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RESEARCH ARTICLE

Gefitinib Represses JAK-STAT Signaling Activated by CRTC1-MAML2 Fusion in Mucoepidermoid Carcinoma Cells

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Abstract: ***Background*:** Gefitinib is well-known as a tyrosine kinase inhibitor targeting non-small-lung-cancer (NSCLC) containing EGFR mutations. However, its effectiveness in treating mucoepidermoid carcinoma (MEC) without such EGFR mutations suggests additional targets.

***Objective*:** The CRTC1-MAML2 (C1-M2) fusion typical for MEC has been proposed to be a gefitinib target.

***Method*:** To test this hypothesis, we developed a set of siRNAs to down-regulate C1-M2 expression. RNA-seq and Western blot techniques were applied to analyze the effects of gefitinib and siC1-M2 on the transcriptome of and the phosphorylation of tyrosine kinases in a MEC cell line H292.

***Results*:** Deep-sequencing transcriptome analysis revealed that gefitinib extensively inhibited transcription of genes in JAK-STAT and MAPK/ERK pathways. Both siC1-M2 and gefitinib inhibited the phosphorylation of multiple signaling kinases in these signaling pathways, indicating that gefitinib inhibited JAK-STAT and MAPK/ERK pathways activated by C1-M2 fusion. Moreover, gefitinib inhibition of EGFR and MAPK/ERK was more effective than that of AKT, JAK2 and STATs, and their dependence on C1-M2 could be uncoupled. Taken together, our results suggest that gefitinib simultaneously represses phosphorylation of multiple key signaling proteins which are activated in MEC, in part by C1-M2 fusion. Gefitinib-repressed kinase phosphorylation explains the transcriptional repression of genes in JAK-STAT and MAPK/ERK pathways.

***Conclusion*:** These findings provide new insights into the efficacy of gefitinib in treating mucoepidermoid carcinoma, and suggest that a combination of gefitinib and other inhibitors specifically against C1-M2 fusion could be more effective.

Keywords: Gefitinib, CRTC1-MAML2 fusion, EGFR, JAK-STAT, mucoepidermoid carcinoma cells, phosphorylation.

1. INTRODUCTION

Epidermal growth factor receptor (EGFR), a 170 kDa transmembrane glycoprotein, belongs to the type I subfamily tyrosine kinase receptors. The binding of a ligand, such as EGF, transforming growth factor-α (TGFα) or amphiregulin (AREG) causes the dimerization of EGFR, stimulating its intrinsic tyrosine kinase activity and the activation of downstream signaling cascades, JAK-STAT1/3/5, PI3K-Akt, Ras-MAPK-ERK, PLCγ-PKC, that are crucial for normal cell growth and proliferation[1-2]. Aberrant activation of EGFR signaling by overexpression or mutation occurs in a wide range of epithelial cancers, particularly prevalent in small cell lung cancers (NSCLC), and targeting EGFR signaling network represents a rationale for novel treatment approaches [3-4].

Gefitinib is well-known as an ATP analogue and tyrosine kinase inhibitor (TKI) targeting aberrant EGFR activity in cancers, representing one of the three EGFR-targeted TKIs as first-line treatment options [5-6]. NSCLC patients containing L858 substitution and exon 19 deletion are highly responsive to gefitinib, and the underlying mechanism is related to the gain-of-function activation of EGFR by these mutations which sensitize EGFR to the ATP analogue [7-9]. Unfortunately, most responsive NSCLC patients become resistant to gefitinib later. The majority of resistance is associated with a secondary EGFR mutation reducing gefitinib affinity [10]. In principle, an unbiased genome-wide study of gefitinib targeted genes and pathways should be helpful in better understanding the gefitinib resistance, and ultimately

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in facilitating the development of more powerful therapeutic strategies.

