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EGFR Tyrosine Kinase Inhibitors Differentially Affects Autophagy in Head and Neck Squamous Cell Carcinoma

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Abstract

Head and neck squamous cell carcinoma (HNSCC) is the sixth most prevalent cancer worldwide. The majority of HNSCCs overexpress Epidermal Growth Factor Receptor (EGFR), an essential receptor tyrosine kinase (RTK) that promotes HNSCC growth and metastasis. Therefore, EGFR has been used as an important therapeutic target to treat HNSCC. Inhibition of EGFR stimulates autophagy in cancer cells. However, the role of autophagy in EGFR inhibitor-induced cancer suppression is still in a debate. Here, we reveal that the first- and the second-generation EGFR tyrosine kinase inhibitors (TKIs) differentially affect HNSCC autophagy. The second-generation EGFR TKIs have much stronger effects on autophagy than the first-generation TKIs. The second-generation EGFR TKIs not only promote autophagy initiation signaling but also block autophagic flux by disturbing the lysosomes function, indicating a novel mechanism by which EGFR TKIs modulate cancer cell autophagy. Blocking the initiation of autophagy does not affect the second-generation EGFR TKI-induced HNSCC growth suppression. This suggests that the anti-growth effect of the second-generation EGFR TKIs on HNSCC is not dependent on autophagy.

Keywords

HNSCC; EGFR; EGFR TKIs; autophagy; Beclin-1

1. Introduction

HNSCC, involving squamous cell carcinomas at the lip, oral cavity, pharynx and larynx, is the sixth most prevalent cancer worldwide [1]. In the United States, about 45,000 new cases diagnosed and 9,000 patients dead due to HNSCC in 2015 [2]. The 5-year relative survival

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Conflict of interests

The authors declare that they have no conflict of interest.

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rate of HNSCC is about 66% [2]. New therapies are urgently required to improve the patients' survival.

EGFR is an essential RTK regulating cell proliferation and differentiation [3]. The majority (80–90%) of HNSCCs overexpress EGFR and the elevated expression correlates with cancer metastasis, recurrence, and poor patient prognosis [4,5]. As a result, the anti-EGFR monoclonal antibody, cetuximab, has been developed and is the currently only anti-EGFR drug approved by Food and Drug Administration to treat HNSCC [6]. However, clinical data has evidenced that only a small percentage of HNSCC patients have major responses to cetuximab [6,7]. EGFR TKIs are also developed in propose of cancer treatment. The first-generation of EGFR TKIs such as Erlotinib and Gefitinib are reversible TKIs and have been used to treat non-small cell lung cancer [8]. The second-generation EGFR TKIs such as Dacomitinib and Afatinib are irreversible TKIs. The effects of these EGFR TKIs on HNSCC patient treatment are in evaluation by ongoing clinical trials [9].

Autophagy is a self-degradative process whereby cellular proteins and organelles are accumulated to form autophagosomes, and finally digested in the lysosomes [10,11]. Inhibition of EGFR can trigger autophagy [12,13], although the consequences of autophagy activation in HNSCC patients' response to anti-EGFR therapies are not clear. Here, we report that the first- and the second-generation EGFR TKIs differentially modulate HNSCC autophagy. The second-generation EGFR TKIs block autophagic flux by disturbing lysosome function, which is a novel mechanism for EGFR TKIs to affect autophagy. Furthermore, blocking of autophagy initiation does not affect the second-generation EGFR TKI-induced HNSCC growth suppression. These results suggest that new considerations should be taken in evaluating the relationships between autophagy and the HNSCC responses to different EGFR TKIs.

2. Material and methods

2.1 Cell culture

HNSCC cell lines UMSCC1 and UMSCC6 were maintained in Dulbecco's Modified Eagle Medium (DMEM) supplemented with 10% fetal bovine serum (FBS), 1X Non-Essential Amino Acids Solution (Gibco), and 1 μ g/ml hydrocortisone.

