Acta Pharmaceutica Sinica B 2022;12(9):3639–3649

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Acta Pharmaceutica Sinica B

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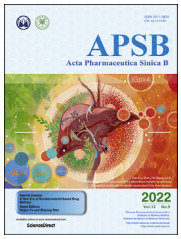

ORIGINAL ARTICLE

FOXO3 mutation predicting gefitinib-induced hepatotoxicity in NSCLC patients through regulation of autophagy

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Received 27 November 2021; received in revised form 13 January 2022; accepted 14 January 2022

KEY WORDS

Gefitinib;
Hepatotoxicity;
Pharmacometabolomic;
Pharmacokinetics;
Pharmacogenomics;
FOXO3;
Autophagy

Abstract Hepatotoxicity is a common side effect for patients treated with gefitinib, but the related pathogenesis is unclear and lacks effective predictor and management strategies. A multi-omics approach integrating pharmacometabolomics, pharmacokinetics and pharmacogenomics was employed in non-small cell lung cancer patients to identify the effective predictor for gefitinib-induced hepatotoxicity and explore optional therapy substitution. Here, we found that patients with rs4946935 AA, located in Forkhead Box O3 (*FOXO3*) which is a well-known autophagic regulator, had a higher risk of hepatotoxicity than those with the GA or GG variant (OR = 18.020, 95%CI = 2.473 to 459.1784, $P = 0.018$) in a gefitinib-concentration dependent pattern. Furthermore, functional experiments identified that rs4946935_A impaired the expression of *FOXO3* by inhibiting the promotor activity, increasing the

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Peer review under responsibility of Chinese Pharmaceutical Association and Institute of Materia Medica, Chinese Academy of Medical Sciences.

https://doi.org/10.1016/j.apsb.2022.02.006

threshold of autophagy initiation and inhibiting the autophagic activity which contributed to gefitinib-induced liver injury. In contrast, erlotinib-induced liver injury was independent on the variant and expression levels of *FOXO3*. This study reveals that *FOXO3* mutation, leading to autophagic imbalance, plays important role in gefitinib-induced hepatotoxicity, especially for patients with high concentration of gefitinib. In conclusion, *FOXO3* mutation is an effective predictor and erlotinib might be an appropriately and well-tolerated treatment option for patients carrying rs4946935 AA.

1. Introduction

The progression-free survival of non-small cell lung cancer (NSCLC) patients with epidermal growth factor receptor (EGFR)-activating mutation has been significantly prolonged due to the application of tyrosine kinase inhibitors (TKIs)[1,2], such as gefitinib. Gefitinib is widely used in NSCLC patients in China and other developing regions due to its definite efficacy, good availability and affordability. However, hepatotoxicity is a common side effect of gefitinib with a more than 50% occurrence in clinical reports[1,2]. According to the WJTOG3405 study[2], 27.6% patients suffering severe hepatotoxicity (grade $\geq$3) from gefitinib that necessitated cessation of treatment[3]. Meanwhile, the serum transaminase levels of patients with gefitinib-induced hepatotoxicity elevated again after gefitinib resumption[4], which may lead to dose reduction, treatment interruption and even treatment failure.

Hepatotoxicity is a major safety concern in gefitinib treatment; however, the mechanism of gefitinib-induced hepatotoxicity remains a relatively under-investigated area[5,6]. Usually, two aspects are involved in drug-induced hepatotoxicity: one is the direct cytotoxicity induced by xenobiotics or their reactive metabolites; the other is associated with indirect toxicity *via* secondary immune reactions[7]. Several non-clinical experiments have been conducted to investigate the possible mechanism of gefitinib-induced hepatotoxicity. It was found that gefitinib concentrations in liver was 10-fold higher than that in the circulation, which may cause direct cytotoxicity[8,9]. Meanwhile, reactive metabolites formation was also observed in the liver cells and microsomes[10,11]. However, there remains a paucity of exploration in the role of gefitinib concentration in hepatotoxicity and no metabolite profiling has been depicted in NSCLC patients[12–14]. Since several reports have demonstrated the association between single nucleotide polymorphisms (SNPs) in cytochrome P450 (CYP) and transporters and the susceptibility to gefitinib-induced hepatotoxicity[15,16], the exposure of gefitinib or its metabolites may contribute to gefitinib-induced hepatotoxicity. Therefore, quantification of gefitinib and its metabolites and analyzing the influence of them on the incidence and severity of hepatotoxicity is of great value in the investigation of predictors and mechanisms of gefitinib-related hepatotoxicity.

Meanwhile, around half of patients were suffered liver injury during treatment of gefitinib at recommended dosage and the time period of development of liver injury induced by gefitinib was 1–23 months[6,17], indicated that host factors play a vital role in gefitinib-induced liver injury. With the development of pharmacogenomics, it is known that genetic structure affects patients' predisposition to drug-induced toxicities. Therefore, genes directly or potentially involved in gefitinib-induced hepatotoxicity, such as enzymes and transporters involved in gefitinib metabolism and transportation, chemokines and factors in immune-mediated reaction, pathways and regulators leading to liver cell death *via* regulating autophagy, apoptosis and necrosis, are worth genotyping. Mutations in these genes may play pivotal role in the variability of hepatotoxicity.

Therefore, we comprehensively explored the main plasma metabolites of gefitinib in NSCLC patients using a targeted metabolomics approach and investigated their effects on the liver injury in this study. Meanwhile, in order to identify mechanistic genetic biomarkers for gefitinib-induced hepatotoxicity, 194 genetic variants involving transporters, metabolic enzymes, immunological and autophagy factors were analyzed in 180 NSCLC patients to speculate into the mechanisms under gefitinib-induced hepatotoxicity and provide more strategies to prevent or treat this adverse effect.

2. Materials and methods

2.1. Patients and study design

This study was initiated in 2013 (NCT01994057). From November 2013 to September 2018, a total of 180 NSCLC patients were enrolled in this study at Sun Yat-sen University Cancer Center (Guangzhou, China). All patients enrolled were above 18 years with EGFR activating mutation. All patients received gefitinib daily (250 mg) without any metabolism inhibitor or agonist. Patients with abnormal liver function before the treatment of gefitinib were excluded in this study. Two milliliters of peripheral blood were collected immediately after one month of gefitinib treatment. All samples were stored at −80 °C until analysis. Hepatotoxicity was recorded and graded according to the Common Terminology Criteria for Adverse Events 4.0[18]. The measurements of alanine transaminase (ALT), aspartate aminotransferase (AST), lactate dehydrogenase, total bilirubin, and alkaline phosphatase were performed in laboratory. The study was approved by the ethical committee of Sun Yat-sen University Cancer Center (Guangzhou, China). The informed consents were obtained from all patients enrolled in this study.

