INTRODUCTION

The survival rate of laryngeal carcinoma (LC) has not markedly improved despite recent advances in treatment modalities; the main reason for this may be treatment failure primarily due to chemoradioresistance. However, the exact mechanism of radioresistance remains unclear. In previous studies, we determined that GLUT1 is involved in the radioresistance of LC. Inhibition of GLUT1 expression using an antisense oligonucleotide may enhance the radiosensitivity of LC. However, the precise mechanism of radioresistance of LC requires further investigation.

Abstract

In this study, we investigated the ability of curcumin alone or in combination with GLUT1 siRNA to radiosensitize laryngeal carcinoma (LC) through the induction of autophagy. Protein levels in tumour tissues and LC cells were measured by immunohistochemistry and Western blotting. In vitro, cell proliferation, colony formation assays, cell death and autophagy were detected. A nude mouse xenograft model was established through the injection of Tu212 cells. We found that GLUT1 was highly expressed and negatively associated with autophagy-related proteins in LC and that curcumin suppressed radiation-mediated GLUT1 overexpression in Tu212 cells. Treatment with curcumin, GLUT1 siRNA, or the combination of the two promoted autophagy. Inhibition of autophagy using 3-MA promoted apoptosis after irradiation or treatment of cells with curcumin and GLUT1 siRNA. 3-MA inhibited curcumin and GLUT1 siRNA-mediated non-apoptotic programmed cell death. The combination of curcumin, GLUT1 siRNA and 3-MA provided the strongest sensitization in vivo. We also found that autophagy induction after curcumin or GLUT1 siRNA treatment implicated in the AMP-activated protein kinase-mTOR-serine/threonine-protein kinase-Beclin1 signalling pathway. Irradiation primarily caused apoptosis, and when combined with curcumin and GLUT1 siRNA treatment, the increased radiosensitivity of LC occurred through the concurrent induction of apoptosis and autophagy.

KEYWORDS
apoptosis, autophagy, curcumin, GLUT1, laryngeal carcinoma, radiosensitivity
Curcumin is aflavouring agent extracted from the Curcuma longa rhizome,6-9 which has attracted a great deal of attention due to its antioxidant, anti-inflammatory, anti-tumour and antifibrosis properties.6 Curcumin can inhibit cell growth and enhance the radio-sensitivity of many cancers, including LC, possibly through the regulation of autophagy.5,9

Many studies have linked autophagy inhibitors with improvement in the radiosensitivity of some solid malignant tumors.10-13 Previous studies have shown that the expression of autophagy-associated genes (ATGs) is reduced in the tissues of laryngeal squamous cell carcinoma and is related to prognosis, lymph node metastasis and T stage.14-16 Increasing evidence indicates a relationship between autophagy and the radiosensitivity of LC, but the detailed mechanism remains unclear.

The PI3K/Akt pathway plays a key role in autophagy regulation, and blockage of PI3K/Akt may enhance the radiosensitivity of some malignant tumors, suggesting that PI3K/Akt affects radiore-sistance through the regulation of autophagy.17-19 The inhibition of glycolysis increases the AMP/ATP content,20 which then activates the AMP-activated protein kinase (AMPK) pathway, which is closely associated with autophagy.21-25 The relationship between AMPK and autophagy may be associated with the serine/threonine-protein kinase (ULK1) complex, a key regulator of autophagy, as a bridge between the upstream energy receptors mTOR and AMPK and downstream autophagosome formation. AMPK/mTOR signalling regulates the activation of ULK1. AMPK also inactivates mTOR,26 along with increases in GLUT1 expression and glucose uptake in thyroid PCCL3 cells25 and blood-brain barrier pericytes.27 Curcumin also activates the AMPK pathway.25 Therefore, we speculate that GLUT1 siRNA and curcumin treatment would activate the AMPK pathway and in-duce activation of ULK1 to enhance autophagy. However, few stud-ies have considered this or the involvement, if any, of autophagy in the radiosensitizing process and whether the PI3K/Akt and AMPK pathways regulate autophagy in this process.

Here, we investigated the expression of GLUT1 and Beclin-1 and LC3-II in human LC tissues using immunohistochemical methods, and analysed the relationships between these markers and LC. We fur-ther investigated whether combining curcumin with GLUT1 siRNA has a synergistic effect on the radiosensitivity of LC and whether the mechanism is through autophagy modulation by the PI3K/Akt or AMPK pathway.

2 | MATERIALS AND METHODS

2.1 | Patient samples

Forty-five fresh tissues from patients with LC admitted to our hos-pital between May 2013 and October 2016 were collected. Twelve fresh paracarcinoma tissue samples were obtained from the nega-tive margin (approximately 0.5 cm from the edge) of patients who underwent partial or total laryngectomy. No patients underwent chemotherapy or radiotherapy preoperatively. All fresh samples were immediately frozen in liquid nitrogen and maintained for subse-quent testing.

