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***Plasmodium* circumsporozoite protein enhances the efficacy of gefitinib in lung adenocarcinoma cells by inhibiting autophagy via proteasomal degradation of LC3B**

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Abstract

Background: Almost all patients with lung adenocarcinoma (LUAD) develop resistance to EGFR-TKIs, which limit the long-term clinical application of these agents. Accumulating evidence shows one of the main reasons for resistance to EGFR-TKIs is induction of autophagy in tumor cells. Our previous study found that circumsporozoite protein (CSP) in *Plasmodium* can suppress autophagy in host hepatocytes. However, it is unknown whether CSP-mediated inhibition of autophagy could improve the anti-tumor effect of EGFR-TKIs.

Methods: We constructed A549 and H1975 cell lines with stable overexpression of CSP (OE-CSP cells). CCK-8, LDH, flow cytometry, and colony analysis were performed to observe the effect of CSP overexpression on cell viability, the apoptosis rate, and stemness. The sensitizing effect of CSP on gefitinib was evaluated *in vivo* using a subcutaneous tumor model in nude mice and immunohistochemical assay. The role of CSP in regulation of autophagy was investigated by laser confocal microscopy assay and Western blotting. A transcriptome sequencing assay and real-time polymerase chain reaction were used to determine the levels of mRNA for autophagy-related proteins. Cyclohexane, MG132, TAK-243, and immunoprecipitation assays were used to detect and confirm proteasomal degradation of LC3B.

Results: OE-CSP A549 and H1975 cells were more sensitive to gefitinib, demonstrating significant amounts of apoptosis and decreased viability. In the OE-CSP group, autophagy was significantly inhibited, and there was a decrease in LC3B protein after exposure to gefitinib. Cell viability and stemness were recovered when OE-CSP cells were exposed to rapamycin. In nude mice with xenografts of LUAD cells, inhibition of autophagy by CSP resulted in suppression of cell growth and more marked apoptosis during exposure to gefitinib. CSP promoted ubiquitin-proteasome degradation of LC3B, leading to inhibition of autophagy in LUAD cells after treatment with gefitinib. When LUAD cells were treated with the

ubiquitin-specific inhibitor TAK-243, cell viability, apoptosis, and growth were comparable between the OE-CSP group and a control group both *in vivo* and *in vitro*.

Conclusions: CSP can inhibit gefitinib-induced autophagy via proteasomal degradation of LC3B, which suggests that CSP could be used as an autophagy inhibitor to sensitize EGFR-TKIs.

Key words: lung adenocarcinoma, circumsporozoite protein, gefitinib, autophagy, ubiquitination.

Background

Lung cancer is a common malignancy with high morbidity and mortality rates and results in 1.8 million deaths each year [1]. Non-small cell lung cancer (NSCLC) accounts for 85% of all lung cancers, and lung adenocarcinoma (LUAD) is the most common subtype. Although a conventional therapeutic strategy based on surgical resection followed by chemoradiotherapy can benefit patients with early-stage LUAD, most patients have advanced LUAD at the time of diagnosis and a poor prognosis after conventional treatment [2]. Epidermal growth factor receptor (EGFR)-tyrosine kinase inhibitor (TKI) therapy is preferred in patients with locally advanced or metastatic NSCLC because of its high specificity, curative effect, and favorable safety profile. EGFR-TKIs have doubled the disease-free survival rate in patients with advanced NSCLC and an *EGFR* mutation [3, 4]. Unfortunately, drug resistance is a crucial issue in the clinical application of EGFR-TKIs. The majority of patients with LUAD rapidly develop resistance during EGFR-TKI therapy and do not benefit from treatment [5]. Many efforts have been made to overcome this resistance, including combining an EGFR-TKI with an anti-neovascularization agent [6] or c-Met inhibitor [7]. Despite some positive overall results, these strategies cannot solve the resistance problem completely. Therefore, there is an urgent need to develop new targets to combat drug resistance.