Several lines of evidences suggest that gefitinib has additional targets other than the mutant EGFRs. For example, there are several separate reports showing clinical responses of pulmonary mucoepidermoid carcinoma (MEC) to gefitinib containing no sensitizing EGFR mutations [11-14]. *In vitro* data showed that MEC cell lines are sensitive to gefitinib [11, 15], which has been either attributed to the inhibition of cAMP/CREB related signaling or the activated AREG-EGFR signaling related to C1-M2 [15-17]. Moreover, higher autocrine production of amphiregulin in NSCLC and HNSCC (head and neck squamous cell carcinoma) in EGFR wild-type cancer cell lines can predict their sensitivity to both gefitinib and cetuximab [18]. Therefore, it is clear that gefitinib targets the wild-type EGFR as well. In addition, gefitinib targets the ERK and STAT5 activation in lung cancer cell lines containing EGFR mutants [8]. The reported gefitinib side effects in NSCLC patients containing EGFR mutants also suggest the presence of additional gefitinib targets [6]. Due to the chemical nature of gefitinib as an ATP analogue, its high binding affinity with the mutant EGFR than the wild type is likely due to the activated substrate pocket constitutively open to ATP and ATP analogues [19, 20]. It is therefore reasonable to propose that gefitinib might inhibit the activity of some other kinases who are in active state, because the active kinase has a higher affinity to ATP and presumably to ATP analogue as well.

MEC is the most common malignant salivary gland tumor [21-22], while the gefitinib targets in MEC cells remain unclear, which limits its potential clinical application. It could be possible that gefitinib targets the activated kinase which has been well documented in MEC. For example, HER-2/neu overexpression, H-RAS mutations, high expression and phosphorylation levels of EGFR and ERK1/2, and high EGFR gene copies are all presented in a significant population of MEC, correlating with poor prognosis [23], tumor grade [24], aggressiveness of tumor behavior [25]. Histological grade of the tumors, and disease-free interval and overall survival [26], respectively.

CRTC1-MAML2 (C1-M2), derived from the chromosomal translocation t (11; 19) between the genes encoding CREB-regulated transcriptional co-activator 1 (CRTC1) and Mastermind-like protein 2 (MAML2), occurs in approximately 30-80% of MEC and is proposed to be targeted by gefitinib [16, 27-30]. The fusion gene constitutively activates both Notch signaling targets and cAMP/CREB-dependent transcription, and the later correlates with its transforming activity [15, 31-33]. The fusion protein binds and activates c-Jun and c-Fos in AP-1 transcription factor complex, as CRTC1 does, which may contribute to the transforming activity of C1-M2 as well [34]. More recently, C1-M2 fusion interaction with MYC protein and activation of AREG-EGFR signaling have been shown to contribute to the cell transformation [35] and proliferation [17].

In this study, unbiased transcriptome analysis shows that gefitinib robustly inhibited the expression of genes in JAK-STAT and MAPK (ERK) signaling pathways during the early time of treatment. Both gefitinib and siC1-M2 inhibited the phosphorylation of EGFR, JAK, STAT, and ERK, which was not only consistent with the hypothesis of kinase targets for gefitinib, but also with its transcriptome impact.

2. MATERIALS AND METHODS

2.1. Plasmid Construction

The CRTC1-MAML2 and AREG sequence was cloned from the cDNA of H292 cell line with EcoRI and XhoI restriction enzyme sites into pIRES-hrGFP-1a Vector (Agilent, Beijing, China) through one step cloning assay (pEASY-T1 cloning kit, Transgene).

2.2. siRNA Design

Control siRNA and siRNAs targeting the CRTC1, MAML2, CRTC1–MAML2(C1-M2) were synthesized by Shanghai GenePharma. These siRNA sequences are:

siCRTC1-MAML2-1: 5'-GGGCCGCGCGGCUCCAGG GUUCCUUGA-3';

siCRTC1-MAML2-2: 5'-GACGCGGGCCGCGCGGCU CCAGGGUUC-3';

siCRTC1-MAML2-3: 5'-CGCGGGCCGCGCGGCUCC AGGGUUCCU-3';

siCRTC1-4: 5'-GACCUGAGCCUGACGCGGGCCGC GCGG3';

siMAML2-5: 5'-CUCCAGGGUUCCUUGAAAAGAAA ACAG3'.

2.3. Cell Culture and Reagents

HeLa (a human cervical cancer cell line) was cultured in Dulbecco's modified Eagle's medium (Gbico), while H292 (mucoepidermoid carcinoma with pulmonary origin) and HCT116 (a human colorectal adenocarcinoma cell) were maintained in RBM1640 (Gbico) supplemented with 10% inactivated fetal bovine serum (BI) and 1% penicillin/streptomycin (HyClone). Cells were grown at 37℃ with 5% CO2. Gefitinib was obtained from Sigma, stored at -20 ℃ and diluted in DMSO before use.

