Mechanism of targeting the mTOR pathway to regulate ferroptosis in NSCLC with different EGFR mutations

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Abstract. Patients with non-small cell lung cancer (NSCLC) harboring epidermal growth factor receptor (EGFR)-activating mutations can be treated with EGFR-tyrosine kinase inhibitors (TKIs). Although EGFR-TKI-targeted drugs bring survival promotion in patients with EGFR mutations, drug resistance is inevitable, so it is urgent to explore new treatments to overcome drug resistance. In addition, wild-type EGFR lacks targeted drugs, and new targeted therapies need to be explored. Ferroptosis is a key research direction for overcoming drug resistance. However, the role and mechanism of regulating ferroptosis in different EGFR-mutant NSCLC types remains unclear. In the present study, H1975 (EGFR T790M/L858R mutant), A549 (EGFR wild-type) and H3255 (EGFR L858R mutant) NSCLC cell lines were used. The expression of ferroptosis markers in these cell lines was detected using western blotting and reverse transcription-quantitative PCR. Cell viability was determined using the MTT assay and reactive oxygen species (ROS) levels were measured using flow cytometry. The results showed that, compared with EGFR wild-type/sensitive mutant cells, EGFR-resistant mutant cells were more sensitive to the ferroptosis inducer, erastin. Furthermore, the mammalian target of rapamycin (mTOR) inhibitor, everolimus (RAD001), induced cell death in all three cell lines in a dose-dependent manner. The ferroptosis inhibitor, ferrostatin-1, could reverse cell death in EGFR-resistant mutant and EGFR wild-type cells induced by RAD001, but could not reverse cell death in EGFR-sensitive mutant cells. Compared with EGFR wild-type/sensitive mutant cells, EGFR-resistant mutant cells were more sensitive to RAD001 combined with erastin. In addition, a high-dose of RAD001 reduced the expression levels of ferritin heavy-chain polypeptide 1 (FTH1), glutathione peroxidase 4 (GPX4) and ferroportin and significantly increased ROS and malondialdehyde (MDA) levels in EGFR-resistant mutant and EGFR wild-type cells. In the present study, GPX4 inhibitor only or combined with RAD001 inhibited the AKT/mTOR pathway in EGFR-resistant mutant cells. Therefore, the results of the present study suggested that inhibition of the mTOR pathway may downregulate the expression of ferroptosis-related proteins in EGFR-resistant and EGFR wild-type NSCLC cells, increase the ROS and MDA levels and ultimately induce ferroptosis.

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Introduction

Non-small cell lung cancer (NSCLC) is one of the most common malignant tumors, and the 5-year survival rate following diagnosis is <15% (1). The epidermal growth factor receptor (EGFR) is associated with the growth and progression of cancer and an activation mutation in the tyrosine kinase domain (exon 21 L858R point mutation or exon-19 deletion) has been identified as an oncogenic driver of NSCLC (2). A study has shown that 30-40% of Asian patients with NSCLC harbor EGFR mutations at the time of diagnosis (3). Although it was demonstrated that the overall response and disease control rates in gefitinib- and erlotinib-treated groups (via targeting of EGFR) were 76.9 vs. 74.4% and 90.1 vs. 86.8%, respectively (4), drug resistance is inevitable, and ~36% of patients with NSCLC have EGFR T790M/L858R resistance mutations (5). Although a second-generation EGFR-tyrosine kinase inhibitor (TKI), afatinib, showed high activity in an EGFR T790M/L858R driven xenotransplantation model, it did not increase the objective response rate in patients with NSCLC with drug-resistant mutations (6). Third-generation EGFR-TKIs, such as CO1686 and AZD9291, are the only effective drugs for treating NSCLC with the EGFR L858R/T790M mutation (7,8). However, because the drug resistance molecular subtypes emerged (such as acquisition of

the EGFR C797S mutation or loss of the T790M mutation), drug resistance is still inevitable after a period of treatment (9). Additionally, EGFR-TKIs may not be superior to chemotherapy in patients with NSCLC with wild-type EGFR (10). A study demonstrated that chemotherapy showed a superiority in terms of PFS (HR, 1.84; 95% CI, 1.35-2.52) and ORR (16.8 vs. 7.2%; relative risk, 1.11; 95% CI, 1.02-1.21) compared with EGFR-TKIs in patients with NSCLC with wild-type EGFR (11).Chemotherapy remains the primary treatment strategy for patients with wild-type EGFR NSCLC (10). Thus, identifying additional treatment strategies for EGFR-resistant mutant and EGFR wild-type NSCLC is important.

Ferroptosis is a newly discovered type of programmed cell death that differs from apoptosis, necrosis and autophagy. Ferroptosis is characterized by iron-dependent cell death, which progresses with the accumulation of lipid peroxide and the generation of reactive oxygen species (ROS) (12). During lipid peroxidation, the activity of glutathione peroxidase 4 (GPX4) decreases, and therefore lipid oxides cannot be metabolized through the glutathione reductase reaction catalyzed by GPX4 (13,14). The redox-active iron form (Fe^{2+}) oxidizes lipids in a manner similar to that of the Fenton reaction to produce large amounts of ROS, causing an increase in malondialdehyde (MDA), which promotes ferroptosis (15). Ferritin is a major intracellular iron storage protein complex that includes ferritin light-chain polypeptide 1 and ferritin heavy-chain polypeptide 1 (FTH1) (16), and increased ferritin expression limits ferroptosis (17). In addition, iron levels are actively regulated in cells through transferrin, which transports iron into the cells, and ferroportin, which exports iron from the cells (18). The degradation of iron storage proteins and changes in iron transporters can increase iron-mediated ROS production, eventually leading to ferroptosis (19). A study has shown that inducing ferroptosis can improve therapeutic effects in gefitinib-resistant lung cancer (20). In addition, another report has shown that inducing ferroptosis enhances the cytotoxicity of erlotinib in NSCLC cells, thereby overcoming erlotinib resistance (21), suggesting that inducing ferroptosis may be an effective treatment for EGFR-TKI-resistant NSCLC.