This study was approved by the Institutional Review Board of The First Affiliated Hospital, College of Medicine, Zhejiang University (number: 2019-566-1). Written informed consent was obtained from all patients before sample collection.

2.2 | Immunohistochemistry

Briefly, samples were fixed, embedded and cut into sections 5μm thick. After deparaffinization and hydration, sections underwent antigen retrieval. Endogenous peroxidase activity was blocked by H2O2, and slides were incubated with primary antibodies against GLUT1, Beclin1 and LC3-II diluted in working solutions (1:50) overnight. Sections were incubated with secondary antibod-ies labelled with streptavidin-horseradish peroxidase. Then, the slides were stained using a 3,3′-diaminobenzidine (DAB) staining kit and subjected to haematoxylin and eosin staining. The slides were photographed under a microscope (Olympus BX41; Olympus Corp., Tokyo, Japan); cells labelled with brownish-yellow granules were identified as positive cells. Five high-magnification fields were randomly selected, and 100 cells were counted in each field. The scoring for the rate of positive cells was as follows: 0, <10%; 1, 10%-25%; 2, 26%-50%; and 3, >50%. The scoring for dye depth was as follows: 0, no staining; 1, light yellow; 2, the depth of staining was between 1 and 3 points; and 3, yellow to brown staining. Immunohistochemical expression was assessed by the total score according to the following formula: the score of the rate of positive cells + the score of dye depth. Total scores of 0-1, 2, 3-4 and 5-6 were considered negative (−), weak-positive (+), positive (++), and strong-positive (+++), respectively.

2.3 | Cell culture and treatments

Tu212 cells were purchased from the Cell Research Institute of the Chinese Academy of Sciences (Shanghai, China). Tu212 cells were maintained in RPMI-1640 (Gibco-BRL, Gaithersburg, MD, USA), supplemented with 10% heat-inactivated foetal bo-vine serum albumin (BSA; Hyclone, Logan, UT, USA), 100 U/mL penicillin and 100 g/mL streptomycin at 37°C in a 5%CO2 at-mosphere. After digesting using trypsin and harvesting after growing to 80%-90% confluence, the cells were seeded in six-well plates and transfected with GLUT1-siRNA (GLUT1-siRNA sequences:sense5′-CUGUGGGCUCUUUCGUUAATT-3′, antisense5′-UUACGAAAAAGCCCAACAGAG-3′) or negative con-trol siRNA (sense5′-UUCUCGCCAAGCUGACGTT-3′, antisense 5′-ACUGUACACGUCGCCAGATT-3′) using Lipofectamine-2000 (Invitrogen, Thermo Fisher Scientific). Twenty-four hours after transfection, the transfected cells were treated with 10 Gy X-ray irradiation, curcumin (20 μM), 3-MA (2 mM), chloroquine (10 μM) or a combination of these treatments for an additional 24 hours.
siRNA for GLUT1 was synthesized by GenePharma (Shanghai, China). X-ray radiation was performed on a linear accelerator (Clinac 23EX, Varian Medical Systems, Orlando, USA); the plate distance was 100 cm, the radiation field area was 35 cm × 35 cm, the X-ray energy was 6 MV, and the dose rate was 500 MU/min. All assays were carried out in triplicate.

2.4 | Clonogenic assay

Briefly, the well surface of a 6 cm plate was covered with a mixture of 1.2% agarose and Dulbecco's modified eagle's medium (DMEM) medium and was allowed to cool for solidification. After transfection with GLUT1-siRNA or control siRNA, cells were exposed to 10 Gy irradiation, curcumin treatment or the combination for 24 hours. The cell suspension was mixed with 0.7% agarose and medium and seeded in a 6 cm plate coated with 1.2% agarose. After solidification of agarose-containing cells, all plates were incubated at 37°C in a 5%CO2 atmosphere for 10 days. Images of the colonies were acquired after crystal violet staining.

2.5 | Flow cytometry

After treatment, cells were harvested and resuspended. Next, 5 µL fluorescein isothiocyanate and 10 µL propidium iodide (Sigma Aldrich Co., St. Louis, MO, USA) were added, and samples were incubated. The proportion of cells undergoing non-apoptotic cell death and apoptotic cell death was determined by flow cytometry using Annexin-FITC kits. Flow cytometry data were analysed using the ModFit LT software (Becton Dickinson, Mountain View, CA, USA). Each experiment was performed in triplicate.

2.6 | CCK8 assay

Cells transfected with GLUT1-siRNA were subjected to 10 Gy irradiation, curcumin treatment or the combination. All cell groups were exposed to phosphate-buffered saline (PBS) or z-VAD-fmk for 48 hours. Subsequently, 10 µL cell counting solution was added. The absorption at 450 nm was measured using a Spectra Plus microplate reader (Molecular Devices Co., Sunnyvale, CA, USA). The relative cell inhibition rate (%) was determined using the following formula: 100 – (sample absorption/control mean absorption) × 100%.

