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Original Article

Gefitinib, an EGFR inhibitor, prevents liver fibrosis development of mice

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Abstract: Liver fibrosis, a serious threat to human health, still lacks of effective anti-fibrosis treatment. Studies have found that EGF plays an important role in liver fibrosis. The purpose of this study was to investigate the effect of Gifitinib, an EGFR inhibitor, on liver fibrosis of mice. Experiment was divided into three groups, the control group, the liver fibrosis group and Gifitinib intervention group. The liver fibrosis model was induced by intraperitoneal injection with carbon tetrachloride (CCl4) three times per week for 8 weeks in liver fibrosis group and Gifitinib intervention group. The mice in Gifitinib intervention group were intraperitoneal injection of Gifitinib after six weeks injection of CCl4. Meanwhile, mice in liver fibrosis group were only intraperitoneal injection with equal volume of solvent. Masson staining was applied to observe the liver histology change. The expression of mRNA and protein were assessed by PCR and western blot. Previously, we found that serum levers of ALT, AST in Gifitinib intervention group were lower than that in liver fibrosis group; Alb levels were higher than that in liver fibrosis group. The protein and mRNA levers of TGF-β1 and α-SMA were gradually increased along with the progress of liver fibrosis and obviously down-regulated by Gifitinib intervention as compared to the liver fibrosis group.

Keywords: Carbon tetrachloride, liver fibrosis, Gifitinib, epidermal growth factor receptor

Introduction

Liver fibrosis is a tissue repair response for a variety of chronic liver injury factor, if the cause is not clear in time and lack of effective intervention will further progress to liver cirrhosis, and even liver cancer [1]. Cirrhosis affects about 1% to 2% of the world's population, due to the lack of effective and specific interventions at present about one million people die of liver cirrhosis the world each year, thus seriously affecting the health of human beings [2]. The abnormal accumulation of extracellular matrix is the main characteristics of liver fibrosis. Hepatic stellate cells, the main source of extracellular matrix, play an important role in the progress of liver fibrosis. When liver damage occurs, hepatic stellate cells activate to muscle fiber cells and secrete extracellular matrix, promoting the development of liver fibrosis [3, 4]. In addition, studies showed that epithelial-mesenchymal transformation (EMT) also play an important role in the process of liver fibrosis, muscle fiber cells are not only come from the stationary phase hepatic stellate cells, *in vitro* cultured hepatocytes and bile duct epithelial cells can accelerate EMT process under the stimulation of transformative growth factor β1 [5, 6].

Receptor tyrosine kinase signaling pathway plays an important role in cell growth, differentiation, metabolism, and migration [7-9], EGFR was one of the most prominent representatives, which have effect on lung, cardiovascular and kidney fibrosis [10]. Mice with overexpression of dominant negative EGFR showed significantly less renal tubular injury after partial nephrectomy or chronic infusion of angiotensin II compared with wild type mice [11-13]. EGFR activation is related to the fibro-proliferative process in the human lung disease and animal

Table 1. PCR primers

Gene	Primer sequence (5'→3')	Product size (bp)	Annealing temperature
β-actin (mus)	Forward: AACAGTCCGCCTAGAAGCAC Reverse: CGTTGACATCCGTAAAGACC	281	57°C
TGF-β1 (mus)	Forward: GCCCTCGGGAGCCACAAACC Reverse: GCAGCAGGAGTCGCGGTGAG	277	60°C
α-SMA (mus)	Forward: CCCTGCTCTGCCTCTAGCACACA Reverse: TCCTGACCACTAGAGGGGGCCA	231	60°C

pulmonary fibrosis [14]. Oral administration of EGFR tyrosine kinase inhibitor can prevent progress of pulmonary fibrosis induced by EGFR activation, partially reverse pulmonary fibrosis, and will not lead to inflammatory cell influx and extra lung injury [14, 15]. However, it is still unknown whether Gifitinib affect the progress of liver fibrosis.

We hypothesized that Gifitinib may be helpful to prevent the development of liver fibrosis. In this study, we evaluated the anti-fibrotic properties of Gifitinib on liver fibrosis induced by carbon tetrachloride (CCl4) in mice.