The phosphatidylinositol 3-kinase (PI3K)/AKT signaling pathway is also associated with tumor development (22). Mammalian target of rapamycin (mTOR), an important serine/threonine protein kinase downstream of PI3K/AKT, regulates the proliferation, survival, invasion and metastasis of tumor cells by activating ribosomal kinases (23). mTOR inhibitors (notably RAD001, an oral rapamycin derivative) have been approved by the US Food and Drug Administration (FDA) for wider use in antitumor clinical treatment, providing more treatment options for patients with pancreatic neuroendocrine tumors (PNETs), estrogen receptor$^+$ + HER2$^-$ breast cancer and other solid tumors (24). A study has shown that RAD001 promotes the death of T790M$^+$ NSCLC cells (25), suggesting that RAD001 is a potential treatment strategy for EGFR-TKI resistant tumors. In addition, inhibition of mTOR overcomes lapatinib resistance by inducing ferroptosis in NSCLC (26), suggesting that the regulation of mTOR may promote ferroptosis and overcome EGFR-TKI resistance. Other studies have shown that the induction of ferroptosis in wild-type EGFR NSCLC enhances the therapeutic effect of cisplatin (27) and overcomes cell resistance to gefitinib (28). A previous report also demonstrated that dual inhibition of the EGFR/mTOR pathway had a significant antitumor effect on wild-type EGFR NSCLC (29). However, whether mTOR inhibition enhances the antitumor effect in EGFR-TKI-resistant mutants and wild-type EGFR NSCLC by inducing ferroptosis and the related mechanisms remain unknown.

The present study investigated the effect and mechanism of targeting the mTOR pathway to regulate ferroptosis in NSCLC with EGFR wild-type, EGFR sensitive mutant cells and EGFR-resistant mutant cells through MTT assay, western blotting, reverse transcription-quantitative PCR (RT-qPCR), ROS assay and MDA assay.

Materials and methods

Cell culture. Human NSCLC (H1975, A549 and H3255) cell lines were purchased from The Cell Bank of Type Culture Collection of The Chinese Academy of Sciences and cultured in RPMI 1640 medium supplemented with 10% fetal bovine serum and 1% penicillin/streptomycin (all from Gibco; Thermo Fisher Scientific, Inc.). Cells were incubated at 37˚C in a 5% CO_2 incubator and routinely passaged every 3 days.

MTT assay. H1975, A549 and H3255 cells were seeded into 96-well plates with $4x10^3$ cells/well and treated with various concentrations of RAD001 (0, 0.25, 0.5, 1, 2, 4, 8 and 16 μM) or ferroptosis regulators, erastin (0, 0.25, 0.5, 1, 2, 4, 8 and 16 μM), ferrostatin-1 (0, 0.125, 0.25, 0.5, 1, 2, 4 and 8 μM) and RSL3 (0, 0.25, 0.5, 1, 2, 4, 8 and 16 μM). After 24 h, these cells were incubated with MTT reagent (20 μl/well) at 37˚C for 4 h. Then the supernatant was replaced with 150 μl/well DMSO. This mixture was shaken for 10 min and the absorbance at 490 nm was measured using a CLARIOstarPlus microplate reader (BMG Labtech GmbH).

Western blotting. The H1975, A549 and H3255 cells with different doses of RAD001 or RSL3 treatment were lysed using RIPA lysis buffer containing the protease and phosphatase inhibitors (Beyotime Institute of Biotechnology). The supernatant was centrifuged at 15,000 x g at 4˚C for 15 min and a BCA protein assay kit (Takara Bio, Inc.) was used to determine the concentration of proteins in the samples. The extracted protein (50 μg/lane) was mixed with 5X sodium dodecyl sulfate (SDS) loading buffer (BioTeke Corporation), which was loaded in a 10% gel (Beyotime Institute of Biotechnology) and the proteins were separated using SDS-polyacrylamide gel electrophoresis, transferred to a polyvinylidene fluoride membrane and the membrane was blocked in 5% non-fat milk at 22-24˚C for 1.5 h. The membranes were then incubated overnight at 4˚C with different primary antibodies (listed below). After washing with TBS-1% Tween 20 for three times, the membranes were incubated with the corresponding secondary antibodies for 1 h at 22-24˚C (listed below). The proteins were visualized using an enhanced chemiluminescence kit (cat. no. P0018AS; Beyotime Institute of Biotechnology) and analyzed with Quantity One software (version 4.6.9; Bio-Rad Laboratories, Inc.). Primary antibodies against Caspase 3/Cleaved-Caspase 3 (1:1,000; cat. no. 9662; Cell Signaling Technology, Inc.), FTH1 (1:1,000; cat. no. 3998; Cell Signaling Technology, Inc.), transferrin (1:1,000; cat. no. ab109503; Abcam), transferrin

Table I. Primer sequences used in reverse transcription-quantitative PCR.

Gene name	Sense primer	Antisense primer
xCT	5'-TGGAACGAGGAGGTGGAGAA-3'	5'-TGGTGGACACAACAGGCTTT-3'
FTH1	5'-CCAGAACTACCACCAGGACTC-3'	5'-GAAGATTCGGCCACCTCGTT-3'
GPX4	5'-GCTGGACGAGGGGAGGAG-3'	5'-GGAAAACTCGTGCATGGAGC-3'
Ferroportin	5'-GAAAATCCCTGGGCCCCTTT-3'	5'-CTCTCGCTGAGGTGCTTGTT-3'
Transferrin	5'-CAGAAGCGAGTCCGACTGTG-3'	5'-CGCTTTTCATATGGTCGCGG-3'
Transferrin receptor	5'-GGACGCGCTAGTGTTCTTCT-3'	5'-CATCTACTTGCCGAGCCAGG-3'
GAPDH	5'-ACCACAGTCCATGCCATCAC-3'	5'-TCCACCACCCTGTTGCTGTA-3'

xCT, cysteine-glutamate antiporter; FTH1, ferritin heavy-chain polypeptide 1; GPX4, glutathione peroxidase 4; GAPDH, glyceraldehyde-3-phosphate dehydrogenase.

