

## ORIGINAL ARTICLE

# M2 macrophages reduce the effect of gefitinib by activating AKT/mTOR in gefitinib-resistant cell lines HCC827/GR

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**Keywords**

$\beta$ -catenin; CCL2; epidermal growth factor receptor; gefitinib resistance; non-small cell lung cancer.

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Thoracic Cancer **11** (2020) 3289–3298**Introduction**

Lung cancer is the leading cause of cancer-related deaths worldwide.<sup>1</sup> With the further study of lung cancer, people gradually understand the relevant knowledge of lung cancer. Lung cancer is classified into two broad histological subtypes; non-small cell lung cancer (NSCLC) and small cell lung cancer (SCLC), accounting for approximately 85% and 15% of cases, respectively.<sup>2</sup> Undoubtedly, there are different treatment modalities that exist in accordance with different pathological characteristics. In particular, the specific blockade of the epidermal growth factor receptor (EGFR) signal transduction pathway has been reported to result in attenuated responses being achieved together with partially prolonged progression-free survival in patients

**Abstract**

**Background:** The biological behavior of cells change after they develop drug resistance, and the degree of resistance will be affected by the tumor microenvironment. Here, we aimed to explore the changes in the biological behavior of tumors and to observe the differences in the release of cytokines and chemokines which can influence the tumor microenvironment. We also aimed to study how TKIs-resistant cell lines recruit macrophages to reduce the sensitivity of the cells following gefitinib administration.

**Methods:** We generated and maintained gefitinib-resistant cell lines to study the differences between gefitinib-sensitive cell lines according to clone formation, cell growth curve analysis, whole-exome sequencing, and qPCR ARRAY technology. We used the WNT/ $\beta$ -catenin inhibitor, WNT/ $\beta$ -catenin activator and over-expression  $\beta$ -catenin lentivirus to observe the changes in CCL2. M2 macrophages and gefitinib-resistant cell lines HCC827/GR were cocultured to detect the viability gefitinib for inducing cell death.

**Results:** The proliferation and migratory activities were much more pronounced in HCC827/GR cells. CCL2 expression was also enhanced and regulated by  $\beta$ -catenin in HCC827/GR. CCL2 promoted the chemotactic ability of M2 macrophages. M2 macrophages reduced the antitumor effect of gefitinib treatment by activating AKT/mTOR.

**Conclusions:** Gefitinib-resistant cell lines have stronger proliferation and migration capabilities, and attract macrophages by releasing more CCL2 to reduce the sensitivity of cells to gefitinib.

harboring *EGFR* mutations uniquely sensitive to tyrosine kinase inhibitors (TKIs).<sup>3–6</sup> However, drug resistance inevitably develops and accelerates disease progression within 12 months.<sup>7</sup> The confirmed mechanisms for developing drug resistance mostly arise from T790M mutation or through the bypass of signal pathway activation.<sup>8–10</sup> In addition, MET<sup>11</sup> and HER2<sup>8</sup> amplification, *BRAF*<sup>12</sup> mutation, *KRAS*<sup>13, 14</sup> mutation, and SCLC transformation<sup>15</sup> will also cause drug resistance. The loss of PTEN plays an important role in the development of TKIs resistance by bypassing the signaling pathway activation.<sup>16</sup> Although PI3K/AKT/mTOR is not a downstream molecule of PTEN, loss of PTEN activity has been reported to enhance the activity of AKT and mTOR, and the activation of these

molecules may be involved in reducing the killing effect of gefitinib on tumor cells.<sup>17–19</sup>

The tumorigenesis and the progression and metastasis of tumors are extremely dependent on their microenvironment.<sup>20–22</sup> Changes in the microenvironment or the induction of inflammation via cytokines or chemokines are sufficient to decrease the ability of killer cells by TKIs.<sup>23, 24</sup> After or during resistance has developed, some cytokines or chemokines released by tumor cells can reshape the tumor microenvironment. There are studies which have confirmed that tumor-associated macrophages are involved in inducing resistance against TKIs. CCL2 has been reported to play an indispensable role in the process of TKI resistance<sup>25, 26</sup> as an important factor for chemotaxis of macrophages.<sup>27–29</sup> Studies have also confirmed that TKIs can increase CCL2 content,<sup>30, 31</sup> however, there are also studies suggesting TKIs inhibit the secretion of CCL2 in M2 macrophages.<sup>32</sup> In addition, there are studies that show that the regulation of CCL2 by TKIs is unclear. Although  $\beta$ -catenin has been proven to promote the expression of CCL2 in colorectal cancer,<sup>33</sup> the relationship between  $\beta$ -catenin activity and CCL2 expression has not been studied in lung cancer. Additionally, it has been reported that TKIs enhance the activity of  $\beta$ -catenin,<sup>34</sup> but there are also studies confirming that TKIs can reduce the activity of  $\beta$ -catenin.<sup>35</sup> There is currently a lack of research on the relationship of TKIs/ $\beta$ -catenin/CCL2, and the mechanism of how macrophages participate in TKI resistance is unclear.

Therefore, the aims of this study were to explore the different cytokines and chemokines released by gefitinib-resistant cells and parental cell lines, screen the cytokines and chemokines which recruit macrophages, and study the impact of macrophages in gefitinib resistance.

## Methods

### Cell lines, reagents, and antibodies

NSCLC cell lines with mutated *EGFR* (19del), HCC827 (Keygenbio, Nanjing, China) were cultured at 37°C in a humidified 5% CO<sub>2</sub> atmosphere. The cell lines were cultured in RPMI-1640 medium. To culture the cell lines, 10% fetal bovine serum was supplemented in the medium. Gefitinib (Selleckchem, Houston, TX, USA), which is a type of EGFR-TKI, was mixed in the medium at increasing concentrations to generate and maintain resistant cell lines for approximately six months. The WNT/ $\beta$ -catenin inhibitor XAV-939 and WNT/ $\beta$ -catenin activator WNT agonist 1 were purchased from Selleckchem (Houston, TX, USA).