Materials and methods

Animal grouping and treatment

Sixty male ICR mice (16-20 g) were purchased from the Shanghai Laboratory Animal Center (Shanghai, China). All animals were housed in animal facilities with free access to standard food and water. Animals were allocated randomly into six groups, the control group (n = 10), the liver fibrosis group (n = 40) and the Gifitinib intervention group (n = 10). The liver fibrosis model was induced by intraperitoneal injection with 1 μg/g body weight of CCl4 (dissolved 1:4 in oil) three times per week for 8 weeks. In order to clearly illuminate the dynamic liver change during the progress of fibrosis, the liver fibrosis group was further divided into four subgroups according to different time points: 2 weeks (n = 10), 4 weeks (n = 10), 6 weeks (n = 10) and 8 weeks (n = 10). Mice in Gifitinib intervention group were intraperitoneal injection with 20 mg/kg/day of Gifitinib, starting at 6 weeks after the first administration of CCl4 and lasting for 2 weeks. After eight weeks, mice of Gifitinib intervention group were all sacrificed. On week 2, 4, 6 and 8 after CCl4 treatment, mice of liver fibrosis group were killed. Blood and liver samples of each group were collected. All experiments were performed in accordance with the guidelines for the care and use of experiment animals by the National Institutes of Health.

Histologic evaluation

The partial liver from each mouse was fixed in 10% buffered formalin and embedded with paraffin. All tissue specimens were then sliced into 4 μm thickness. To further evaluate histological change, Masson's staining was utilized. Furthermore, the histological semiquantitative scoring system (SSS) of hepatic fibrosis [16] was employed to evaluate level of liver fibrosis. Images of each group were assessed blindly by an experienced pathologist.

Serum biochemical analysis

The mice of each group were anesthetized with urethane (1.0 g/kg, intraperitoneal injection (i.p.)), the blood samples from abdominal aorta were centrifuged at 2500 rpm at 4°C for 10 min and serum levels of alanine aminotransferase (ALT), aspartate aminotransferase (AST) and albumin (Alb) were measured using an automated clinical analyzer. After finishing with the serum collection, mice were sacrificed and liver samples were then immediately stored at -80°C for further analysis. The largest right liver lobe of each mouse was fixed in 10% buffered formalin solution for histologic evaluation.

RNA isolation and reverse transcription-polymerase chain reaction (RT-PCR)

Total RNA was extracted from samples of snap-frozen liver using RNAiso Plus reagent (Aidlab Biotechnologies Co., China) according to the manufacturer's instructions. The isolated RNA was reverse transcribed to cDNA and then amplified through polymerase chain reaction (PCR) in a final reaction system which contained 12.5 μl of 2× Master Mix, 8 μl of RNase-free dH20, 2.5 μl of cDNA sample, 1 μl of forward primers and 1 μl of reverse primers. The cycle conditions were as follows: 94°C for 30 s, annealing for 30 s, and 72°C for 45 s, for a total of 30 cycles. The annealing temperatures and