receptor, CD71 (1:1,000; cat. no. ab84036; Abcam), ferroportin (1:1,000; cat. no. ab235166; Abcam), GPX4 (1:1,000; cat. no. ab125066; Abcam), phosphorylated (p-)mTOR (1:500; cat. no. 2971; Cell Signaling Technology, Inc.), mTOR (1:500; cat. no. 2972; Cell Signaling Technology, Inc.), AKT (1:1,000; cat. no. 9272; Cell Signaling Technology, Inc.) and anti-glyceraldehyde-3-phosphate dehydrogenase (GAPDH; 1:1,000; cat. no. ab9485; Abcam), and secondary antibodies against goat anti-rabbit IgG (1:1,000; cat. no. WLA023; Wanleibio Co., Ltd.) and goat anti-mouse IgG (1:1,000; cat. no. WLA024; Wanleibio Co., Ltd.) were used.

Reverse transcription-quantitative PCR (RT-qPCR). H1975, A549 and H3255 cells were seeded into a 6-well microplate at a density of $5x10^5$ cells/well. Total RNA from the cells in each group was extracted using TRIzol reagent (Thermo Fisher Scientific, Inc.) according to the manufacturer's instructions. After RNA quality was tested, cDNA was synthesized according to the manufacturer's protocols using a PrimeScript II Reverse Transcriptase kit (Takara Bio, Inc.). qPCR was then performed using a LightCycler 480 system (Roche Diagnostics) and a SYBR Green Master Kit (Takara Bio, Inc.). The reaction conditions were as follows: 95˚C for 3 min, followed by 40 cycles of 95˚C for 30 sec, 57˚C for 30 sec and 72˚C for 30 sec. GAPDH mRNA was used to normalize relative mRNA levels. The relative quantification of the PCR product was calculated, using the $2^{-\Delta\Delta Cq}$ method (30). Primer sequences are listed in Table I.

ROS assay. H1975, A549 and H3255 cells with different doses of RAD001 treatment were seeded into a 6-well microplate at a density of $3x10^5$ cells/well. After 24 h of treatment with RAD001 (0, 0.5, 1, 8 μM), the cells were collected, 10 μmol/l DCFH-DA was added and the reaction mixture and was incubated at 37˚C for 20 min. The cells were then washed three times with serum-free cell culture medium of RPMI 1640 and the rate of ROS was detected using a flow cytometer (FACSCanto; BD Biosciences). The results were analysed using BD FACSDiva software (BD Biosciences).

MDA assay. H1975, A549 and H3255 cells with different doses of RAD001 treatment were seeded into a 6-well microplate at a density of $3x10^5$ cells/well. After 24 h of treatment with RAD001 (0, 0.5, 1, 8 μM), the cells were collected and the levels of intracellular MDA were measured using a Lipid Peroxidation MDA Assay Kit (cat. no. S0131S; Beyotime Institute of Biotechnology) according to the manufacturer's instructions.

Statistical analysis. All data were analyzed using SPSS 17.0 (SPSS, Inc.) and GraphPad Prism (version 6.01; Dotmatics) software. Statistical data are presented as the mean ± standard deviation. One-way analysis of variance was used to compare the means among three or more groups. Dunnett's test was used for all comparisons against a single control. P-values were based on two-tailed statistical analyses. $P<0.05$ was considered to indicate a statistically significant difference.

Results

Different EGFR genotypes of NSCLC have a different sensitivity to ferroptosis inducers. To explore the sensitivity of wild-type EGFR and EGFR-mutated NSCLC cells to ferroptosis, the expression levels of ferroptosis markers in H1975, A549 and H3255 cells were first detected. When compared with H3255 (EGFR L858R mutation) cells, the expression levels of FTH1, GPX4 and the transferrin receptor were higher and the level of transferrin was lower in H1975 (EGFR T790M/L858R) and A549 (EGFR wild type) cells (Fig. 1A). However, compared with H3255, the expression levels of xCT and ferroportin were notably higher in A549, but not H1975. When the A549, H1975 and H3255 cells were treated with the ferroptosis inducer, erastin, cell death was induced in a dose-dependent manner. Compared with H3255/A549 cells, H1975 cells were more sensitive to erastin (IC_{50}: 15.66 μM vs. 6.989 μM vs. 13.83 μM, Fig. 1B). These results suggest that the NSCLC cell lines with wild-type EGFR and EGFR T790M/L858R mutations were more prone to erastin-induced ferroptosis.

Inhibition of mTOR may induce ferroptosis in EGFR-TKI resistant mutant and EGFR wild-type NSCLC. To explore whether RAD001 regulates ferroptosis in NSCLC with different EGFR mutations, H3255 (EGFR-TKI-sensitive), H1975 (EGFR-TKI-resistant) and A549 (EGFR wild-type) cells were treated with RAD001 at different concentrations. It was found that as the drug concentration increased, cell viability gradually decreased in a dose-dependent manner. Compared