2.4. Cell Transient Transfection

Transfections were carried out using Lipofectamine 2000 Reagent or Lipofectamine RNAiMAX Reagent (Invitrogen) according to the manufacturer's instruction. Each experiment was performed in triplicate and repeated at least 3 times.

2.5. Real-time Quantitative Reverse Transcription PCR

Trizol (Invitrogen) was used to extract total RNAs from the relevant sample. RNA was reverse transcribed into cDNA using M-MLV Reverse Transcriptase (Vazyme). Real-time PCR was performed with the Step One Real-Time PCR System using the SYBR Green PCR Reagents Kit (Yeasen). Glyceraldehyde-3-phosphate dehydrogenase (GAPDH) was used as an internal control for the normalization of gene expression. The primers are provided in Supplemental Table 1. Q-PCR data represents the mean values from at least three independent experiments.

2.6. Western Blotting Analysis

Protein samples were loaded into 10% or 12% SDS-PAGE gels depending on molecular weight and transferred onto 0.45 mm PVDF membranes. The membranes were blocked with 5% skim milk (in a buffer containing 10 mM Tris, pH 8.0, 150 mM NaCl, 0.05% Tween 20) for an hour, incubated overnight with primary antibody at 4℃ and then incubated with horseradish peroxidase-conjugated secondary antibody for 1h at room temperature. Membranes were then visualized through chemiluminescence. We also have quantitated some of the WB bands by the software Image J.

Antibodies: The following antibodies were purchased from commercial sources including anti-AKT1 (Polyclonal Antibody, AB clonal; A7270), anti-EGFR (Polyclonal Antibody, AB clonal; A11351); anti- ERK1/2 (Monoclonal Antibody, Protein tech; 66192-1-lg); anti-JAK2 (Polyclonal Antibody, Protein tech; 17670-1-AP); anti-STAT1 (Polyclonal Antibody, AB clonal; A0027); anti-STAT3 (Polyclonal Antibody, AB clonal; A1192); anti-STAT5 (Polyclonal Antibody, AB clonal; A7733); anti-β-actin (Monoclonal Antibody, AB clonal; AC004); anti-phospho-EGFR (Polyclonal Antibody, AB clonal; AP0301); anti-phospho-JAK2 (Polyclonal Antibody, Flarebio; CSB-PA000562); anti-phospho-STAT3 (Polyclonal Antibody, AB Clonal; AP0070); anti-phospho-STAT5 (Polyclonal Antibody, Bioss; Bs-1659R); anti-phospho-STAT1 (Polyclonal Antibody, Bioss; Bs-1657R); anti-phospho-AKT1 (Polyclonal Antibody, AB Clonal; AP0140); and anti-phospho-AKT1 (Polyclonal Antibody, AB Clonal; AP0472).

2.7. Statistical Analyses of Experimental Data

Statistical analysis was performed using Student's t test in the Statistical Product and Service Solutions (SPSS) software. The results are expressed as the means ± SD, and a probability of $p < 0.05$ was considered to be significant.

2.8. RNA Extraction and Sequencing

2.8.1. RNA Extraction

Trizol (Invitrogen) was used to extract total RNAs from the control and siRNA-transfected and gefitinib-treated H292 cells. The RNA was further treated with RQ1 DNase (Promega, Madison, WI, USA) to remove DNA. The quality and quantity of the RNA were determined by measuring the absorbance at 260 nm/280 nm (A260/A280) using Smartspec Plus (BioRad, USA). The integrity of RNA was further verified by 1.5% agarose gel electrophoresis.

2.8.2. Library Preparation

For each sample, 10μg of the total RNA was used for RNA-seq library preparation. Polyadenylated mRNAs were purified and concentrated with oligo (dT)-conjugated magnetic beads (Invitrogen, Carlsbad, CA, USA) before directional RNA-seq library preparation. The purified mRNAs were then iron fragmented at 95°C followed by end repair and 5' adaptor ligation. Then, reverse transcription was performed with RT primer harboring 3' adaptor sequence and randomized hexamer. The cDNAs were purified, amplified, and stored at -80°C until they were used for sequencing.