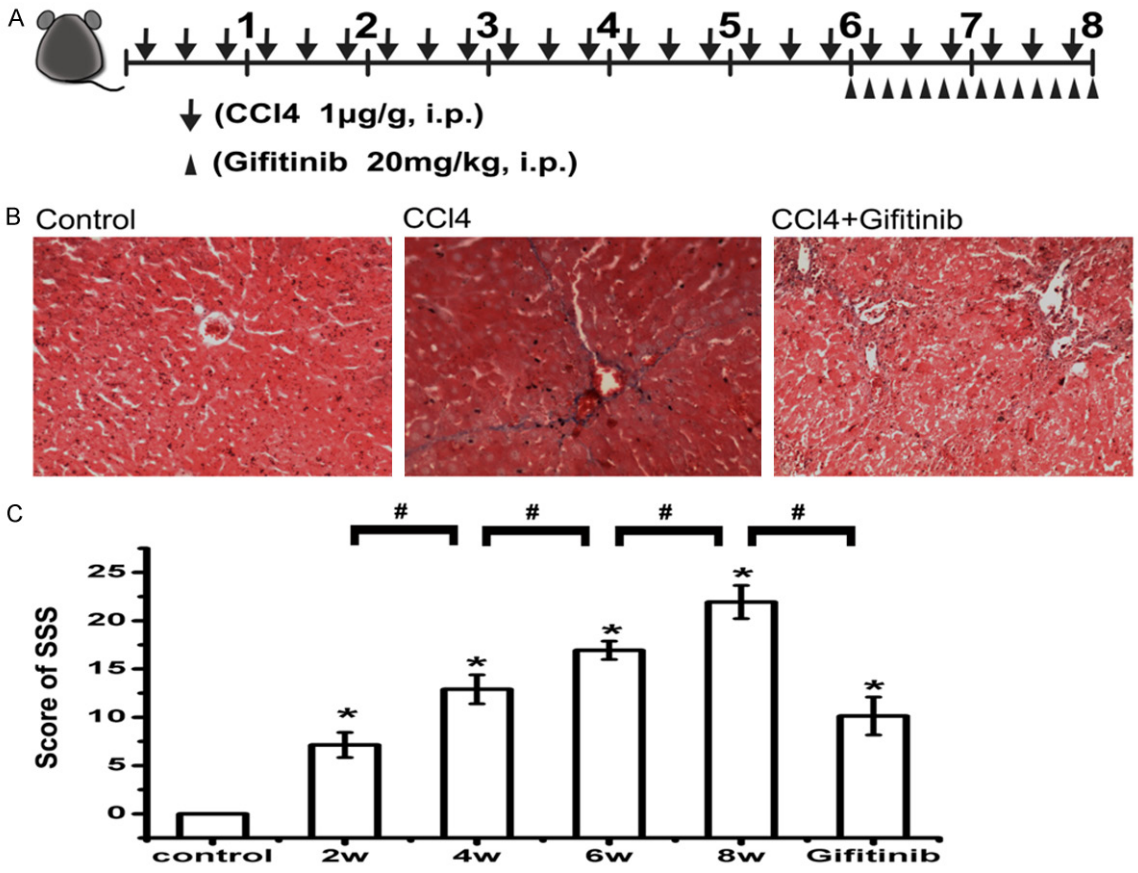

Figure 1. Effects of Gifitinib on histological Effects of Gifitinib on histological characteristics in mice. A. Mice in the liver fibrosis group were intraperitoneal injection with CCl4 (1 μg/g body weight dissolved 1:4 in corn oil) three times a week for 8 weeks. Mice in Gifitinib intervention group were intraperitoneal injection with 20 mg/kg/day of Gifitinib, starting at 6 weeks after the first administration of CCl4 and lasting for 2 weeks. B. Masson's trichrome staining was used to detect the accumulated collagen in liver sections from vehicle, CCl4, CCl4+Gifitinib at 8 weeks. C. The histological semiquantitative scoring system (SSS) of hepatic fibrosis was utilized to evaluate level of liver fibrosis. Data represent the mean ± SD of 6 mice (*Significant compared to control group, *P < 0.05; #Significant compared between the subgroups of liver fibrosis group and Gifitinib intervention group, #P < 0.05).

primer sequences used in this assay are listed in **Table 1**.

Protein isolation and Western blotting

Total liver proteins were isolated using lysis buffer which contained protease inhibitors and then concentration of protein was detected by BCA protein assay kit (Beyotime, Shanghai) according to the manufacturer's protocol. After heat denaturation with boiling water for 10 min, total proteins samples (25 μg/lane) were subjected to sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) and transferred onto the nitrocellulose membrane. The membrane was blocked using 5% bovine serum albumin for 90 min at 37°C and subsequently incubated with primary antibodies against TGF-β1 (Santa Cruz, sc-52893), α-SMA (Boster, BM0002), EGFR (Cell Signaling, 3265), pEGFR (Cell Signaling, 3777) and β-actin (Anbo, E0012) overnight at 4°C. After being washed 4×7 min with Tris-Buffered saline (TBST; 50 mM Tris-HCl, 150 mM NaCl, and 0.05% Tween), the membrane was incubated with secondary horseradish peroxidase-conjugated antibody at 37°C for 90 min. Then antibody-bound proteins were visualized with a chemiluminescent kit (Millipore Corporation, Billerica, USA).