2.7 | Immunofluorescence

After incubation for 48 hours, cells were washed three times with PBS and fixed with 4% paraformaldehyde. Subsequently, cells were permeabilized using 0.2% Triton X-100 and blocked using 5% BSA. The cells were subsequently incubated overnight at 4°C with an antibody against LC3 (1:500). Then, the cells were stained with secondary antibody for 1 hour and with 4',6-diamidino-2-phenylindole (DAPI) for 5 minutes. Immunofluorescence signals from LC3-positive cells were obtained by confocal laser scanning microscopy (LSM 800; Carl Zeiss, AG, Oberkochen, Germany).

2.8 | Western blotting

Total proteins from cells and tumour tissues were extracted using radioimmunoprecipitation assay lysis buffer. Protein samples (30 µg) were subjected to sodium dodecyl sulphate-polyacrylamide gel electrophoresis. After transferring proteins to a polyvinylidene fluoride (PVDF) membrane, membranes were incubated with primary antibodies against GLUT1 (Abcam, Cambridge, USA), p-PI3K (CST, Cambridge, USA), p-Akt (CST, Cambridge, USA), Akt (CST, Cambridge, USA), Beclin-1 (Proteintech, Wuhan, China), LC3-II (Proteintech, Wuhan, China), p62 (Abcam, Cambridge, USA), caspase 3 (RD), caspase 9 (Abcam, Cambridge, USA), AMPK (Abcam, Cambridge, USA), p-AMPK (Abcam, Cambridge, USA), p-ULK1 (Abcam, Cambridge, USA), p-ULK1 (Affinity), ULK1 (Abcam, Cambridge, USA), mTOR (Abcam, Cambridge, USA) or p-mTOR (Abcam, Cambridge, USA). Glyceraldehyde-3-Phosphate Dehydrogenase (GAPDH) served as a control. After washing with a TBST solution, membranes were incubated with secondary antibodies. Signal was developed using an enhanced chemiluminescence assay kit (Beyotime Biological Technology Co. Ltd., Shanghai, China) and analysed semi-quantitatively using the ChemiDoc XRS + System (Bio-Rad Laboratories, Hercules, CA, USA).

2.9 | Xenograft model

Four-week-old female athymic nude mice (BALB/c) were housed. Approximately 0.2 mL (2 × 10^7/mL) Tu212 cells transfected with GLUT1-siRNA or control siRNA were inoculated subcutaneously into the right flank of mice. Mice were randomly divided into 11 groups and treated with 10 Gy X-ray irradiation, curcumin, 3-MA or their combination (n = 6). The groups were as follows: Tu212, Tu212 + 10 Gy, Tu212 + Curcumin, Tu212 + Curcumin +10 Gy, Tu212 + GLUT1-siRNA, Tu212 + GLUT1-siRNA + 10 Gy, Tu212 + GLUT1-siRNA + Curcumin, Tu212 + GLUT1-siRNA + Curcumin +10 Gy, Tu212 + 3-MA, Tu212 + 3-MA + 10 Gy, and Tu212 + 3-MA + GLUT1-siRNA + Curcumin +10 Gy. X-ray radiation was performed on a linear accelerator (Clinac 23EX, Varian Medical Systems). The source-skin distance was 100 cm, the radiation field area was 35 cm × 35 cm, the X-ray energy was 6 MV, and the dose rate was 500 MU/min. Tumour growth rates were determined by measuring the two orthogonal dimensional diameters of each tumour three times a week. Tumour volumes were calculated according to the formula V = 4/3πxa² × b (with a = short axis, and b = long axis). When the tumours grew to a mean average volume of 100 mm³, drugs were injected intraperitoneally three times at 2-day intervals (3-MA: 40 µg/each, curcumin:2 mg/each; GLUT1-siRNA:100 µg/each). For the X-ray irradiation group,
each mouse was subjected to concurrent 10 Gy X-ray irradiation after the first drug treatment. Two weeks later, mice were euthanized under general anaesthesia using pentobarbital, and the tumours were excised. After weighing, the tumour tissues were stored at -80°C until further analysis.

### 2.10 Transmission electron microscopy

After 48 hours of treatment, the tumour tissues were fixed in 2.5% glutaraldehyde, post-fixed in 1% osmium tetroxide, and gradually dehydrated in ethanol and acetone. After embedding into epoxy resin, tumours were cut into sections and stained with uranyl acetate and lead citrate. Autophagy levels were determined by transmission electron microscopy (Thermo Fisher Scientific, Waltham, MA, USA).