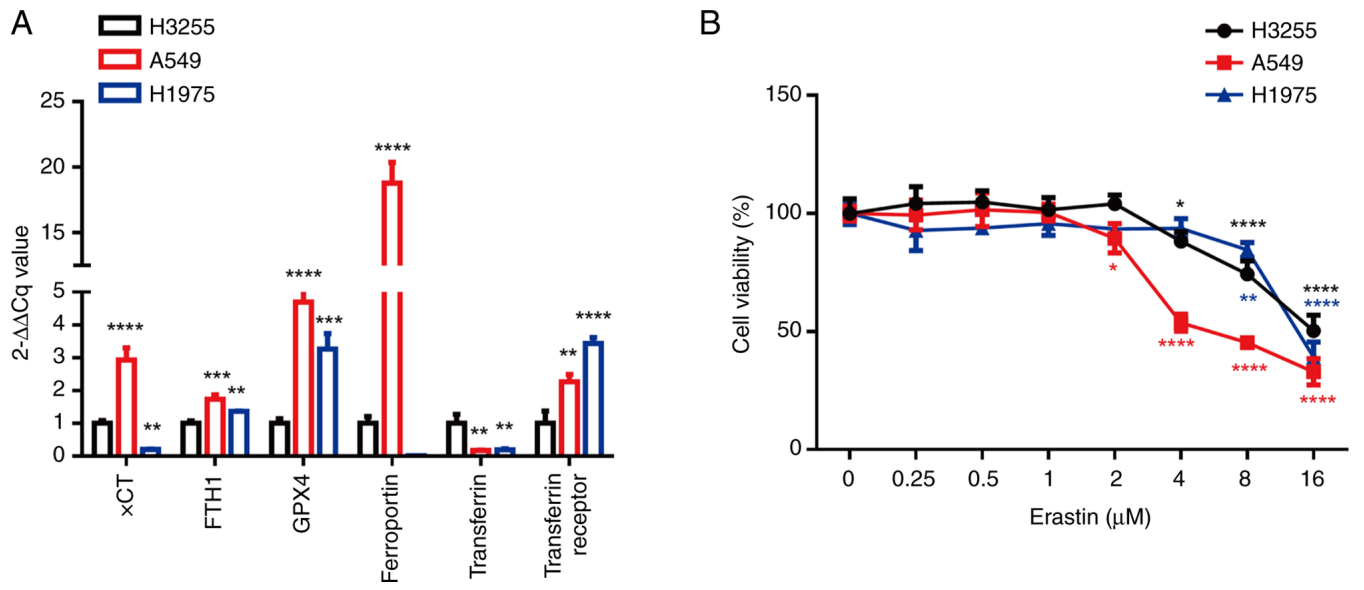

Figure 1. Different epidermal growth factor receptor mutant non-small cell lung cancer cell lines have a different sensitivity to a ferroptosis inducer. (A) mRNA expression levels of ferroptosis markers (xCT, FTH1, GPX4, ferroportin, transferrin and the transferrin receptor) in A549, H1975 and H3255 cells were analyzed using the $2^{-\Delta\Delta Cq}$ method. **P<0.01, ***P<0.001 and ****P<0.0001 vs. H3255 cells using Dunnett's test. (B) A549, H1975 and H3255 cells were treated with different concentrations of erastin (0, 0.25, 0.5, 1, 2, 4 and 8 μM) and the MTT assay was used to detect the cell viability after 24 h. *P<0.05, **P<0.01, and ****P<0.0001 vs. the control group (0 μM of Erastin) using Dunnett's test. xCT, cysteine-glutamate antiporter; FTH1, ferritin heavy-chain polypeptide 1; GPX4, glutathione peroxidase 4.

with H3255 cells, H1975 and A549 cells were more sensitive to RAD001 (IC_{50}: 4.474 mM vs. 8.869 μM vs. 50.79 μM; Fig. 2A). Since ferroptosis is a form of apoptosis independent cell death, it was first tested whether different concentrations of RAD001 induce cell apoptosis, and then explored which concentration of RAD001 might induce ferroptosis. The three cell types were treated with low- and high-dose RAD001. Only 0.5 and 1 μM RAD001 treatment in A549 cells induced an increase in cleaved-Caspase3/Caspase3 (Fig. 2B-D). The three cell lines were then treated with RAD001, ferrostatin-1 (Fer-1) and RAD001 + Fer-1, respectively. The results showed that compared with RAD001 (1 μM) control, Fer-1 reduced the cell death induced by RAD001 (1 μM) in H1975 and A549 cells obviously. The difference was statistically significant. However, Fer-1 did not reverse cell death in H3255 cells (Fig. 2E-G).

mTOR inhibitor combined with an ferroptosis inducer promotes ferroptosis in EGFR resistant mutant and wild type EGFR NSCLC cells. The H3255, A549 and H1975 cells were treated with RAD001, erastin (ferroptosis inducer) and RAD001 + erastin, respectively. After 24 h, the results showed that erastin induced H1975, A549 and H3255 cell death in a dose-dependent manner. In addition, compared with H3255/A549 cells, H1975 cells were more sensitive to RAD001 combined with erastin. The IC_{50}s of RAD001 (0.5 μM) + erastin were 15.15, 15.35 and 4.106 μM in H3255, A549 and H1975 cells, respectively. The IC_{50}s of RAD001 (1 μM) + erastin were 15.87, 15.46 and 1.653 μM in H3255, A549 and H1975 cells, respectively (Fig. 3A-C).

Inhibiting mTOR promotes ferroptosis in EGFR resistant mutant and wild-type EGFR NSCLC cells by inducing lipid peroxidation. H3255, A549 and H1975 cells were treated with low or high concentrations of RAD001 (Fig. 4A-C). The results showed that 0.5, M and 8 μM of RAD001 inhibited mTOR activity by 56, 43 and 40% in A549 cells, respectively. In addition, 0.5, 1 and 8 μM of RAD001 inhibited mTOR activity by 5, 14 and 22% in H1975 cells, respectively. However, these concentrations of RAD001 did not inhibit mTOR activity in H3255 cells. It was found that 8 μM RAD001 significantly decreased the expression of GPX4, FTH1 and ferroportin by 43, 31 and 22%, respectively, in A549 cells and by 41, 29 and 26% in H1975 cells, respectively. However, RAD001 did not markedly alter GPX4, FTH1 or ferroportin expression in H3255 cells. Following treatment with different concentrations of RAD001 in A549, H3255 and H1975 cells for 4 h, high concentrations (8 μM) of RAD001 significantly induced the accumulation of ROS by 52, 33 and 55% in these three types of cells, respectively (Fig. 4D). Moreover, 8 μM RAD001 increased the level of MDA by 23% in A549 cells and by 31% in H1975 cells, respectively. However, 8 μM of RAD001 did not significantly alter MDA in H3255 cells (Fig. 4E). These results suggested that RAD001 induced lipid peroxidation in EGFR-resistant mutant and wild-type EGFR NSCLC cells by inhibiting mTOR, leading to ferroptosis.