2.2 Reagents

EGFR TKIs Gefitinib, Erlotinib, Dacomitinib, and Afatinib were purchased from Selleckchem (Houston, TX). Cell proliferation reagent WST-1, Dimethyl Sulfoxide (DMSO), Chloroquine, Spautin-1, and Flavopiridol were from Sigma-Aldrich (St. Louis, MO). Antibodies to LC3, phosphor-ULK1 (Ser317), phosphor-ULK1 (Ser757), total ULK1, phosphor-p70 S6K, Cathepsin D were from Cell Signaling Technology (Danvers, MA). Antibodies to Beclin-1, GAPDH, Tubulin, and total p70 S6K were from Santa Cruz Biotechnology (Santa Cruz, CA). Antibody to actin was from MP Biomedicals (Santa Ana, CA). Secondary antibodies were from Jackson ImmunoResearch Laboratories (West Grove, PA). DQ-Red BSA was obtained from ThermoFisher Scientific (Waltham, MA).

2.3 Cell viability assay

Cells were seeded in 96-well plates and 24hr later treated with different EGFR TKIs in concentrations ranging from 0 to 10 μ M for 72hr. The cell viability was then tested by using WST-1 cell viability assay kits according to the manufacturer's instructions. The relative cell viability was normalized with respect to the viability of the vehicle control (DMSO, 1:1000) treatment group. Briefly, after TKIs treatment, the cells were incubated with WST-1 (10 μ l) in the cell culture medium (100 μ l) for 2hr. The absorbance of each sample solution was then measured at 450nm against a background control as blank using a microplate reader (BioTek™ Synergy™ H1 Hybrid Multi-Mode). The wavelength of 650 nm was used as the reference wavelength.

2.4 Immunofluorescence

Cells on coverslips were fixed with 4% paraformaldehyde (PFA) in PBS for 15 min and permeabilized with 0.5% Triton X-100 in PBS for 10 min at room temperature. Cells were then blocked with 3% BSA in PBS for 1hr at room temperature and incubated with primary antibody overnight at 4°C, washed with 0.1% Triton X-100 in PBS, incubated with fluorescence-labeled secondary antibodies for 1hr at room temperature. Cells were then washed and incubated with DAPI for 1 min to stain the cell nucleus. Cells were examined under a Zeiss LSM 710 laser scanning fluorescence confocal microscope with a 63 $\times$ NA Plan-Apochromat lens. Images were acquired and processed using ZEN 2012 software (Carl Zeiss Microimaging) and ImageJ (NIH).

2.5 DQ-Red BSA Digestion

Cells were seeded in 24-well plates with coverslips. When attached to the coverslips, cells were treated with different EGFR TKIs for 24 hrs. Then cells were incubated with DQ-Red BSA (10 μ g/ml) for 2hr at 37°C. Cells were then washed with PBS to remove excess DQ-Red BSA, fixed with 4% PFA, and subjected to confocal microscopy analysis to measure the fluorescence intensity of DQ-Red BSA.

2.6 Statistics

All data analysis was performed using SigmaPlot. Bar graphs represent means $\pm$ SEM., as indicated. Statistical significance was assessed using the Student *t*-test.

3. Results

3.1 The second-generation EGFR TKIs are more efficient than the first-generation TKIs in inhibiting HNSCC growth

To compare the effects of different EGFR TKIs on suppression of HNSCC cells growth, two HNSCC cell lines UMSCC1 and UMSCC6 were treated with different EGFR TKIs. Both adding of the first- and the second-generation EGFR TKIs decreased cell viability compared with the DMSO vehicle control (Fig. 1A and 1B). This result confirmed that EGFR-inhibition can suppress HNSCC cell growth. Furthermore, the TKI dose-response curves indicated that the second-generation EGFR TKIs Dacomitinib and Afatinib are more effective in suppressing HNSCC cells growth than the first-generation EGFR TKIs Gefitinib

and Erlotinib (Fig. 1A and 1B). This is consistent with that the second-generation EGFR TKIs are more efficient to inhibit EGFR activation (Fig. 1C–1F).