Autophagy is a highly conserved survival process that is precisely regulated to degrade aging organelles or malfunctioning proteins in response to stress, hypoxia, or starvation [8]. Interestingly, autophagy has opposite functions under different conditions. In normal somatic cells, autophagy degrades misfolded proteins to prevent malignant transformation. However, in an established tumor, autophagy can act as a survival mechanism that prevents tumor cell death when exposed to harsh conditions and chemotherapy [9-11]. Moreover, autophagy is upregulated in response to treatment with an EGFR-TKIs and plays a critical role in resistance to TKIs [12, 13]. Combined treatment with an EGFR-TKI and an autophagy inhibitor may be an

effective approach when basal autophagy is upregulated in cancer cells [10, 14, 15]. There is some evidence indicating that inhibition of autophagy is a potential target that could be used to overcome resistance to EGFR-TKIs [16].

Circumsporozoite protein (CSP) is a surface protein of the *Plasmodium* sporozoite and has a critical role in the motility of the sporozoite and its ability to invade a host [17]. After sporozoites invade a host hepatocyte, CSP is shed and released into the cytosol to modulate intracellular signaling pathways that assist in the development and survival of parasites [18]. Our previous study found that CSP could suppress autophagy in hepatocytes, thereby helping the parasite to evade interferon-gamma-mediated death [19]. This finding raised the interesting possibility that CSP might increase the sensitivity of LUAD cells to EGFR-TKIs. Therefore, the aim of the present study was to explore the role of CSP-mediated inhibition of autophagy in EGFR-TKI resistance in LUAD cells.

Material and methods

Cell culture and reagent

LUAD cells (A549 and H1975) were cultured in Dulbecco's Modified Eagle Medium (Hyclone Laboratories, Logan, UT, USA) with 10% fetal bovine serum (Gibco Laboratories, Gaithersburg, MD, USA), 1% penicillin-streptomycin, and 2 mM L-glutamine (Sangon Biotech, Shanghai, China) at 37°C in a humidified atmosphere of 5% CO₂.

Cell viability assay

Cells (2×10³) were seeded in 96-well plates and cultured for 12 h. For detection of viability, the cells were treated with various concentrations of gefitinib for 24 h or 25 μM gefitinib for the indicated time points. Cell viability was examined using a CCK8 kit (Beyotime Biotechnology, Jiangsu, China).

LDH assay

Cellular cytotoxicity was detected using an LDH assay kit (Beyotime Technology) according to the manufacturer's instructions. Briefly, cells (5×10^3) were seeded in 96-well plates and treated with gefitinib for 24 h. After treatment with gefitinib, the LDH release rate was detected at 490 nm, and the percentage of LDH released was calculated for cellular cytotoxicity.

Apoptosis assay

Cells (5×10^5) were seeded in 12-well plates and treated with 25 μ M gefitinib, 0.25 μ g/mL rapamycin, 1 μ M TAK-243 (MedChemExpress, Monmouth Junction, NJ, USA), or combined treatment for 24 h. Annexin V (BioLegend, San Diego, CA, USA) and propidium iodide (Beyotime Technology) were stained on the surface of the cells. The data were analyzed using FlowJo software (version 10.0; Ashland, OR, USA).

Colony formation assay

Cells (1×10^3) were seeded in 6-well plates and cultured for 12 h. The cells were then treated with 5 μ M gefitinib for 7 days. The clones were fixed with 4% paraformaldehyde and stained with 0.1% crystal violet solution. The colony formation assay was repeated three times. All data were analyzed using ImageJ software (National Institutes of Health, Bethesda, MD, USA).

Construction of cells with stable overexpression of CSP

The CSP coding sequence of *Plasmodium yoelii* 265BY was amplified and cloned into pCDH-CMV. 293FT cells were transfected with recombinant pCDH-CMV-CSP plasmid and psPAX2 and pMD2.G packaging plasmids to produce lentiviruses. A549 and H1975 cells were infected with control or CSP-overexpressing lentiviruses at a multiplicity of infection of 15 for 48 h. Cells were limited-diluted and monoclonal-cultured during puromycin challenge (3 μ g/mL, Gibco) to select infection-positive cells. The control and OE-CSP cells were cultured in Dulbecco's Modified Eagle Medium supplemented with 10% fetal bovine serum and 1 μ g/mL

puromycin.