The following primary antibodies were used: anti-GAPDH (Abcam, ab181602), mTOR (CST, mAb #2983),

Phospho-mTOR (Ser2448) (CST, mAb #5536). AKT (Abcam, ab32505), p-AKT (Abcam, ab192623).

### Whole-exome sequencing

Whole-exome sequencing of DNA was performed from the gefitinib-resistant cell lines HCC827/GR and the gefitinib-sensitive cell lines HCC827. Genomic DNA was sheared and the sample libraries were prepared according to the TruSeq DNA Sample Preparation Guide (Illumina, San Diego, CA, USA). The captured, purified, and amplified libraries targeting the exomes were subjected to paired-end sequencing (2 × 100 bp) on the Illumina HiSeq 2000 and aligned against the human genome 19 (hg19) using the Burrows Wheeler Aligner with default settings. These were conducted according to the manufacturer's instructions.

### Western blot analysis

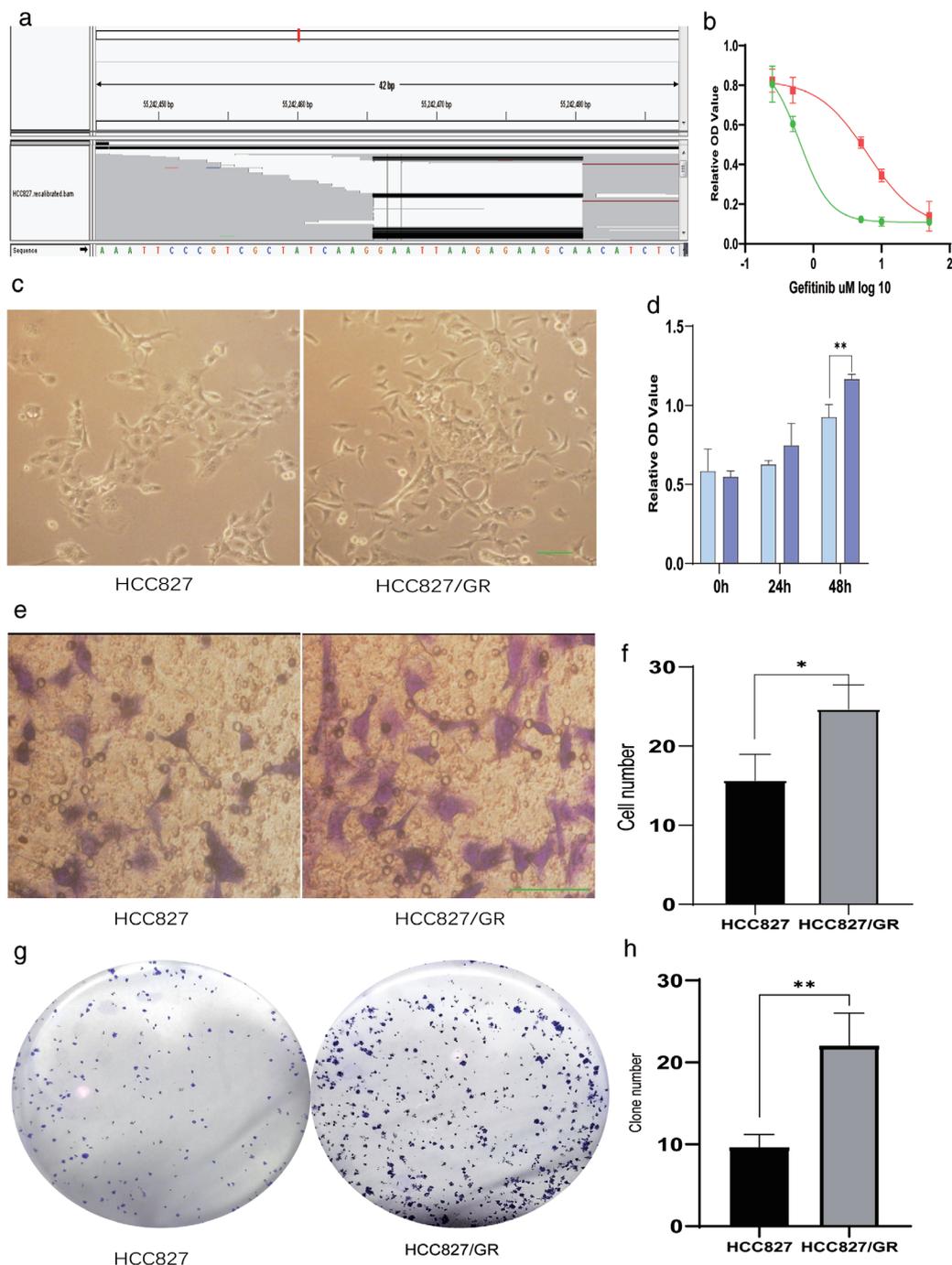
Protein extraction from cell was performed using the RIPA buffer (Beyotime Institute of Biotechnology, Nanjing, China) and protease inhibitor (Beyotime Institute of Biotechnology, Nanjing, China). Protein concentration was determined through the BCA protein assay kit (Bio-Rad). Sodium dodecyl sulfate (SDS)-polyacrylamide gel electrophoresis (PAGE) (Beyotime Institute of Biotechnology, Nanjing, China) was performed to separate the different proteins, and proteins were transferred to polyvinylidene difluoride (PVDF) membranes. Membranes were blocked in 5% milk for one hour and incubated with specific primary antibodies overnight, followed by incubation with HRP-labeled secondary antibodies for two hours. Protein bands were detected by chemiluminescence.

