Original Paper

The Effect of and Mechanism Underlying Autophagy in Hepatocellular Carcinoma Induced by CH12, a Monoclonal Antibody Directed Against Epidermal Growth Factor Receptor Variant III

Wen Xu^{a,b} Fei Song^a Biao Wang^a Kesang Li^a Mi Tian^a Min Yu^a
Xiaorong Pan^a Bizhi Shi^a Jianwen Liu^b Jianren Gu^a Zonghai Li^a Hua Jiang^a

^aState Key Laboratory of Oncogenes & Related Genes, Shanghai Cancer Institute, Renji Hospital, Shanghai Jiaotong University School of Medicine, Shanghai, ^bState Key Laboratory of Bioreactor Engineering & Shanghai Key Laboratory of New Drug Design, School of Pharmacy, East China University of Science and Technology, Shanghai, China

Key Words

Ch12 • EGFRvIII • Autophagy • Hepatocellular carcinoma

Abstract

Background/Aims: Epidermal growth factor receptor variant III (EGFRvIII), the most frequent EGFR variant, is constitutively activated without binding to EGF and is correlated with a poor prognosis. CH12, a human-mouse chimeric monoclonal antibody, has been developed in our laboratory and selectively binds to overexpressed EGFR and EGFRvIII. A previous study had reported that EGFR could influence autophagic activity, and autophagy is closely related to tumor development and the response to drug therapy. In this study, we aimed to elucidate the effect of CH12 on autophagy and efficacy of combining CH12 with an autophagy inhibitor against EGFRvIII-positive tumors. **Methods:** EGFRvIII was overexpressed in liver cancer, glioblastoma and breast cancer, and the change in the autophagy-relevant protein levels was analyzed by western blot assays, LC3 punctate aggregation was analyzed by immunofluorescence. The interaction of Beclin-1 and Rubicon was assessed by co-immunoprecipitation (Co-IP) after CH12 treatment. The efficacy of ATG7 or Beclin-1 siRNA in combination with CH12 in Huh-7-EGFRvIII cells was assessed by CCK-8 assays. The autophagy and apoptosis signaling events in Huh-7-EGFRvIII cells upon treatment with control, CH12, siRNA or combination for 48 h were assessed by western blot assays. **Results:** Our results showed that, in cancer cell lines overexpressing EGFRvIII, only the liver cancer cell lines Huh-7 and PLC/PRF/5 suggested autophagy activation. We then investigated the mechanism of autophagy activation after EGFRvIII overexpression. The results showed that EGFRvIII interacted with Rubicon, an autophagy inhibition protein, and released Beclin-1 to form the inducer complex, thus contributing to autophagy. In addition,

CH12, via inhibiting the phosphorylation of EGFRvIII, promoted the interaction of EGFRvIII with Rubicon, further inducing autophagy. *In vitro* assays suggested that knocking down the expression of the key proteins ATG7 or Beclin-1 in the autophagy pathway with siRNA inhibits tumor cell proliferation. Combining autophagy-related proteins 7 (ATG7) or Beclin-1 siRNA with CH12 in Huh-7-EGFRvIII cells showed better inhibition of cell proliferation. **Conclusion:** EGFRvIII could induce autophagy, and CH12 treatment could improve autophagy activity in EGFRvIII-positive liver cancer cells. The combination of CH12 with an autophagy inhibitor or siRNA against key proteins in the autophagy pathway displayed more significant efficacy on EGFRvIII-positive tumor cells than monotherapy, and induced cell apoptosis.

© 2018 The Author(s)
Published by S. Karger AG, Basel

Introduction

Autophagy is an important defense and protection mechanism in organisms. Autophagy is the process by which cellular organelles and proteins are engulfed by a double-walled membrane that splits off the non-ribosome region of the rough endoplasmic reticulum to form an autophagosome. The autophagosome then fuses with a lysosome to form an autolysosome where the engulfed cellular material is degraded to meet the metabolic needs of the cells themselves and promote the catabolic process of some organelles [1]. The two dominant autophagic pathways in mammals are macroautophagy and molecular chaperone-mediated autophagy (CMA) [2, 3]. There is no obvious selectivity when removing proteins through macroautophagy [4]. The core components of autophagosomes are composed of autophagy-related proteins (ATGs) in a specific order. The Beclin-1 complex comprises Beclin-1, Class III PI 3-kinase (VPS34), ATG14 and the serine/threonine PI3 kinase regulatory subunit 4 (PIK3R4, VPS15), and it is an important conditional complex in autophagosome formation and the autophagy initial stage. Light Chain 3 (LC3, Atg8 in yeast) is an autophagy protein downstream of Beclin 1 and a marker of autophagosome formation such that cytoplasmic LC3-I transforms esterified LC3-II on the autophagosome membrane [5-8]. In CMA, the molecular chaperone HSC70 recognizes soluble cytoplasmic protein via the KFERQ sequence (accounting for 30% of soluble cytoplasmic protein *in vivo*) with certain selectivity. After the formed molecular chaperone-substrate complex binds to a specific receptor on the lysosomal membrane, lysosome-associated membrane protein (LAMP), the substrate then enters the lysosomal cavity and is degraded into its components, which will be reused by cells [9-12].