2.2. Cell line culture and reagents

LO2, a human fetal hepatocyte cell line[19], was widely used to assay the hepatotoxicity *in vitro*[20,21]. LO2 and SMCC7721 cell lines were cultured in RPMI 1640 medium (GIBCO) containing 10% fetal bovine serum at 37 °C under a humidified atmosphere with 5% CO_2. M523595 (M1, Toronto Research Chemicals), M537194 (G235, Toronto Research Chemicals), M387783 (G236, Toronto Research Chemicals) and M605211 (M2, Nayuansu, Shanghai, China), the main metabolites of gefitinib[22], were obtained from commercial companies. Gefitinib, erlotinib, hydroxychloroquine (HCQ) and Baf-A1 were provided by Selleck

Chemicals (Selleck). All the solvents for liquid chromatography were purchased from Tedia Company Inc (Fairfield, OH, USA).

2.3. Determination of gefitinib and its main metabolites in plasma samples of patients

Gefitinib and its main plasma metabolites were determined with liquid chromatography with tandem mass spectrometry[23]. In brief, gefitinib and its main metabolites were extracted from plasma with *tert*-butyl methyl ether in room temperature and separated on an X-Terra RP18 Column (50 mm × 2.1 mm, 3.5 μm, Waters) followed analysis in tandem mass spectrometry system.

2.4. DNA extraction and genotyping

DNA was extracted through TIANGEN Blood Genome Extraction Kit (TIANGEN, Beijing, China) based on the manufacturer's instructions. All 194 tag SNPs (Supporting Information Table S1), involved in transporters, metabolic enzymes, inflammatory, immunological and autophagy-related factors, were selected with HaploView 4.2 and analyzed by using a previously published Agena MassARRAY System technique (Agena, CA, USA)[24].

2.5. Plasmid construction and cell transfection

Stable knock-out of Forkhead Box O3 (*FOXO3*) was accomplished by clustered regulatory interspaced short palindromic repeats/CRISPR-associated protein 9 systems with a small guide RNA (Supporting Information Table S2). The overexpression of *FOXO3* (NM_201559) was constructed on pCDH1 plasmid. LO2 and SMCC7721 cells were transfected with Lipofectamine 3000 according to manufacturer's instructions (Invitrogen, BSN, USA).

2.6. Cell survival assay

LO2 and SMC7721 cells were used to assess direct cytotoxicity of gefitinib or erlotinib *in vitro* with Cell Counting Kit-8 (CCK-8; Invigentech, CA, USA) according to manufacturer's instructions. Briefly, 5×10^3 cells/well were seeded into 96-well plates (Corning, NY, USA) and cultured overnight. The final concentrations of gefitinib were 3.125, 6.25, 12.5, 25, 50 and 100 μmol/L, respectively. The cell viability was assayed by CCK-8 based on manufacturer's instructions after incubation for 72 h.

2.7. Western blot

Total protein was extracted from cells by using a RIPA Kit (Beyotime, China), separated on 10% SDS-PAGE gels and transferred to PVDF membrane (Millipore, Bedford, MA, USA) according to standard procedures. Anti-GAPDH (WB, 1:10000, ab181602) was purchased from Abcam (Cambridge, USA). Anti-FOXO3 (WB, 1:1000, 10849-1-AP) was obtained from Proteintech (Rosemont, USA). Anti-LC3B (WB, 1:1000, L7543) was purchased from Sigma−Aldrich (Missouri, USA).

2.8. Quantitative RT-PCR

Total RNA was isolated by using Trizol Reagent (Thermo Fisher, USA) from cells or patients' samples according to the manufacturer's instructions. The total RNA was reversely transcript into cDNA with the PrimeScript RT Reagent Kit (RR036A, Takara, Japan) and followed quantitative RT-PCR by using SYBR Green Master Mix (RR820A, Takara, Japan) in 7500 apparatus (Applied Biosystems, USA). The primers for RT-PCR were listed in Table S2.

2.9. Luciferase reporter assay

We generated the reporter plasmids by inserting a 135-bp region into the GV272 promoter vector (GENCHEM, Shanghai, China) for FOXO3_rs4946935_G of the major allele and FOXO3_rs4946935_A of the minor allele (Table S1). The activity of luciferase was detected by the Dual Luciferase Reporter Assay System (Promega, WI, USA). The expression efficiency was analyzed by ratios of firefly luciferase activity value and *Renilla* luciferase activity value.

2.10. Transmission electron microscopy

For transmission electron microcopy, cells were fixed with 2.5% glutaraldehyde (pH 7.3) in 4 °C for 12 h. Cell suspension was centrifuged in 4 °C for 5 min and followed resuspension with 2.5% glutaraldehyde. The cells were stained with plumbous nitrate and uranyl acetate before examined under a JEM-1230 Transmission Electron Microscope (JEOL, Tokyo, Japan).

2.11. Statistical analysis

All statistical analyses were performed in R 3.6.0 software and GraphPad 7.0 (CA, USA) with appropriate statistical methods. The association between gefitinib/metabolites and hepatotoxicity/clinical confounders were analyzed in ggpur 0.4.0 and visualized with ggplot2 3.3.3. The association between SNPs and hepatotoxicity were analyzed by SNPassoc 2.0.2. The multivariate logistic regression, 95% confidence intervals (CIs) and odds ratios (ORs) were conducted with packages Glmnet 3.0.2. All reported *P* values are two-sided, and no adjustment has been made for multiple comparisons.

3. Results

3.1. Patients' characteristics

A total of 180 patients were enrolled between November 2013 and September 2018 in Sun Yat-sen University Cancer Center. The characteristics of enrolled patients at baseline were shown in Table 1. The 180 subjects had a median age of 57 years old, including 111 (61.7%) females and 69 (38.3%) males. Multiple subjects developed gefitinib-induced hepatotoxicity. Cumulatively, 87 patients (48%) suffered from at least grade 1 hepatotoxicity and 45 patients (25%) developed ≥2 grade hepatotoxicity (Fig. 1A). No statistical significance was found between gefitinib-induced hepatotoxicity and clinical confounders (Supporting Information Table S3).