### Cell growth curve analysis

We used cell counting kit-8 (CCK-8; Dojindo Molecular Technologies Inc., Shanghai, China) assay to detect cellular proliferation. The cells were transferred to a 96-well plate with 1000 cells/well for CCK-8 assay and incubated for 1.5 hours before being measured at 450 nm on a microplate reader.

### RNA isolation and RT-qPCR

Total RNA was isolated using TRIzol Reagent (Thermo Fisher Scientific) in accordance with the manufacturer's instructions. Using the PrimeScript RT reagent kit (GeneCopoeia, Inc., USA), we synthesized complementary DNA from 1  $\mu$ g of total RNA. Relative quantification of the transcripts was performed, and data were calculated



**Figure 1** Biological characteristics were different in HCC827/GR cells. **(a)** Whole-exome sequencing showed the *EGFR* (19 del E746-A750) deletion mutation. **(b)** Mean relative OD value of parental HCC827 and HCC827/GR cells. IC<sub>50</sub> analysis of dose–response curves showed that the IC<sub>50</sub> value of HCC827/GR cells was higher compared to that of the parental cell line HCC827 —●—, HCC827; —■—, HCC827/GR. **(c)** Cell morphology was changed. Magnification, 100 $\times$ . Scale bar, 100  $\mu$ m. **(d)** Cell growth was measured by cell counting kit-8 and is presented as mean  $\pm$  SD. HCC827/GR cells showed faster growth than parental HCC827 cells at 48 hours (HCC827 vs. HCC827/GR =  $0.927 \pm 0.079$  vs.  $1.167 \pm 0.029$ ,  $P = 0.0079$  by two-tailed Student's *t*-test) □, HCC827; ■, HCC827/GR. **(e)** Transwell migration assay image showed that HCC827/GR cells possessed stronger migration ability. Magnification, 400 $\times$ . Scale bar, 100  $\mu$ m. **(f)** Transwell migration assay data analyses demonstrated that there was a greater number of cells migrating to the other side compared to that in parental HCC827 cells ( $P = 0.0257$  by two-tailed Student's *t*-test). **(g)** and **(h)** Cell clone formation experiments image and assay data confirmed that the colony-forming ability of HCC827/GR was significantly higher than that of the parental cells ( $P = 0.0075$ ).

using the  $\Delta\Delta C_t$  method. PCR was performed using the SYBR Green Master Mix (GeneCopoeia, Inc., USA) on the LightCycler 480 device (Roche Diagnostics). GAPDH expression level was used to normalize gene expression of other transcripts. Nonspecific products were not observed. Primer sequences were as followed:

CCL2 F: CCTTCATTCCCAAGGGCTC  
 CCL2R: CTTCTTTGGGACACTTGCTGC  
 CD206F: TACTGAACCCCCACAACCTGC  
 CD206R: ACCAGAGAGGAACCCATTCG  
 IL-10F: CCAGACATCAAGGCGCATGT  
 IL-10R: GATGCCTTCTCTTGGAGCTTATT  
 ELISA

The concentrations of CCL2 were detected using a commercial human CCL2 ELISA kit (MULTISCIENCES (LIANKE) BIOTECH,CO.,LTD, Hangzhou, China), in accordance with the manufacturer’s instructions.

**Transfection**

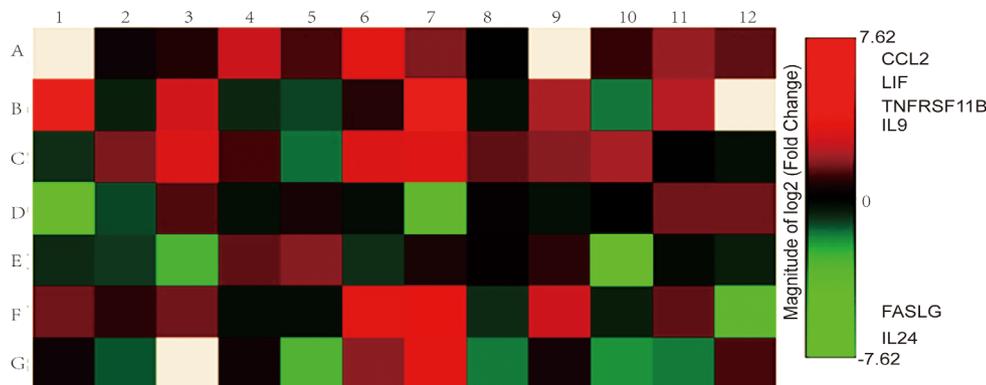
The experiment was carried out according to the manufacturer’s protocol to transfect the lentivirus encoding  $\beta$ -catenin protein and nontargeting-scramble control vector. The lentivirus was purchased from Biosune (Shanghai, China). The culture medium was changed into complete medium after 24 hours. The overexpressed cells were selected using 0.25  $\mu$ g/mL puromycin and were confirmed by western blot.