Statistical analysis

All data were expressed as mean ± standard deviation (SD). Statistical significances were determined using one-way analysis of variance (ANOVA) or the least significant difference (LSD)

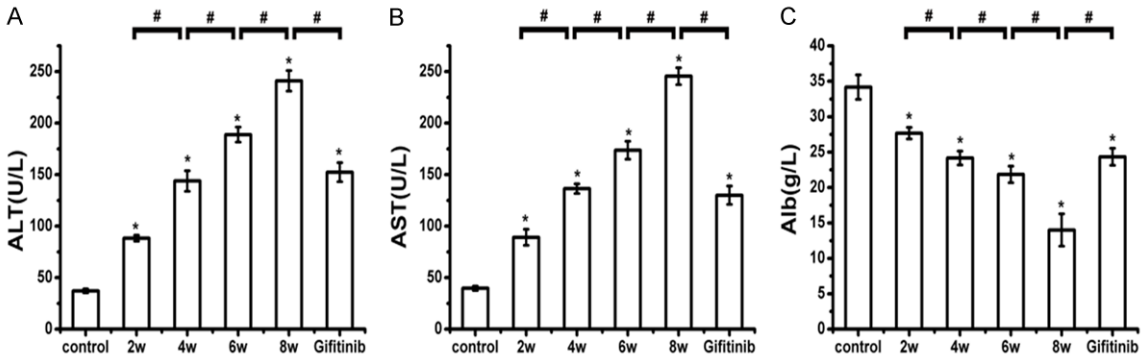

Figure 2. Effects of Gifitinib on serum concentrations of ALT, AST and Alb. The activities of alanine aminotransferase (ALT) (A) aspartate aminotransferase (AST) (B) and albumin (Alb) (C) were assayed by using an automated blood chemistry analyzer. (*Significant compared to control group, *P < 0.05; #Significant compared between the subgroups of liver fibrosis group and Gifitinib intervention group, #P < 0.05).

test. SPSS19.0 software (IBM, USA) was used for statistical analyses; $P < 0.05$ was considered to have statistical significance.

Results

Effects of Gifitinib on histological characteristics in mice

In order to test whether EGFR inhibitor can ameliorate liver fibrosis, we injected the EGFR tyrosine kinase inhibitor Gifitinib to mice with liver fibrosis induced by CCl4. Repeated injection of CCl4 with mice induced progressive liver fibrosis. Masson stain was adopted to show the accumulation of collagen fiber. As showed in **Figure 1**, in the control group the structure of hepatic lobule was normal, hepatic cells were arranged radically and rare fiber staining could be found. However, after 8 weeks of CCl4 injection, hyperplasia of collagen fibers was found, fibers formatted around central veins and pseudolobule was developed. While liver specimens of Gifitinib intervention group showed obviously much less collagen fiber compared to the 8 weeks liver fibrosis subgroup. The histological semiquantitative scoring system (SSS) of hepatic fibrosis was utilized to evaluate level of liver fibrosis. The score of SSS was found to increase gradually in the liver fibrosis group along with the time and a significantly higher score of SSS was found in the liver fibrosis subgroup as compared to the control group (P < 0.05). However, Gifitinib was found to significantly reverse those changing tendency induced by CCl4 with distinct decreased score of SSS.

Effects of Gifitinib on serum concentrations of ALT, AST and Alb

As shown in **Figure 2**, serum concentration of ALT and AST were gradually increased in the liver fibrosis group along with the time and a gradually decreased level of Alb was also observed as compared to the control group (P < 0.05). However, Gifitinib was found to reverse those changes of serum markers observed in the liver fibrosis group with distinct decreased ALT and AST levels and increased Alb level.