### 2.11 TUNEL assay

Tumour sections were stained with the in situ Cell Death Detection Kit-POD (Roche, Shanghai, China). In brief, tissue sections were fixed using the fixation solution and incubated with the blocking buffer. The sections were incubated for 60 minutes at 37°C and in the dark with a TUNEL reaction mixture and subsequently with 100 µL termination solution at room temperature for 10 minutes. Then, they were incubated with 50 µL streptavidin-HRP conjugate, stained with 200 µL DAB solution, mounted and observed under a fluorescence microscope.

### 2.12 Statistical analyses

Data are expressed as the mean ± SEM and were analysed using the SPSS 25.0 software (IBM Corp., Armonk, NY, USA). The variance was similar between the groups that are being statistically compared. Statistical significance was determined using one-way ANOVA; *P* < .05 was considered statistically significant.

### 3 RESULTS

#### 3.1 GLUT1 expression is negatively associated with Beclin-1 and LC3-II in LC

Immunohistochemical analyses showed that GLUT1 expression that occurred in a diffuse pattern localized in the cell membrane was robustly enhanced in LC compared to paracarcinoma tissue (Figure S1A). In all, 42 of 45 LC patients (93.3%) presented with GLUT1-positive expression, while 50% (6/12) were positive in paracarcinoma samples (Table S1; *P* = 0.0015). However, Beclin-1 and LC3-II were predominantly expressed in the cytoplasm and showed low levels in tumour tissue compared to paracarcinoma in LC (Figure S1B). The positive rate of Beclin-1, LC3-II expression was 71.1% (32/45), 73.3% in LCs, respectively. The positive rate of Beclin-1, LC3-II expression was 75% (9/12), 100% in paracarcinoma samples, respectively. There was a significant negative correlation between GLUT1 expression status and Beclin-1 or LC3-II (Figure S1C). Thus, there may be a negative association between ATGs and GLUT1 status in LC.

#### 3.2 Combination of curcumin with GLUT1 siRNA enhances the radiosensitivity of Tu212 cells

Exposure to radiation robustly reduced clone formation (Figure 1A), and the combination of curcumin or GLUT1 siRNA with irradiation was expected to exert a similar effect. However, curcumin, GLUT1 siRNA and their combination following irradiation at a dose of 10 Gy enhanced the cytotoxicity on Tu212 cells compared to any single treatment. The colony-forming ability was further suppressed by the combination of irradiation, curcumin and GLUT1 siRNA; clonogenic survival was substantially reduced compared to cells exposed to any combination of two treatments (Figure 1A; Figure S2). Immunoblotting showed that treatment with GLUT1 siRNA or curcumin alone or in combination robustly decreased GLUT1 expression. Although irradiation at a dose of 10 Gy increased GLUT1 expression, the combination of GLUT1 siRNA or curcumin with irradiation at 10 Gy significantly reduced GLUT1 expression (Figure 1B,C). Additionally, another GLUT1 siRNA (siRNA2) was chosen to prove our data. The results showed that combination of curcumin with GLUT1 siRNA enhances the radiosensitivity of Tu212 cells (Figure S3). Taken together, the results of our clonogenic survival assay and immunoblot analysis indicate that low GLUT1 expression was associated with reduced clonogenic survival and stronger radiosensitivity. This suggests that the combination of curcumin with GLUT1 siRNA enhances the radiosensitivity of LC, partly through modulation of GLUT1 expression.

#### 3.3 Combination of curcumin and GLUT1 siRNA induces both apoptotic and non-apoptotic cell death

As shown in Figure 3, 10 Gy irradiation, curcumin and GLUT1 siRNA, as individual treatments, significantly increased the number of apoptotic cells compared to the control, untreated control and NC groups (Figure 2A; *P* < 0.001). Both curcumin and GLUT1 siRNA treatment after irradiation resulted in more apoptotic cells than irradiation alone (Figure 2A; *P* < 0.001). The maximum number of apoptotic cells was produced in the group treated with a combination of 10 Gy irradiation, curcumin and GLUT1 siRNA (Figure 2A). Curcumin, GLUT1 siRNA and irradiation, individually and as a combination, promoted the expression of apoptosis-related proteins, including cleaved caspase3/9, with the highest levels seen in the group treated with the combination of all three treatments (Figure
We also observed that curcumin and GLUT1 siRNA individually or in combination induced non-apoptotic cell death compared to the NC and irradiation alone groups, while irradiation did not affect this process, with or without curcumin or GLUT1 siRNA treatment (Figure 2B). Additionally, combination of curcumin and GLUT1 siRNA2 induces both apoptotic and non-apoptotic cell death (Figures S5 and S6). These observations suggest that curcumin or GLUT1siRNA treatment induces apoptotic and non-apoptotic cell death, whereas irradiation only accelerates the apoptotic process in Tu212 cells.