**Transwell assay**

The cells were digested and collected from the culture flask. They were then resuspended in serum-free medium and counted. A total of 100  $\mu$ L of cell suspension was taken and added to the transwell chamber, and 600  $\mu$ L of medium containing 20% fetal bovine serum were added to the lower

**Table 1** The detection of gene mutation status in HCC827/GR cell lines

Reference gene	Mutation	Chromosome	Reference base	Alteration base	Mutation occurrence
EGFR	T790M	chr7	C	T	No
ERBB2	S310Y	chr17	C	A	No
ERBB2	S310F	chr17	C	T	No
ERBB2	V777L	chr17	G	C	No
BRAF	V600E	chr7	T	A	No
BRAF	G469V	chr7	G	T	No
BRAF	G464V	chr7	G	T	No
MET	V1110I	chr7	G	A	No
MET	H1112R	chr7	A	G	No
MET	D1246H	chr7	G	C	No
MET	Y1248H	chr7	T	C	No
MET	M1268T	chr7	T	C	No
MET	H1112Y	chr7	C	T	No
MET	V1110I	chr7	G	A	No
MET	V1110L	chr7	G	A	No
KRAS	G12D	chr12	G	A	No
KRAS	G12S	chr12	G	A	No
KRAS	G12C	chr12	G	T	No

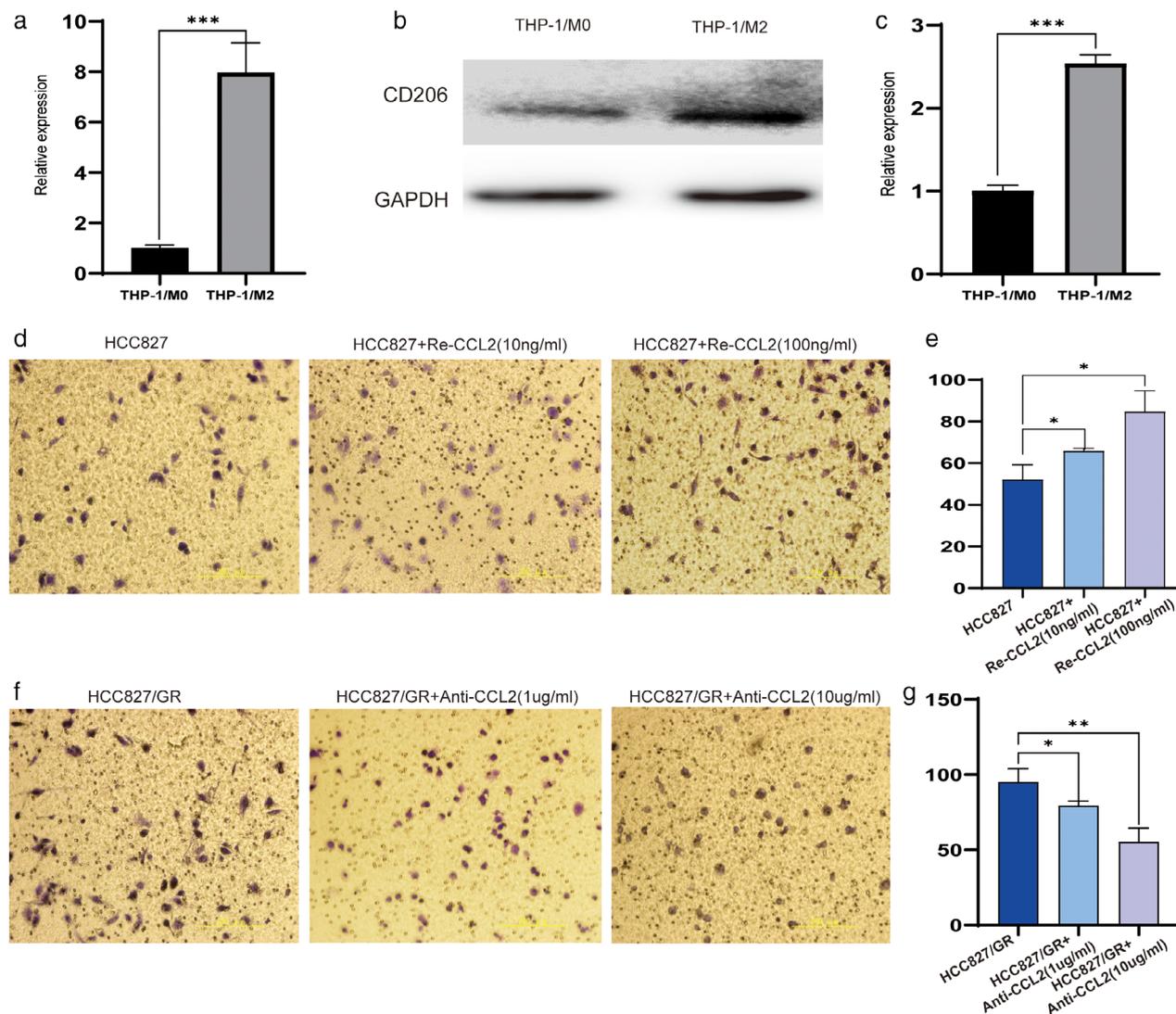


**Figure 2** The difference of CCL2 between HCC827/GR and HCC827. The qPCR array showed the difference of cytokines and chemokines between the gefitinib-resistant cell line HCC827/GR and parental cell line HCC827. The data are presented as the ratio of HCC827/GR to HCC827.

chamber of the 24-well plate. The cells were cultured for 48 hours. The chamber was removed and washed twice with calcium-free PBS and fixed with methanol for 30 minutes, and the cells were dried. The cells were then stained with 0.1% crystal violet for 20 minutes, the upper nonmigrated cells were gently wiped off using a cotton swab and were washed three times with PBS. The cells were viewed under an optical phase contrast microscope, three fields of view were selected and the number of cells passing through the chamber membrane was counted.