Effect of Gifitinib on fibrosis-related genes in CCL4-treated mice

In order to further evaluate the antifibrotic efficacy of Gifitinib, the key fibrotic genes markers such as TGF-β1 and α-SMA were examined by RT-PCR. We observed that both TGF-β1 and α-SMA mRNA are increased gradually in liver fibrosis group compared to the control group (P < 0.05). However, gene level of TGF-β1 and α-SMA in Gifitinib intervention group were significantly less compared with the liver fibrosis group (P < 0.05) (**Figure 3**).

Influence of Gifitinib on the expression of α-SMA, TGF-β1, EGFR and pEGFR in CCl4-treated mice

As showed in **Figure 4**, expression of α-SMA and TGF-β1 were significantly increased in liver fibrosis group compared to the control group. However, treatment of Gifitinib significantly inhibited those elevations. In order to evaluate the effect of Gifitinib on EGFR in mice, the

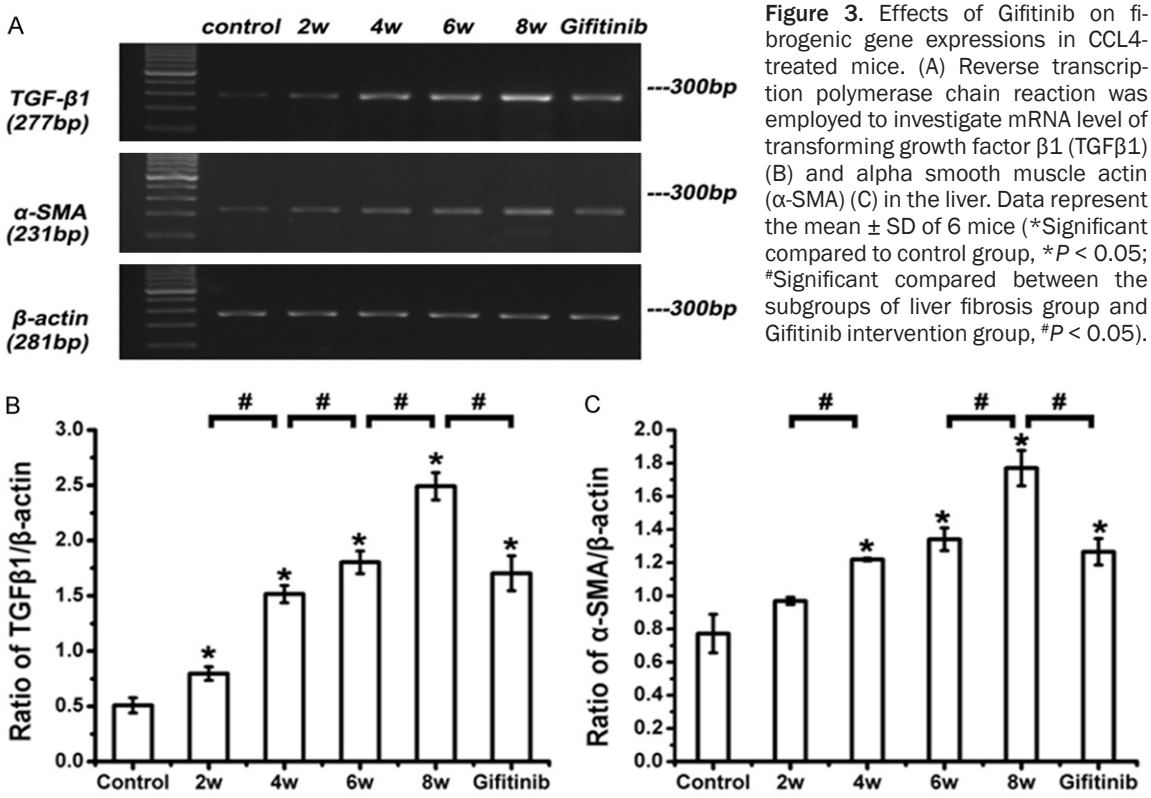

Figure 3. Effects of Gifitinib on fibrogenic gene expressions in CCL4-treated mice. (A) Reverse transcription polymerase chain reaction was employed to investigate mRNA level of transforming growth factor β1 (TGFβ1) (B) and alpha smooth muscle actin (α-SMA) (C) in the liver. Data represent the mean ± SD of 6 mice (*Significant compared to control group, $^{*}P < 0.05$; #Significant compared between the subgroups of liver fibrosis group and Gifitinib intervention group, $^{\#}P < 0.05$).