2.8.3. Sequencing Method

The cDNA libraries were subsequently quantified and sequenced on the Illumina NextSeq 500 platform using the pair-ends protocol to generate 2×150nt reads.

2.9. RNA-Seq Raw Data Clean and Alignment

Raw reads containing more than 2-N bases were first discarded. Then adaptors and low-quality bases were trimmed from raw sequencing reads using FASTX-Toolkit (Version 0.0.13). The short reads less than 16nt were also dropped. After that, clean reads were aligned to the GRCH38 genome by tophat2 [36] with 4 mismatches. Uniquely mapped reads were used to calculate reads number and RPKM value (RPKM represents reads per kilobase and per million) for each gene.

2.10. Differentially Expressed Genes (DEG) Analysis

The edgeR [37] were used to determine the differentially expressed genes, based on the fold change (fold change ≥2 or ≤0.5) and P-value ($P \leq 0.01$).

2.11. Functional Enrichment Analysis

Hypergeometric test and Benjamini-Hochberg FDR controlling procedure were used to define the enrichment of each GO term. Pathway analysis was performed using KEGG database (http://www.genome.jp/kegg).

3. RESULTS

3.1. A Set of siRNAs Effectively Target C1-M2 Fusion Transcript in the Absence and Presence of Gefitinib Treatment

The advantages of applying siRNAs to silence the interested genes not only include time-saving, flexibility, no permanent perturbation of the physiological state of cells, but also its potentials as therapeutic agents [38]. The most prominent disadvantage of siRNA down-regulation is the experimental variation when compared to the stable cell lines using shRNA and CRISPR/Cas9 [39]. To overcome this problem, we developed five siRNAs directly targeting the C1-M2 fusion site and the adjacent sequence (CRTC1 exon1 or MAML2 exon2) (Fig. **1a**), and applied all of them in parallel as biological repeats to secure the major conclusions of this study.

Previous studies have shown that shRNA targeting the fusion site was not successful in knocking down the C1-M2 fusion transcript [17, 32]. To assess the effectiveness and specificity of these five siRNAs, we detected the levels of C1-M2 fusion transcript using primer pairs #c, and exon1-exon2 junction of the non-fusion CRTC1 and MAML2 using primer pairs #a and #b, in the pure H292 cell culture by quantitative real-time PCR (qRT-PCR) (Supplementary Fig. **1A**) shows that all these five siRNAs effectively down-regulated the C1-M2 fusion transcript level. None of these siRNA showed an appreciable effect on CRTC1 non-fusion transcript level, but some down-regulated MAML2 level (Supplementary Fig. **1B-C**). We speculated that the biased location of seed region of siRNAs at MAML2 exon2 but not

