Acta Biochim Biophys Sin 2022, 54(4): 514–523
https://doi.org/10.3724/abbs.2022023
Advance Access Publication Date: 13 April 2022
Original Article

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HMGB1-mediated autophagy promotes gefitinib resistance in human non-small cell lung cancer

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Received 28 June 2021 Accepted 15 November 2021

Abstract

Non-small cell lung cancer (NSCLC) ranks the first in incidence and mortality among malignant tumors in China. Molecular targeted therapies such as gefitinib, an oral inhibitor of the epidermal growth factor receptor tyrosine kinase, have shown significant benefits in patients with advanced NSCLC. However, most patients have unsatisfactory outcomes due to the development of drug resistance, and there is an urgent need to better understand the pathways involved in the resistance mechanisms. In this study, we found that HMGB1 is highly expressed in drug-resistant cells and confers to gefitinib resistance in NSCLC cells via activating autophagy process. Gefitinib upregulates HMGB1 expression in time-dependent and dose-dependent manners in human NSCLC cells. RNA interference-mediated knockdown of HMGB1 reduces PC9GR cell viability, induces apoptosis, and partially restores gefitinib sensitivity. Mechanistic analyses indicate that elevated HMGB1 expression contributes to gefitinib resistance by inducing autophagy. Thus, our results suggest that HMGB1 is an autophagy regulator and plays a key role in gefitinib resistance of NSCLC.

Key words gefitinib resistance, high-mobility group box 1, autophagy, non-small cell lung cancer

Introduction

Lung cancer, of which about 85% of cases are non-small cell lung cancer (NSCLC), is one of the most common and lethal malignancies worldwide [1,2]. However, most patients are diagnosed at an advanced stage of NSCLC, at which point the prognosis is unfavorable and the 5-year survival rate is only about 15% [3]. Several molecular targeted drugs have been approved for the treatment of NSCLC, including the epidermal growth factor receptor (EGFR) tyrosine kinase inhibitor gefitinib, which is used as a first-line treatment for advanced NSCLC [4]. However, the emergence of drug resistance in most patients is the major cause of failure of clinically targeted therapies [5,6]. Multiple mechanisms of acquired resistance to EGFR-targeting therapies have been described, including EGFR threonine to methionine (T790M) mutation, MET amplification, PIK3CA mutation, and AXL activation [7–10]. However, the mechanism of resistance in the remaining cases is still poorly understood and requires further study.

One protein implicated in the resistance to several drug targeted therapies is high-mobility group protein box 1 (HMGB1) which is a highly conserved protein with A-box and B-box DNA-binding domains. It plays critical roles in the regulation of transcription and maintenance of chromosome structure and stability [11,12]. In addition to its nuclear functions, HMGB1 is also secreted into the extracellular milieu where it interacts with various receptors to trigger the expression of genes related to inflammation and infection, including cytokines, chemokines, and growth factors [13,14]. Many studies have demonstrated that HMGB1 is overexpressed in various cancers, including breast, gastric, and colorectal cancers [15–17]. Recent studies indicated that HMGB1 also promotes anticancer drugs resistance of cancer cells [18,19]. However, the relationship between HMGB1 expression levels and the resistance to anticancer therapies, as well as the underlying mechanisms involved have not been fully elucidated.

Since its discovery in 1963, autophagy has been found to play crucial fundamental roles in the maintenance of cell homeostasis and survival. Given the role of autophagy in many key biological processes, it is not surprising that autophagy plays an important role in many diseases. Deregulation of autophagy may lead to the

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development of metabolic disorders, including insulin resistance, diabetes, obesity, atherosclerosis, and osteoporosis [20–23]. In recent years, a large body of evidence has indicated that autophagy plays dual roles in drug resistance. The pro-death or pro-survival roles of autophagy are highly dependent on the tumor type and treatment characteristics. For example, inhibition of autophagy enhanced the therapeutic effects of cisplatin and 5-fluorouracil in colon cancer [24], while TIPE2 suppressed cisplatin resistance by inducing autophagy via mTOR signaling pathway [25]. Our group has reported an important relationship between HMGB1 and autophagy [26], and our previous studies also partially explored the mechanism of EGFR-TKI gefitinib resistance in NSCLC patients [27–29]. Nevertheless, the specific relationship among gefitinib resistance, autophagy and HMGB1 remains unclear.

In this study, we investigate the expression of HMGB1 in human NSCLC cell lines and tissues. We demonstrate for the first time that the HMGB1 expression level is linked to gefitinib sensitivity by inducing autophagy in NSCLC patients. Our findings thus may identify potential new therapeutic targets for NSCLC.

Materials and Methods

Specimen and cell culture

A total of 42 NSCLC specimen tissues were collected by CT-guided percutaneous lung biopsy or fiberoptic bronchoscopy lung biopsy from advanced NSCLC patients who had either an exon 19 deletion (19DEL) or an exon 21-point mutation (L858R) in their EGFRs, and none of these patients received radiotherapy or chemotherapy before surgery. And 24 of them were collected from patients before EGFR-TKIs treatment (BT group) and the others were collected after the acquisition of resistance to EGFR- TKIs (AR group). This study was approved by the Research Ethics Committee of the Second Affiliated Hospital of Nanjing Medical University and informed consent was obtained from all subjects. These specimens were immediately snap-frozen in liquid nitrogen and stored at −80°C until use. The clinical information of the patients is summarized in Table 1.

Human NSCLC cell line (PC9) was purchased from the Institute of Biochemistry and Cell Biology of the Chinese Academy of Sciences (Shanghai, China). The PC9GR cell line was previously established in our own laboratory: the parental PC9 cells were cultured with increasing concentrations of gefitinib from 5 nM to 5 μM for 6 months [28]. PC9 and PC9GR cells were cultured in RPMI medium (Gibco, Carlsbad, USA) containing 10% FBS in a 37°C incubator in 5% CO_2.

Real-time PCR analysis

TRIzol (Invitrogen, Carlsbad, USA) was used to extract the total RNA from NSCLC cells. RNA concentration and purity were determined using a UV nanodrop 2000 spectrophotometer (Thermo Fisher Scientific, Waltham, USA). Primers of *HMGB1* and *GAPDH* (internal reference) were designed according to the gene sequence provided by GenBank. According to the manufacturer's instructions, RNA (1 μg) was reverse-transcribed using a PrimeScript RT Reagent Kit (Takara, Dalian, China). Real-time PCR analyses were performed with SYBR Premix Ex Taq (Takara). The primer sequences used in qPCR were as follows: *HMGB1* forward, 5′-TCAAAGGAGAACATCCTGGCCTGT-3′ and reverse, 5′-CTGCT TGTCATCTGCAGCAGTGTT-3′; *GAPDH* forward, 5′-AGAGGCAG GGATGATGTTCTG-3′ and reverse, 5′-GACTCATGACCACAGTCCA TGC-3′. Relative mRNA expression levels were calculated using the $2^{-\Delta\Delta Ct}$ method.