3.2. Association between hepatotoxicity and concentrations of gefitinib/metabolites

The concentrations of gefitinib and its four metabolites were all available for 180 patients (Table 1). As is shown in Fig. 1B−F, no analyte was associated with gefitinib-induced hepatotoxicity,

Table 1 Characteristic information of patients.

Variables	No. of patients (%)
	Gefitinib (n = 180)
Median age (range), year	57 (28−88)
Median height (range), cm	162.5 (150.0−181.0)
Median weight (range), kg	60.7 (38−94)
Median BSA (range), m^2	1.68 (1.33−2.21)
Sex	
Male	69 (38.3)
Female	111 (61.7)
Smoking	
Never	150 (83.3)
Ever	30 (16.7)
Stages	
IIIb	10 (5.6)
IV	170 (94.4)
EGFR mutation types	
19 del	102 (56.7)
21 L858R	72 (40.0)
Other	6 (3.3)
Gefitinib, mean ± SD, ng/mL	246.286 ± 141.988
M1*, mean ± SD, ng/mL	150.431 ± 122.246
M2*, mean ± SD, ng/mL	12.323 ± 7.341
G235*, mean ± SD, ng/mL	6.510 ± 4.765
G236*, mean ± SD, ng/mL	1.362 ± 0.884

*The metabolites of gefitinib.

indicating that in the general subjects, concentration monitoring cannot predict hepatotoxicity induced by gefitinib.

3.3. *FOXO3 variant (G>A rs4946935) was an independent risk factor for gefitinib-induced hepatotoxicity in a gefitinib concentration dependent pattern*

Among 194 SNPs, only G>A rs4711998 in *IL17*, C>T rs4795896 in *CCL11*, G>A rs4946935 in *FOXO3* and G>A rs12722604 in *IL2RA* were associated with gefitinib-induced hepatotoxicity by SNPassoc 2.0.2 with WGassociation analysis (Fig. 2A). For G>A rs4711998 (Fig. 2B), patients with GG genotype (76.2%) had a higher risk of gefitinib-induced hepatotoxicity than those with GA (43.1%) and AA (46.9%) genotypes (P = 0.024). For C>T rs4795896 (Fig. 2C), the hepatotoxicity in CC carriers (65.3%) was significantly higher than those in TC (42.1%) and CC carriers (41.7%) (P = 0.017). The percentage of gefitinib-induced hepatotoxicity were 51.6%, 38.7% and 90% for rs4946935 GG, GA and AA carriers (P = 0.0038), respectively (Fig. 2D). For G>A rs12722604 (Fig. 2E), patients with AG genotype (58.5%) had a higher risk of gefitinib-induced hepatotoxicity than those with GG (44.4%) and AA (45.5%) genotypes (P = 0.042).

To identify the risk factor for gefitinib-induced hepatotoxicity, multivariate logistic regression analysis was accomplished with clinical confounders, including sex, BSA, stages, *EGFR* mutation types and smoking status. As is shown in Fig. 2F, only G>A rs4946935 was significantly associated with gefitinib-induced liver injury. The AA carriers were more prone to develop hepatotoxicity than GG carriers, with the OR of 18.020 (95% CI = 2.473 to 459.184, P = 0.018) by multivariate logistic analysis.

Furthermore, plasma AST/ALT level was positively and significantly correlated with gefitinib concentration in rs4946935 AA carriers (Fig. 2G, Supporting Information Fig. S1C and S1D) but not in general patients (Fig. S1A and S1B), providing the rational of dose reduction in patients carrying rs4946935 AA genotype. Taken together, *FOXO3* variant (G>A rs4946935) was an potential and independent risk factor for gefitinib-induced hepatotoxicity probably in a gefitinib concentration dependent pattern.

3.4. *rs4946935_A impaired the expression of FOXO3*

G>A rs4946935 was one of the tagSNPs (Fig. 3A) selected by HaploView 4.2 and located in intron 3 of *FOXO3* on chromosome 6 (Fig. 3B). To study whether rs4946935 affected the mRNA expressions of *FOXO3*, the associations was analyzed between the expression of *FOXO3* and rs4946935 by expression quantitative trait loci according to Genotype Tissue Expression (GTEx) database. We found that rs4946935_A significantly impaired *FOXO3* mRNA expression in human spleen and brain caudate (Fig. 3C). To further address the relationship between rs4946935 and *FOXO3* mRNA levels in NSCLC, we have tested whether the variant have an impact on the levels of *FOXO3* mRNA in patients. The results showed that *FOXO3* levels in rs4946935 AA carriers were significantly lower than those in GA and GG carriers (Fig. 3D), suggesting that rs4946935 might be characterized as a functional variant. Since rs4946935 was located in the third intron of *FOXO3*, we hypothesized that the variant impaired expression of *FOXO3 via* suppressing the promotor activity. To investigate whether rs4946935_A has an impact on the promoter activity of *FOXO3* in an allele-specific manner, relative luciferase activities of the rs4946935_A and rs4946935_G of *FOXO3* were detected in LO2 cells. A significant reduction of luciferase activity was observed for rs4946935_A of *FOXO3* compared to that for rs4946935_G (Fig. 3E, P = 0.0012), indicating that rs4946935_A impaired the expression of *FOXO3* by inhibiting the promotor activity of *FOXO3*.

3.5. *Overexpression of FOXO3 protected hepatocytes from cytotoxicity of gefitinib*

To investigate the role of FOXO3 in gefitinib-induced hepatotoxicity, we conduct cell survival assay in *FOXO3* knock out (KO) and -overexpression (OE) LO2 and SMCC7721 cells treated with gefitinib. The results showed that overexpression of FOXO3 in LO2 cells significantly increased the half maximal inhibitory concentration (IC_{50}) of gefitinib (103.9 μmol/L, P < 0.0001) while knockout of *FOXO3* decreased (21.49 μmol/L, P = 0.0001; Fig. 3F−H), which was consistent with those in SMCC7721 cells (Supporting Information Fig. S2). Collectively, higher expression level of FOXO3 could be protective in hepatotoxicity under gefitinib culture.

3.6. *Gefitinib-induced hepatotoxicity was facilitated by FOXO3 mutation via autophagy inhibition*

To interrogate the downstream effects induced by FOXO3 in cells, the associations were analyzed between the expression of FOXO3 and autophagy-related genes in 226 liver samples according to GTEx database. As shown in Fig. 4A, FOXO3 was significantly correlated to the expressions of *ATG3*, *ATG4A*, *ATG5*, *ATG7*, *ATG10*, *ATG12*, *ATG14*, *ATG16L1* and *MAPLC3B*, implying that FOXO3 played a pivotal role on regulating autophagy in liver.