To verify the existence of non-apoptotic cell death, we used a caspase inhibitor, z-VAD-fmk, to block apoptosis and pyroptosis. Cell viability decreased sharply under irradiation, curcumin or GLUT1siRNA treatment compared to the NC group. The combination of irradiation with either curcumin or GLUT1 siRNA and the combination of all three treatments resulted in lower proliferative activity than a single-drug arm. The addition of z-VAD-fmk completely impeded the proliferation-inhibiting effects of irradiation and moderately restricted the effect in the groups treated with curcumin and GLUT1 siRNA alone as well as in combination. However, the addition of z-VAD-fmk, irradiation after curcumin, GLUT1 siRNA or combined treatment did not enhance their cytotoxic effects on Tu212 cells (Figure 3A). z-VAD-fmk administration may significantly limit the pro-apoptotic effects of radiotherapy, curcumin and GLUT1 siRNA treatments individually(Figure 3B,C). However, non-apoptotic cell death mediated by curcumin and GLUT1 siRNA was notably inhibited by z-VAD-fmk, while z-VAD-fmk did not affect the proportion of non-apoptotic cells in the control, NC siRNA and NC siRNA + 10 Gy groups. AMC-HN-8 cells were employed to perform clonogenic survival assay, apoptosis assay and apoptosis inhibitor assay. The results also were in keeping with the data obtained from TU212 cells (Figures S7-S9). Taken together, these findings suggest that curcumin and GLUT1siRNA may induce apoptotic and non-apoptotic cell death, whereas irradiation preferentially promotes apoptosis and has no effect on non-apoptotic cell death.

### 3.4 Curcumin and GLUT1 induce autophagy of LC cells

Immunoblotting showed that the levels of Beclin-1, LC3II/I and p62 were unchanged in cells transfected with NC siRNA or exposed to irradiation (Figure 4A; Figure S10), whereas treatment with curcumin and GLUT1 siRNA alone or in combination significantly increased Beclin-1 expression and the LC3II/I ratio, as well as reducing the level of p62 (Figure 4A; Figure S10). However, with irradiation, GLUT1 siRNA and curcumin alone and in combination induced changes in ATGs (Figure 4A; Figure S10). Immunofluorescence analysis also indicated that the accumulation of LC3 was increased in the absence of GLUT1 and in the presence of curcumin, and greater accumulation was observed in cells exposed to a combination of curcumin and GLUT1siRNA (Figure 4B). When administered along with irradiation, the accumulation of LC3 was not influenced by GLUT1 siRNA or curcumin alone or in combination. These results indicate that curcumin and GLUT1 siRNA cause autophagic events independent of irradiation in LC cells.
Non-apoptotic cell death induced by combined treatment with curcumin and GLUT1 siRNA depends on autophagy

We observed that inhibition of autophagy using 3-MA did not affect the numbers of apoptotic or non-apoptotic cells, while 3-MA enhanced irradiation cytotoxicity. 3-MA alone or in combination with irradiation had no impact on non-apoptosis, suggesting that autophagy inhibition-mediated radiosensitivity with irradiation was not associated with non-apoptotic cell death (Figure 5A). Compared to cells exposed to the combination of curcumin and GLUT1 siRNA after irradiation, the addition of 3-MA increased the apoptotic cell population, while also suppressing non-apoptotic cell death induced by the combination of curcumin and GLUT1 siRNA after irradiation(Figure 5A). In addition, Beclin-1 expression and the LC3II/I ratio were reduced, while p62 expression was upregulated by the addition of 3-MA (Figure 5C; Figure S10). Irradiation did not affect 3-MA-mediated changes in Beclin-1, LC3II/I ratio and p62 expression (Figure 5B; Figure S10). Moreover, the sharp increase in autophagy due to the combination of curcumin and GLUT1 siRNA after irradiation was robustly blocked by 3-MA (Figure 5B; Figure S11). These results indicated that the non-apoptotic cell death induced by curcumin and GLUT1 siRNA is a form of autophagy with or without irradiation (ie autophagy-associated cell death).

Curcumin and GLUT1 siRNA increase the radiosensitivity of LC by promoting autophagy in vivo

Both tumour volume and weight were remarkably reduced when mice were subjected to irradiation or the intratumoural injection of curcumin or GLUT1 siRNA, compared to the control group (Figure 6A,B). Curcumin and GLUT1 siRNA alone or in combination along with irradiation further inhibited tumour growth compared to a single-drug arm (Figure 6A,B). Although 3-MA alone did not affect tumour growth, it limited the tumour size and weight when combined with irradiation (Figure 6A,B). Combination of 3-MA, curcumin and GLUT1 siRNA after irradiation exhibited the most potent inhibitory effect on tumour growth (Figure 6A,B).
curcumin and GLUT1siRNA individually; the combination of irradiation with curcumin or GLUT1siRNA; and the combination of curcumin and GLUT1siRNA induced the expression of cleaved caspase3/9 and promoted apoptosis, particularly combination therapy (Figure 6C; Figure S12A,B). 3-MA exposure alone did not affect apoptosis; however, 3-MA after irradiation or combined curcumin and GLUT1siRNA...
after irradiation also showed stronger pro-apoptotic effects on tumour cells (Figure 6C; Figure S12A,B). Thus, inhibition of autophagy amplified by curcumin and GLUT1siRNA increased the radiosensitivity of LC in vivo, potentially by promoting apoptosis.