expression of EGFR and phosphorylated EGFR in liver were also examined using western blotting. The levels of EGFR and pEGFR in liver fibrosis group were significantly higher than those in the control group (P < 0.0 5). However, in Gifitinib intervention group, the expression of EGFR and pEGFR were significantly lower than the 8 week liver fibrosis subgroup (P < 0.05). Moreover, linear correlation analysis showed a positive correlation between the expression of EGFR/pEGFR and TGF-β1 (rs = 0.904, 0.961, P < 0.05).

Discussion

Liver fibrosis, a serious threat to human health, there is still lack of effective anti-fibrosis treatment that is available for human use, and numerous efforts are directed at the development of liver antifibrotic therapies. It is mainly characterized by abnormal excessive deposition of extracellular matrix (ECM) and collagen and studies showed that quiescence HSC activated to myofibroblast-like cells play a vital role in this progress [1-4, 17]. Various cytokines especially TGF-β1 was found to mediate the progression of liver fibrosis. TGF-β1 can inhibit the synthesis of ECM degradation enzymes, as plasminogen activator inhibitor type 1 (PAI 1) and tissue inhibitor of metalloproteinase (TIMPs), and is key for fibroblasts recruitment, myofibroblast differentiation, epithelial-mesenchymal transition (EMT) and extracellular matrix deposition [18]. In our study, the liver fibrosis model was successfully established by intraperitoneal injection with CCl4 in mice, as evidenced by histological evaluation and serum markers in parallel with the upregulated expression of TGF-β1 and α-SMA.

Receptor tyrosine kinase signaling pathway plays an important role in cell growth, differentiation, metabolism, and migration [7, 9, 19], EGFR is one of the most prominent representatives. Studies showed that EGFR support the fibrosis progress of lung and oral administration of EGFR tyrosine kinase inhibitor can prevent progress of pulmonary fibrosis induced by EGFR activation, partially reverse pulmonary fibrosis, and will not lead to inflammatory cell influx and extra lung injury [14, 15]. Furthermore, recent researches have shown that EGFR pathway plays a critical role in TGF-β function since EGFR was important for transcription of PAI-1 in