We further compared the expression of autophagy-related genes between *FOXO3*-KO and -OE in LO2 cells treated with

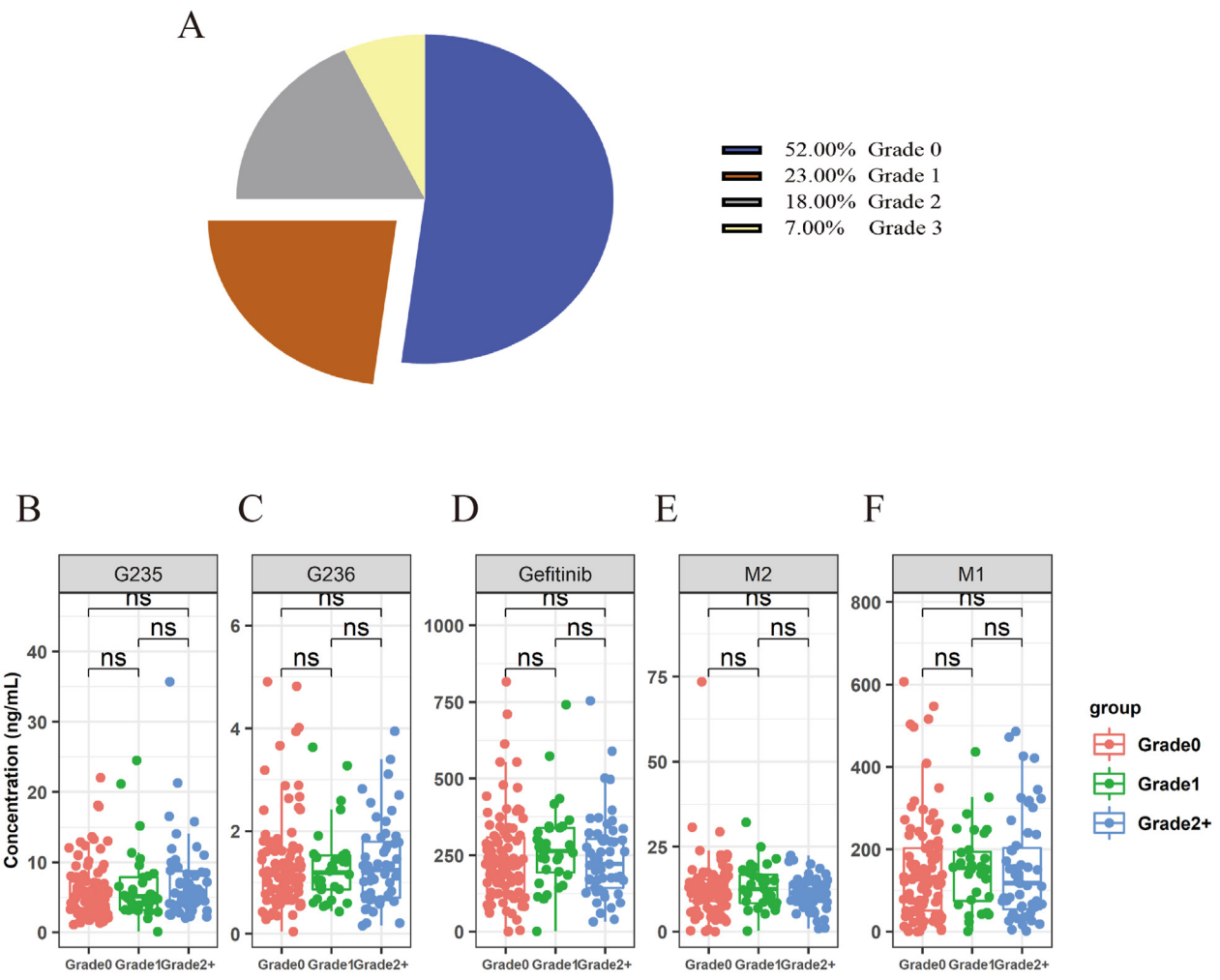

Figure 1 The concentration of gefitinib/metabolites was not associated with gefitinib-induced hepatotoxicity in the general subjects ($n = 180$). (A) The grades of hepatotoxicity induced by gefitinib in NSCLC patients; (B−F) Neither the concentration of gefitinib nor the metabolites was associated with hepatotoxicity in the general subjects ($n = 180$). ns: no significant.

gefitinib. As expected, the expressions of autophagy-related genes were up-regulated in cells with FOXO3 overexpression, while opposite expression pattern was observed in FOXO3 knockout cells ($P < 0.05$, Fig. 4B). Meanwhile, LC3-II/I expression in *FOXO3*-KO and -OE cells were measured as well. Our results showed that LC3-II/I expression level in *FOXO3*-KO was lower than that in -OE and -WT cells treated with gefitinib (Fig. 4C). In addition, overexpression of FOXO3 increased activity of autophagy and decreased the threshold of autophagy initiation as indicated by LC3-II/I expression level (Fig. 4C). Furthermore, transmission electron microcopy analysis revealed that the drop of autophagic activity in *FOXO3*-KO cells was underlined by a reduction of the number of autophagosomes after incubation with gefitinib (Fig. 4D).

To study whether inhibition of autophagy affected the cytotoxicity of gefitinib in *FOXO3*-OE cells, we co-incubated HCQ or Baf-A1 and gefitinib in *FOXO3*-OE cells. By CCK8 assays, we found that inhibition of autophagy significantly suppressed proliferation of *FOXO3*-OE hepatocytes under incubation of gefitinib (Fig. 4E), and increased the cytotoxicity of gefitinib (Fig. 4F). Collectively, rs4946935_A impaired the expression of *FOXO3*, thus contributing to gefitinib-induced hepatotoxicity through autophagy inhibition.

3.7. G>A rs4946935 correlated with the expression of autophagy-related genes

To investigate whether G>A rs4946935 is correlated with the expression of autophagy-related genes in an allele specific manner, we carried out a correlation analysis between G>A rs4946935 and the autophagy-related genes mentioned above using data derived from GTEx database. Accordingly, results showed that the expression of autophagy-related genes in rs4946935_A carriers, except for *ATG5* and *ATG7*, were lower compared to those in rs4946935_G carriers in human liver tissue (Supporting Information Fig. S3), indicating that G>A rs4946935 correlated with the expression of autophagy-related genes in liver. Taken together, these results implied that rs4946935_A induced down-regulation of autophagic activity by impairing expression of *FOXO3*.