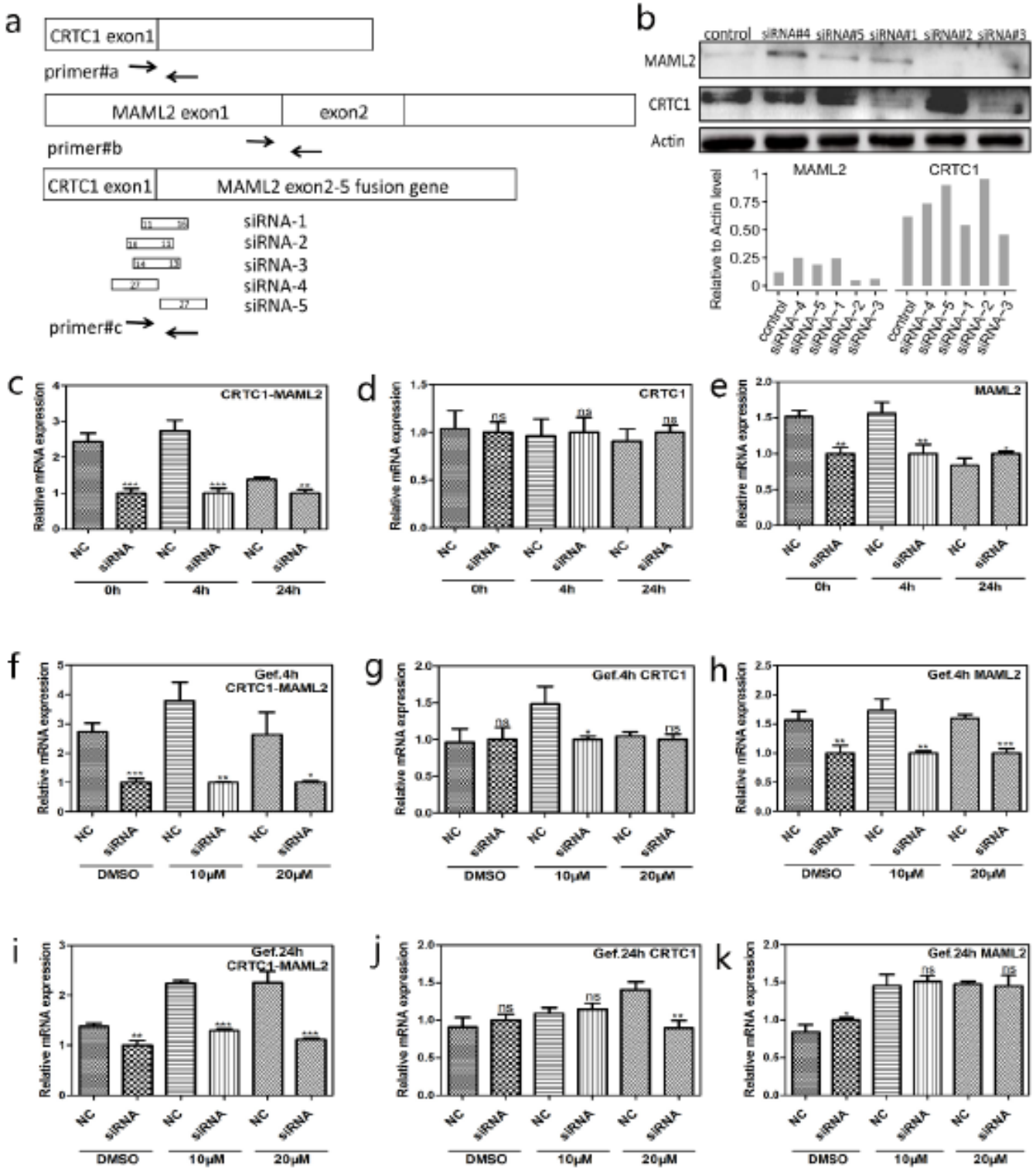

Fig. (1). A set of siRNAs effectively targeted C1-M2 fusion transcript in the absence and presence of gefitinib treatment. (a) The CRTC1-MAML2 fusion gene consists of exon1 of CRTC1 and the exon2-5 of MAML2. #a, #b, and #c are primers to identify CRTC1, MAML2, and CRTC1-MAML2 transcripts by quantitative real-time PCR (qRT-PCR) respectively and specially. Here we synthesized three siRNAs that directly target the fusion site of C1-M2, with one having equal base pairs on both fused exon (siRNA-3), and two biased to either the fused CRTC1 exon (siRNA-2) or MAML2 exon (siRNA-1). We also synthesized two target the adjacent sites on either CRTC1 (siRNA-4) or MAML2 (siRNA-5) exons. (b) The effect of these siRNAs on protein levels of MAML2 and CRTC1 was investigated in H292 cells transient-transfected with siRNA-1, siRNA-2, siRNA-3, siRNA-4, and siRNA-5 siRNA for 48h by western blotting analysis. (c-k) Dectection of MAML2, and CRTC1-MAML2 transcripts levels in H292 cells were treated with siRNA-1in the presence of DMSO for 0h, 4 h and 24 h (c-e), or treatment gefitinib for 4 h (f-h) and for 24 h (i-k), using in Quantitative Real-time PCR (qRT-PCR). *P<0.05; **P<0.01; ***P<0.001.