In addition to tumour growth inhibition, curcumin and GLUT1siRNA alone or combined with irradiation promoted autophagy by increasing Beclin-1 level and LC3II/I ratio, while reducing p62 expression. However, irradiation alone had no effect on autophagy in tumour tissues (Figure 6C,D; Figure S12B). After treatment with curcumin and GLUT1 siRNA alone or in combination with irradiation, changes in ATGs were further enhanced and the number of autophagosomes was significantly increased (Figure 6C,D; Figure S12B). Although 3-MA exposure alone or after irradiation did not alter autophagy activity (Figure 6B,D; Figure S12B), addition of 3-MA

|          | Curcumin | 10 Gy | GLUT-1 siRNA | NC siRNA |
|----------|----------|-------|--------------|----------|
| Beclin1  | +        |       | +            | -        |
| LC3 I    | +        |       | +            | -        |
| LC3 II   | +        |       | +            | -        |
| p62      | -        |       | +            | +        |
| GAPDH    | -        |       | -            | -        |

**FIGURE 4** The alteration of autophagy status of laryngeal carcinoma cells induced by GLUT-1 siRNA and Curcumin treatment. (A) Cells were treated by 10 Gy, GLUT-1 siRNA and Curcumin alone, or the two/three combination. Autophagy-associated genes including Beclin1, LC3II/I and p62 were determined by Western blotting. (B) LC3 expression and distribution as determined by immunofluorescent staining. The point accumulation of LC3-labelled cells was stained in red. Blue, 4′,6-diamidino-2-phenylindole (DAPI); Red, LC3. Data are expressed as the mean ± SEM (n = 3)
markedly inhibited curcumin/GLUT1 siRNA-induced autophagy after irradiation, producing fewer autophagic bodies (Figure 6C,D; Figure S12B). These results suggested that curcumin/GLUT1siRNA-mediated autophagy is the key determinant underlying the radiosensitivity of LC cells in vivo.

3.7 The AMPK-mTOR-ULK1 pathway is required for curcumin and GLUT1 siRNA-mediated autophagy

Next, we determined that the PI3K/Akt pathway in Tu212 cells and tumour tissues was unaffected by any of the applied treatments, alone or in combination (Figure 7A,B; Figure S13A,B). Although, as an inhibitor of class III PI3K, 3-MA robustly inhibited the PI3K/Akt pathway, the combination of 3-MA with irradiation or the combination of 3-MA after 10 Gy irradiation, curcumin and GLUT1siRNA did not affect this pathway (Figure 7A,B; Figure S13A,B). Thus, autophagy induced by curcumin and GLUT1siRNA is independent of the PI3K/Akt pathway.

Autophagy signalling, including increases in Beclin-1 expression and the LC3II/I ratio and a decrease in p62 expression, was activated by curcumin and GLUT1siRNA alone or in combination compared to control cells; this occurred along increases in phosphorylated AMPK and ULK1$^{ser555}$ and decreases in phosphorylated mTOR and ULK1$^{ser757}$ (Figure 7C). Thus, the AMPK-mTOR-ULK1 pathway was activated after treatment with curcumin and GLUT1siRNA with activation of autophagy (Figure 7C; Figure S13).

Following ablation of AMPK, we found that the AMPK-mTOR-ULK1 signalling axis was depressed by inhibition of p-AMPK and ULK1$^{ser555}$ and induction of phosphorylated ULK1$^{ser757}$ and mTOR; however, the levels of Beclin-1, LC3II/I and p62 expression were unaffected (Figure 7C; Figure S13C). However, AMPK siRNA transfection significantly suppressed curcumin and GLUT1 siRNA-induced autophagy by limiting the AMPK-mTOR-ULK1 pathway, thereby decreasing the activity of p-Beclin-1 (Figure 7C; Figure S13C).

In contrast, irradiation alone did not alter the autophagy activity or the AMPK-mTOR-ULK1 pathway compared to the control groups transfected with or without AMPK siRNA (Figure 7C; Figure S13C). There were also no effects of irradiation on the activation of AMPK-mTOR-ULK1 mediated by curcumin and GLUT1siRNA and subsequent autophagy events in the presence of NC siRNA or AMPK siRNA (Figure 7C; Figure S13C). These results indicate that autophagy was evoked by the AMPK-mTOR-ULK1 signalling pathway after curcumin and GLUT1siRNA alone or in combination.