3.8. G>A rs4946935 was disassociated with erlotinib-induced hepatotoxicity

As mentioned above, patients with gefitinib-induced hepatotoxicity may lead to treatment interruption or even treatment failure. Even though erlotinib itself have the potential to cause liver injury,

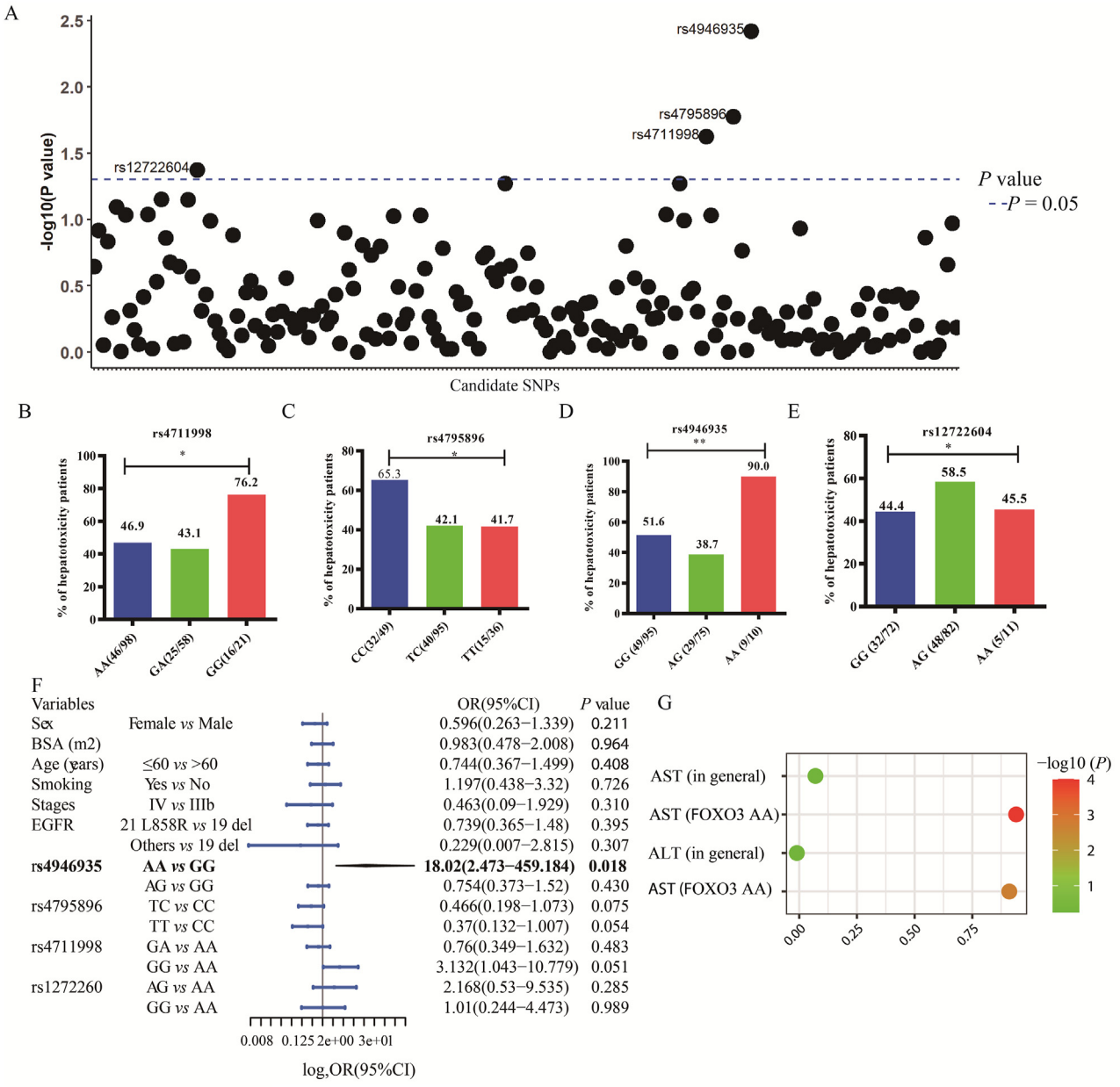

Figure 2 G>A rs4946935 was associated with gefitinib-induced hepatotoxicity. (A) Among 194 SNPs, only G>A rs4711998 in *IL17*, C>T rs4795896 in *CCL11*, G>A rs4946935 in *FOXO3* and G>A rs12722604 in *IL2RA* were associated with gefitinib-induced hepatotoxicity; (B) G>A rs4711998, located in *IL17A*, was associated with gefitinib-induced hepatotoxicity; (C) C>T rs4795896, located in *CCL11*, was associated with gefitinib-induced hepatotoxicity; (D) G>A rs4946935, located in *FOXO3*, was correlated with gefitinib-induced hepatotoxicity; (E) G>A rs1272260, located in *IL2RA*, was correlated with gefitinib-induced hepatotoxicity; (F) G>A rs4946935, located in *FOXO3*, was significantly associated with gefitinib-induced hepatotoxicity by multivariate logistic regression; (G) Plasma AST/ALT level was significantly correlated with the concentration of gefitinib in *FOXO3* AA carriers. *$P < 0.05$; **$P < 0.01$.

previous case reports and clinical practice revealed that erlotinib is a suitable treatment substitution for patients who are intolerant to gefitinib-induced liver injury[3,25], indicating that gefitinib and erlotinib do not share the same mechanism of liver injury. Therefore, we sought to explore whether G>A rs4946935 was associated with erlotinib-induced liver injury. Under appropriate inclusion and exclusion criteria presented in Supporting Information, 22 NSCLC patients treated with erlotinib were enrolled. Among them, 6 patients (27.3%) suffered at least grade 1 hepatotoxicity. And accordingly, G>A rs4946935 was not associated with erlotinib-induced hepatotoxicity (Supporting Information Fig. S4A) and erlotinib-induced liver injury was independent on the levels of FOXO3 *in vitro* (Fig. S4B), implying that rs4946935, to some extent, specific to gefitinib-induced hepatotoxicity. And the mechanism of erlotinib-induced liver injury might be different from that of gefitinib. To further investigate the role of FOXO3 in erlotinib-induced hepatotoxicity, we detected the effect of erlotinib on autophagy in LO2 cells with or