Table 1. Clinical characteristics of the 24 patients with EGFR-mutant before ERGR-TKI treatment (BT group) and 18 patients with acquired resistance to ECFR-TKIs (AR group)

Clinical characteristics	BT group ($n=24$)	AR group ($n=18$)
Sex		
Male	9	8
Female	15	10
Age		
<60	13	12
≥60	11	6
Histology		
Adenocarcinoma	21	18
Non-adenocarcinoma	3	0
Smoking		
Never	13	11
Ever	11	7
Stage		
IIIB	5	3
IV	19	15
EGFR		
19DEL	16	5
L858R	8	4
T790M	0	9

Cell transfection

At logarithmic growth phase, NSCLC cells were inoculated in a 6-well plate. Cells were transfected with HMGB1-siRNA or its control siRNA, HMGB1-shRNA or its control shRNA (GenePharma, Shanghai, China) using Lipofectamine 2000 (Invitrogen) according to the manufacturer's instructions, and then cultured at 37°C and 5% CO_2 for 48 h. HMGB1 was subcloned into the pCDNA3.1 vector (Invitrogen) and cells were transfected with pCDNA-HMGB1 vector or empty vector using Lipofectamine 2000 (Invitrogen), and then cultured at 37 °C and 5% CO_2 for 48 h. The siRNA sequences are as follows: HMGB1 siRNA, 5′-GCAGCCCUAUGAAAGAAATT-3′; control siRNA, 5′-UCCAAGUAGAUUCGACGGCGAAGTG-3′; HMGB1-shRNA, 5′-GGGAGGAGCAUAAGAAGAATT-3′; control shRNA, 5′-UUCUCCGAACGUGUCACGUTT-3′.

Cell viability assay

Cell viability was measured using Cell Counting Kit-8 (CCK8) assay kit (Promega, Madison, USA) according to the manufacturer's protocol. Briefly, cells at the logarithmic growth phase were collected and seeded in a 96-well plate. Cells were treated with 20 μL of CCK8 dye at 0, 24, 48, 72 and 96 h, with or without gefitinib (5 μM) treatment for 48 h. Absorbance was then measured with a microplate reader at 450 nm.

Colony formation assay

Cells at the logarithmic growth phase were collected and seeded in a 6-well plate. Cells were incubated at 37°C in a 5% CO_2 incubator for 1–2 weeks. After the formation of visible colonies, cells were fixed with methanol for 20 min and stained with 0.1% crystal violet

(Sigma-Aldrich, St Louis, USA), then dry naturally after wash with running water. Experimental results were obtained by counting the stained colonies.

Apoptosis assay

Cells were transfected with si-HMGB1 or si-NC, and cultured for 48 h. Then cells were collected and cell suspensions were prepared. Cells were incubated with FITC-Annexin V and propidium iodide (PI) for 15 min in the dark at room temperature. Cell apoptosis was analyzed by flow cytometry on a FACS Calibur flow cytometer (BD Biosciences, Franklin Lakes, USA).

Western blot analysis

Western blot analysis was performed as described previously [30]. PC9 and PC9GR cells were collected and extracted using RIPA extraction reagent (Beyotime, Shanghai, China). Protein samples were subject to 10% SDS-PAGE and then transferred to a PVDF membranes. Membranes were subsequently blocked in 5% skim milk powder at 4°C for 2 h, followed by incubation with antibodies against EGFR, p-EGFR, ERK, p-ERK, AKT, p-AKT, HMGB1, LC3I-II, P62, BAX, Bcl-2 and PCNA (1:1000) overnight at 4°C. Then membranes were incubated with the corresponding HRP-conjugated secondary antibody (1:3000; Cell Signaling Technology, Boston, USA) at 4°C for 2 h. After extensive wash, protein bands on membranes were detected using ECL chromogenic substrate (Beyotime) and quantified by densitometry (ImageJ SAAinc). GAPDH was used as the loading control. Antibodies against LC3I-II, P62 were purchased from Proteintech (Rosemont, USA) and other antibodies were purchased from Cell Signaling Technology.

Animal experiments

All male nude mice (4–6 weeks old) were purchased from the Animal Experimental of the Nanjing Medical University. The mice were weighed and randomly divided into four groups ($n = 4$). PC9GR cells transfected with sh-HMGB1 were suspended in PBS at a concentration of 2×10^7 cells/mL, and injected subcutaneously into the armpit of nude mice in a volume of 100 μL, untreated cells were used as the control. Gefitinib was injected via gavage at a dose of 25 mg/kg every day for 14 total doses. Tumor volumes were measured every other day and calculated using the formula: $V = 0.5 \times (l \times w^2)$, in which l is length, and w is width. At 15 days after injection, mice were euthanized, and the subcutaneous growth of each tumor was examined. Tumors were weighed and measured, and then subject to qRT-PCR, western blot, H&E staining, and immunohistochemical analyses. The animal experiments were approved by the Committee on the Ethics of Animal Experiments of Nanjing Medical University.

Statistical analysis

Statistical analyses and data plotting were performed using Prism 7.0 software. Statistical analysis in the form of a Student's *t*-test (two-tailed) was performed using SPSS 16.0 software. *P* values less than 0.05 were considered statistically significant.

Results

Overexpression of HMGB1 is correlated with acquired resistance to gefitinib

To explore the relationship between HMGB1 and gefitinib resistance in NSCLC, we examined HMGB1 expression in human NSCLC cell lines PC9 and PC9GR respectively, which are sensitive and resistant to gefitinib. qRT-PCR and western blot analysis showed that HMGB1 level was higher in PC9GR cells than in PC9 cells (Figure 1A). Then, we examined HMGB1 expression during the development of resistance to EGFR-TKIs in advanced NSCLC patients, which were divided into two groups (Table 1): before EGFR-TKI treatment (BT group) and after acquired resistance to EGFR-TKIs (AR group). qRT-PCR analysis showed that HMGB1 expression in the AR group was significantly higher than that in the BT group (Figure 1B). These findings indicated that HMGB1 overexpression may represent a novel indicator for gefitinib treatment. Next, western blot analysis results showed that after gefitinib treatment, there was no significant change in the phosphorylation of EGFR, ERK, or AKT in PC9GR cells, while the phosphorylation of EGFR, ERK, and AKT in PC9 cells was significantly inhibited (Figure 1C). Then, the gefitinib resistance of PC9 and PC9GR cells were confirmed by CCK8 assay, and we found that the half-maximal inhibitory concentrations (IC_{50}) of gefitinib for PC9 and PC9GR cells were 8.78 μM and 56.18 μM respectively, which corresponds to a 6.39-fold difference in drug sensitivity (Figure 1D). These data suggested that HMGB1 expression level may be associated with gefitinib sensitivity in NSCLC cells.