Autophagy, as a highly conserved mechanism of eukaryotic cells, plays an important role in maintaining homeostasis, cell differentiation, growth and development, and the response to internal and external stimuli [1]. In recent years, autophagy has attracted great interest and attention from tumor biologists, and there is growing evidence to indicate that autophagy is closely correlated with tumorigenesis and the response of tumor cells to treatment [13, 14]. On the one hand, autophagy can remove damaged organelles to avoid the occurrence of harmful free radicals and mutations, thus avoiding greater damage. It has been found that the autophagy-induced gene *Beclin-1* is missing or mutated in various human tumors, including breast tumors, lung tumors, and prostate tumors, thereby losing its inhibition on tumors [15-17]. On the other hand, enhanced autophagy can also be detected in tumor cells after anti-hormonal treatment or chemoradiotherapy [13, 14, 18]. It is controversial regarding whether this enhanced autophagy caused by antitumor therapy leads to the death of tumor cells or maintains the survival of tumor cells, but it mainly depends on the type of tumor cells, treatment method, tumor environment and treatment characteristics [19]. Because of the dual relationship between tumor and autophagy, autophagy inhibitors and inducers cannot be simply used in the clinical treatment of tumors.

EGFR is a receptor tyrosine kinase (RTK). EGFR plays a very important role in the occurrence and development of tumors and is an important treatment target for tumors. Recent studies have shown that EGFR influences autophagy activity in many aspects. On the one hand, EGFR can affect autophagy by regulating mTOR, the key target of macroautophagy

through the PI3K and Ras/Raf pathways, which can interact with ULK1 and affect ULK1's participation in the Beclin-1 complex, thereby influencing the formation and maturation of autophagosomes [20-23]. On the other hand, EGFR-mediated Beclin-1 phosphorylation can inhibit the formation of the autophagy initiation complex, which is also an important pathway that influences autophagy. Recent studies have suggested that EGFR influences the binding of Beclin-1 to autophagy inhibitor protein Rubicon, thereby affecting the initiation of autophagy [24]. EGFRvIII is the most common mutation type of EGFR that is not expressed in normal tissues but is highly expressed in multiple malignant tumors, mostly in brain glioma, non-small cell lung tumor, head and neck squamous cell carcinoma and hepatocellular carcinoma. EGFRvIII expression is closely correlated with the occurrence, development, malignant transformation, metastasis and prognosis of tumors [25-27]; therefore, it is considered an ideal target for the treatment of tumors. EGFRvIII has the property of self-sustained phosphorylation activation independent of ligand binding [28], and it can cause constitutive activation of the PI3K/AKT signaling pathway and perform signal transduction by activating the Ras/ERK pathway [29, 30]. This detailed study investigated the type of influence EGFRvIII expression brings to autophagy, and in which pathways.

Currently there are three available monoclonal antibodies targeting EGFR: cetuximab (C225), panitumumab and nimotuzumab. Interestingly, macroautophagy induced by different anti-EGFR antibodies may produce diametrically opposite effects on the growth of tumor cells. For example, cetuximab can antagonize its pro-apoptotic effect by downregulating HIF-1 α and Bcl-2 and activating macroautophagy induced by the Beclin-1/h VPS34 complex [31], whereas panitumumab can induce macroautophagy to inhibit the proliferation of colon tumor cells [32]. Therefore, macroautophagy has dual effects on the antibody treatment of tumors. How to kill tumor cells by inducing macroautophagy with antitumor drugs and how to eliminate the tumor-promoting effect of macroautophagy while promoting its antitumor effect are issues that need to be addressed during the monoclonal antibody therapy of tumors.

CH12, a monoclonal antibody directed against EGFRvIII, can significantly inhibit the growth of EGFRvIII-positive hepatocellular carcinoma cells, glioblastoma, and breast cancer in nude mice [33-35]. Results from a mechanistic study showed that EGFRvIII protein and its phosphorylation levels are significantly reduced in hepatocellular carcinoma cells after treatment with CH12. In this study, we aimed to solve the following issues: the influence of EGFRvIII expression on the autophagy of tumor cells; the effect of CH12 on the autophagy of EGFRvIII-positive hepatocellular carcinoma cells; and the significance of such an effect in the antitumor process of CH12. The results from this study are expected to provide a certain experimental basis for the clinical application of the CH12 antibody.

Materials and Methods

Cell culture

Human hepatocellular carcinoma cells Huh-7 and PLC/PRF/5 were obtained from the American Type Culture Collection (ATCC, USA). The human glioblastoma cells U2511MG and U87MG and the human breast cancer cells BT474 and SKBR3 were also obtained from the ATCC. All hepatocellular carcinoma cells and GBM cells were cultured in Dulbecco's modified Eagle's medium (DMEM) (Gibco, USA) supplemented with 10% fetal bovine serum (FBS) (Serana, Australia). All breast cancer cells were cultured in 1640 medium (Gibco, USA) supplemented with 10% FBS. Cells were maintained at 37°C in a humidified atmosphere of 5% CO₂. Cancer cells exogenously overexpressing EGFRvIII were established according to previously reported methods [25].