3.2 The second-generation EGFR TKIs have stronger effects on autophagy than the first-generation EGFR TKIs

Next, the HNSCC cell autophagy induced by these different EGFR TKIs was detected and compared. As shown in Fig 2A, the LC3 puncta (red) indicated the formation of autophagosomes. Both Dacomitinib- and Afatinib-treatment robustly enhanced LC3 puncta formation (Fig. 2A and 2B), indicating a strong effect of the second-generation EGFR TKIs on autophagy. In contrast, Gefitinib and Erlotinib only induced weak accumulation of LC3 puncta (Fig. 2A and 2B).

The production of LC3II and its final degradation at lysosomes reflect the levels of autophagic flux [14]. To determine the effects of EGFR TKIs on the autophagic flux, the levels of LC3II before and after inhibition of its lysosomal degradation by lysosome inhibitor chloroquine were detected via Western blot. Compared with DMSO or the first-generation EGFR TKIs, the second-generation EGFR TKIs significantly enhanced LC3II levels without chloroquine treatment (Fig. 2C and 2D). This is consistent with that the second-generation EGFR TKIs induced more LC3 puncta accumulation (Fig. 2A). The chloroquine treatment dramatically increased LC3II levels in cells treated with DMSO or the first-generation EGFR TKIs, but not further enhance LC3II levels in cells treated with the second-generation EGFR TKIs (Fig. 2C and 2D). This suggests that the second-generation EGFR TKIs may affect LC3II degradation and autophagic flux.

EGFR can modulate autophagy by regulating Beclin-1, a key component in the initiation of autophagy [13,15]. However, knockdown of Beclin-1 by siRNA in UMSCC1 cells did not decrease basal LC3II levels and did not block the Dacomitinib-induced increase of LC3II levels (Fig. 2E). This suggests that the effect of Dacomitinib on autophagy is Beclin-1-independent.

3.3 The second-generation EGFR-TKIs affect autophagy by enhancing autophagy initiation signaling and disturbing lysosome function

To determine the mechanisms by which the second-generation EGFR TKIs modulate autophagy, the autophagy initiation signaling and the autophagy maturation were detected. ULK-1 (Unc-51 Like Autophagy Activating Kinase 1) plays important roles in the initiation of autophagy [16]. The Ser757 phosphorylation of ULK-1 by mTOR (the mechanistic target of rapamycin) inhibits autophagy initiation [17,18,19]. Decrease of ULK-1 Ser757 phosphorylation indicates the inhibition of mTOR and the promotion of autophagy initiation. The second-generation EGFR TKIs induced significant decrease of ULK-1 Ser757 phosphorylation (Fig. 3A and 3B), indicating the release of ULK-1 from the inhibition by mTOR. In contrast, the first-generation EGFR TKIs did not affect ULK-1 Ser757 phosphorylation (Fig. 3A and 3B). The activation levels of mTOR was further measured by detecting the phosphorylation of p70 S6 Kinase (p70 S6K), a downstream substrate of mTOR. The second-generation EGFR TKIs significantly decreased p70 S6K phosphorylation (Fig. 3A and 3C), indicating inhibition of mTOR activity. These results

indicate that the second-generation EGFR TKIs can promote autophagy initiation by decreasing mTOR activation and blocking ULK-1 Ser757 phosphorylation.

To define the effect of the second-generation EGFR TKIs on autophagy maturation, the autolysosome formation was detected. The matured autophagosome will be fused with lysosome to form autolysosome, which enables the digest of autophagosome component [20]. The colocalization of LC3 and the lysosome marker lysotracker indicates the formation of autolysosome. The percentage of LC3 colocalized with lysotracker did not change in the second-generation EGFR TKIs-treated cells compared with DMSO or the first-generation TKIs-treated cells (Fig. 3D and 3E). This indicates that the autolysosome formation is not blocked by the second-generation EGFR TKIs.