Immunofluorescence assay

Cells were seeded on the surface of 8-mm × 8-mm slides and cultured for 12 h. Cells were infected with the RFP/GFP-LC3B adenovirus after treatment with polybrene to neutralize the negative charge on the cell surface. The medium was replaced with Hank's balanced salt solution (HBSS). Twenty-four hours later, the cells were harvested, fixed with 4% paraformaldehyde, and underwent nuclear staining with DAPI. DAKO Fluorescence Mounting Medium (Agilent Technologies Inc, Santa Clara, CA, USA) was used to avoid fluorescence quenching, and the slides were mounted for fluorescence microscopy.

***In vivo* xenograft model**

Control cells or OE-CSP cells (5×10^6) were mixed with Matrigel in a volume ratio of 1:1 and subcutaneously injected into nude mice (Charles River Laboratories, Beijing, China). When the tumor volume was approximately 100 mm³, the mice were divided into control-NC, CSP-NC, control-gefitinib, and CSP-gefitinib groups. The control-NC and CSP-NC groups were fed olive oil every other day, while the control gefitinib and CSP-gefitinib groups were treated with suspension buffer containing gefitinib (150 mg/kg) and olive oil. Mice were treated for 3 weeks with TAK-243 [20] (intravenous administration at 2 doses/week, 12.5mg/kg) or plus gefitinib to detect the role of the ubiquitin-proteasome system. The long (a) and short (b) tumor diameters were measured with a Vernier caliper, and the computational formula used for tumor volume was $v = a \cdot b^2 / 2$. When the tumor volume approached 2000 mm³, the tumors were extracted, photographed, and fixed with 4% paraformaldehyde for immunohistochemical assay. All methods were carried out in accordance with the approved Guide for the Care and Use of Laboratory Animals of the Army Medical University.

Immunohistochemical assay

The immunohistochemical assay protocol was performed as described in a previous study [21]. Briefly, the tumor tissues were embedded in paraffin, sectioned, and dewaxed, after which the antigen was restored in citrate buffer. The sections were incubated with TUNEL, Ki67, and LC3B to detect cell apoptosis, proliferation, and autophagy levels *in vivo*. Sections were then incubated with specific secondary horseradish peroxidase-conjugated antibodies (Dako, Carpinteria, CA, USA), photographed, and scored.

Western blot

Cells were treated with gefitinib, cycloheximide, MG132, or HBSS for the indicated time period and then lysed using RIPA lysis buffer (Sangon Biotech) on ice. After the protein concentration was determined by BCA kit (Beyotime Technology), certain volume of protein was mixed with 4x Laemmli Sample Buffer (Bio-rad, CA, USA) for boiling water bath for 10 minutes for degeneration. The protein was separated by 10% sodium dodecyl sulfate-polyacrylamide gel electrophoresis and transferred onto polyvinylidene fluoride (PVDF) membranes (Millipore, Burlington, MA, USA). The PVDF membrane was blocked with 1% protein-free blocking buffer (Sangon Biotech). Strips of the appropriate size were cut out according to the marker and then incubated overnight at 4°C with specific antibodies, namely, LC3B, β -actin, ATG3, ATG5, ATG7 (all from Cell Signaling Technology, USA), SQSTM1, and Beclin 1 (Abcam, UK). After washing TBST buffer, the PVDF strips were incubated with secondary horseradish peroxidase-conjugated antibodies for 2 h at room temperature. The protein bands were visualized using an electrochemiluminescence kit (Takara Bio, Shiga, Japan), and their relative density was analyzed using ImageJ software.

For the immunoprecipitation assay, the GFP-LC3B plasmid was transfected into control cells and OE-CSP cells using a Lipofectamine 3000 kit (Thermo Fisher, Waltham, MA, USA). Six hour later, the cells were treated with MG132 or TAK-243

plus MG132 for 24 h and then collected, lysed, equalized, and incubated with GFP antibody (ratio 100:1) for 2 h at 4°C. The protein was harvested and purified using a Capturem IP & Co-IP kit (Takara Bio) according for the manufacturer's instructions. The protein was then separated by Western blotting and incubated with GFP or ubiquitin antibody (Cell Signaling Technology, Beverly, MA, USA).