Reagents

Erlotinib was purchased from Abcam (USA). The chimeric mAb CH12 (IgG1) was produced in dihydrofolate reductase-deficient CHO DG44 cells as previously described at 20 mg/mL [33]. EGFR, p-EGFR, P62, LC3B, ATG7, Beclin-1, Rubicon, PARP and Caspase 3 antibody were purchased from Cell signaling technology (MA, USA).

EGFRvIII stable expression cell establishment

The EGFRvIII plasmid and the control plasmid were purchased from Genepharma Company (China). The cells were infected with lentivirus that expresses EGFRvIII and selected in puromycin (Invitrogen, USA). The clones were confirmed by western blotting. Stable cell lines were maintained in DMEM supplemented with 10% FBS, and 2 µg/ml puromycin.

Western blot assay

Collected cells were washed, and cell pellets were resuspended in mammalian protein extraction reagent (M-PER) (Thermo Fisher Scientific, USA). The extracted protein was quantified using a BCA Kit (Thermo Fisher Scientific, USA), separated on SDS-PAGE gels at 8%-14% polyacrylamide according to protein weight and blotted onto a PVDF nitrocellulose membrane (Bio-Rad Laboratories, USA). The membrane was blocked in 5% skim milk in PBST for 1 h and then probed with primary antibodies overnight at 4°C. The following primary antibodies were used: the p62 antibody was purchased from Epitomics; the phosphor-EGFR, EGFR, GAPDH antibodies were purchased from Santa Cruz Biotechnology, and the mTOR, phosphor-mTOR, LC3B, Beclin-1, Beclin-2, phosphor-Beclin-1, VPS34, Rubicon, and ATG14 antibodies were obtained from Cell Signaling Technology. After washing the membranes in PBST, they were incubated with the appropriate secondary antibodies for 1 h at room temperature, washed three times in PBST and then visualized with enhanced chemiluminescence reagent following the manufacturer's instructions (Thermo, USA). Software IMAGE LAB (Bio-Rad, Hercules, CA, USA) and IMAGEJ (National Institutes of Health, USA) were used for the analysis and densitometry quantification of the protein levels.

Immunofluorescence

The human hepatocellular carcinoma cell lines Huh-7 and PLC/PRF/5 with or without EGFRvIII overexpression were plated on glass-bottomed cell culture dishes (Nest, USA) at 50% confluency, and then were washed in PBS, fixed in 4% paraformaldehyde (PFA) at room temperature for 15 min, permeabilized in 0.2% Triton X-100 for 15 min, and blocked with 1% bovine serum albumin (BSA) in PBS for 1 h. Next, cells were stained with primary LC3 antibody at 4°C overnight and were incubated with Alexa 488-conjugated donkey anti-mouse secondary antibodies (Jackson ImmunoResearch, USA) at 37°C for 1 h. Autophagosome formation was determined by confocal microscopy (1000×) (Olympus, Japan).

Co-immunoprecipitation

Briefly, prepared cell lysates were incubated with the indicated antibodies (5 µg/500 µl) at 4°C overnight, followed by the addition of protein G sepharose beads (Sigma, USA). After 4 h, the beads were collected, washed three times, resolved by SDS/PAGE, and immunoblotted with the indicated antibodies.

CCK-8 assay

The CCK-8 assay was used to determine the cell viability following treatment with ATG7 or Beclin-1 siRNA in combination with CH12 in Huh-7-EGFRvIII cells. Huh-7-EGFRvIII cells were seeded at a density of 2,000 cells/well in 96-well plates and were treated with CH12 at a concentration of 200 µg/mL or siRNA at a concentration of 100 nM or the combination for 48 h. Cell viability was assessed using the Cell Counting Kit-8 (CCK-8) (Dojindo, China) according to the manufacturer's instructions.

Statistical analysis

All data are presented as the mean ±SD of at least three triplications. Parametric data in different groups were examined for the homogeneity of variance and the data with equal variances were compared using one-way ANOVA followed by least significant difference (LSD) method for the inter-multiple group comparisons; non-parametric data or data with unequal variances were compared using Kruskal-Wallis rank test followed by Dunnett T3 method for inter-multiple group comparisons (GraphPad Software, San Diego, CA). Significant differences of parametric data between two groups were determined by two-tailed unpaired Student's *t*-test. Non-parametric data in two groups were determined by Mann-Whitney U test. *P* < 0.05 was considered a statistically significant difference.