Gefitinib induces high expression of HMGB1 and activates autophagy in sensitive and resistant NSCLC cells

We next investigated the relationship between gefitinib treatment and the cellular levels of HMGB1 and autophagy in PC9 and PC9GR cells. Western blot analysis of cells treated with 5 μM gefitinib for 0, 12, 24, 36, and 48 h or with 0, 5 and 10 μM gefitinib for 48 h showed that HMGB1 expression was increased by gefitinib treatment in both cell lines in dose- and time-dependent manners (Figure 2A,B), which is consistent with the correlation observed between HMGB1 expression level and differential gefitinib sensitivity of PC9 and PC9GR cells. qRT-PCR also confirmed that HMGB1 mRNA level was elevated after treatment with gefitinib (Figure 2C). To determine whether gefitinib treatment affects autophagy, we analyzed the expressions of LC3-II and P62. Notably, western blot analysis revealed that untreated PC9GR cells expressed higher basal level of LC3II and lower basal level of P62 compared with untreated PC9 cells, consistent with a higher basal level of autophagy, and that gefitinib increased LC3-II expression and concomitantly decreased P62 expression in both cell lines (Figure 2A,B). These results indicated that gefitinib not only increases the expression of HMGB1 but also induces autophagy in NSCLC cells.

Knockdown of HMGB1 inhibits the proliferation and increases the apoptosis of PC9GR cells

To determine whether HMGB1 expression directly contributes to gefitinib sensitivity in NSCLC cells, we transfected PC9GR cells with an HMGB1-targeting siRNA. We confirmed that HMGB1 level was effectively suppressed (~1.6-fold) by si-HMGB1 transfection in PC9GR cells by qRT-PCR and western blot analysis (Figure 3A). Knockdown of HMGB1 was found to increase the sensitivity of PC9GR cells to gefitinib, as indicated by a significant loss of cell viability (Figure 3B), decrease in colony formation (Figure 3C) in si-HMGB1-transfected PC9GR cells compared with the control PC9GR cells. As shown in Figure 3D, HMGB1 knockdown significantly increased the PC9GR cell apoptotic rate from 5.17% to 20.42%

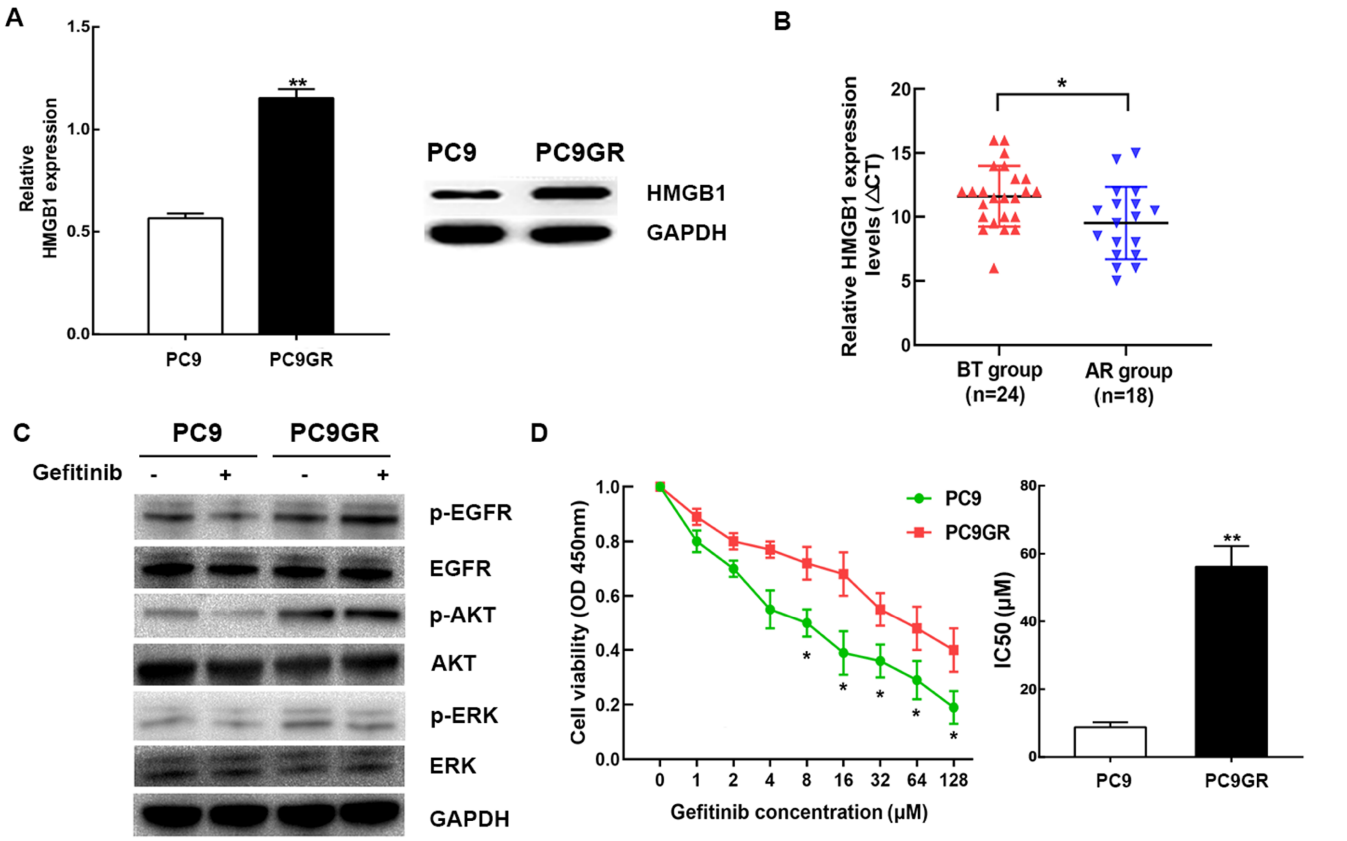

Figure 1. Overexpression of HMGB1 is correlated with acquired resistance to gefitinib (A) HMGB1 expression level was determined by qRT-PCR and western blot analysis. (B) HMGB1 was detected in BT group and AR group by qRT-PCR. *GAPDH* was used as an internal reference gene. (C) The expressions of HMGB1, p-EGFR, p-ERK, p-AKT, total EGFR, ERK, AKT of PC9 and PC9GR cells with or without gefitinib treatment were measured by western blot analysis. GAPDH was used as the loading control. (D) Gefitinib-sensitive cells PC9 and gefitinib-resistant cells PC9GR were treated with increasing concentrations of gefitinib for 48 h. The inhibition rate was determined by CCK8 assay, and the value of IC_{50} was calculated. Data are representative of three independent experiments. $^{*}P<0.05$, $^{**}P<0.01$.