Then we detected the effects of the second-generation EGFR TKIs on lysosome function. Cathepsin D (CTSD) is a lysosomal protease activated at low pH, and its precursor form (46 kDa) is processed into a mature form (32 kDa) in acidic lysosomes [21]. The ratios of CTSD's mature form/precursor were significantly dropped down by the second-generation EGFR TKIs-treatment (Fig. 3F and 3G), which indicates a decrease of CTSD precursor procession and a disturbance of lysosome function. Furthermore, the DQ-Red BSA digestion assay was used to detect the lysosome function. DQ-Red BSA is so heavily labeled that the fluorophore is self-quenched. Hydrolysis of this compound at lysosomes by proteases results in dequenching and producing of brightly fluorescent fragments [22]. The fluorescence intensity of DQ-BSA at lysosomes was significantly decreased by the second-generation EGFR TKIs-treatment (Fig. 3H and 3I), indicating less DQ-Red BSA hydrolysis at lysosomes. These results indicate that the second-generation EGFR TKIs can disturb the lysosome function. This suggests that the enhanced LC3II levels in the second-generation EGFR TKIs-treated cells are partially due to the less degradation of LC3II at lysosomes. In contrast, the first-generation EGFR TKIs did not affect CTSD precursor procession and DQ-Red BSA digestion (Fig. 3F–I). This indicates that disturbing lysosome function is a specific effect caused by the second-generation but not the first-generation EGFR TKIs.

The acidic environment is required for the normal function of lysosome. To determine whether the second-generation EGFR TKIs can disturb the acidic environment of lysosome, Lysosensor GreenDND-189 was used to stain the EGFR TKIs-treated cells. Lysosensor Green DND-189 (pK_a 5.2) is brightly fluorescent at acidic PH and is almost nonfluorescent at neutral or basic PH. Compared with DMSO or the first-generation TKIs, the second-generation EGFR TKIs did not decrease Lysosensor Green DND-189 fluorescent (Fig. 3J). This indicates that the second-generation EGFR TKIs do not disturb the acidic environment of lysosome. The change of CTSD precursor procession in the second-generation EGFR TKIs-treated cells (Fig. 3F) may not due to change of the acidic environment of lysosome.

3.4 Blocking autophagy initiation does not affect Dacomitinib-induced HNSCC growth suppression

To determine whether autophagy affects the efficiency of the second-generation EGFR TKIs in suppressing HNSCC growth, Spautin-1 was used to block the autophagy initiation. By promoting the degradation of Vps34 PI3 kinase complexes, Spautin-1 inhibits autophagy initiation [23]. As shown in Fig. 4A and 4B, Spautin-1 significantly decreased Dacomitinib-

induced autophagy. Adding of Spautin-1 did not change the effect of Dacomitinib on blocking HNSCC cells growth (Fig. 4C). This indicates that the autophagy induced by Dacomitinib is not related to its function to suppress HNSCC growth. Flavopiridol is a pan-cyclin-dependent kinase (CDK) inhibitor and it has synergistic effects with other anti-tumor drugs to inhibit tumor growth in an autophagy-dependent manner [24]. Flavopiridol had a synergistic effect with Dacomitinib to further block the HNSCC growth (Fig. 4C). This suggests that autophagy is not directly related to the anti-cancer effect of Dacomitinib alone but it can affect the synergistic effect of Dacomitinib with other anti-cancer drugs such as Flavopiridol.

4. Discussion

Inhibition of EGFR signaling induces autophagy in many types of cancers [13,15]. Activation of EGFR leads to phosphorylation of Beclin-1 and this suppresses the initiation of autophagy [13]. The first-generation EGFR TKIs inhibit EGFR activation and promote autophagy initiation in a Beclin-1-dependent manner [13,15]. However, our current study indicates that knockdown of Beclin-1 cannot block the second-generation EGFR TKIs-induced autophagy (Fig. 2E). This suggests that the first- and the second-generation EGFR TKIs affect cancer cell autophagy by different mechanisms. Furthermore, the second-generation EGFR TKIs not only promote the autophagy initiation signaling, but also block autophagic flux by disturbing lysosome function (Fig. 3). These results suggest a novel mechanism by which EGFR TKIs regulate autophagic flux.