Results

Enhancement of macroautophagy activity in EGFRvIII-positive hepatocellular carcinoma cells

After the overexpression of EGFRvIII, changes in the macroautophagy activity of different cell lines were detected. Western blot results showed that a significant increase in LC3I/II conversion and downregulation of P62 were found in Huh-7 and PLC/PRF/5 hepatocellular carcinoma cell lines, indicating that the overexpression of EGFRvIII promoted autophagy in these two cell lines (Fig. 1A). However, in U251MG and U87MG glioma cell lines, as well as in BT474 and SKBR3 breast tumor cell lines, the overexpression of EGFRvIII had no effect on their autophagy status. Next, we detected the punctate aggregation of LC3 in Huh-7 and PLC/PRF/5 cells and their EGFRvIII-overexpressed cell lines with immunofluorescence. The results showed that the overexpression of EGFRvIII increased punctate aggregation of LC3 in Huh-7 and PLC/PRF/5 cells, which is a marker for enhancement in autophagy (Fig. 1B). The quantification analysis of LC3 puncta per cell in parent Huh-7 and PLC/PRF/5 cells and their EGFRvIII-positive sublines was showed. These results suggested that the overexpression of EGFRvIII promoted autophagy in hepatocellular carcinoma cell lines.

EGFRvIII promotes autophagy by interacting with Rubicon

To explore the effect of EGFRvIII on autophagy, we detected changes in autophagy-related proteins by western blotting. The results showed that the expression levels of Beclin-1, p-Beclin-1 and VPS34 showed no changes (Fig. 2A). To determine whether the binding proteins of Beclin-1 were changed, we investigated the Beclin-1 complex. The IP results suggested that VPS34, which binds to Beclin-1, was slightly increased, whereas Rubicon was significantly decreased (Fig. 2B). According to previous studies, the binding of EGFR to Rubicon in breast tumor cells can release Beclin-1 and promote autophagy [24]. We detected the

Fig. 1. EGFRvIII overexpression in Huh-7 and PLC/PRF/5 cells induces autophagy. (A) Western blot analysis of the change in P62 and LC3II/LC3I expression after EGFRvIII overexpression. (B) Immunofluorescence showed the effect of the overexpression of EGFRvIII on inducing LC3 punctate aggregation in parent Huh-7 and PLC/PRF/5 cells and their EGFRvIII-positive sublines. LC3 puncta per cell were quantified in parent Huh-7 and PLC/PRF/5 cells and their EGFRvIII-positive sublines. All data are presented as the mean $\pm$ SE of three repetitions. $P < 0.05$ was considered statistically significant. * $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$ versus the parent group.

Fig. 2. Analysis of autophagy-relevant protein expression and their interaction after EGFRvIII overexpression. (A) Western blot analysis of the autophagy-relevant protein after EGFRvIII overexpression in parent Huh-7 and PLC/PRF/5 cells and their EGFRvIII-positive sublines. (B) IP assay of Beclin-1 interaction with its relevant proteins Rubicon, VPS34 and ATG14, and Rubicon interaction with EGFRvIII and Beclin-1 in Huh-7 and Huh7-EGFRvIII cells.

Fig. 3. CH12 treatment induces autophagy in Huh-7-EGFRvIII and PLC/PRF/5-EGFRvIII cells. (A) Western blot analysis of the change in P62 and LC3I/LC3II expression after CH12 treatment in Huh-7-EGFRvIII and PLC/PRF/5-EGFRvIII cells. (B, C) Immunofluorescence showed the effect of CH12 on inducing LC3 punctate aggregation in Huh-7-EGFRvIII and PLC/PRF/5-EGFRvIII cells. LC3 puncta per cell were quantified in cells. All data are presented as the mean $\pm$ SE. $P < 0.05$ was considered statistically significant. * $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$ versus the control.

interacting proteins of Rubicon, and the results showed that after expression, EGFRvIII could bind to Rubicon, whereas the binding of Rubicon to Beclin-1 was decreased, promoting the formation of the Beclin-1 initiation complex and activating autophagy (Fig. 2C).

Fig. 4. Analysis of autophagy-relevant protein expression and their interaction with EGFRvIII-positive liver cancer cells after CH12 treatment. (A) Western blot analysis of autophagy-relevant protein expression in Huh-7-EGFRvIII and PLC/PRF/5-EGFRvIII cells after CH12 treatment. (B) IP assay of Beclin-1 interaction with its relevant proteins Rubicon, VPS34 and ATG14, and Rubicon interaction with EGFRvIII and Beclin-1 in Huh-7-EGFRvIII cells after CH12 treatment. All data are presented as the mean $\pm$ SE. $P < 0.05$ was considered statistically significant. * $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$ versus the control.

CH12 further activates autophagy after treating EGFRvIII-positive hepatocellular carcinoma cells

To investigate the influence of CH12 treatment on the autophagy of EGFRvIII-positive hepatocellular carcinoma cells, we detected changes in autophagy-related proteins by western blotting, and the results showed that after CH12 treatment, LC3I/II conversion was significantly increased, and p62 was downregulated (Fig. 3A). Next, we detected the punctate aggregation of LC3 in Huh-7-EGFRvIII and PLC/PRF/5-EGFRvIII cells after treatment with CH12, and the results showed that CH12 treatment increased the punctate aggregation of LC3 and promoted autophagy (Fig. 3B). The quantification analysis of LC3 puncta per cell in parent Huh-7 and PLC/PRF/5 cells and their EGFRvIII-positive sublines was showed.