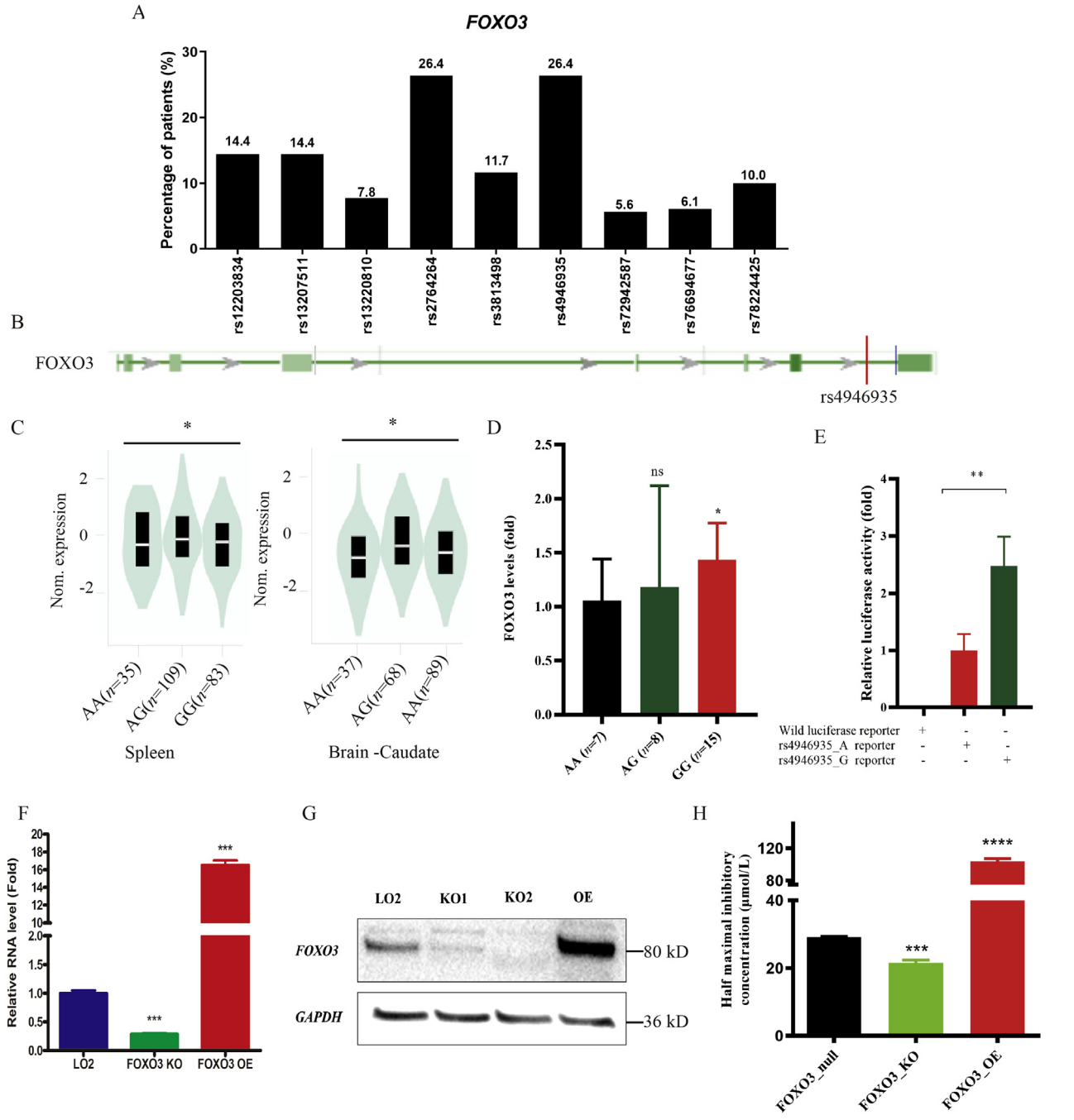

Figure 3 rs4946935_A impaired the expression of *FOXO3*. (A) All tag SNPs of *FOXO3* in HCB; (B) G>A rs4946935 was located in intron 3 of *FOXO3* on Chromosome 6; (C) *FOXO3* rs4946935 was correlated to expression levels of FOXO3 in human spleen and brain caudate according to GTEx database; (D) *FOXO3* rs4946935 was correlated to expression levels of FOXO3 in NSCLC patients; (E) Luciferase activity of rs4946935_ A and _G of *FOXO3* reporter vectors in LO2 cells; (F–H) Overexpression of *FOXO3* significantly increased the IC_{50} of gefitinib while knockout of *FOXO3* decreased in LO2 cells. Data represent mean ± SD of three or more independent experiments; $^{*}P < 0.05$; $^{**}P < 0.01$; $^{***}P < 0.001$; $^{****}P < 0.0001$; ns: no significance.

without *FOXO3*-KO or -OE. We found that the effects of erlotinib and gefitinib on autophagy were similar in LO2 cells (Fig. S4C), suggesting that the role of autophagy in TKIs-induced liver injury was difference. Collectively, our results are consistent with the previous clinical reports and erlotinib could be an appropriately and well-tolerated treatment option for patients for whom carrying rs4946935 AA.

4. Discussion

Gefitinib-induced hepatotoxicity sometimes leads to treatment failure and unnecessary medical costs in NSCLC patients with *EGFR* sensitive mutations. Up to date, the mechanisms behind gefitinib-induced hepatotoxicity is still unclear. Here, we performed pharmacometabolomic and pharmacogenomic

