113 cells treated with AG1487 in concentration 200 nM for 72 h.

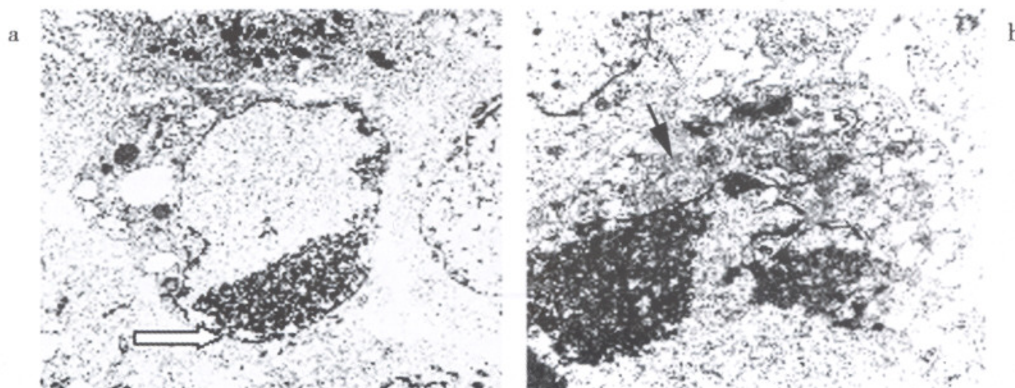

Figure 4. Effects of AG1487 on apoptosis of Tca8113 cells. a. Nuclear condensation (⇨), vesicle formation (→) occurring during apoptosis of Tca8113 cells exposed to AG1487 at concentration of 100 nM ($\times$7,000). b. Apoptotic cell exhibiting swelling of endoplasmic reticulum (→) of Tca8113 cells exposed to AG1487 at concentration of 200 nM ($\times$15,000).

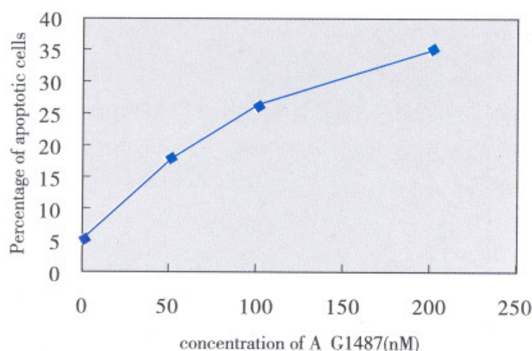

Figure 5. The relationship between percentage of apoptotic cells and different concentrations of AG1487 in Tca8113 cells.

4 Discussion

Dimerization and phosphorylation of the epidermal growth factor (EGF) receptor are the initial and essential events of EGF or TGF-α-induced signal transduction, so selective inhibition of tyrosine kinase activity is one of promising strategy for cancer treatment. Tyrphostin AG1487, as one of the phosphotyrosine kinase inhibitors, has been used in a few of studies and showed it can induce inactive, unphosphorylated EGF receptor – erbB-2 heterodimers, thereby sequester EGF receptor from signaling interactions with other erbB coreceptors[15]. These effects lead to the activation of ERKs-MAPK (mitogen-activated kinase) be inhibited[12,16]. Biochemical and cell fractionation studies have indicated that activation of ERKs and translocation from the cytoplasm to the nucleus are required for growth factor-induced gene expression and cell cycle entry[17]. At present study, we demonstrated expression of activated ERK1/2 could be effectively inhibited by tyrphostin AG1487. And this inhibitory effect was in a does-dependant pattern. The result of immunochemical staining showed intensity of activated ERK1/2 reactivity in nucleus marked decreased, it means translocation process of activated ERK1/2 from cytoplasm to nucleus was inhibited by tyrphostin AG1487. All these results suggested small molecule inhibitor of EGF receptor tyrosine kinase, AG1487, could effectively inhibit the ERK-MAP kinase pathway and translocation process of activated ERK1/2 in Tca8113 cells.

Besides EGF receptor regulating the growth and progression of human oral squamous cell carcinoma, the link between EGFR signaling and the cell cycle has also been identified[18]. A few of studies demonstrated both therapeutic mAbs and small molecule inhibitors of EGF receptor tyrosine kinase induced a remarkable G1 cell cycle arrest, accompanied by a decrease in the percentage of cells in the S phase in malignant tumor cells[13,14], and this was associated with increased expression of p27 (KIP1), which binds to and inactivates cyclin-dependet kinase-2 activity and produces cell cycle arrest in G1 phase[14]. At present study, the percentage of Tca8113 cells in G1 phase was studied after treated with tyrphostin AG1487 at different concentrations and for different time, the results showed the percentage of cells in G1 phase was correlated to the concentrations and treating time. Along with the increasing of concentrations and prolonging of treating time, the percentage of G1 cells gradually increased. When exposing Tca8113 cells to 100 nM AG1487 for 72 h, the percentage of cells in G1 phase reached to maximum (77.57%). Further analysis suggested these results were in dose- and time-dependent patterns.

Apoptosis is a physiological mechanism of cell death that occurs during normal development and under certain pathological conditions[19,20]. Several types of regulatory pathway can contribute to the cellular decision to enter apoptosis. These include DNA damage, dominant death signals from "death receptors" such as Fas, tumor necrosis factor receptor, and block the signaling pathways from growth factor receptor-activated kinase cascades[21]. Activation of the EGF receptor provides a potent survival signal in many cell types. PI3K and ERK1/2 are signaling molecules on two major downstream pathways initiated by the activation of EGF receptor[22,23]. A few of studies demonstrated small molecule inhibitor of the EGF receptor tyrosine kinase, for example ZD-1839 and AG1487, could induce apoptosis of malignant tumor cells *in vivo* or *in vitro*[16,24]. And this effect was also dose dependent. At present study, the percentage of apoptotic cells were calculated after exposing Tca8113 cells to different concentrations of AG1487. The results showed along with the increasing of concentrations of AG1487, the percentage of cells proceeding apoptosis also increased. And the morphologic changes of apoptotic cells, such as vesicle formation in the cytoplasm, nuclear condensation were also influenced by the concentration of tyrphostin AG1487. All these results suggested tyrphostin AG1487 could induce apoptosis of Tca8113 cells and the effect was in a dose dependent pattern.

In summary, EGF receptor signaling pathways play an important role in regulating cellular processes in Tca8113 cells. Small molecule inhibitor of EGF receptor tyrosine kinase, AG1487, could effectively inhibit ERK1/2-MAPK pathway and re-

duce the expression of activated ERK1/2 in a doesdependent manner. Cell cycle kinetic analyses demonstrate AG1487 induce a remarkable G1 cell cycle arrest, followed by a modest apoptotic response. All these results suggest the EGFR kinase specific inhibitor is of potential to be developed into drugs for squamous cell carcinoma treatment.

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