4 DISCUSSION

The present study demonstrated an association between GLUT1 expression and the T stage of LC. GLUT1 expression was significantly increased after irradiation compared to control cells. These results are similar to those of our previous studies$^{3,5,28}$ and suggest a correlation between elevated GLUT1 expression and radiosensitivity of
**FIGURE 6** Effect of GLUT-1 siRNA and Curcumin on radiosensitivity of laryngeal carcinoma in vivo. (A) Innoculated cells were prior treated with siRNA. Mice bearing these cells were then treated with irradiation or curcumin. Tumour size and mean weight were imaged and calculated in the different groups. (B) Tumour weight in each group. (C) The expression of Beclin1, LC3II/I, p62 and caspase 3/9 in tumour tissues was assessed by Western blotting. (D) The number of autophagosomes in tumour tissues was assessed by transmission electron microscopy. Arrows indicate autophagic bodies; n = 6 per group; *P < 0.05; **P < 0.01. Data are expressed as the mean ± SEM (n = 3)
|          | 3-MA |          | GLUT-1 siRNA | Curcumin |          | 10 Gy | GLUT-1 siRNA | Curcumin |          |
|----------|------|----------|--------------|----------|----------|-------|--------------|----------|----------|
| p-Pi3K   | -    | -        | +            | -        | -        | +     | +            | -        | -        |
| Pi3K     | -    | -        | +            | -        | -        | +     | +            | -        | -        |
| p-Akt    | -    | -        | +            | -        | -        | +     | +            | -        | -        |
| Akt      | -    | -        | +            | -        | -        | +     | +            | -        | -        |
| GAPDH    | -    | -        | +            | -        | -        | +     | +            | -        | -        |

**Second Column**

|          | 3-MA |          | GLUT-1 siRNA | Curcumin |          | 10 Gy | GLUT-1 siRNA | Curcumin |          |
|----------|------|----------|--------------|----------|----------|-------|--------------|----------|----------|
| p-Pi3K   | -    | -        | +            | -        | -        | +     | +            | -        | -        |
| Pi3K     | -    | -        | +            | -        | -        | +     | +            | -        | -        |
| p-Akt    | -    | -        | +            | -        | -        | +     | +            | -        | -        |
| Akt      | -    | -        | +            | -        | -        | +     | +            | -        | -        |
| GAPDH    | -    | -        | +            | -        | -        | +     | +            | -        | -        |

**Third Column**

|          | 10 Gy |          | GLUT-1 siRNA | Curcumin |          | GLUT-1 siRNA | Curcumin |          |
|----------|-------|----------|--------------|----------|----------|--------------|----------|----------|
| p-AMPK   | -     | +        | +            | -        | -        | +            | -        | -        |
| AMPK     | -     | +        | +            | -        | -        | +            | -        | -        |
| p-ULK1 (ser555) | - | + | + | - | + | + | - | + | + |
| p-ULK1 (ser757) | - | + | + | - | + | + | - | + | + |
| ULK1     | -     | +        | +            | -        | -        | +            | -        | -        |
| p-mTOR   | -     | +        | +            | -        | -        | +            | -        | -        |
| mTOR     | -     | +        | +            | -        | -        | +            | -        | -        |
| p-Becnin1| -     | +        | +            | -        | -        | +            | -        | -        |
| Beclin1  | -     | +        | +            | -        | -        | +            | -        | -        |
| LC3 I    | -     | +        | +            | -        | -        | +            | -        | -        |
| LC3 II   | -     | +        | +            | -        | -        | +            | -        | -        |
| p62      | -     | +        | +            | -        | -        | +            | -        | -        |
| GAPDH    | -     | +        | +            | -        | -        | +            | -        | -        |
As a well-known antioxidant, curcumin has both radiosensitizing and radioprotective properties. We first observed that the combination of GLUT1 siRNA and curcumin significantly increased the radiosensitivity of LC in vitro and in vivo, possibly by modulating GLUT1 expression.