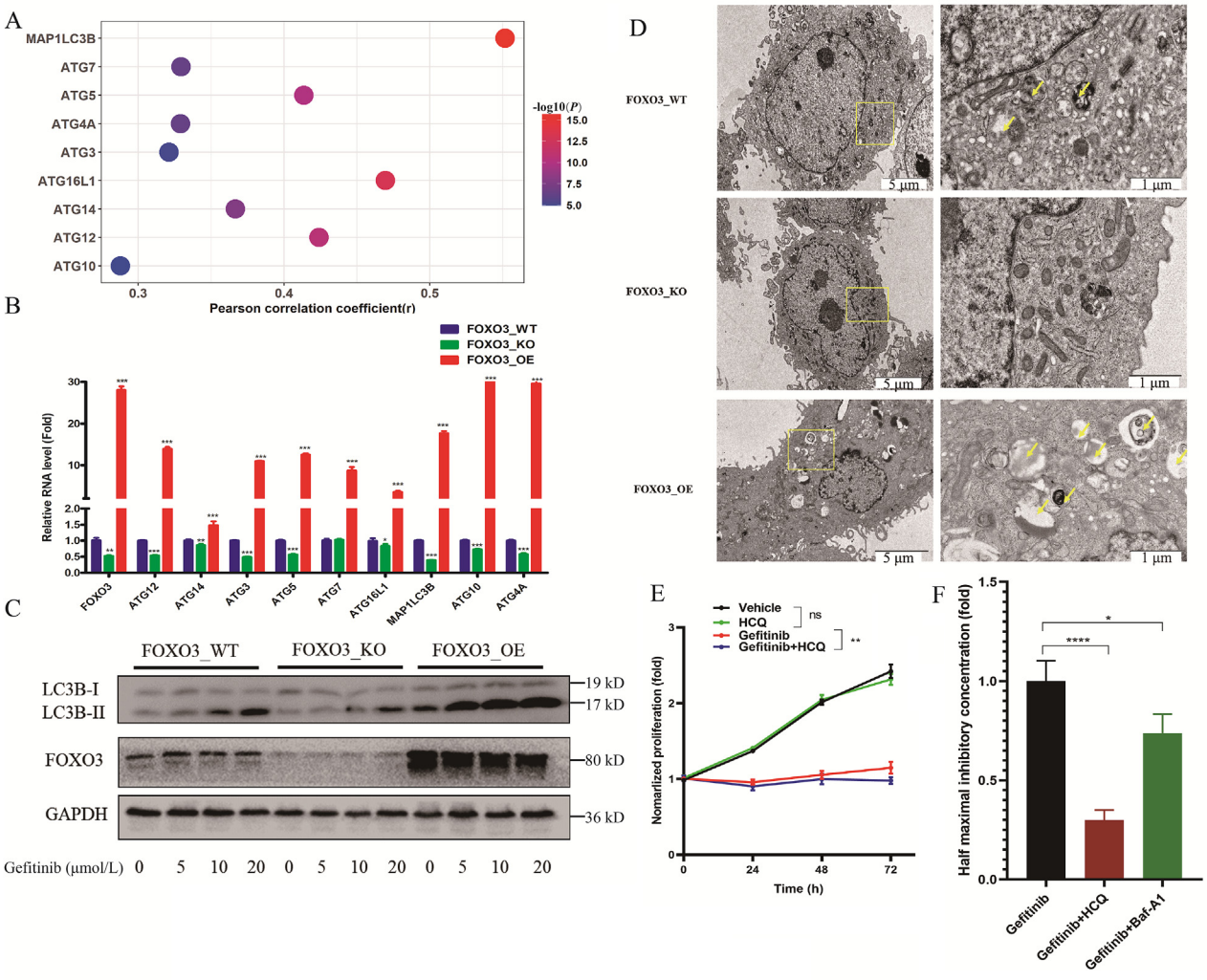

Figure 4 gefitinib-induced hepatotoxicity was *FOXO3*-dependent by inhibiting autophagy. (A) The expression of FOXO3 was correlated with autophagy-related genes in liver tissue according to GTEx dataset; (B) FOXO3 directly regulates the expression of *ATG3*, *ATG4A*, *ATG5*, *ATG7*, *ATG10*, *ATG12*, *ATG14*, *ATG16L1*, and *MAP1LC3B* after treatment with gefitinib in LO2 cells; (C) Overexpression of *FOXO3* decreased the threshold of autophagy initiation as indicated by LC3-II/I expression level; (D) Electron micrographs of *FOXO3*-knock out and -overexpression LO2 cells under incubation of gefitinib; (E) Inhibition of autophagy significantly suppressed proliferation of FOXO3 overexpression hepatocytes under incubation of gefitinib; (F) Inhibition of autophagy significantly increased the cytotoxicity of gefitinib in FOXO3 overexpression hepatocytes. Data represent mean ± SD of three or more independent experiments; $^{*}P < 0.05$; $^{**}P < 0.01$; $^{***}P < 0.001$; $^{****}P < 0.0001$; ns: no significance.

investigations to explore the possible clue and found that the mutation (rs4946935, G>A) in *FOXO3*, an autophagic regulator, was significantly correlated with gefitinib-induced hepatotoxicity. This variant significantly impaired the expression of FOXO3 and facilitated gefitinib-induced hepatotoxicity *via* autophagy inhibition which was verified *in vitro*. Meanwhile, in FOXO3 rs4946935 AA carriers, the plasma level of AST/ALT displayed a gefitinib-concentration dependent pattern, indicating that a reduced dosage of gefitinib may result in safe and successful control in rs4946935 AA carriers. Furthermore, consistent with clinical practice, rs4946935 was not a common predictor in gefitinib- and erlotinib-induced hepatotoxicity validated in another 22 NSCLC patients.

Whether the hepatic toxicity is gefitinib/its metabolites concentration-dependent is still unclear. Although several preclinical studies found that gefitinib-induced hepatotoxicity is direct hepatocyte cytotoxicity and reactive metabolites formation in the liver cells and microsomes have been found[9,11], the relationship between concentration and effect was inconsistent with those in clinical investigations[12–14]. Additionally, several studies found that common SNPs in *CYP3A4*, *CYP2D6*, *ABCB1* and *ABCG2* did not affect hepatotoxicity[26,27], implying that exposure of gefitinib/its metabolites did not influence the toxicity. In this study, no correlation between the concentrations of gefitinib and its metabolites and hepatotoxicity were found in general sample, but in patients carrying *FOXO3* rs4946935 AA, the plasma levels of AST and ALT were gefitinib-concentration dependent. In contrast, reduced dosing of gefitinib resulted in safe and successful control in a patient who once developed hepatotoxicity[28]. All these data indicated that reduced dosing of gefitinib might result a safe and successful control in rs4946935 AA carriers.