Inhibition of GPX4 inhibits AKT/mTOR and promotes ferroptosis in EGFR resistant mutant NSCLC cells. To clarify the regulatory relationship between mTOR and GPX4, H3255, A549 and H1975 cells were treated with the GPX4 inhibitor, RSL3. The results showed that RSL3 notably inhibited cell viability in a dose-dependent manner. Furthermore, compared with A549 and H1975 cells, H3255 cells were the most sensitive to RSL3 (Fig. 5A). H1975, A549 and H3255 cells were also treated with different concentrations of RAD001 in combination with RSL3. Subsequent, western blotting showed that high-dose RAD001 markedly inhibited the AKT/mTOR pathway in H1975 and A549 cells compared with H3255 cells. High-dose RSL3 inhibited

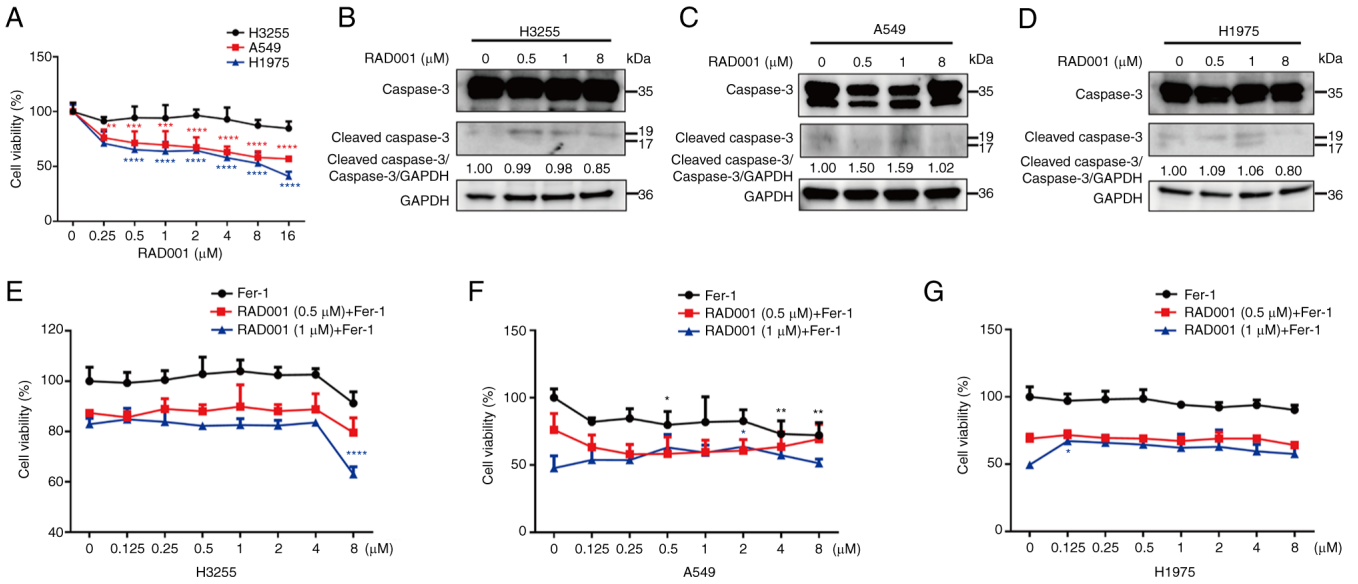

Figure 2. RAD001 may regulate ferroptosis in EGFR T790M/L858R and wild type EGFR non-small cell lung cancer. (A) H3255, A549 and H1975 cells were treated with different concentrations of RAD001 (0, 0.25, 0.5, 1, 2, 4, 8 and 16 μM). After 24 h, cell viability was detected using MTT. **P<0.01, ***P<0.001 and ****P<0.0001 vs. the control group (0 μM of RAD001) using Dunnett's test. (B) H3255, (C) A549 and (D) H1975 cells were treated with different concentrations of RAD001 (0, 0.5, 1 and 8 μM) for 24 h and cleaved-Caspase3/Caspase3 was detected using western blotting. GAPDH was used as an internal standard. (E) H3255, (F) A549 and (G) H1975 cells were treated with different concentrations of RAD001 (0, 0.5 and 1 μM) combined with Fer-1 (0, 0.125, 0.25, 0.5, 1, 2, 4 and 8 μM). After 24 h, the cell viability was detected using MTT. *P<0.05, **P<0.01, and ****P<0.0001 vs. the control group (0 μM of Fer-1) using Dunnett's test. EGFR, epidermal growth factor receptor; GAPDH, glyceraldehyde-3-phosphate dehydrogenase; Fer-1, ferrostatin-1.

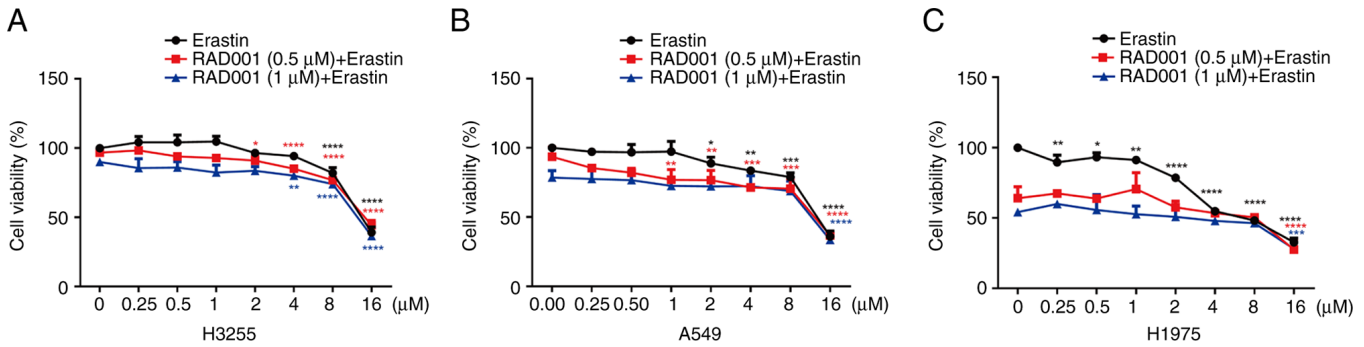

Figure 3. RAD001 combined with erastin promotes ferroptosis in EGFR T790M/L858R and wild-type EGFR non-small cell lung cancer. (A) H3255, (B) A549 and (C) H1975 cells were treated with different concentrations of RAD001 (0, 0.5 and 1 μM) combined with erastin (0, 0.25, 0.5, 1, 2, 4, 8 and 16 μM). After 24 h, the cell viability was detected using MTT. *P<0.05, **P<0.01, ***P<0.001 and ****P<0.0001 vs. the control group (0 μM of erastin) using Dunnett's test. EGFR, epidermal growth factor receptor.