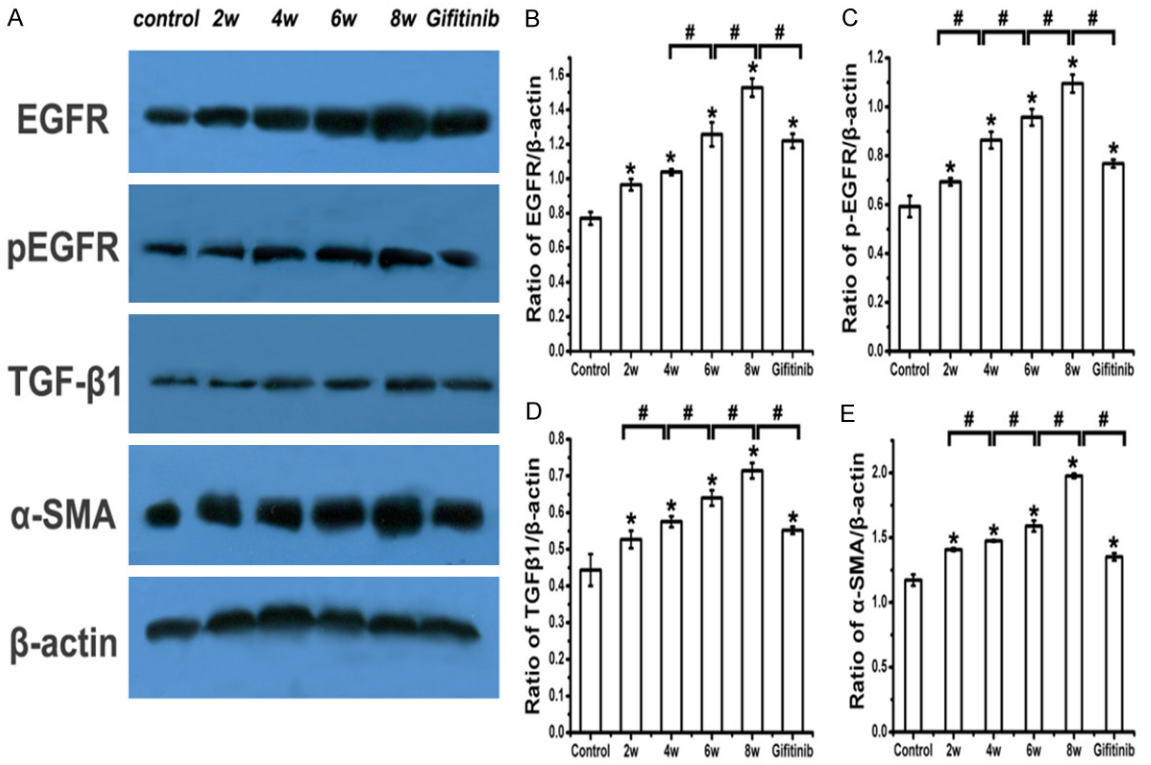

Figure 4. Effect of Gifitinib on the expression of α-SMA, TGF-β1, EGFR and pEGFR in CCL4-treated mice. (A) Western blotting was assessed to investigate protein level of epidermal growth factor receptor (EGFR) (B) phosphoepidermal growth factor receptor (pEGFR) (C), transforming growth factor β1 (TGFβ1) (D) and alpha smooth muscle actin (α-SMA) (E) in the liver. Data represent the mean ± SD of 6 mice (*Significant compared to control group, *P < 0.05; #Significant compared between the subgroups of liver fibrosis group and Gifitinib intervention group, #P < 0.05).

smooth muscle cells [20] and Cox-2 expression in human airway epithelial cells [21] induced by TGF-β1. In addition, many growth factors, including ligands that combine with EGFR can enhance the motility of fibroblasts [22]. Addition of EGF to the fibroblast cultures can stimulate many other matrix proteins including glycosaminoglycan in vitro [23, 24]. EGF has potential to change migration speed of fibroblast [24]. In our study, we found that EGFR tyrosine kinase inhibitor Gifitinib also showed an antifibrotic efficacy to the pathogenesis of liver fibrosis. Our data showed that the increased aminotransferases levels and expression of TGF-β1 and α-SMA induced by CCl4 and histological change improved in the Gifitinib intervention group, suggesting that Gifitinib could ameliorate the progress of liver fibrosis in mice induced by CCl4 to some extent. Furthermore, the expression of EGFR was upregulated in CCl4 fibrosis, and the phosphorylation type of EGFR was also showed the similar elevated tendency, suggesting the involvement of EGFR in the regulation of liver fibrogenesis. Thus, EGFR might be a potential novel marker for assessing the progress of fibrosis. Moreover, the expression of EGFR/pEGFR and TGF-β1 was showed to have a positive linear correlation, suggesting that a cross talk between EGFR and TGF-β1 signaling, a potential mechanism of the protective role of Gifitinib in liver fibrosis.

In conclusion, we show that EGFR/pEGFR were upregulated in the progress of liver fibrosis and inhibition of EGFR activity through injection of Gifitinib suppressed the gene and protein expression of α-SMA and TGF-β1 in CCl4 induced liver fibrosis model of mice. This study indicates that EGFR act as a driver of TGFβ1 dependent liver fibrosis and a potential biomarker for fibrogenesis, thus providing new insights into the regulation effect of Gifitinib.

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Disclosure of conflict of interest

None.

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