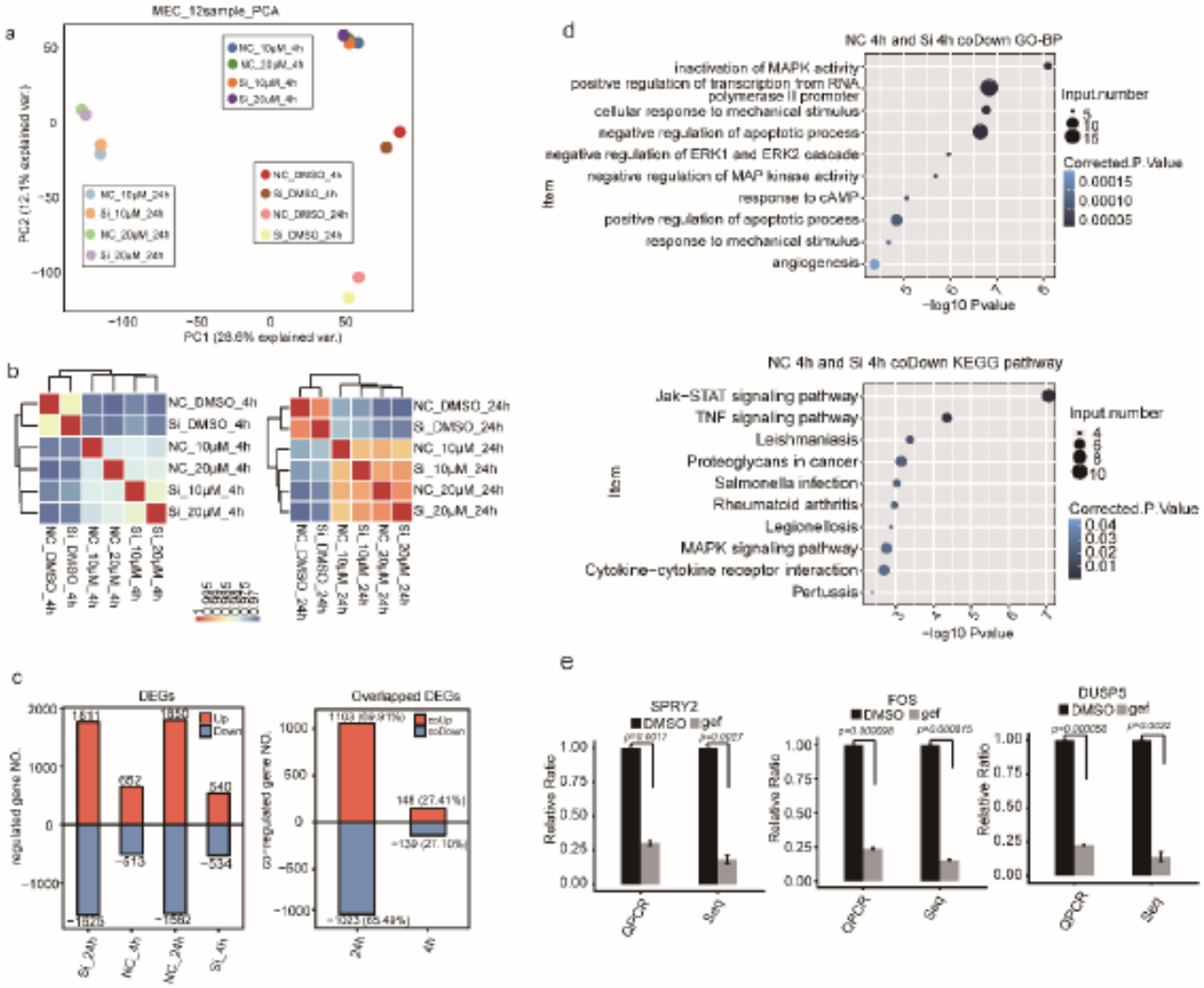

Fig. (2). Gefitinib effectively inhibits JAK-STAT/MAPK/TNF pathways and inhibits TNF/TGF/VEGF/ERB/JAK-STAT pathways more reactive responds to siC1-M2 treatment. (a) Principle component analysis (PCA) of 12 samples. (b) Sample cluster analysis of correlation treated with gefitinib for 4 h (left) and 24 h (right). (c) Bar plot of total number of DEGs regulated by gefitinib (left panel) and co-DEGs (overlap of Si and NC, right panel). (d) Bubble plot of GO-BP terms and KEGG pathways enriched in the genes co-down regulated by gefitinib under 4 h inhibitor treatment. (e) Quantitative Real-time PCR (qRT-PCR) assays validated several signaling pathway genes responding to inhibitor treatment at 4 h.

on CRTC1 that could at least in part contribute to the observed discrepancy of siRNA-targeted down-regulation of CRTC1 and MAML2.