CH12 promotes autophagy by inhibiting the phosphorylation of EGFR and increasing the binding of EGFRvIII to Rubicon

To determine the effect of CH12 on autophagy, we detected changes in autophagy-related protein levels by western blotting. The results suggested the expression levels of Beclin-1, p-Beclin-1 and VPS34 showed no changes (Fig. 4A). Given that EGFRvIII influences autophagy by affecting the binding of Rubicon to Beclin-1 as demonstrated above, we detected changes in the binding proteins of Beclin-1 after CH12 treatment with erlotinib as the control. The IP results showed that CH12 significantly inhibited the phosphorylation of EGFRvIII. After treatment with CH12 and erlotinib, the interaction between EGFRvIII and Rubicon was significantly enhanced, significantly reducing the binding of Rubicon to Beclin-1 (Fig. 4B).

Knocking down important proteins of autophagy-related pathways to inhibit autophagy promotes the death of hepatocellular carcinoma cells

To determine the influence of autophagy on the proliferation of EGFRvIII-positive hepatocellular carcinoma cell lines, we knocked down ATG7 and Beclin-1, important proteins for the autophagy process, with siRNA while using CH12 in combination, and observed the survival rate of cells. The results showed that after knocking down autophagy-related proteins, autophagy was significantly inhibited, and the survival rate of cells was significantly

Fig. 5. The combination of CH12 with ATG7 or Beclin-1 siRNA suppressed the growth of Huh-7-EGFRvIII cells in vitro. (A) CCK-8 assay examining the efficacy of ATG7 or Beclin-1 siRNA in combination with CH12 in Huh-7-EGFRvIII cells. Huh-7-EGFRvIII cells were treated with CH12 at a concentration of 200 μ g/mL or siRNA at a concentration of 100 nM or the combination for 48 hours. Data are expressed as the cell viability in triplicate experiments (Bars, SD). (B, C) Immunoblots assessing autophagy and apoptosis signaling events in Huh-7-EGFRvIII cells upon treatment with Control, CH12, siRNA or the combination for 48 hours. All data are presented as the mean $\pm$ SE. $P < 0.05$ was considered statistically significant. * $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$ versus the control, # $P < 0.05$, ## $P < 0.01$, ### $P < 0.001$ versus single treatment.

reduced (Fig. 5A). Western blot results showed that treating Huh-7-EGFRvIII cells with interfering siRNA of ATG7 and Beclin-1 combined with CH12 could more significantly induce the apoptosis of cells (Fig. 5B,C).

Discussion

Autophagy, a highly conserved mechanism of eukaryotic cells, plays an important role in maintaining homeostasis, cell differentiation, growth and development, as well as in the response to internal and external stimuli [1]. Autophagy is closely correlated with tumorigenesis and the response of tumor cells to treatment [13, 14]. Autophagy is a double-edged sword. On the one hand, autophagy can remove damaged organelles to avoid the occurrence of harmful free radicals and mutations, thus avoiding greater damage. It has been found that the autophagy-induced gene *Beclin-1* is missing or mutated in various human tumors, thereby losing its inhibition on tumors [15-17]. On the other hand, enhanced autophagy can also be detected in tumor cells after anti-hormonal treatment or chemoradiotherapy [13, 14, 18]. It is controversial whether this enhanced autophagy caused by antitumor therapy leads to the death of tumor cells or maintains the survival of tumor cells; however, it mainly depends on the type of tumor cell and treatment method [19].

EGFR plays a very important role in the occurrence and development of tumors, and recent studies have shown that EGFR influences autophagy activity in many aspects. On the one hand, EGFR could affect the PI3K/AKT/mTOR pathway, and mTOR is a key regulatory protein for the formation and maturation of autophagosomes [20-23]; on the other hand, EGFR-mediated phosphorylated Beclin-1 could inhibit the formation of the autophagy initiation complex, which is also an important pathway that influences autophagy. Recent studies have shown that EGFR can bind to Rubicon, which influences the binding of Beclin-1 to Rubicon, thereby affecting the initiation of autophagy. EGFRvIII is a common mutation type of EGFR that is closely correlated with the occurrence, development, malignant

transformation, metastasis and prognosis of tumors [28-31]. This study first explored the influence of EGFRvIII on autophagy. Immunofluorescence data showed that in EGFRvIII-overexpressed hepatocellular carcinoma cells, the conversion of LC3I to LC3II was increased, and the punctate aggregation of LC3 was more obvious, which is a marker for enhancement in autophagy (Fig. 1). We also analyzed the expression levels of the autophagy initiator proteins p-Beclin-1, Beclin-1, Beclin-2 and VPS34 and found that EGFRvIII did not affect their expression levels (Fig. 2 A). Thus, we further analyzed the interacting protein of Beclin-1, and the results showed that EGFRvIII can interact with Rubicon, which reduced the binding of Beclin-1 to Rubicon and promoted the binding of Beclin-1 to VPS34, thereby activating autophagy (Fig. 2B).