AKT/mTOR pathway notably in H1975 cells and the combination of RAD001 and RSL3 further enhanced the inhibitory effect on the AKT/mTOR pathway in this type of cells; however, this effect was not observed in A549 cells, possibly due to the low mTOR expression in A549 cells (Fig. 5B-D).

Discussion

TKIs have greatly changed the clinical prospects of patients with NSCLC with EGFR activation mutations (31). Although disease control is prolonged and the tumor response rate is high, all patients eventually progress after EGFR-TKI treatment (32). Therefore, there is an urgent need to develop novel treatment strategies for these patients. In addition, if there are no co-mutations in driving genes such as anaplastic lymphoma kinase and ROS1, it is difficult for patients with NSCLC with wild-type EGFR to benefit from targeted therapy. In the present study, compared with H3255 cells harboring the EGFR L858R mutation, H1975 cells with the EGFR T790M/L858R mutation and A549 cells with the EGFR wild-type mutation were more sensitive to the ferroptosis inducer, erastin, suggesting that targeted ferroptosis may be a potential treatment against EGFR T790M/L858R resistant mutant and wild-type NSCLC. In addition, A549 cells harbor the KRAS G12S mutation. KRAS mutation is a common carcinogenic driver mutation that accounts for ~35% of lung adenocarcinomas (33). Although sotorasib (AMG510), a drug targeting the KRAS G12C mutation, has been approved by the FDA as a second-line treatment for locally advanced or metastatic NSCLC with the KRAS G12C mutation (34), there are no approved targeted drugs for other KRAS mutation types (35). A study has shown that KRAS mutations