The effect of these siRNAs on protein levels of MAML2 and CRTC1 was also investigated, showing that siRNA-2 and siRNA-3 reduced MAML2 protein level, while siRNA-1 and siRNA-3 reduced CRTC1 protein expression (Fig. **1b**). The antibody-recognized antigen of MAML2 was located at 5' portion of the protein spanning exon1 and exon2, the detected protein could represent the C1-M2 fusion and MAML2 protein level. However, antibody of CRTC1 recognized C-terminal antigen, therefore the siRNA-reduced expression of CRTC1 protein level might reflect a feedback regulation of CRTC1 expression by C1-M2 knock-down.

We next transfected the most effective siRNA-1 to reduce the expression of fusion transcript in the presence and absence of gefitinib treatment (Fig. **1**). siRNA-1 effectively down-regulated C1-M2 fusion expression in H292 in the absence and presence of gefitinib at three different growth time-points (Fig. **1c**, **f** and **i**). siRNA-1 did not reduce the CRTC1 transcript level (Fig. **1d**, **g** and **j**), but reduced the level of MAML2 transcript at a relatively smaller extent (Fig. **1e**, **h** and **k**).

3.2. Gefitinib Effectively Inhibits JAK-STAT/ MAPK/ TNF Pathways and TNF-β/TGF-β /VEGF/ERB Pathways Become Gefitinib-Targeted Upon siC1-M2 Treatment

In order to unbiasedly identify genes and functional pathways targeted by gefitinib in the MEC cell line H292, we sequenced the polyA-selected mRNAs/lncRNAs in H292 cells in the presence and absence of the inhibitor, and in the presence or absence of siRNA treatment (down-regulated CRTC1-MAML2). After removing adaptor sequences and low-quality sequencing reads, we obtained a total of 24,091,168-33,800,994 reads high-quality reads from each

sample (Supplemental Table 2A). When these reads were mapped onto the human GRCH38 genome using Tophat2, 66.88%-84.27% were aligned and about 96.54% were uniquely aligned (Supplemental Table 2B). The uniquely mapped reads were highly enriched in CDS regions and 3′UTR regions (Supplemental Table 2C), and the total genes detected with mean RPKM ≥ 1 are from 11,304 to 11,849 (Supplemental Table **2A**-**D**).

We first analyzed the global influence of different factors including inhibitor concentration and working hour, as well as the presence of siC1-M2, on the expression of H292 cells. Principle component analysis (PCA) was performed to identify the major factors, showing that culture time and inhibitor treatment were two major factors globally influenced the gene expression pattern (Fig. **2a**). Sample cluster analysis according to expression correlation coefficients and were then performed. At 4 h of inhibitor treatment, the expression patterns in siRNA-treated and control samples were separable (Fig. **2b**, left). At 24 h treatment, the effect of siRNA down-regulation was overridden by the inhibitor treatment (Fig. **2b**, right). Overall, gene expression at 10 M and 20 M were very similar to each, and therefore treated as biological repeats in the following analysis.

We then analyzed differentially expressed genes (DEGs) reflecting transcriptional regulation, in response to gefitinib treatment at 4 h and 24 h using edgeR method (standard: fold change≥2 or ≤0.5, p-value≤0.01). 1074-3436 DEGs for 4 h and 24 h, respectively, were found to respond the inhibitor treatment regardless of the presence of siC1-M2; the number of DEGs at 4 h were much smaller than 24 h (Fig. **2c**, left, Supplemental Table **2E**). When DEGs were overlapped with each other, demonstrating that gefitinib-responsive genes at 24 h were highly similar, while quite different at 4 h (Fig. **2c**, right, Supplemental Table **2F**).