### Clone formation

Cells were collected with 0.05% trypsin and inoculated into six-well plates at a concentration of 1000 cells per well. They were incubated at 37°C for 12–14 days. After removing the culture medium, the cells were fixed in 4% paraformaldehyde for 15 minutes, stained with crystal violet for five minutes, washed once with PBS and then dried. The cells were observed under an optical phase contrast microscope and the data were analyzed.



**Figure 3** HCC827/GR enhanced the migration of macrophages via CCL2. **(a)** The transcription of CD206 in THP-1/M2 after induction by PMA and IL-4,  $P = 0.0005$ . **(b)** The protein expression of CD206 tested by western blot in THP-1/M2 after induction by PMA and IL-4. **(c)** The transcription of IL-10 in THP-1/M2 after induction by PMA and IL-4,  $P < 0.0001$ . **(d and e)** Transwell migration assay image and assay data confirmed that the migration ability of HCC827 treated with CCL2 recombinant protein was significantly higher than that of HCC827 with dose-dependence, HCC827 vs. Re-CCL2 (10 ng/mL) =  $52 \pm 7.21$  vs.  $65.67 \pm 1.53$ ,  $P = 0.0325$ ; HCC827 vs. Re-CCL2 (100 ng/mL) =  $52 \pm 7.21$  vs.  $84.67 \pm 10.02$ ,  $P = 0.0102$ . **(f and g)** Transwell migration assay image and assay data confirmed that the migration ability of HCC827/GR treatment with CCL2 blocking antibodies was significantly lower than that of HCC827/GR with dose-dependence, HCC827/GR versus anti-CCL2(1  $\mu$ g/mL) =  $95 \pm 9.0$  versus  $79.33 \pm 3.21$ ,  $P = 0.0469$ , HCC827/GR versus anti-CCL2 (10  $\mu$ g/mL) =  $95 \pm 9$  versus  $55.33 \pm 9.29$ ,  $P = 0.006$ . Scale bar, 200  $\mu$ m.

## Statistical analysis

Data were analyzed using GraphPad Prism V8 (GraphPad Software, Inc., California, USA) and were represented as mean  $\pm$  standard deviation (SD). Student's *t*-test was used to compare means of two groups and  $P < 0.05$  was considered statistically significant.

## Results

The proliferation and migration abilities were much more pronounced in HCC827/GR cells.

Whole-exome sequencing confirmed that the cell line HCC827 contains an EGFR (19 del E746-A750) deletion mutation (Fig 1a). We successfully generated the EGFR-TKI-resistant cell line HCC827/GR. Cells were routinely cultured with EGFR inhibitors under increasing concentrations for six months and TKI resistance was continuously maintained by means of the processed culture medium containing TKI inhibitors at certain concentrations. IC50 of the EGFR-TKI-resistant cell line HCC827/GR (IC50 = 0.6303) was more than 10 times compared to that of HCC827 (IC50 = 6.277) (Fig 1b). There were also changes in cell morphology (Fig 1c) and cells were much more dispersed from cell to cell and spindle-shaped structures became much more obvious.

Cell growth curve and transwell migration assay were performed to determine the differences between the two groups in order to investigate the proliferation and migration ability of cells in HCC827/GR. The results also showed that the cell proliferation of HCC827/GR was enhanced compared with that of HCC827 (Fig 1d). The migration ability of HCC827/GR significantly increased compared to that of HCC827 (Fig 1e and f). In addition, cell clone formation experiments were also conducted, and

we found that HCC827/GR clonal ability was also enhanced (Fig 1g and h).

Whole exon sequencing showed the difference in gene mutation status between HCC827/GR and HCC827 cell lines.

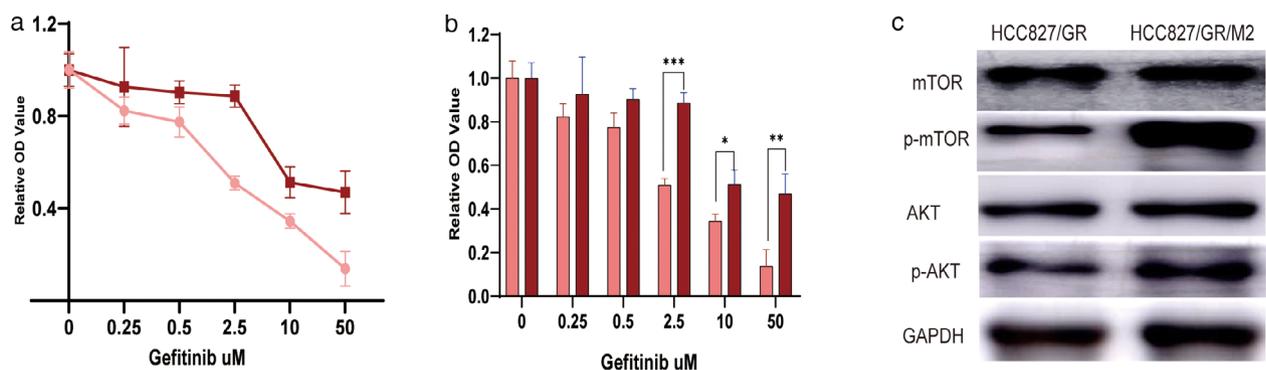
The detection of the gene mutation status of HCC827/GR cell lines was conducted, and we observed whether there was a change in resistance-related mutation. Through the analysis of the whole exon sequencing results, it was found that the HCC827/GR cell line had no definite changes in terms of resistance-related mutation. We were able to demonstrate the partial results of mutation (Table 1), including *T790M* (Fig S1a), *ERBB2* (Fig S1b), *BRAF* (Fig S1c), *MET* (Fig S2a), and *KRAS* (Fig S2b). Since no clear resistance-related mutations were found, the next step was to conduct protein molecular level experiments to determine the possible mechanism of gefitinib resistance.