Transcriptome sequencing and quantitative reverse transcription PCR

A549 cells (1×10^6) were seeded in 6-well plates and treated with HBSS for 24 h. The cells were lysed using TRIzol buffer, and transcriptome sequencing analysis was performed by Sangon Biotech. The heatmap was created using R package pheatmap v1.0.12. Real-time quantitative reverse transcription polymerase chain reaction was performed to confirm the transcriptional level of ATG proteins. Briefly, the cells were treated with rapamycin, lysed with TRIzol buffer, and reverse-transcribed using a PrimeScript™ RT reagent kit with gDNA Eraser (Takara Bio). Real-time polymerase chain reaction (PCR) was then performed using a TB Green Fast qPCR kit (Takara Bio). The primers used in this study were as follows: ATG3, forward primer: 5'-GACCCCGGTCCTCAAGGAA-3', reverse primer: 5'-TGTAGCCC ATTGCCATGTTGG-3'; ATG5, forward primer: 5'-AAAGATGTGCTTCGAGATGTGT-3', reverse primer: 5'-CACTTTGTCAGTTACCAACGTCA-3'; ATG7, forward primer: 5'-ATGATCCCTGTAACTTAGCCCA-3', reverse primer: 5'-CACGGAAGCAAACAAC-3'; LC3B, forward primer: 5'-GATGTCCGACTTATTCGAGAGC-3', reverse primer: 5'-TTGAGCTGTAAGCGCCTTCTA-3'; Beclin-1, forward primer: 5'-CCATGCAGGTGAGCTTCGT-3', reverse primer: 5'-GAATCTGCGAGAGACACCATC-3'; SQSTM1, forward primer: 5'-GCACCCCAATGTGATCTGC-3', reverse primer: 5'-CGCTACACAAGTC GTAGTCTGG-3'.

Statistical analysis

All data are presented as the mean $\pm$ standard deviation. The proliferation, apoptosis, colony assay, and Western blotting experiments were repeated three times. The clones and relative grayscale values were analyzed using ImageJ software. The study data were analyzed and compared using SPSS software (version 19.0; IBM Corp., Armonk, NY, USA). Differences between groups were examined by one-way analysis of variance and unpaired two-tailed Student's *t*-tests. Differences were considered statistically significant at $p < 0.05$.

RESULTS

Overexpression of CSP increased the sensitivity of NSCLC cells to gefitinib *in vitro* and *in vivo*

The anti-tumor activity of CSP was investigated by measuring changes in cell proliferation, apoptosis, and stemness in A549 and H1975 (LUAD) cell lines when CSP was overexpressed. Exogenous CSP expression was first verified by RT-qPCR (Fig. S1). Cell viability was evaluated by CCK8 assay or LDH assay when cells were treated with various concentrations of gefitinib for 24 h or at different time points (Fig. 1a-1c, 1f-1h). OE-CSP A549 and H1975 cells were more sensitive to gefitinib, and their viability was significantly lower than that of control cells. The survival rate of OE-CSP cells decreased in a concentration-dependent and time-dependent manner. The findings for cell apoptosis and colony formation were consistent with those for cell viability. When cells were treated with gefitinib, the viable and non-viable apoptotic cell rates were significantly higher in the OE-CSP group (Fig. 1d, 1i). The clone rate (%) was also lower in the OE-CSP group than in the control group (Figure 1e and 1j). Inhibition of cell proliferation by CSP was then confirmed in a subcutaneous tumor model. Nude mice were inoculated subcutaneously with control cells or OE-CSP cells and started on treatment with gefitinib on alternate days when the tumor volume reached approximately 100 mm³. After treatment with gefitinib, the growth of xenografts was significantly more suppressed, and the tumor diameter was

252 remarkably smaller in the OE-CSP group than in the control group (Fig. 2a, 2b).
253 Immunohistochemistry results showed that gefitinib caused pronounced cell death as
254 a result of induction of cell apoptosis and inhibition of cell proliferation, with a greater
255 amount of TUNEL (Fig. 2c) and a lower Ki67 index in the OE-CSP group (Fig. 2d).
256 These findings indicate that CSP enhanced the sensitivity of LUAD cells to gefitinib
257 and inhibited tumor cell growth both *in vivo* and *in vitro*.