Autophagy is a cell defense process against xenobiotics and universally participates in drug-induced hepatotoxicity[29−32]. Autophagy protects hepatocytes from cytotoxicity through clearing reactive metabolites protein adducts[33], eliminating the damaged mitochondria[34] and maintaining the turnover of endoplasmic reticulum in liver cells[35]. FOXO3, an autophagic regulator serving as a surveillance mechanism, detects and corrects autophagy flux disruptions and plays a pivotal role in multiple cell self-defense process in skeletal muscles[36] and bones[37], especially in liver[38−40]. Ni et al.[41] found that FOXO3 mediated liver cell self-defense by up-regulating autophagy-related genes expression, including *ATG5* and *ATG6* (Beclin 1), protecting hepatocytes from alcohol-induced steatosis and liver injury in mice. In our study, the protective effect of FOXO3 was verified in *FOXO3*-OE LO2 and SMCC7721 cells treated with gefitinib. Knockout of *FOXO3* decreased autophagic activity and the number of autophagosomes and facilitated cytotoxicity while overexpression of FOXO3 strongly upregulated the expression of autophagy-related genes, including *ATG3*, *ATG4A*, *ATG5*, *ATG7*, *ATG10*, *ATG12*, *ATG14*, *ATG16L1* and *MAPLC3B*. In mammalian cells, *ATG7* and *ATG10* were activated once autophagy was upregulated, which initiated the formation of the ATG16L1−ATG5−ATG12 elongation complex[42]. ATG7, ATG4 and ATG3 participated in the cleavage and lipidation of LC3 to generate the LC3-I and then LC3-II[42], which facilitated the forming phagophore. In this study, we found that *FOXO3* rs4946935AA was correlated with lower expression of some ATG factors, such as ATG10, which is a reflective of a relatively lower autophagic activity level compared to GG carriers. Since cells with deficient autophagy, are primed to undergo apoptosis[43], *FOXO3* rs4946935 AA could facilitate gefitinib-induced hepatocyte cell death *via* autophagy inhibition and apoptosis. Notably, among the patients enrolled, a considerable amount of rs4946935 GA and GG carriers suffered hepatotoxicity from gefitinib as well, suggesting other factors involved in gefitinib-induced liver injury besides *FOXO3* rs4946935 AA. Collectively, rs4946935_A was a risk factor of gefitinib-induced hepatotoxicity through inhibiting FOXO3-dependent autophagy.

We and Luo et al.[20] back-to-back independently found autophagy was involved in gefitinib-induced hepatotoxicity. Luo et al. found that PLK1, an autophagic regulator, facilitated gefitinib-induced hepatotoxicity by increasing the autophagic activity. However, in their experiment, no alteration of FOXO3 level was found in LO2 cells after gefitinib incubation. Suspected reason behind this phenomenon is that, in Luo et al.'s study, samples were collected 24 h after incubation of gefitinib[20], while FOXO3 expression change and regulation may happen within 24 h. Since when basal autophagy is inhibited, like patients carrying FOXO3 deficit mutation, FOXO3, at the center of a homeostatic feedback loop, poises cells for suppression of autophagy following cytokine deprivation[43]. In addition, our recent results showed that the variants in PLK1 were not associated with liver injury induced by gefitinib in NSCLC patients (Supporting Information Fig. S5). Certainly, our findings need more prospective clinical trials to verify.

Indeed, hepatotoxicity was the main reason of drug switching in EGFR−TKI treatments[44]. In previous clinical case reports, several case studies suggested that erlotinib was a well-tolerated and effective alternative treatment option for patients suffered from gefitinib-induced hepatotoxicity[3,25], implied that the mechanisms of hepatotoxicity induced by gefitinib/erlotinib might be different. In the study, we identified G>A rs4946935 as a specific indicator only for gefitinib-induced but not for erlotinib-induced hepatotoxicity. And erlotinib-induced liver injury was independent on the levels of FOXO3 *in vitro* as well, partially explaining the clinical puzzle that erlotinib can be served as a substitute for those who are intolerant to gefitinib even though both of them target EGFR. The differences of the chemical structure or the metabolic pathways of gefitinib and erlotinib may be the one of possible reasons of these differences between gefitinib- and erlotinib-induced hepatotoxicity. For example, erlotinib was identified as an inhibitor to CYP3A4 and CYP3A5 in a time- and concentration-dependent manner *via* the glutathione-reactive metabolites[45], while gefitinib inhibited BCRP and P-gp[46]. Although inhibition of *EGFR* signaling induces autophagy in tumor cells[47], erlotinib[48] stimulated mitochondrial-depended apoptosis and necrosis in liver cells, which was not observed in hepatocyte treated with gefitinib through transmission electron microcopy analysis. Collectively, because they do not share a common mechanism in drug-induced hepatotoxicity, erlotinib might be an appropriate option for patients with rs4946935 AA who discontinued gefitinib treatment due to hepatotoxicity.

5. Conclusions

This study is the largest clinical cohort to investigate the predictors and mechanisms of gefitinib-induced hepatotoxicity and to explore optional therapy substitution with a multi-omics approach. Our results indicate that the variation of *FOXO3* is a potential and mechanistic biomarker for gefitinib-induced hepatotoxicity through its regulation of autophagy. Erlotinib rather than gefitinib might be a rational treatment option for patients carrying rs4946935 AA. Hopefully, through considering genotype profile of patients, personalized strategies could be adopted to prevent gefitinib-induced hepatotoxicity.

Acknowledgments

This work was supported by the National Natural Science Foundation of China (Grant Nos. 81973398, 81473283, 81730103, 81573507 and 82020108031), The National Key Research and Development Program (Grant Nos. 2017YFC0909300 and 2016YFC0905000, China), Guangdong Provincial Key Laboratory of Construction Foundation (Grant No. 2017B030314030, China), Science and Technology Program of Guangzhou (201607020031, China), National Engineering and Technology Research Center for New drug Druggability Evaluation (Seed Program of Guangdong Province (No. 2017B090903004, China), the 111 project (Grant: B16047, China), China Postdoctoral Science Foundation (Grant Nos. 2019M66324, 2020M683140 and 2020M683139). The authors are grateful to Kornel Tomczyk (Faculty of Foreign Language Education, Sun Yat-sen University, Guangzhou, China) and Keyue Wang (Faculty of life science and medicine, King's College of London, UK) for their help with the language review of this article. We would also like to thank the patients who gracefully donated their samples and time to this project.

Author contributions

Data curation, Yan Huang and Wei Feng; Formal analysis, Shu Liu and Wenfeng Fang; Funding acquisition, Min Huang and Li Zhang; Methodology, Shaoxing Guan and Xi Chen; Project administration, Min Huang and Li Zhang; Software, Wei Zhuang, Youhao Chen and Yunpeng Yang; Supervision, Xueding Wang; Validation, Shaoxing Guan and Heng Liang; Visualization,

Hongyun Zhao, Guohui Wan; Writing—original draft, Shaoxing Guan and Xi Chen; Writing—review & editing, Shaoxing guan, Xiaoxu Zhang, Fei wang, Qibiao Su, and Xueding Wang.

Conflicts of interest

The authors report no conflicts of interest, financial or otherwise.

Appendix A. Supporting information

Supporting data to this article can be found online at https://doi.org/10.1016/j.apsb.2022.02.006.

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