CCL2 mRNA expression was higher in HCC827/GR than that of HCC827.

The dynamic interaction between tumor cells and the surrounding microenvironment is crucial for changing the response to TKI treatment and can aid in promoting the development of drug resistance. Cytokines and chemokines play vital roles in reshaping the tumor microenvironment. We then used the qPCR ARRAY technology to screen the differences between cytokines and chemokines and between gefitinib-resistant and parental cell lines, and to explore which changes were able to reshape the tumor microenvironment. PCR ARRAY analyses of cytokines and chemokines were conducted in cell lines HCC827/GR and HCC827. CCL2 mRNA expression was higher in HCC827/GR than that in HCC827 (Fig 2).

CCL2 promoted the chemotactic ability of M2 macrophages.

We identified whether THP1 was converted to M2 macrophages after induction by PMA and IL-4. In the



**Figure 4** The HCC827/GR cocultured with THP-1/M2 reduced the sensitivity of killer cells by gefitinib monotherapy. (a) The different abilities of killer cells in the gefitinib administration group (termed G group) and gefitinib administration group cocultivation of M2 macrophages (termed G/M2 group)  $\bullet$   $\circ$ , G;  $\blacksquare$   $\square$ , G/M2. (b) The assay data of the relative vitality treated with single gefitinib administration between the HCC827/GR cocultured with THP-1/M2 (G/M2 group) and HCC827/GR without coculture with THP-1/M2 (G group)  $\square$ , G;  $\blacksquare$ , G/M2. (c) p-AKT and p-mTOR expression were high in gefitinib-resistant cell lines after coculture with THP-1/M2.

transcription and protein expression of CD206, in which M2 specific markers were detected, the results showed that CD206 were upregulated (Fig 3a and b). We determined that the transcription of IL-10 was also upregulated in induced cells (Fig 3c). CD206 combined with IL-10 was used to verify that THP1 was converted to M2 macrophages (termed THP-1/M2).

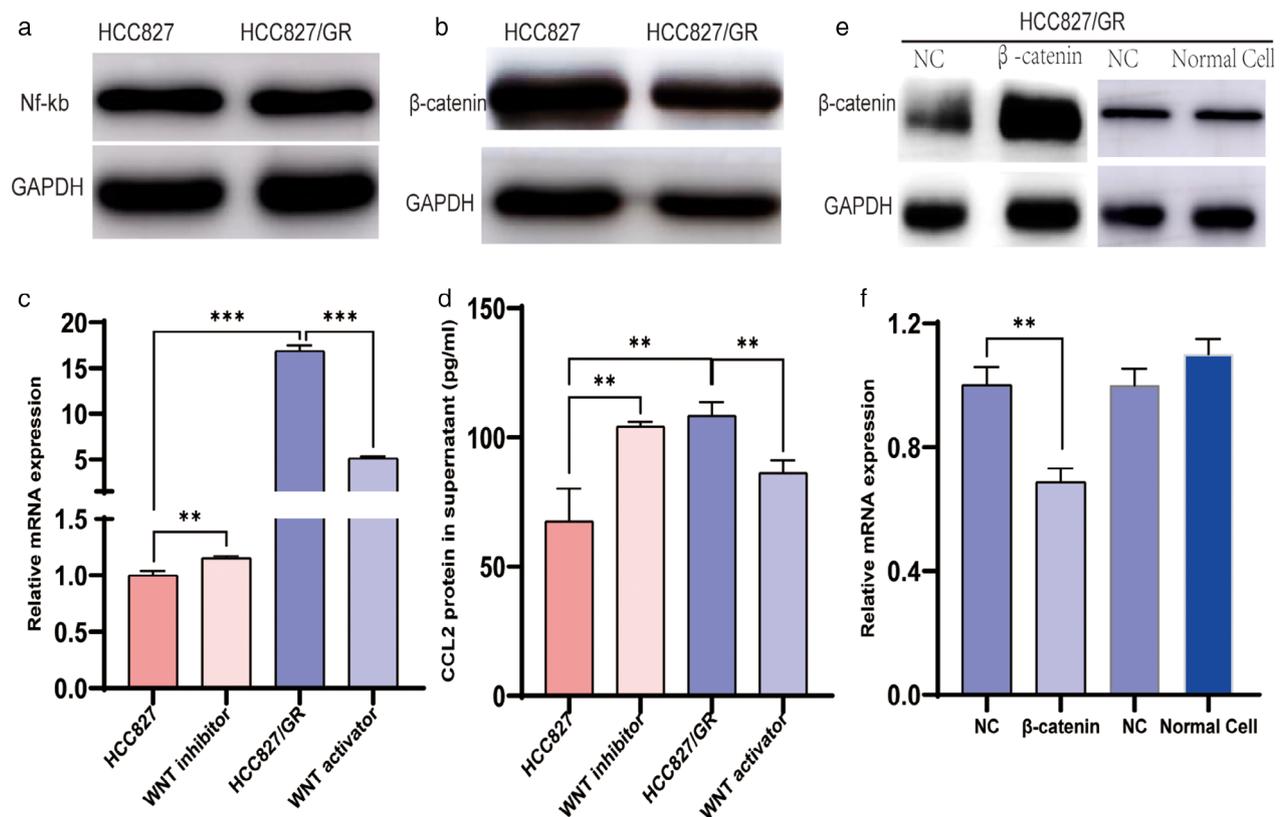
Cell line analyses using HCC827, and which was cocultured with THP-1/M2 macrophages, were also conducted to test the migration ability treated with or without CCL2 recombinant protein. The application of CCL2 recombinant protein enhanced the chemotaxis of M2 macrophages in the HCC827 cell line (Fig 3d and e) with dose-dependence. We performed experiments on THP-1 /M2 macrophages coculture with gefitinib-resistant cell line HCC827/GR to observe the migration ability of the cells when treated with or without CCL2 blocking antibodies. Blocking CCL2 reduced macrophage migration ability (Fig 3f and g) with dose-dependence.