258 **CSP inhibited the autophagy induced by gefitinib**

259 Given that treatment with gefitinib contributed to induction of autophagy in several
260 tumor models, we then compared autophagy in the OE-CSP and control groups after
261 exposure to gefitinib. As shown in Fig. 3a and 3b, conversion of the autophagy-related
262 protein (ATG) LC3-I to LC3-II increased after treatment with gefitinib in the control
263 group but was significantly attenuated in the OE-CSP group. Furthermore, when cells
264 were infected with GFP/RFP-LC3B adenovirus, there was a significant decrease in
265 the number of LC3B puncta in the OE-CSP group in comparison with the control
266 group when cells were treated with HBSS, which is a classical pathway for induction
267 of autophagy (Fig. 3c, 3d). The LC3B protein levels measured at different time points
268 after treatment with HBSS also confirmed inhibition of autophagy in the OE-CSP
269 group (Fig. 3e, 3f). Inhibition of autophagy in OE-CSP cells was also observed *in vivo*;
270 subcutaneous tumors were isolated after gefitinib therapy, and immunohistochemical
271 assays demonstrated that the immunohistochemistry score for LC3B protein was
272 much lower in the OE-CSP group than in the control group (Fig. 3g). These findings
273 demonstrated that overexpression of CSP could suppress the autophagy induced by
274 gefitinib.

275 **CSP sensitized the inhibitory effect of gefitinib by suppressing autophagy**

276 To investigate whether the ability of CSP to promote sensitivity to gefitinib was
277 mediated by inhibition of autophagy, we treated cells with rapamycin, an inhibitor of
278 the Akt/mTOR pathway, to induce autophagy. As shown in Fig. 4a and 4b, the LC3B

protein level was markedly increased in the OE-CSP group after treatment with rapamycin, indicating that rapamycin reverses the CSP-mediated suppressive effect on autophagy. As expected, although the sensitivity of the two LUAD cell strains that overexpressed CSP to gefitinib increased markedly, there was no difference in cell viability (Fig. 4c, 4d) or the rate of apoptosis (Fig. 4e, 4f) between the control and OE-CSP groups after co-treatment with rapamycin and gefitinib. Moreover, the colony formation assay did not reveal a statistically significant difference in the number of clones between the OE-CSP and the control group in response to treatment with gefitinib plus rapamycin, demonstrating that the reduced stemness behavior of cells in the OE-CSP group was rescued by rapamycin (Figure 4g, 4h). These findings suggest that the sensitization to gefitinib in the OE-CSP group was mediated by repression of autophagy.

CSP enhanced degradation of LC3B via the ubiquitin-proteasome pathway

The mRNA levels of ATG from the OE-CSP and control A549 cells were sequenced and compared to explore the mechanism underlying the ability of the LC3B protein to downregulate CSP. As shown in Fig. 5a and 5b, overexpression of CSP had no significant effect on ATG mRNA levels overall. A subsequent real-time PCR experiment further confirmed this result, suggesting that the decrease in LC3B protein in OE-CSP cells was not mediated at the transcription stage. The ubiquitin-proteasome and autophagy-lysosome pathways are the two main routes of protein degradation in eukaryotes. Interestingly, autophagy was inhibited in OE-CSP cells, suggesting that the autophagy-lysosome pathway is not involved in reduction of the LC3B protein level. Therefore, we examined whether the decreased expression of LC3B was induced by the ubiquitin-proteasome pathway. The half-life of LC3B was detected by Western blotting at different time points after the cells were treated with cycloheximide. We found that the half-life of LC3B was significantly shorter in the OE-CSP group than in the control group after treatment with HBSS (Fig. 5c), and that

the protein was aggregated when cells were treated with the proteasome inhibitor MG132 (Fig. 5d). Moreover, the ubiquitination level of LC3B in the OE-CSP group was much higher than that in the control group (Fig. 5e). Our data suggest that degradation of LC3B protein by CSP may be mediated by the autophagy-lysosome pathway.