Sunitinib, a multi-targeted RTK inhibitor, also affects autophagy by disturbing lysosome function [25]. Sunitinib is a weak base (pK_a 8.95), which accumulates in lysosomes and neutralizes the acidic PH of lysosome [25]. However, Dacomitinib- or Afatinib-treatment did not disturb the acidic environment of lysosome (Fig. 3J). This indicates that the second-generation EGFR TKIs and Sunitinib use different mechanisms to affect lysosome function. In the second-generation EGFR TKIs-treated cells, the Lysosensor Green DND-189 fluorescence was even brighter than in control cells, and there were more stained vesicles (Fig. 3J). This suggests that the second-generation EGFR TKIs may affect the lysosome homeostasis.

The different effects of the first- and the second-generation EGFR TKIs on autophagy may due to their different ways to bind EGFR. The first-generation EGFR TKIs bind EGFR reversibly. While the second-generation EGFR TKIs form irreversible covalent attachments to the EGFR kinase domain. Thus they inhibit EGFR activation more efficiently than the first-generation EGFR TKIs [26]. This is supported by our results that the second-generation EGFR TKIs can completely block EGFR activation with much lower drug concentrations than the first-generation TKIs (Fig. 1C–1F). Though Dacomitinib or Afatinib almost blocked all EGFR activation at 0.1 μ M, higher drug concentrations can further decrease HNSCC growth (Fig. 1A and 1B). This suggests the effect of these second-generation EGFR TKIs on cancer growth suppression is not just dependent on inhibiting EGFR activation. EGFR belongs to the ErbB family receptor tyrosine kinases [4]. Though the second-generation EGFR TKIs are much more effective to inhibit EGFR than the other ErbB family members, they still can attenuate the activation of other ErbB kinases such as ErbB2 and

ErbB4 [26]. This may also contribute to the effects of the second-generation EGFR TKIs on cancer growth suppression and autophagy.

There are some reports suggest that autophagy can affect cancer cell responses to anti-EGFR therapies. Inhibition of autophagy promotes the anti-EGFR therapies-induced cell death of non-small cell lung cancer and colorectal cancer [27,28]. However, inhibiting autophagy initiation did not affect Dacomitinib-induced HNSCC cell growth suppression (Fig. 4C). This suggests that the correlation of autophagy with cancer responses to anti-EGFR therapies can be cancer cell types different. It is possible that inhibiting autophagy can promote the anti-tumor effects of the second-generation TKIs in other types of cancer cells. The second-generation EGFR TKIs block autophagic flux by disturbing lysosome function (Fig. 3). It is possible that the disturbance of lysosome function rather than the blocking of autophagic flux contributes to the second-generation EGFR TKIs-induced HNSCC growth suppression.

The CDK inhibitor Flavopiridol is a potent anti-tumor drug that can convert the BH3 mimetic GX-015-070-induced autophagy to apoptosis [24]. Thus it has synergistic effect to inhibit tumor growth when used together with GX-015-070 [24]. The combination of Flavopiridol and the second-generation EGFR TKI Dacomitinib has synergistic effect to suppress the growth of HNSCC cells (Fig. 4C). This suggests a novel strategy to improve the efficiency of anti-EGFR therapies.

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Highlights

- The first- and the second-generation EGFR TKIs differentially modulate autophagy.
- The second-generation EGFR TKIs disturb lysosome function.
- Dacomitinib-induced HNSCC growth suppression is not dependent on autophagy.