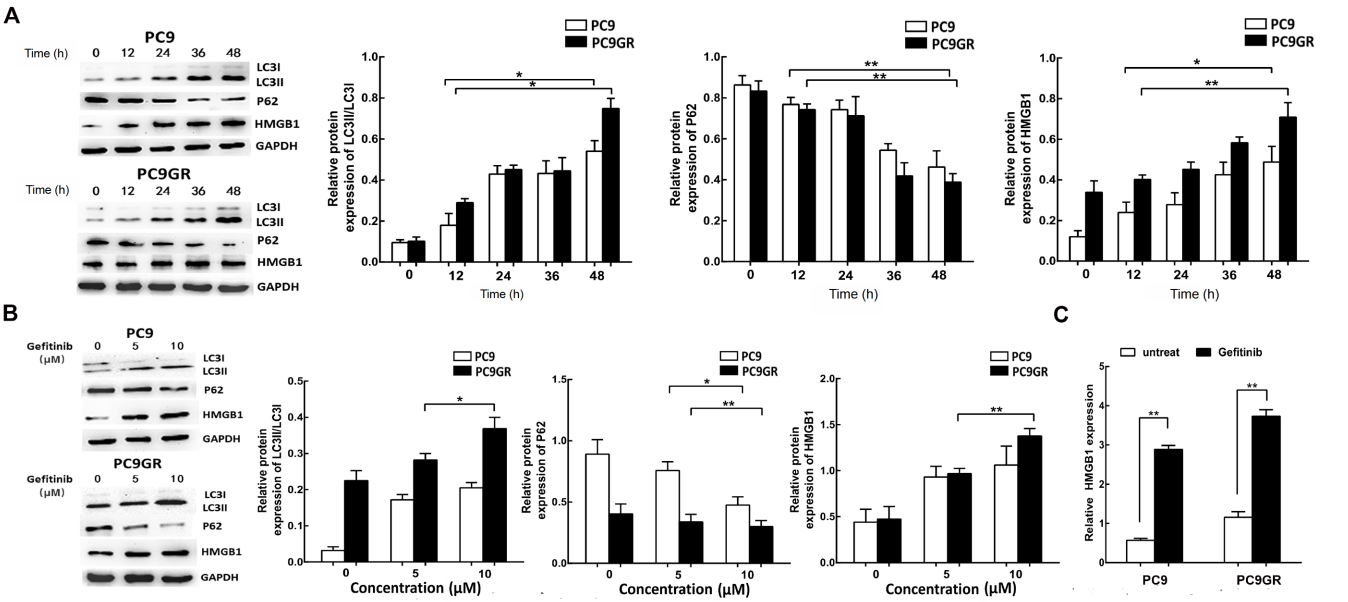

Figure 2. Gefitinib induces HMGB1 expression in time- and dose-dependent manners (A) PC9 and PC9GR cells were treated with gefitinib (5 μM) for 0, 12, 24, 36 and 48 h, HMGB1, LC3II/LC3I and P62 protein levels were analyzed by western blot analysis. (B) PC9 and PC9GR cells were treated with gefitinib of 0, 5 and 10 μM for 48 h, HMGB1, LC3II/LC3I and P62 protein levels were analyzed by western blot analysis. (C) PC9 and PC9GR cells were treated with gefitinib (5 μM) for 48 h, HMGB1 mRNA level was analyzed by qRT-PCR. GAPDH was used as an internal reference gene. $^{*}P<0.05$, $^{**}P<0.01$.