Ubiquitin inhibition by TAK-243 rescues the sensitizing effect of CSP on gefitinib

After confirmation that overexpression of CSP can sensitize LUAD cells to inhibition by gefitinib via enhancement of ubiquitin degradation of LC3B, we examined cell viability, apoptosis, and stemness by inhibiting the cell ubiquitin-activating enzyme using TAK-243, a small-molecule inhibitor that disrupts all ubiquitin signaling and global protein ubiquitination [20]. Given the cytotoxicity of TAK-243 in tumor cells, we first treated A549 cells with various concentrations of TAK-243 to detect cell viability and selected a concentration of 1 μ M because it did not interfere with cell survival (Supplementary Fig. 2). As shown in Fig. 6a, the ubiquitylation level of LC3B decreased significantly in OE-CSP cells after they were treated with TAK-243. The viability (Fig. 6b) and stemness (Fig. 6c) of OE-CSP cells were comparable with those of control cells after treatment with gefitinib plus TAK-243, as was the cell apoptosis rate (Fig. 6d). To further confirm the role of ubiquitylation in the response of CSP-sensitized lung cancer cells to gefitinib, we intravenously injected TAK-243 twice a week during treatment with gefitinib. Injection of TAK-243 significantly inhibited the anti-tumor efficiency of gefitinib in the OE-CSP group, and the tumor diameter was larger than that in the gefitinib group (Fig. 6e).

Discussion

Clinical trials using autophagy-targeting drugs either as monotherapy or in combination therapy with EGFR-TKIs are undergoing. Although some studies have

shown these autophagy inhibitors to have promising effects in patients with intractable disease, side effects such as retinopathy and gastrointestinal problems due to agents such as hydroxychloroquine and chloroquine are an issue, and the appropriate indications for these drugs are not yet determined [16, 22, 23]. An increasing number of studies have found that attenuated parasites [24], blood-stage malarial parasites [25], anti-parasitic agents [26-28], and parasite proteins [29, 30] had anti-tumor activity in various tumor models. These reports point to the possibility of identifying anti-tumor strategies from the perspective of parasites. In this study, we found that overexpression of *Plasmodium* CSP in the cytoplasm of LUAD cells could suppress the autophagy induced by gefitinib and play an active role in enhancing apoptosis and suppressing proliferation and stemness of LUAD cells via synergistic interaction. Moreover, we noticed that overexpression of CSP did not induce any cytotoxicity in LUAD cells either *in vitro* or *in vivo*. This might be related to its ability to protect cells from death caused by invasion of sporozoites [18]. Therefore, suppression of CSP-mediated autophagy and low cell toxicity indicate that it is a promising sensitizer for EGFR-TKIs.

Autophagy and proteasomes are the most common pathways for degradation of protein and cooperate to maintain cell and protein homeostasis [31]. However, the difference is that the ubiquitin-proteasome system mainly degrades soluble monomers and misfolded proteins, whereas autophagy predominantly degrades protein aggregates and damaged organelles [32, 33]. In the past, autophagy and the proteasome were deemed to be independent of each other, whereas accumulating evidence now shows crosstalk between these degradation pathways [34], including sharing and co-degradation of substrates [35-38]. In this study, we demonstrated that the ATG protein LC3B was downregulated by CSP via the ubiquitin-proteasome system but could not identify the mechanisms regulating LC3B ubiquitination in OE-CSP LUAD cells. LC3B is usually degraded in autophagolysosomes during the process of autophagy; however, in this study, cellular autophagy was inhibited in

OE-CSP LUAD cells, suggesting degradation of autophagy is not responsible for the decreased amount of LC3B protein. Recent studies found that the LC3B protein level in cytoplasm was also controlled by ubiquitin enzyme and deubiquitinase [39, 40]. Therefore, more research is needed to determine whether or not CSP can regulate the action of these enzymes.