Due to the important function and tumor specificity of EGFRvIII, antibodies targeting EGFRvIII have been actively developed. The clinical data of mAb806 in Phase I suggested significant clinical efficacy and low toxicity [36, 37]. The human-mouse chimeric monoclonal antibody CH12, independently developed by our laboratory also showed a significant effect on EGFRvIII-overexpressed tumor models. This current study further explored the influence of CH12 on autophagy. The data showed that after treating Huh-7-EGFRvIII cells with CH12, the autophagy activity was significantly enhanced (Fig. 3 A, B). To determine how CH12 influenced the autophagy process, based on a former study on the relationship between EGFRvIII and autophagy, we further analyzed EGFRvIII and the autophagy inhibitor protein Rubicon while using erlotinib as a positive control to inhibit the phosphorylation of EGFR. The results of signaling pathways showed that EGFRvIII and erlotinib could inhibit the phosphorylation of EGFR and EGFRvIII. When the phosphorylation activity was reduced, the binding of EGFRvIII to Rubicon was increased, reducing the binding of Beclin-1 to Rubicon and promoting autophagy (Fig. 4A,B).

To determine the influence of enhanced autophagy on the proliferation of EGFRvIII-positive hepatocellular carcinoma cell line, we inhibited the autophagy process using interfering RNA targeting Beclin-1 and ATG7, and the results showed that when autophagy was inhibited, tumor proliferation was weakened, and the combined use of CH12 had a better inhibitory effect. The results of signaling pathways showed that the combined use of CH12 with interfering RNA against autophagy process proteins can inhibit autophagy and promote the apoptosis of cells (Fig. 5).

Previous studies have reported that the combined use of sorafenib and the autophagy inhibitor chloroquine achieved good effect in both the *in vitro* and *in vivo* treatment of hepatocellular carcinoma [38]. Meanwhile, in non-small cell lung tumor with the expression of wild-type EGFR, the combined use of chloroquine and erlotinib could overcome the resistance to erlotinib, and the use of gefitinib in combination can also increase the toxicity of gefitinib. The clinical phase I experiment of the combined use of chloroquine and erlotinib showed that the combined use of the two has good tolerance [39-41]. Therefore, the combination of CH12 and an autophagy inhibitor to treat EGFRvIII-positive hepatocellular carcinoma is worth further exploration.

Conclusion

Previous data from our lab suggested that CH12 could downregulate phosphorylation of EGFRvIII and its downstream activation of AKT and ERK in hepatocellular tumor and contributed to cell growth inhibition [33]. And this study suggested that EGFRvIII could enhance the autophagy activity of hepatocellular carcinoma, and CH12 promoted the interaction of EGFRvIII with Rubicon via inhibiting the phosphorylation of EGFRvIII, induced autophagy and attenuated its anti-proliferation effect in some extent. The combination of CH12 with an autophagy inhibitor or siRNA against key proteins in the autophagy pathway displayed more significant efficacy on EGFRvIII-positive hepatocellular cancer cells than monotherapy, and further induced cell apoptosis (Fig. 6). Therefore, the treatment effect of

Fig. 6. The mechanism diagram of anti-tumor effect of CH12. Previous data from our lab suggested that CH12 could down-regulate phosphorylation of EGFRvIII and its downstream activation of AKT and ERK in hepatocellular tumor and contributed to cell growth inhibition (33). And this study suggested that CH12 promoted the interaction of EGFRvIII with Rubicon via inhibiting the phosphorylation of EGFRvIII, induced autophagy and attenuated its anti-proliferation effect in some extent. The combination of CH12 with an autophagy inhibitor or siRNA against key proteins in the autophagy pathway displayed more significant efficacy on EGFRvIII-positive hepatocellular cancer cells than monotherapy, and further induced cell apoptosis.

the combined use of CH12 and the autophagy inhibitor on EGFRvIII-positive hepatocellular carcinoma *in vivo* remains to be verified in further studies.

Abbreviations

EGFR (epidermal growth factor receptor); Co-IP (co-immunoprecipitation); CMA (chaperone-mediated autophagy); ATG (autophagy-related protein); LAMP (lysosome-associated membrane protein); RTK (receptor tyrosine kinase); ATCC (American Type Culture Collection); DMEM (Dulbecco's modified Eagle's medium); FBS (fetal bovine serum); BSA (bovine serum albumin).

Acknowledgements

The study was funded by the Supporting Programs of the National Natural Science Foundation (No. 81372468 and No. 81672724), the Shanghai Science and Technology Development Funds (No. 14431903500) and the research fund of the State Key Laboratory of Oncogenes and Related Genes (91-17-04).

Disclosure Statement

The authors declare that they have no competing interests.