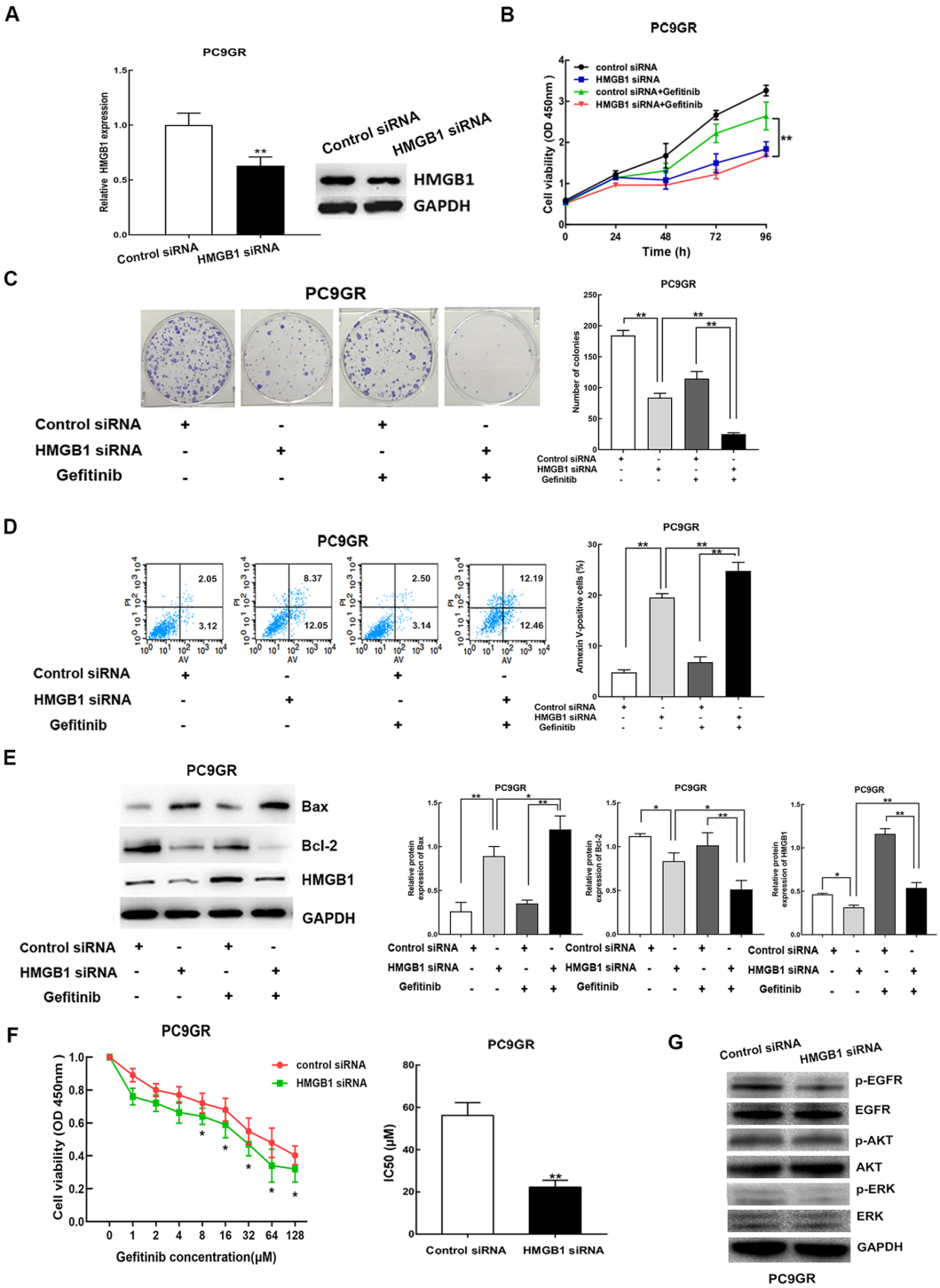

Figure 3. Knockdown of HMGB1 inhibits the proliferation and increases the apoptosis of PC9GR cells (A) PC9GR cells were transfected with control siRNA and HMGB1 siRNA for 48 h, and HMGB1 protein and mRNA levels were detected by western blot analysis and qRT-PCR respectively. (B) PC9GR cells were treated with gefitinib (5 μM) for 48 h, then cell viability was analyzed by CCK8 assay. (C) Colony formation of cell proliferation was analyzed with gefitinib treatment. (D) PC9GR cells were treated with gefitinib (5 μM) for 48 h, cell apoptosis was analyzed by flow cytometry with membrane linkage protein-V/PI staining. (E) PC9GR cells were treated with gefitinib (5 μM) for 48 h, and BAX and BCL-2 expression levels were analyzed by western blot analysis. (F) The inhibition rate was determined by CCK8 assay. Data are representative of three independent experiments, and the values of IC_{50} are calculated. (G) The expressions of p-EGFR, p-AKT, p-ERK, total EGFR, AKT, and ERK in HMGB1-depleted PC9GR cells were examined by western blot analysis. GAPDH was used as the loading control. $^{*}P < 0.05$, $^{**}P < 0.01$.

compared with the control cells. To further confirm the effects of HMGB1 knockdown on apoptosis, we performed western blot analysis of the pro-apoptotic protein BAX and the anti-apoptotic protein BCL-2. Consistent with the results of the flow cytometric

apoptosis assay, HMGB1 knockdown induced upregulation of BAX expression and downregulation of BCL-2 expression in si-HMGB1-expressing PC9GR cells compared with the control PC9GR cells (Figure 3E). To further determine the role of HMGB1 in gefitinib resistance, we examined PC9GR cell viability after gefitinib treatment for 48 h, and the results showed that HMGB1 knockdown decreased the IC_{50} value from 56.18 μM to 22.24 μM, corresponding to a 2.52-fold increase in sensitivity (Figure 3F). We next examined whether HMGB1 regulates ERK and AKT downstream signaling. HMGB1 depletion resulted in remarkable decrease in the phosphorylation levels of EGFR, AKT, and ERK in PC9GR cells (Figure 3G). Overall, these data confirmed that HMGB1 can make drug-resistant cells sensitive to gefitinib *in vitro*.

Upregulation of HMGB1 facilitates the gefitinib resistance of PC9 cells *in vitro*

To further explore the biological functions of HMGB1 in the sensitivity of NSCLC cells, we determined whether overexpression of HMGB1 in PC9 cells could render them sensitivity to gefitinib. PC9 cells were transfected with an HMGB1 overexpression plasmid. Indeed, transfection with an HMGB1 overexpression plasmid promoted PC9 cell proliferation with or without gefitinib treatment