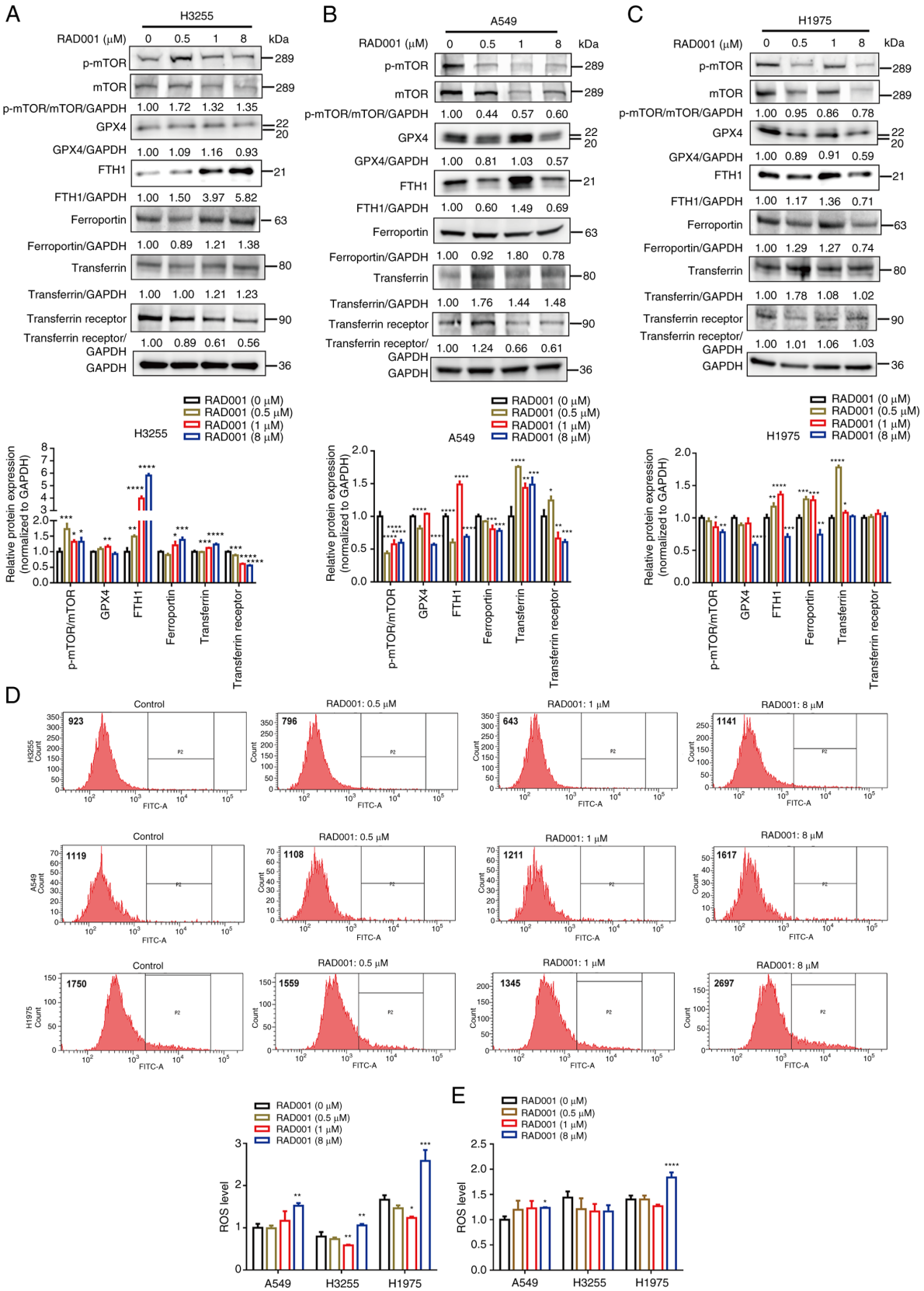

Figure 4. Effect of RAD001 on ferroptosis related proteins and ROS in EGFR T790M/L858R and wild-type EGFR non-small cell lung cancer. (A) H3255, (B) A549 and (C) H1975 cells were treated with different concentrations of RAD001 (0, 0.5, 1 and 8 μM). After 24 h, the expression levels of p-mTOR, mTOR, GPX4, FTH1, ferroportin, transferrin and the transferrin receptor were detected using western blotting. GAPDH was used as an internal standard. *P<0.05, **P<0.01, ***P<0.001 and ****P<0.0001 vs. the control group (0 μM of RAD001) using Dunnett's test. (D) H3255, A549 and H1975 cells were treated with different concentrations of RAD001 (0, 0.5, 1 and 8 μM) for 4 h. The ROS levels were detected using flow cytometry. *P<0.05, **P<0.01 and ***P<0.001 vs. the control group (0 μM of RAD001) using Dunnett's test. (E) H3255, A549, and H1975 cells were treated with different concentrations of RAD001 (0, 0.5, 1, and 8 μM) for 4 h. The accumulation of lipid peroxidation was detected using an MDA kit *P<0.05 and ****P<0.0001 vs. the control group (0 μM of RAD001) using Dunnett's test. EGFR, epidermal growth factor receptor; GAPDH, glyceraldehyde-3-phosphate dehydrogenase; ROS, reactive oxygen species; MDA, malondialdehyde; p-, phosphorylated; mTOR, mammalian target of rapamycin; FTH1, ferritin heavy-chain polypeptide 1; GPX4, glutathione peroxidase 4.

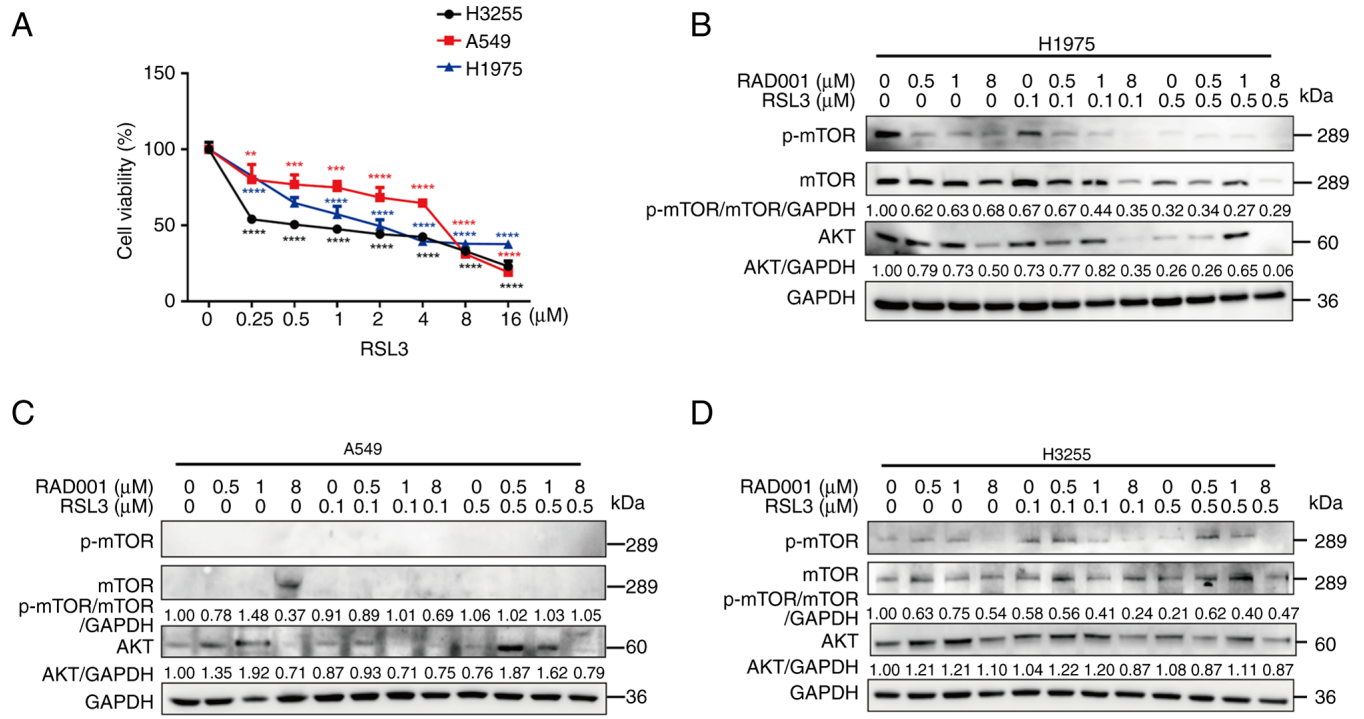

Figure 5. Inhibition of GPX4 inhibits mTOR/AKT pathway of EGFR T790M/L858R and wild-type EGFR non-small cell lung cancer cells. (A) H3255, A549 and H1975 cells were treated with different concentrations of RSL3 (0, 0.25, 0.5, 1, 2, 4, 8 and 16 μM) for 24 h. The cell viability was detected using MTT. **P<0.01, ***P<0.001 and ****P<0.0001 vs. the control group (0 μM of RSL3) using Dunnett's test. (B) H1975, (C) A549 and (D) H3255 cells were treated with different concentrations of RAD001 (0, 0.5, 1 and 8 μM) combined with RSL3 (0, 0.1 and 0.5 μM). After 24 h, the expression of p-mTOR, mTOR and AKT were detected using western blotting, and GAPDH expression was used as an internal standard. EGFR, epidermal growth factor receptor; GAPDH, glyceraldehyde-3-phosphate dehydrogenase; p-, phosphorylated; mTOR, mammalian target of rapamycin.

are associated with EGFR-TKI resistance (36). In NSCLC, 15-30% of patients with adenocarcinoma have functional mutations in the KRAS gene, which means that the tumors in these patients do not respond to EGFR-TKIs (37). The targeted regulation of ferroptosis has a significant antitumor effect on EGFR-TKI resistant NSCLC treated with EGFR-TKIs such as gefitinib (20). Therefore, based on the findings of the present and aforementioned studies, the regulation of ferroptosis may be an effective treatment strategy against tumors harboring KRAS G12S and EGFR-TKI resistance.