Bioimmunotherapy is another novel treatment for cancer that has a significant curative effect by activating the autoimmune response against tumor cells [41][42]. Use of parasites as antigens to induce the host immune response and exert an anti-tumor effect is now a promising area of research. Experiments have shown that attenuated *Plasmodium* sporozoites and *Toxoplasma gondii* or *Toxoplasma* antigen have an inhibitory effect in lung cancer [43], melanoma [44], pancreatic cancer [45, 46], ovarian cancer [47], and colon cancer [48]. CSP has become a prime vaccine candidate for malaria based on its importance in the physiological processes of the sporozoite. Previous studies have confirmed that the abundant epitopes of CSP trigger the immune response [49] and induce robust CD8⁺ T-cell responses that are capable of eliminating developing parasites in hepatocytes, resulting in protective immunity [50]. The findings of this study have led to the interesting hypothesis that overexpression of CSP in lung cancer cells could induced a CSP peptide-specific CD8⁺ T-cell response that combats tumor growth. Unfortunately, this hypothesis could not be tested in this study because immunodeficient mice were used but will be explored in the future.

Conclusion

In this study, we found that CSP enhanced drug sensitivity and inhibited the autophagy induced by gefitinib in NSCLC cells and was mediated by the ubiquitin-proteasome system.

Authors' contributions

LX and ZJ were responsible for the *in vitro* experiments. LQX, ZD, and DXF performed the nude mice experiments. YGX, LXQ and LMY analyzed all the data. LX and ZJ wrote the manuscript. ZH and DJG designed the research and reviewed and edited the manuscript. All authors read and approved the final manuscript.

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Availability of data and materials

The datasets used and/or analyzed in this study are available from the corresponding author on reasonable request.

Ethics approval and consent to participate

Not applicable.

Consent for publication

Not applicable.

Competing interests

The authors declare they have no competing interests.

Abbreviations

CSP, circumsporozoite protein; EGFR, epidermal growth factor receptor; HBSS, Hank's balanced salt solution; LDH, lactate dehydrogenase; NSCLC, non-small cell lung cancer; OE, overexpressing; PCR, polymerase chain reaction; TKI, tyrosine kinase inhibitor.

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Fig. 1 Overexpression of circumsporozoite protein (CSP) facilitated cytotoxicity caused by gefitinib. Control and OE-CSP A549 (a) and H1975 (f) cells (2×10^3) were seeded in 96-well plates and treated with gefitinib. Cell viability was assessed at 12 h

intervals. A549 **(b)** and H1975 **(g)** cells were treated with the indicated concentration of gefitinib for 24 h, and their viability was determined using a CCK8 kit and the lactate dehydrogenase assay **(c, h)**. Next, control and OE-CSP A549 **(d)** and H1975 **(i)** cells (5×10^5) were stained with Annexin V and propidium iodide after treatment with gefitinib. The apoptosis rate in the early and late stages was detected by flow cytometry. Control and OE-CSP A549 **(e)** and H1975 **(j)** cells (1×10^3) were then fixed in 4% paraformaldehyde and stained with 0.1% crystal violet solution. After incubation for 7 days, the colony number was analyzed using ImageJ software. The clone rate (%) was normalized to the control group (DMSO). The data are presented as the mean $\pm$ standard deviation of three independent experiments (ns, not statistically significant, * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$).

Fig. 2 Overexpression of CSP enhances gefitinib-mediated inhibition of cell proliferation *in vivo*. Control and OE-CSP A549 (a) and H1975 (b) cells (5×10^6) were subcutaneously injected into nude mice and gefitinib was started when the tumor volume approached 100 mm³. The growth curves for the four groups were drawn and the final tumors were photographed. The tumor tissues were subjected to immunohistochemistry staining for TUNEL (c) and Ki67 (d). Scale bar, 50 μ m (* $p < 0.05$, ** $p < 0.01$).

Fig. 3 Overexpression of CSP inhibited cell autophagy both *in vivo* and *in vitro*. The autophagy-related protein LC3B was detected after control and OE-CSP A549 (a) and H1975 (b) cells were treated with gefitinib. The relative density was examined using ImageJ software. Control and OE-CSP A549 (c) and H1975 (d) cells (1×10^5) were transfected with GFP/RFP-LC3B adenovirus and treated with HBSS for 24 h.