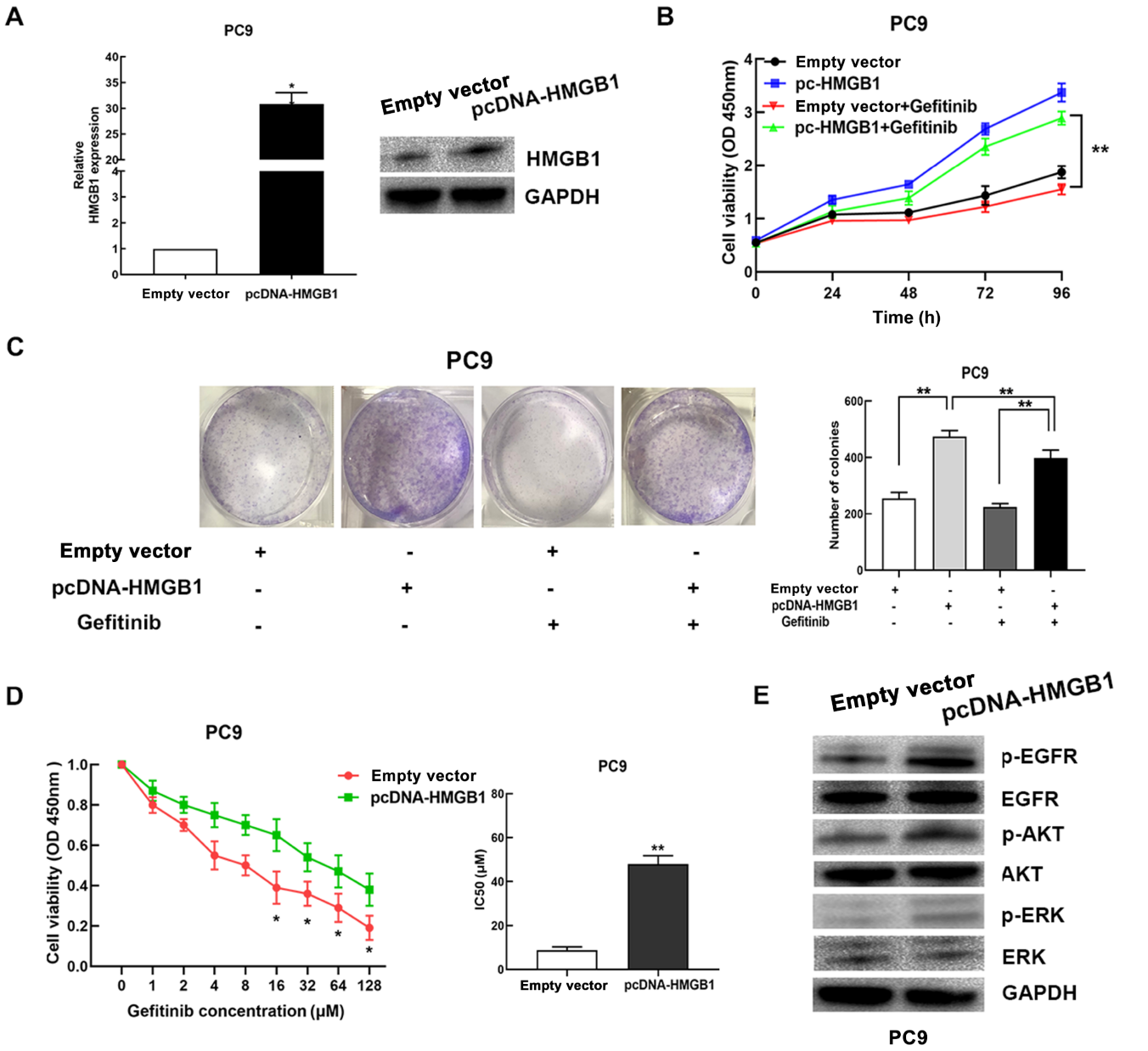

Figure 4. Upregulation of HMGB1 facilitates the gefitinib resistance of PC9 cells *in vitro* (A) PC9 cells were transfected with empty vector or pcDNA-HMGB1 for 48 h, and HMGB1 expression were detected by qRT-PCR and western blot analysis. (B) PC9 cells were treated with gefitinib (5 μM) for 48 h, then cell viability was analyzed by CCK8 assay. (C) Colony formation of cell proliferation was analyzed with gefitinib treatment. (D) The inhibition rate was determined by CCK8 assay. Data are representative of three independent experiments, and the values of IC_{50} are calculated. (E) The expression levels of p-EGFR, p-AKT, p-ERK, total EGFR, AKT and ERK in HMGB1-overexpressing PC9 were examined by western blot analysis. GAPDH was used as the loading control. *$P<0.05$, **$P<0.01$.

(Figure 4A). Similar results were obtained in colony-forming assays (Figure 4B,C). The IC_{50} values of gefitinib in inhibiting cell viability of control PC9 cells and HMGB1-overexpressing PC9 cells were 8.78 μM and 47.93 μM respectively, corresponding to a 5.4-fold decrease in gefitinib sensitivity (Figure 4D). Similarly, HMGB1 overexpression dramatically increased the phosphorylation levels of EGFR, ERK, and AKT signaling in PC9 cells (Figure 4E). Taken together, these data suggested that upregulation of HMGB1 expression reduces the sensitivity of PC9 cells to gefitinib.

Gefitinib-induced upregulation of HMGB1 expression induces autophagy

Having demonstrated that gefitinib upregulates HMGB1 expression and autophagy in PC9 and PC9GR cells, we next determined whether HMGB1 can directly modulate autophagy flux. To this end, we examined the abundance of autophagosomes and the expression of autophagy-related proteins in the control cells and si-HMGB1-transfected PC9GR cells with or without gefitinib treatment. Transmission electron microscopy revealed relatively few autophagosomes in si-HMGB1-expressing cells compared with the control cells, but they were more abundant in PC9GR cells after gefitinib treatment (Figure 5A). Similarly, LC3-II/LC3-I expression levels were decreased and P62 expression level was elevated in PC9GR cells with HMGB1 knockdown (Figure 5B), indicating that HMGB1 expression level directly affects autophagy. Meanwhile, the ratio of LC3-II to LC3-I protein was higher in PC9GR cells than in PC9 cells, while P62 expression was lower in PC9GR cells than in PC9 cells (Figure 5C). These findings indicated a direct role for HMGB1 in autophagy regulation in NSCLC cells.

HMGB1 knockdown enhances the sensitivity of PC9GR cells to gefitinib *in vivo*

Finally, we determined whether our *in vitro* findings in the role of HMGB1 are also observed *in vivo* using a mouse xenograft model of