References

- 1 Yang Z, Klionsky DJ: Eaten alive: a history of macroautophagy. *Nat Cell Biol* 2010;12:814-822.
- 2 Yang Z, Klionsky DJ: Mammalian autophagy: core molecular machinery and signaling regulation. *Curr Opin Cell Biol* 2010;22:124-131.
- 3 Cuervo AM: Chaperone-mediated autophagy: selectivity pays off. *Trends Endocrinol Metabol* 2010;21:142-150.
- 4 Mizushima N, Levine B, Cuervo AM, Klionsky DJ: Autophagy fights disease through cellular self-digestion. *Nature* 2008;451:1069-1075.

- 5 Maiuri MC, Ciriollo A, Kroemer G: Crosstalk between apoptosis and autophagy within the Beclin 1 interactome. *EMBO J* 2010;29:515-516.
- 6 Salminen A, Kaarniranta K, Kauppinen A: Beclin 1 interactome controls the crosstalk between apoptosis, autophagy and inflammasome activation: impact on the aging process. *Ageing Res Rev* 2013;12:520-534.
- 7 He C, Levine B: The beclin 1 interactome. *Curr Opin Cell Biol* 2010;22:140-149.
- 8 Funderburk SF, Wang QJ, Yue Z: The Beclin 1-VPS34 complex—at the crossroads of autophagy and beyond. *Trends Cell Biol* 2010;20:355-362.
- 9 Cuervo AM, Dice JF: A receptor for the selective uptake and degradation of proteins by lysosomes. *Science* (80-) 1996;273:501.
- 10 Salvador N, Aguado C, Horst M, Knecht E: Import of a cytosolic protein into lysosomes by chaperone-mediated autophagy depends on its folding state. *J Bio Chem* 2000;275:27447-27456.
- 11 Agarraberes FA, Dice JF: A molecular chaperone complex at the lysosomal membrane is required for protein translocation. *J Cell Sci* 2001;114:2491-2499.
- 12 Bandyopadhyay U, Sridhar S, Kaushik S, Kiffin R, Cuervo AM: Identification of regulators of chaperone-mediated autophagy. *Mol Cell* 2010;39:535-547.
- 13 Kondo Y, Kanzawa T, Sawaya R, Kondo S: The role of autophagy in cancer development and response to therapy. *Nature RevCancer* 2005;5:726-734.
- 14 Kondo Y, Kondo S: Autophagy and cancer therapy. *Autophagy* 2006;2:85-90.
- 15 Liang XH, Jackson S, Seaman M, Brown K, Kempkes B, Hibshoosh H, Levine B: Induction of autophagy and inhibition of tumorigenesis by beclin 1. *Nature* 1999;402:672-676.
- 16 Aita VM, Liang XH, Murty V, Pincus DL, Yu W, Cayanis E, Kalachikov S, Gilliam TC, Levine B: Cloning and genomic organization of beclin 1, a candidate tumor suppressor gene on chromosome 17q21. *Genomics* 1999;59:59-65.
- 17 Qu X, Yu J, Bhagat G, Furuya N, Hibshoosh H, Troxel A, Rosen J, Eskelinen E-L, Mizushima N, Ohsumi Y: Promotion of tumorigenesis by heterozygous disruption of the beclin 1 autophagy gene. *J. Clin Invest* 2003;112:1809-1820.
- 18 Eisenberg-Lerner A, Kimchi A: The paradox of autophagy and its implication in cancer etiology and therapy. *Apoptosis* 2009;14:376-391.
- 19 Baehrecke EH: Autophagy: dual roles in life and death? *Nature Rev Mol Cell Biol* 2005;6:505-510.
- 20 Kim KW, Hwang M, Moretti L, Jaboin JJ, Cha YI, Lu B: Autophagy upregulation by inhibitors of caspase-3 and mTOR enhances radiotherapy in a mouse model of lung cancer. *Autophagy* 2008;4:659-668.
- 21 Eshleman JS, Carlson BL, Mladek AC, Kastner BD, Shide KL, Sarkaria JN: Inhibition of the mammalian target of rapamycin sensitizes U87 xenografts to fractionated radiation therapy. *Cancer Res* 2002;62:7291-7297.
- 22 Weihua Z, Tsan R, Huang W-C, Wu Q, Chiu C-H, Fidler IJ, Hung M-C: Survival of cancer cells is maintained by EGFR independent of its kinase activity. *Cancer Cell* 2008;13:385-393.
- 23 Cao C, Subhawong T, Albert JM, Kim KW, Geng L, Sekhar KR, Gi YJ, Lu B: Inhibition of mammalian target of rapamycin or apoptotic pathway induces autophagy and radiosensitizes PTEN null prostate cancer cells. *Cancer Res* 2006;66:10040-10047.
- 24 Tan X, Thapa N, Sun Y, Anderson RA: A kinase-independent role for EGF receptor in autophagy initiation. *Cell* 2015;160:145-160.
- 25 Wang H, Jiang H, Zhou M, Xu Z, Liu S, Shi B, Yao X, Yao M, Gu J, Li Z: Epidermal growth factor receptor vIII enhances tumorigenicity and resistance to 5-fluorouracil in human hepatocellular carcinoma. *Cancer Lett* 2009;279:30-38.
- 26 Nishikawa R, Ji X-D, Harmon RC, Lazar CS, Gill GN, Cavenee WK, Huang H: A mutant epidermal growth factor receptor common in human glioma confers enhanced tumorigenicity. *Proc Nat Acad Sci* 1994;91:7727-7731.
- 27 Diedrich U, Lucius J, Baron E, Behnke J, Pabst B, Zoll B, Diedrich U: Distribution of epidermal growth factor receptor gene amplification in brain tumours and correlation to prognosis. *J. Neurol* 1995;242:683-688.
- 28 Wikstrand C, Reist C, Archer G, Zalutsky M, Bigner D: The class III variant of the epidermal growth factor receptor (EGFRvIII): characterization and utilization as an immunotherapeutic target. *J Neurovirol* 1998;4:148-158.
- 29 Narita Y, Nagane M, Mishima K, Huang HS, Furnari FB, Cavenee WK: Mutant epidermal growth factor receptor signaling down-regulates p27 through activation of the phosphatidylinositol 3-kinase/Akt pathway in glioblastomas. *Cancer Res* 2002;62:6764-6769.