Fluorescence was then detected using an immunofluorescence method. The puncta of GFP-LC3B and RFP-LC3B were counted using ImageJ software. Scale bar, 5 μ m. The protein level of LC3B in A549 (e) and H1975 (f) cells was detected using Western blotting after they had been treated with HBSS for 0, 6, 12, and 24 h. (g) Expression of LC3B protein in A549 and H1975 tumor tissues after treatment with gefitinib was measured by immunohistochemistry. Data are shown as the mean $\pm$ standard deviation of three independent experiments (ns, not statistically significant, * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$). HBSS, Hank's balanced salt solution.

Fig. 4 Inhibitory effect of gefitinib sensitized by CSP was mediated via suppression of autophagy. Control and OE-CSP A549 (a) and H1975 (b) cells (1×10^6) were seeded in 6-well plates and treated with gefitinib alone or gefitinib plus rapamycin. The LC3B was detected by Western blotting. The viability of (c) A549 and (d) H1975 cells was examined using a CCK8 kit and a lactate dehydrogenase assay. For apoptosis, 5×10^5 control and OE-CSP (e) A549 and (f) H1975 cells were treated with gefitinib alone or gefitinib plus rapamycin and stained with Annexin V and propidium iodide. The

apoptosis rate was then detected using flow cytometry. Colony formation assays were also performed for (g) A549 and (h) H1975 cells as above and the clones were counted using ImageJ software. The clone rate (%) was normalized to the control group (DMSO). The data are presented as the mean $\pm$ standard deviation of three independent experiments (ns, not statistically significant, *p < 0.05, **p < 0.01, ***p < 0.001).

Fig. 5 Overexpression of CSP promotes proteasomal degradation of LC3B. **a** Control and OE-CSP A549 cells (1×10^6) were seeded in 6-well plates and treated with HBSS. They were then lysed with TRIzol buffer and transcriptome sequencing was performed. **b** The mRNA level of the autophagy-related proteins was confirmed using quantitative real-time polymerase chain reaction. **c** Control and OE-CSP A549 cells were treated with cycloheximide and the protein was collected 0, 6, 12, and 24 h later. The LC3B protein level was then detected and the half-life curve was drawn. **d** After treatment with MG132 for 0, 6, 12, and 24 h, the LC3B protein level was detected using Western blotting. **e** Control and OE-CSP A549 cells (1×10^6) were transfected with GFP-LC3B plasmid; they were then lysed with Western lysis buffer and the GFP-LC3B protein was purified using a Capturem IP & Co-IP kit. The ubiquitin level was detected. The data are presented as the mean $\pm$ standard deviation of three independent experiments (ns, not statistically significant, * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$).

Fig. 6 Ubiquitin inhibition by TAK-243 rescues the sensitizing effect of CSP on gefitinib.

a Control and OE-CSP A549 cells (1×10^6) were transfected with GFP-LC3B plasmid and then treated with TAK-243 and MG132 for 24 h. The GFP-LC3B was purified and

the level of ubiquitin was detected by Western blotting. Cell viability after treatment with TAK-243 and gefitinib was examined by **(b)** a CCK8 kit and lactate dehydrogenase assay. **c** Cell stemness ability and **(d)** apoptosis were also assessed using the method described above. The clone rate (%) was normalized to the control group (DMSO). **(e)** Nude mice were then treated with TAK-243 plus gefitinib when the tumor volume was approaching 100 mm³. The data are presented as the mean ± standard deviation of three independent experiments (ns, not statistically significant, **p < 0.01, ***p < 0.001).

Supplementary Figures

Supplementary Fig. 1 Expression of exogenous CSP in lung adenocarcinoma cells was confirmed by real-time quantitative reverse transcription polymerase chain reaction. (a) A549, (b) H1975. (**p < 0.01, ***p < 0.001).

Supplementary Fig. 2 Cell viability was inhibited after treatment with a high dose of TAK-243. Control and OE-CSP cells (2×10^3) were seeded in 96-well plates and treated with the indicated concentration of TAK-243 for 24 h. Cell viability was assessed using a CCK8 kit. (ns, not statistically significant, **p < 0.01, ***p < 0.001).