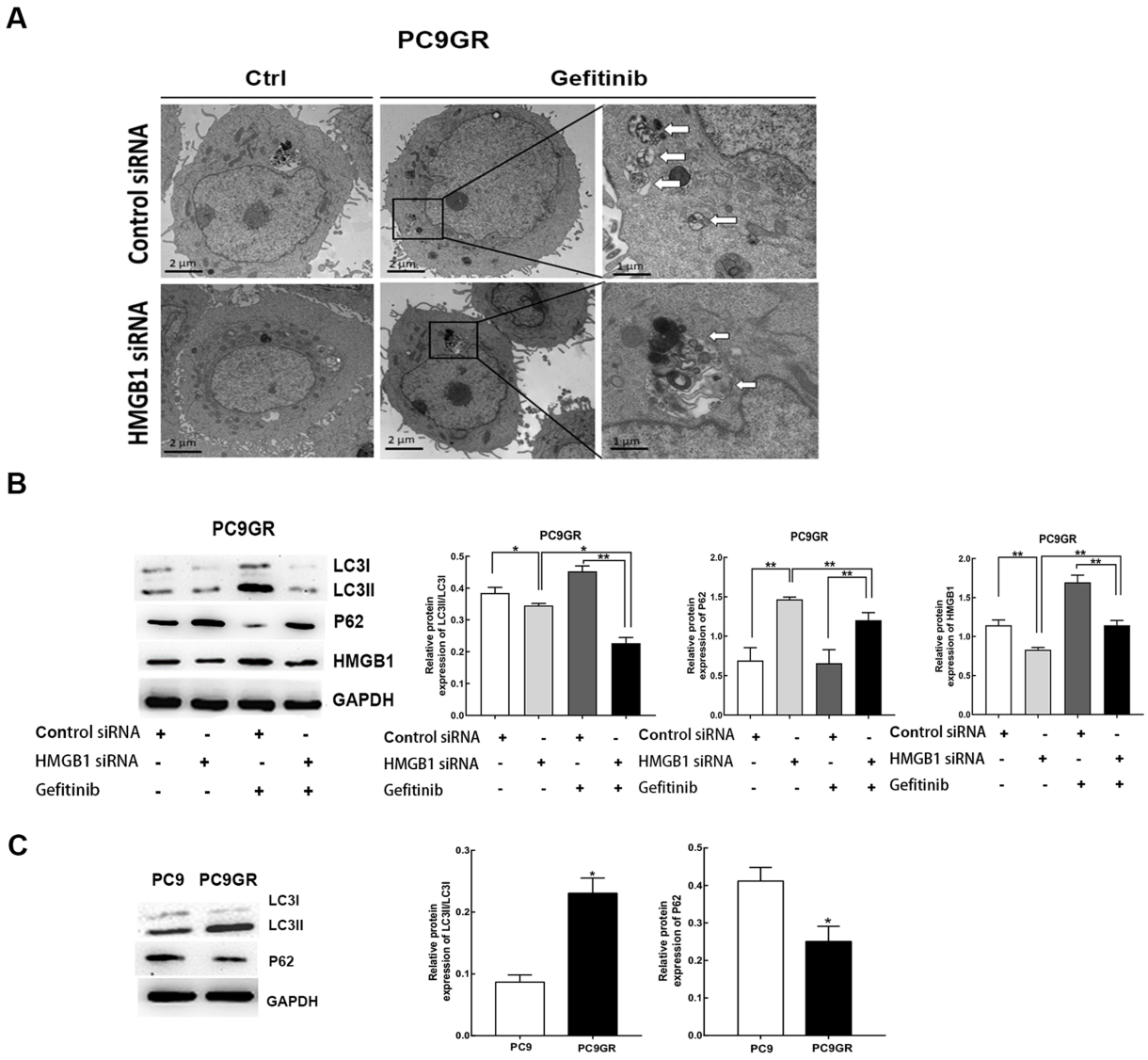

Figure 5. Gefitinib-induced upregulation of HMGB1 expression induces autophagy (A) Autophagosome formation was identified by TEM (indicated by the white arrow), and the enlarged image shows the characteristics of autophagosomes. As described in the Materials and Methods. (B) PC9GR cells were transfected with control or HMGB1 siRNA for 48 h and treated with or without gefitinib (5 μM) for 48 h. Protein levels of LC3II/LC3I and P62 were detected by western blot analysis. (C) Western blot analysis was used to detect LC3II/LC3I and P62 levels in PC9 and PC9GR cells. GAPDH was used as the loading control. $^{*}P < 0.05$, $^{**}P < 0.01$.

NSCLC. PC9GR cells were transfected with control shRNA or HMGB1-targeting shRNA and injected subcutaneously into groups of male nude mice to establish a mouse xenograft model. As shown in Figure 6, tumors formed in the sh-HMGB1 group were smaller and grew more slowly than those tumors formed in the control group (Figure 6A–D). qRT-PCR analysis of tumors excised from