Abnormal activation of mTOR is common in a variety of cancer types such as lung adenocarcinoma, lymphoma and melanoma (38-40), and is related to its role as a key effector downstream of several carcinogenic pathways, such as PI3K/AKT and RAS/RAF/MEK/ERK, as well as tumor inhibitory pathways, such as p53 and LKB1 (38,41-44). A study has shown that mTOR is an important target for the regulation of ferroptosis in many types of tumor cells, like breast cancer and prostate cancer (45). The results of the present study showed that, compared with EGFR L858R mutant NSCLC, EGFR T790M/L858R and EGFR wild-type NSCLC were more sensitive to the mTOR inhibitor, RAD001. RAD001-induced cell death in these two cell types was reduced by the ferroptosis inhibitor, Fer-1. Data from a clinical study suggest that mTOR inhibitors, as single drugs, typically have relatively moderate therapeutic effects, possibly due to the lack of strong cytotoxic effects induced by these inhibitors (40). RAD001 has been approved for the treatment of advanced renal cell carcinoma, PNET and advanced breast cancer (46). However, designing an appropriate combination therapy to enhance the cytotoxicity induced by mTOR inhibition remains an unmet demand in clinical research (40). Recent preclinical studies have further proven that co-targeting mTOR and ferroptosis may be a promising cancer treatment strategy (45,47,48). The results of the present study showed that compared with EGFR L858R mutant NSCLC cells/EGFR wild-type NSCLC cells, EGFR T790M/L858R mutant NSCLC cells were more sensitive to RAD001 combined with the ferroptosis inducer, erastin, suggesting that the combination of mTOR inhibitor and erastin may be a potential treatment strategy for EGFR-resistant mutant NSCLC cells.

mTOR complex 1 mediates cystine-induced GPX4 protein synthesis, inhibits lipid peroxidation and protects cells from ferroptosis (40). The results of the present study showed that RAD001 markedly reduced mTOR activity in EGFR T790M/L858R mutant and wild-type NSCLC cells, resulting in decreased GPX4 protein levels and increased ROS levels. Therefore, the molecular mechanisms underlying the inhibition of mTOR-induced ferroptosis may be related to the accumulation of lipid peroxidation caused by the reduction in GPX4. In addition, the results of the present study demonstrated that the inhibition of mTOR not only changed the expression of GPX4 protein but also reduced the expression of the iron storage protein, FTH1, and the iron transporter, ferroportin. Since FTH1 and ferroportin have important roles in the regulation of iron metabolism in cells, their reduction can lead to iron metabolism disorders and induce ferroptosis (49). The results of the present study indicated that inhibition of mTOR induced ferroptosis in EGFR T790M/L858R mutant and wild-type NSCLC cells

by inducing lipid peroxidation and iron metabolism disorder. In addition, the effect of GPX4 regulation on the AKT/mTOR pathway was observed and it was found that GPX4 inhibition effectively inhibited the AKT/mTOR pathway in H1975 cells. This may be related to the positive regulatory relationship between AKT/mTOR and GPX4 (50,51). Notably, compared with H1975 and A549 cells, H3255 cells were the most sensitive to the GPX4 inhibitor, RSL3, and the least sensitive to erastin, which may be related to the different mechanisms of these drugs. Erastin exerts a significant antitumor effect on wild-type EGFR cells by inducing ROS-mediated caspase-independent cell death (52). Erastin binds directly to voltage-dependent anion channel 2 and produces ROS in an NADH-dependent manner, leading to mitochondrial damage. In certain tumor cells with activated mutations, erastin induces cell death via the RAS/RAF/MEK pathway (53). Another study has shown that RSL3 inactivated GPX4, induced ROS production by lipid peroxidation and rapidly induced ferroptosis in RAS-mutant cells (54). Therefore, for NSCLC with different EGFR mutations, different drugs that regulate ferroptosis may be selected to achieve the most effective antitumor effects. There were certain limitations to the present study. In this study, H1975 (EGFR T790M/L858R), H3255 (EGFR L858R) and A549 (EGFR wild type) cells were selected to test our hypothesis. Experiments performed on other EGFR-mutant NSCLC types of cells would strengthen the significance of the present results and conclusion. The experiments using *in vivo* animal models could also provide more robust results. Moreover, small cell lung cancer (SCLC), squamous cell lung cancer or large cell neuroendocrine lung carcinoma cell lines were not screened. Bebber *et al* (55) reported that non-neuroendocrine SCLC is vulnerable to ferroptosis. In our ongoing study, it was found that, compared with neuroendocrine SCLC, non-neuroendocrine SCLC cells with upregulated GPX4 expression were not sensitive to mTOR inhibitors. Therefore, targeting mTOR may not induce ferroptosis by inhibiting GPX4 in SCLC. We will further explore the role and molecular mechanism of targeting mTOR to regulate ferroptosis in NSCLC, SCLC and large cell neuroendocrine lung carcinoma.

In conclusion, the sensitivity of different EGFR mutation types to ferroptosis was described in the present study and the induction of ferroptosis by targeting mTOR was confirmed to be a potential treatment strategy for EGFR T790M/L858R and wild-type EGFR NSCLC.

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Availability of data and materials

The data generated in the present study may be requested from the corresponding author.

Authors' contributions

YC, HL, CJW and RZ designed the study; RZ and TXW performed the experiments; RZ and CYH analyzed the data; CJW and RZ wrote the manuscript. YLJ performed flow cytometry. All authors read and approved the final version of the manuscript. CJW and RZ confirm the authenticity of all the raw data.

Ethics approval and consent to participate

Not applicable.

Patient consent for publication

Not applicable.

Competing interests

The authors declare that they have no competing interests.

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