Figure 1. The second-generation EGFR TKIs more efficiently inhibited HNSCC cells growth than the first-generation TKIs

UMSCC1 (A) and UMSCC6 (B) cells were treated with vehicle control (DMSO, 1:1000) or four different EGFR TKIs at the concentrations as indicated for 72 hrs. The cell viability was then measured with WST-1. Error bars indicate mean $\pm$ SEM from three independent experiments. The IC50 indicates the concentration of EGFR TKIs where the viability of cells is reduced by half compared with vehicle control. The effect of different EGFR TKIs on inhibiting EGFR activation was detected by EGFR phospho-tyr1068 antibody in UMSCC1 cells. (C) EGFR activation after Dacomitinib treatment. (D) EGFR activation after Afatinib treatment. (E) EGFR activation after Gefitinib treatment. (F) EGFR activation after Erlotinib treatment.

Figure 2. The second-generation EGFR TKIs had stronger effects on autophagy than the first-generation TKIs

(A) UMSCC1 cells were treated with vehicle control (DMSO, 1:1000) or 1 μ M different EGFR TKIs as indicated for 24 hrs, fixed and stained with anti-LC3 (red) and DAPI (blue), scale bar: 10 μ m. (B) Quantification of the LC3 fluorescence from at least 50 cells. (C) UMSCC1 cells were treated with vehicle control (DMSO, 1:1000) or 1 μ M different EGFR TKIs as indicated for 24 hrs, then treated with or without 80 μ M chloroquine (CQ) for 2 hrs. Whole cell lysates were analyzed for LC3II levels by Western blot. (D) Quantification of LC3II levels. (E) UMSCC1 cells were transfected with control or Beclin-1 siRNA, and then treated with Daacomitinib (1 μ M) for 24 hrs. The LC3II levels were then detected by Western blot. Error bars indicate mean $\pm$ SEM from three independent experiments. *, $p < 0.05$; **, $p < 0.01$.

Figure 3. The second-generation EGFR TKIs enhanced autophagy initiation signaling and disturbed lysosome function

(A) UMSCC1 cells were treated with vehicle control (DMSO, 1:1000) or 1 μ M different EGFR TKIs as indicated for 24 hrs. Levels of pULK-1 (S757) and pP70 S6K were measured by Western blot. (B) Quantification of pULK-1 (S757) levels. (C) Quantification of pP70 S6K levels. Quantification of pULK-1 (S757) and pP70 S6K was normalized with total ULK-1 or P70 S6K level. UMSCC1 cells were transfected with GFP-LC3 and treated with EGFR TKIs, and then the colocalization of GFP-LC3 and lysotracker was detected in (D) and quantified in (E). (F) Levels of CTSD precursor and mature CTSD were measured by Western blot. (G) Quantification of the expression ratios of mature CTSD with precursor. The ratio in DMSO vehicle control-treated cells was set up as 1. (H) UMSCC1 cells were treated with EGFR TKIs and then incubated with DQ-Red BSA as indicated, and then the fluorescence of DQ-Red BSA was detected by confocal microscopy. (I) Quantification of DQ-Red BSA fluorescence from at least 50 cells. (J) Lysosensor Green DND-189 staining of UMSCC1 cells after treated with different EGFR TKIs. The values shown on graphs represent the mean $\pm$ SEM from three independent experiments. *, $p < 0.05$; **, $p < 0.01$; ***, $p < 0.001$.

Figure 4. Inhibition of autophagy initiation did not affect Dacomitinib-induced HNSCC growth suppression

(A) UMSCC1 cells were treated with vehicle control (DMSO, 1:1000), spautin-1 (7.5 μ M), Dacomitinib (1 μ M), or their combination as indicated for 24 hrs, and then the LC3II levels were detected by Western blot. (B) Quantification of LC3II levels. (C) UMSCC1 cells were treated with vehicle control (DMSO, 1:1000), spautin-1 (7.5 μ M), Dacomitinib (1 μ M), Flavopiridol (0.1 μ M), or their combinations as indicated for 5 days, and then cell viability was measured with WST-1. The values shown on graphs represent the mean $\pm$ SEM from three independent experiments. **, $p < 0.01$; ***, $p < 0.001$. Dac indicates Dacomitinib. Sp indicates spautin-1. Fla indicates Flavopiridol.