M2 macrophages reduced the antitumor effect of the treatment of gefitinib.

After cocultivation of M2 macrophages and gefitinib-resistant cell lines HCC827/GR for 72 hours, the upper chamber (M2 macrophages) was discarded, and the lower-layer cells (gefitinib-resistant cell lines) were collected in order to detect the sensitivity of gefitinib to killer cells.

The experimental protocol was as follows: (i) gefitinib administration group (termed the G group): gefitinib (0, 0.25, 0.5, 2.5, 10, 50  $\mu\text{mol/L}$ ); and (ii) gefitinib administration group: cocultivation of M2 macrophages (termed the G/M2 group): gefitinib (0, 0.25, 0.5, 2.5, 10, 50  $\mu\text{mol/L}$ ). The assay data of the relative vitality showed that the G/M2 group (cocultured with THP-1/M2) increased the resistance of gefitinib at 2.5  $\mu\text{mol/L}$  ( $P = 0.0003$ ), 10  $\mu\text{mol/L}$  ( $P = 0.0171$ ), 50  $\mu\text{mol/L}$  ( $P = 0.0083$ ) compared to the G group (without cocultured with THP-1/M2) (Fig 4a and b).

In order to clarify the specific mechanism underlying the reduction of the sensitivity of gefitinib after



**Figure 5** CCL2 expression was higher and regulated by  $\beta$ -catenin in HCC827/GR. (a) NF- $\kappa$ B proteins were detected by western blot. (b) The  $\beta$ -catenin proteins expression were reduced in HCC827/GR. (c) RT-qPCR analysis of CCL2 mRNA in cell line HCC827 treated with WNT/ $\beta$ -catenin inhibitors XAV-939 (50  $\mu\text{M}$ ) and in HCC827/GR treated with WNT/ $\beta$ -catenin activators WNT agonist 1 (50 nM). (d) ELISA analysis of CCL2 in cell supernatant in cell line HCC827 treated without or with WNT/ $\beta$ -catenin inhibitors XAV-939 (50  $\mu\text{M}$ ) and in the gefitinib-resistant cell line HCC827/GR treated without or with WNT/ $\beta$ -catenin activators WNT agonist 1 (50 nM). (e) The lentivirus successfully expressed  $\beta$ -catenin protein compared with nontargeting-scramble control vector. (f)  $\beta$ -catenin overexpression reduced the CCL2 transcription.  $n = 3$ , error bars are  $\pm$  standard deviation. Gene expression was normalized to GAPDH expression.

coculture of THP-1/M2 macrophages and gefitinib-resistant cell lines, we detected AKT, p-AKT and mTOR, p-mTOR expression, in gefitinib-resistant cell lines after coculture with THP-1/M2. We found that the activity of p-mTOR and p-AKT were increased (Fig 4c) in gefitinib-resistant cell lines after coculture with THP-1/M2 macrophages, which may be the reason for the reduced sensitivity of gefitinib after coculture with macrophages. Therefore, the reduction of macrophages in tumor tissue may become a potential method for reducing the resistance of gefitinib.

CCL2 expression was higher and regulated by  $\beta$ -catenin in HCC827/GR.

We found that CCL2 was elevated in HCC827/GR, as tested by qPCR array, and we subsequently further studied which gene regulates CCL2 protein expression. The literature confirmed that NF-KB could regulate the expression of CCL2 in normal tissue cells or in tumor cells. Therefore, we tested the changes in the NF-KB in drug-resistant cell lines, but we found that there was no significant change in the NF-KB protein in HCC827/GR cell lines (Fig 5a). In the present study, we detected changes in the  $\beta$ -catenin protein expression in the HCC827/GR cell lines. The results showed that  $\beta$ -catenin protein expressions were reduced in HCC827/GR cell lines (Fig 5b).

Since the expression of  $\beta$ -catenin was downregulated and CCL2 transcription was upregulated, we speculated that there may be a negative regulatory relationship between  $\beta$ -catenin and CCL2. To test this hypothesis, we used WNT/ $\beta$ -catenin activators to observe changes in CCL2 transcription in gefitinib-resistant cells (HCC827/GR) where  $\beta$ -catenin expression was relatively low. As a mirror experiment, we used an inhibitor to inactivate WNT/ $\beta$ -catenin and then observed the changes in CCL2 transcription in parental cell lines (HCC287).

CCL2 mRNA transcription was higher in HCC827/GR than that of in the HCC827 (HCC827 =  $1.000 \pm 0.02240$ , HCC827/GR =  $16.88 \pm 0.3388$ ,  $***P < 0.0001$ , Fig 5c). CCL2 mRNA was upregulated by WNT/ $\beta$ -catenin inhibitors XAV-939 (50  $\mu$ M) in cell line HCC827 (HCC827 =  $1.000 \pm 0.02240$ , WNT inhibitor =  $1.151 \pm 0.01162$ ,  $**P = 0.0039$ , Fig 5c) and was downregulated by WNT/ $\beta$ -catenin activators WNT agonist 1 (50 nM) in gefitinib cell line HCC827/GR (HCC827/GR =  $16.88 \pm 0.3388$ , WNT activator =  $5.148 \pm 0.1219$ ,  $***P < 0.0001$ , Fig 5c).