- 30 Mischel PS, Cloughesy T: Using molecular information to guide brain tumor therapy. *Nature Clin Pract Neurol* 2006;2:232-233.
- 31 Li X, Fan Z: The epidermal growth factor receptor antibody cetuximab induces autophagy in cancer cells by downregulating HIF-1 α and Bcl-2 and activating the beclin 1/hVps34 complex. *Cancer Res* 2010;70:5942-5952.
- 32 Giannopoulou E, Antonacopoulou A, Matsouka P, Kalofonos HP: Autophagy: novel action of panitumumab in colon cancer. *Anticancer Res* 2009;29:5077-5082.
- 33 Jiang H, Wang H, Tan Z, Hu S, Wang H, Shi B, Yang L, Li P, Gu J, Wang H: Growth suppression of human hepatocellular carcinoma xenografts by a monoclonal antibody CH12 directed to epidermal growth factor receptor variant III. *J Biol Chem* 2011;286:5913-5920.
- 34 Xu W, Bi Y, Zhang J, Kong J, Jiang H, Tian M, Li K, Wang B, Chen C, Song F: Synergistic antitumor efficacy against the EGFRvIII+HER2+ breast cancers by combining trastuzumab with anti-EGFRvIII antibody CH12. *Oncotarget* 2015;6:38840-38853.
- 35 Xu W, Bi Y, Kong J, Zhang J, Wang B, Li K, Tian M, Pan X, Shi B, Gu J: Combination of an anti-EGFRvIII antibody CH12 with Rapamycin synergistically inhibits the growth of EGFRvIII+PTEN-glioblastoma. *Oncotarget* 2016;7:24752-24765.
- 36 Scott AM, Lee FT, Tebbutt N, Herbertson R, Gill SS, Liu Z, Skrinos E, Murone C, Saunderson TH, Chappell B, Papenfuss AT, Poon AM, Hopkins W, Smyth FE, MacGregor D, Cher LM, Jungbluth AA, Ritter G, Brechbiel MW, Murphy R, Burgess AW, Hoffman EW, Johns TG, Old LJ: A phase I clinical trial with monoclonal antibody ch806 targeting transitional state and mutant epidermal growth factor receptors. *Proc Natl Acad Sci USA* 2007;104:4071-4076.
- 37 Cleary JM, Reardon DA, Azad N, Gandhi L, Shapiro GI, Chaves J, Pedersen M, Ansell P, Ames W, Xiong H, Munasinghe W, Dudley M, Reilly EB, Holen K, Humerickhouse R: A phase 1 study of ABT-806 in subjects with advanced solid tumors. *Invest New Drugs* 2015;33:671-678.
- 38 Shi Y-H, Ding Z-B, Zhou J, Hui B, Shi G-M, Ke A-W, Wang X-Y, Dai Z, Peng Y-F, Gu C-Y: Targeting autophagy enhances sorafenib lethality for hepatocellular carcinoma via ER stress-related apoptosis. *Autophagy* 2011;7:1159-1172.
- 39 Zou Y, Ling Y-H, Sironi J, Schwartz EL, Perez-Soler R, Piperdi B: The autophagy inhibitor chloroquine overcomes the innate resistance of wild-type EGFR non-small-cell lung cancer cells to erlotinib. *J Thor Oncol* 2013;8:693-702.
- 40 Tang M-C, Wu M-Y, Hwang M-H, Chang Y-T, Huang H-J, Lin AM-Y, Yang JC-H: Chloroquine enhances gefitinib cytotoxicity in gefitinib-resistant nonsmall cell lung cancer cells. *PLoS ONE* 2015;10:e0119135.
- 41 Goldberg SB, Supko JG, Neal JW, Muzikansky A, Digumarthy S, Fidias P, Temel JS, Heist RS, Shaw AT, McCarthy PO: A phase I study of erlotinib and hydroxychloroquine in advanced non-small-cell lung cancer. *J Thor Oncol* 2012;7:1602-1608.