As a type of programmed cell death, a higher proportion of apoptotic cells plays an important role in radiosensitivity. Curcumin, GLUT1 siRNA, and the curcumin/GLUT1 siRNA combination may increase the expression of apoptosis-related proteins and the proportion of apoptotic cells. Our results are similar to previous studies, showing that increased apoptosis is associated with radiosensitivity. We found that curcumin, GLUT1 siRNA, and particularly their combination markedly increased the degree of non-apoptotic cell death after irradiation. Moreover, we found that non-apoptotic cell death was associated with the radiosensitivity of LC. Treatment with the broad-spectrum caspase inhibitor z-VAD-fmk, curcumin, or GLUT1 siRNA and their combination continued to reduce the activity of Tu212 cells after irradiation, indicating that curcumin and GLUT1 siRNA-induced cell death was not dependent on caspase. That is, this death process was different from programmed apoptosis and pyroptosis. Meanwhile, z-VAD-fmk significantly reduced the apoptosis rate after irradiation. As an inhibitor of caspase, z-VAD-fmk blocked the progression of apoptosis and pyroptosis. However, despite the ability of z-VAD-fmk to reduce apoptosis, it had only a slight inhibitory effect on non-apoptotic death induced by curcumin, GLUT1 siRNA, or their combination. As to z-VAD-fmk-induced the down-regulation of non-apoptotic cell death in curcumin or GLUT1 siRNA-exposed cells, we hold the opinion that curcumin or GLUT1 siRNA not only induced apoptosis, but also induced pyroptosis, another type of non-apoptotic cell death. Flexicaulin and vitamin K2 inhibit the proliferation of colorectal carcinoma and breast cancer cells through non-apoptotic cell death. These results suggest that curcumin and GLUT1 siRNA induce apoptosis and non-apoptotic cell death, while irradiation mainly induced apoptosis of LC cells.

As a type of non-apoptotic cell death, autophagy-associated cell death has a potent anti-cancer effect. Here, 3-MA significantly reduced non-apoptotic cell death induced by curcumin/GLUT1 siRNA after irradiation. Meanwhile, it also changed the expression of ATGs. However, it did not change the apoptotic events in LCs. These findings suggest that non-apoptotic cell death induced by curcumin combined with GLUT1 siRNA is dependent on autophagy.

The role of autophagy in radioresistance is paradoxical. Autophagy is a complicated process that can be activated by multiple signalling pathways, mainly through energy signals via AMPK. AMPK activation can phosphorylate Ser555 of ULK1 and inhibit mTOR signalling, effectively blocking the phosphorylation of ULK1 at Ser757,21,22 which induces the activation of ULK1, and subsequently, Beclin-1 and Vps34, resulting in up-regulation of autophagy intensity. Thus, the AMPK-mTOR-ULK1 pathway plays an
important role in autophagy.\textsuperscript{23} AMPK activation also significantly up-regulates the GLUT1 content in thyroid PCCL3 cells.\textsuperscript{24} Curcumin promotes autophagy by activating the AMPK pathway in lung adenocarcinoma cells.\textsuperscript{25} However, the crosstalk among GLUT1, curcumin and the AMPK pathway in LC remains vague. In the present study, both GLUT1siRNA and curcumin alone or in combination increased AMPK activity, thereby inhibiting mTOR-dependent ULK1 inactivation, leading to the activation of autophagy. AMPK depletion robustly limited their effects in promoting the mTOR-ULK1-Beclin-1 pathway and autophagic events. Therefore, GLUT1siRNA and curcumin-mediated autophagy depends on the AMPK-mTOR-ULK1-Beclin-1 signalling cascade. However, irradiation had no effect on this pathway.

In conclusion, treatment with GLUT1siRNA alone or in combination with curcumin resulted in profound improvement of the radiosensitivity of LC cells after irradiation. Curcumin and GULT1siRNA alone or in combination not only promoted apoptosis of LC cells, but also induced autophagy-associated cell death through activation of AMPK/mTOR/ULK1 signalling-mediated autophagy with or without irradiation treatment (Figure S14). This sensitization may have been due to increases in apoptotic and non-apoptotic cell death. In addition, non-apoptotic cell death is dependent on autophagy status, which is induced by activation of the AMPK-ULK1-mTOR-Beclin-1 pathway.

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CONFLICT OF INTEREST
The authors declare no conflict of interest.

AUTHOR CONTRIBUTION
Li-Bo Dai: Conceptualization (supporting); Data curation (lead); Funding acquisition (lead); Investigation (equal); Methodology (equal); Resources (equal); Writing-original draft (lead). Jiang-Tao Zhong: Data curation (equal); Investigation (equal); Methodology (equal); Resources (equal); Software (equal). Li-Fang Shen: Data curation (equal); Investigation (equal); Methodology (equal); Resources (equal); Software (equal). Shui-Hong Zhou: Conceptualization (lead); Funding acquisition (lead); Software (lead); Supervision (lead); Writing-review & editing (lead). Zhong-Jie Lu: Data curation (equal); Formal analysis (equal); Methodology (equal); Resources (equal); Software (equal). Yang-Yang Bao: Data curation (equal); Methodology (equal); Resources (equal). Jun Fan: Conceptualization (equal); Data curation (equal); Investigation (equal); Methodology (equal); Resources (equal); Validation (equal); Visualization (equal).

DATA AVAILABILITY STATEMENT
The data used to support the findings of this study are available from the corresponding author upon request.

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