The amount of CCL2 in the supernatant was important for its function. Therefore, we used ELISA to detect the change of CCL2 from the effect of WNT/ $\beta$ -catenin signaling pathway. CCL2 protein in cell supernatant was higher in HCC827/GR than that of in the HCC827 (HCC827 vs. HCC827/GR,  $67.51 \pm 7.341$  vs.  $108.5 \pm 2.974$ ,  $**P = 0.0067$ , Fig 5d). CCL2 protein in cell supernatant was upregulated by WNT/ $\beta$ -catenin inhibitors XAV-939

(50  $\mu$ M) in cell line HCC827 ( $67.51 \pm 7.341$  vs.  $104.4 \pm 0.9027$ ,  $**P = 0.0076$ , Fig 5d) and was downregulated by WNT/ $\beta$ -catenin activators WNT agonist 1 (50 nM) in gefitinib cell line HCC827/GR (HCC827/GR vs. WNT activator,  $108.5 \pm 2.974$  vs.  $86.28 \pm 2.793$ ,  $**P = 0.0056$ , Fig 5d). The content of CCL2 in the supernatant of the cells decreased when the WNT/ $\beta$ -catenin was activated in gefitinib-resistant cells (HCC827/GR), and the cell CCL2 supernatant content was increased when the WNT/ $\beta$ -catenin was inhibited.

In order to further verify the regulatory effect of  $\beta$ -catenin on CCL2, we used lentiviral overexpression  $\beta$ -catenin to observe the changes of CCL2. We found that overexpression of  $\beta$ -catenin (Fig 5e) reduced the expression of CCL2 mRNA transcription (NC group vs.  $\beta$ -catenin group  $1.001 \pm 0.058$  vs.  $0.6872 \pm 0.044$ ,  $**P = 0.0018$ , Fig 5f).

## Discussion

We successfully constructed gefitinib-resistant HCC827/GR cell lines, and the malignant biological behavior of gefitinib-resistant cell lines was significantly enhanced, which was manifested by cell proliferation, migration, and cloning formation ability assay. The gefitinib-resistant cell lines which we constructed did not demonstrate a clear mutation resulting in resistance, but we found that the chemokine CCL2 released by the gefitinib-resistant cell lines attracted many more M2 macrophages and these M2 macrophages could reduce killer cell ability by treatment with TKIs. Our results also showed that  $\beta$ -catenin protein expression was reduced, which promoted the release of CCL2 by HCC827/GR.

CCL2 has been shown to be induced by NF-KB gastrointestinal stromal tumor,<sup>36</sup> and in hormone-dependent breast cancer,<sup>37</sup> as well as in other cells.<sup>38, 39</sup> The lack or inhibition of NF-KB has been shown to reduce the production of proinflammatory factors, such as CCL2. However, we did not find significant differences in NF-KB protein expression in HCC827/GR cell lines. We speculate that CCL2 may come from the regulation of other signaling pathways. We found that  $\beta$ -catenin protein expression was reduced in HCC827/GR cell lines. Our results are consistent with other studies on gefitinib-resistant cell lines (PC9/GR).<sup>35</sup> However, there was one study which reported that erlotinib induced the expression of  $\beta$ -catenin via dependence on Notch3.<sup>34</sup> Our study used gefitinib and their study used erlotinib, which may account for the differences in results. Two drugs may have different effects on  $\beta$ -catenin or Notch3 expressions. In order to clarify the relationship between CCL2 and  $\beta$ -catenin, we used WNT/ $\beta$ -catenin inhibitor, activator, and overexpression of

$\beta$ -catenin lentivirus to observe changes in CCL2. We found that  $\beta$ -catenin was a negative regulator of CCL2.

The tumor microenvironment is complex, and may be responsible for the low response rate when treated with combination therapy in patients. For example, tumor-associated macrophages have been reported to play an important role in reducing the sensitivity of gefitinib in terms of killer cell ability.<sup>25, 26</sup> Similar to our study, the killing effect was significantly reduced when treated with gefitinib and when gefitinib-resistant cell lines were cocultured with macrophages. This might partly explain why TKIs cannot achieve the same therapeutic effects in clinical trials and patients. The reduction of the infiltration of macrophages in the tumor microenvironment might be an important measure for improving killer cell ability when treated with combination therapy in patients.

The current literature has confirmed that CCL2 plays an important role in chemotactic macrophages.<sup>27–29</sup> Therefore, we suspect that tumor cells also secrete CCL2, which can promote macrophage infiltration into tumor tissue, except for CCL2 secreted by macrophages themselves. Our study found that gefitinib-resistant cell lines release more CCL2 than gefitinib-sensitive cell lines. We therefore speculated that gefitinib-resistant cell lines may attract more macrophages to infiltrate tumor tissues. The results reported in this study show that macrophages are capable of reducing the sensitivity of gefitinib on killer cell ability by blocking CCL2, and reducing the macrophage infiltration of tumor tissues might be a promising way of decreasing resistance to TKI therapy in patients.

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## Disclosure

The authors declare that they have no competing interests.

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## Supporting Information

Additional Supporting Information may be found in the online version of this article at the publisher's website:

**Figure S1.** The results of whole-exome sequencing for T790M, ERBB2, BRAF. (a) T790M; (b) ERBB2, including S310Y, S310F, V777L; (c) BRAF, including V600E, G469V, G464V.

**Figure S2.** The results of whole exon sequencing, MET, KRAS. (a) MET, including V1110I, H1112R, D1246H, Y1248H, M1268T, H1112Y, V1110I, V1110L. (b) KRAS, including G12D, G12S, G12C.