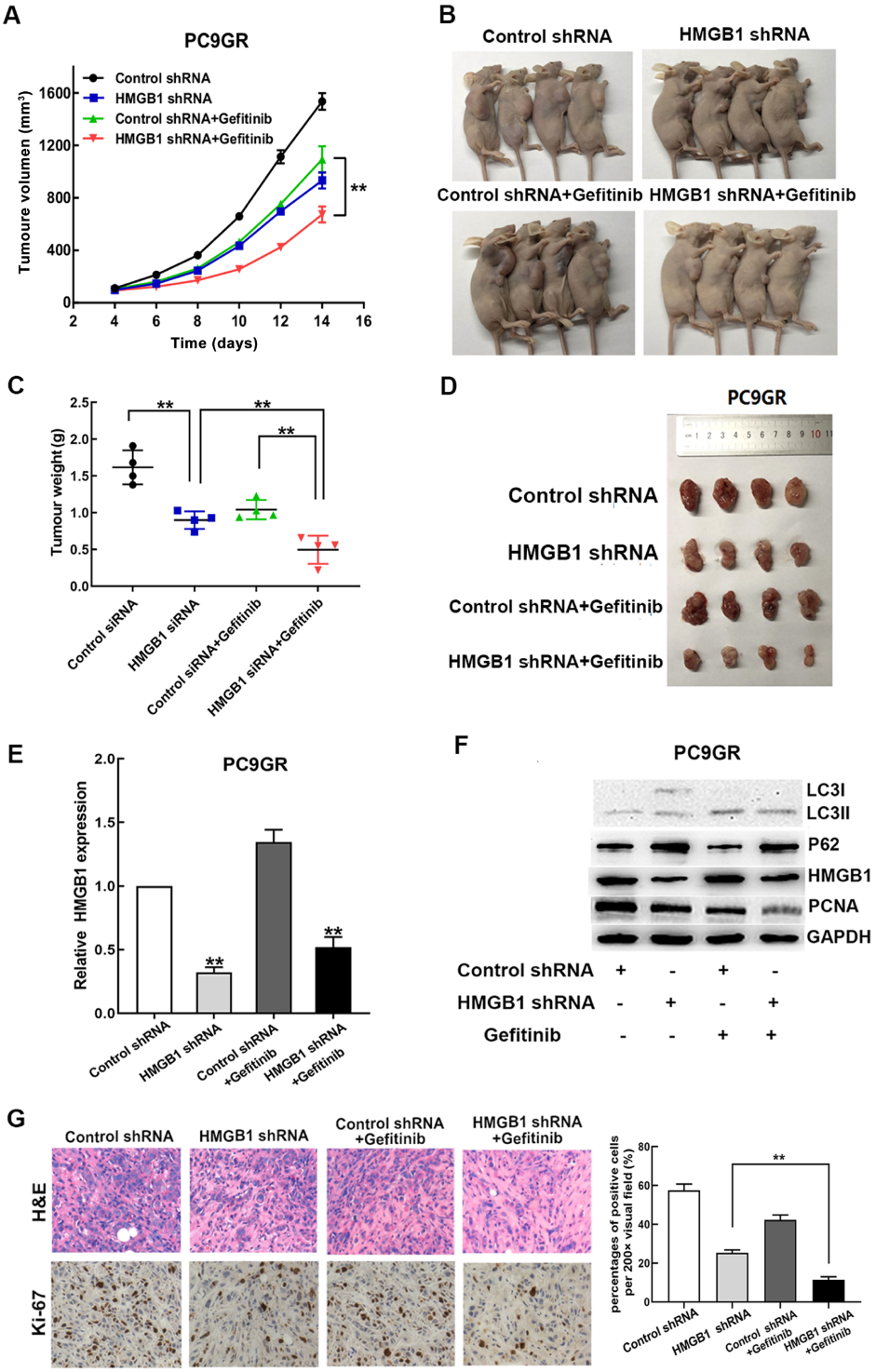

Figure 6. Inhibition of HMGB1 increases the sensitivity to gefitinib chemotherapy *in vivo* (A,B) Male nude mice were inoculated with 5 x 10^6 PC9GR tumor cells and treated with gefitinib (25 ml/kg) on day 4. Tumor growth was monitored for 14 days (n = 4). (C) Two weeks after gefitinib treatment, the tumor weight of mice were recorded. (D) Two weeks after gefitinib treatment, images of tumor xenografts were recorded. (E) HMGB1 expression level in two groups was determined by qRT-PCR. (F) The expressions of autophagy-related molecules, HMGB1 and PCNA, in PC9GR cells were measured by western blot analysis. GAPDH was used as the loading control. (G) Ki-67 protein levels in tumor tissues from control shRNA or HMGB1 shRNA PC9GR cells were determined by IHC staining. Data are presented as the mean ± SD from three independent experiments. *$P < 0.05$, **$P < 0.01$.

mice confirmed that HMGB1 mRNA level was lower in the sh-HMGB1 tumors compared with that in the control group (Figure 6E). Moreover, expression of autophagy-related proteins and HMGB1 were detected by western blot analysis, which is consistent with our previous results (Figure 6F). Finally, we examined the expression of the proliferation marker Ki-67 in tumor sections by immunohistochemical staining, and found that Ki-67 level was lower in the sh-HMGB1 group than in the control group (Figure 6G). These results confirmed that HMGB1 is an important regulator of gefitinib resistance in NSCLC *in vivo*.

Discussion

Increasing pieces of evidence have confirmed that gefitinib is an EGFR-targeting small molecule, and has been proposed as the first-line treatment for EGFR-mutant NSCLC patients. However, the emergence of acquired resistance is almost inevitable and the mechanism of EGFR-TKI acquired resistance is very complex. Recently, several studies have confirmed that mutations in EGFR are varied and closely related to the occurrence and development of drug resistance. The reported reasons for EGFR-TKI acquired resistance are EGFR 20 exon T790M mutation, MET amplification, HER2 amplification and phenotypic transformation of tumor cells [31,32], however, there is still a lack of reliable and effective interventions for EGFR-TKI resistance. Based on previous studies of our research group, we explored the mechanism of gefitinib resistance in non-small cell lung cancer [27–29]. In the present study, we demonstrated that elevated HMGB1 expression participates in the regulation of gefitinib resistance in NSCLC cells by inducing autophagy. Most notably, we found that gefitinib-resistant cells expressed higher level of HMGB1 and had higher basal autophagy rates compared with gefitinib-sensitive cells, and that HMGB1 level directly modulated autophagy activity *in vitro* and *in vivo*.

HMGB1 is present not only in the nucleus but also in the extracellular environment. Intracellular HMGB1 participates in DNA replication and transcription and in the maintenance of chromosomal structure and stability. HMGB1 overexpression correlates with multiple characteristics of malignant tumors, including unlimited replication potential, angiogenesis, and avoidance of apoptosis. Indeed, HMGB1 has been proved to be a successful therapeutic target in experimental models of diverse infectious and inflammatory diseases. Numerous strategies have been employed to restrain the activity and release of HMGB1 in anti-cancer therapeutics [33]. Although the expression level of HMGB1 is related to the resistance of different anti-cancer drugs [34–36], the relationship between HMGB1 and gefitinib resistance in NSCLC patients has never been investigated. We established a gefitinib-resistant cell line named PC9GR, which has been described and reported [28] to investigate the molecular mechanism of HMGB1-mediated gefitinib resistance. We demonstrated that elevated HMGB1 expression participates in the regulation of gefitinib resistance in NSCLC cells and tissues. In addition, we found that knockdown of HMGB1 expression could significantly inhibit the proliferation and migration, and increase the apoptosis of gefitinib-resistant cells. HMGB1 knockdown decreased the IC_{50} value in PC9GR cells. These results suggest that HMGB1 plays an important role in the process of gefitinib resistance in NSCLC cells, but the mechanism need to be further explored.

Autophagy is a complex multistep process and plays an important role in regulating cell homeostasis under physiological and pathological conditions. Aberrant autophagy function has been associated with resistance to several anticancer drugs. Cancer cells may respond to anticancer drug treatment by activating either cellular survival or death pathways. Many studies have shown that antitumor agents activate protective autophagy in NSCLC cells, causing acquired drug-resistance [37], and autophagy inhibitors have also been widely reported to overcome clinical EGFR-TKI resistance [38–40]. Our previous study supported an important role for HMGB1 in autophagy [26]. In this study, we observed changes in autophagy-related protein expression and autophagy flux in NSCLC cells after gefitinib treatment. Interestingly, basal level of LC3-II was higher in the gefitinib-resistant cell line than in the sensitive cell line. Our data confirmed that HMGB1 expression promotes autophagy, as reflected by an increase in LC3-I to LC3-II ratio and p62 degradation. The relative scarcity of autophagosomes in si-HMGB1-transfected PC9GR cells compared with control PC9GR cells, as observed by TEM, was one of the first clues that HMGB1 may be involved in regulating autophagy as a mechanism of gefitinib resistance. In this study, we demonstrated that HMGB1 induced autophagy and that knockdown of this protein partially restored gefitinib sensitivity in drug-resistant NSCLC cells. Inhibition of HMGB1 expression in NSCLC cells also significantly inhibited tumor growth in combination with gefitinib treatment. Our results thus identify a role for autophagy in gefitinib resistance of NSCLC cells, and suggest that autophagy inhibitors may help to overcome the resistance of NSCLC to molecular targeted drugs.

In summary, HMGB1 could promote gefitinib resistance through promoting the proliferation and inhibiting apoptosis of NSCLC cells by regulating autophagy process. Our findings suggest that HMGB1 and/or autophagy inhibitors may improve the efficacy of many cancer therapies and suppress the development of drug resistance.

Funding

This work was supported by the grants from the National Natural Science Foundation of China (Nos. 81672307 and 81871871 to Z.W.), the Key Research and Development Plan (Social development) of Science and Technology Department of Jiangsu Province (No. BE2019760 to Z.W.), the Medical Innovation Team Foundation of the Jiangsu Provincial Enhancement Health Project (No. CXTDA2017021 to Z.W.), and the Postgraduate Research & Practice Innovation Program of Jiangsu Province (No. KYCX19_1170 to Z.C.).

Conflict of Interest

The authors declare that they have no conflict of interest.

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