To understand the potential biological functions targeted by gefitinib-regulated gene transcription, we performed GO functional clustering and KEGG pathway analysis. Genes commonly repressed by gefitinib at 4 h treatment were enriched in MAPK/ERK, JAK-STAT, TNF signaling pathway including genes such as MYC, FOS, SPROUTY homolog SPRY1, SPRY2, SPRY4, and dual specificity phosphatase DUSP1, DUSP4, DUSP5, DUSP6 (Fig. **2d**, **e**). Genes whose transcription up-regulated by the inhibitor were not enriched in these pathways (Supplementary Fig. **2A**). Functional enrichment of genes specifically responding to gefitinib under siC1-M2 condition showed that TNF signaling, TGF- signaling, VEGF signaling and ERB signaling became sensitive to the inhibitor when C1-M2 level was down-regulated by siRNA (Supplementary Fig. **2B**-**C**). Taken together, the transcriptome analysis suggested that gefitinib could effectively target multiple signaling pathways regardless of the C1-M2 fusion transcript level. Down-regulation of C1-M2 level may sensitize the response by additional cell signaling pathways.

3.3. CRTC1-MAML2 Activation of JAK-STAT and EGFR Pathway Could be Uncoupled from AREG Expression

We then assayed the potential contribution of signaling pathways regulated by C1-M2 in the observed transcriptome analysis shown above. Consistent the reported involvement of AREG-EGFR pathway [17], AREG expression level was down-regulated in some samples with C1-M2 knock-down (Fig. **3a**, Supplementary Fig. **3A**). We also demonstrated that the phosphorylation level of EGFR and ERK1/3 (p42/44 MAPK) was down-regulated by multiple siC1-M2 (Fig. **3b**). In addition, P-JAK2, P-STAT1 and P-STAT3 were down-regulated by some siC1-M2. Their response patterns were distinct from P-EGFR and P-ERK, suggesting an independent regulation of these signaling pathways by C1-M2 (Supplementary Fig. **3B**).

To further dissect AREG transcription and EGFR signaling, the levels of AREG mRNA and P-EGFR were examined in H292, HeLa, HCT116 and A549; the later three cell lines do not contain EGFR mutation either. As expected, C1-M2 fusion transcript was only presented in H292, but not the other three cell lines (Fig. **3c**, left). MAML2 was expressed in A549 at a comparable as in H292, however, its expression level was an order of magnitude lower in the other two cell lines, although the expression level of CRTC1 was similar in all four cell lines (Supplementary Fig. **3C**). Expression of AREG was similarly high in H292, HCT116 and A549, indicating that AREG transcription could be driven by factors other than C1-M2 (Fig. **3c**, right). Moreover, P-EGFR level in HeLa and A549 and HCT116 were comparable to that in H292, and the total EGFR level was much lower in HCT116 than the others. P-STAT3 and STAT3 levels in HCT116 were lower as well (Fig. **3d**). Therefore, we conclude that AREG expression does not correlate with EGFR activation in these cell lines.

We then enforced the expression of C1-M2 and AREG in HCT116 and HeLa cells to see whether the ectopic expression of C1-M2 and AREG could activate EGFR signaling (Supplementary Fig. **3D**). C1-M2 increased the protein level of EGFR in HCT116, while AREG decreased (Fig. **3e**). Neither C1-M2 nor AREG changed the levels of STAT3 and P-STAT3 in HCT116 (Fig. **3e**). In HeLa cell, both C1-M2 and AREG could increase the levels of total EGFR and P-EGFR, as well as P-STAT3, while the level of STAT3 kept unchanged (Supplementary Fig. **3E**).

In order to explore the contribution of AREG in EGFR activation, we examined the AREG expression level when C1-M2 was ectopically expressed in HeLa and HCT116, demonstrating that the expression of AREG was successfully promoted by C1-M2 expression in HeLa cells, but repressed in HCT116 cells (Fig. **3f**). This further supported that C1-M2 activation of EGFR expression in HCT116 could be independent of AREG-EGFR signaling.

3.4. The Combinatory Effect of Gefitinib and siC1-M2 on Inhibition of EGFR/JAK-STAT/ERK/AKT Signaling

Gefitinib-repressed expression of genes in JAK-STAT and MAPK/ERK signaling pathways, both are downstream of EGFR signaling. To dissect whether repression of gene expression in JAK-STAT and MAPK/ERK pathways by gefitinib was due to the inhibition of EGFR signaling, we analyzed the phosphorylation level of EGFR and related downstream signaling proteins, showing that the levels of P-EGFR, P-STAT3, P-AKT, as well as were down-regulated by gefitinib treatment at 10-20 M, while

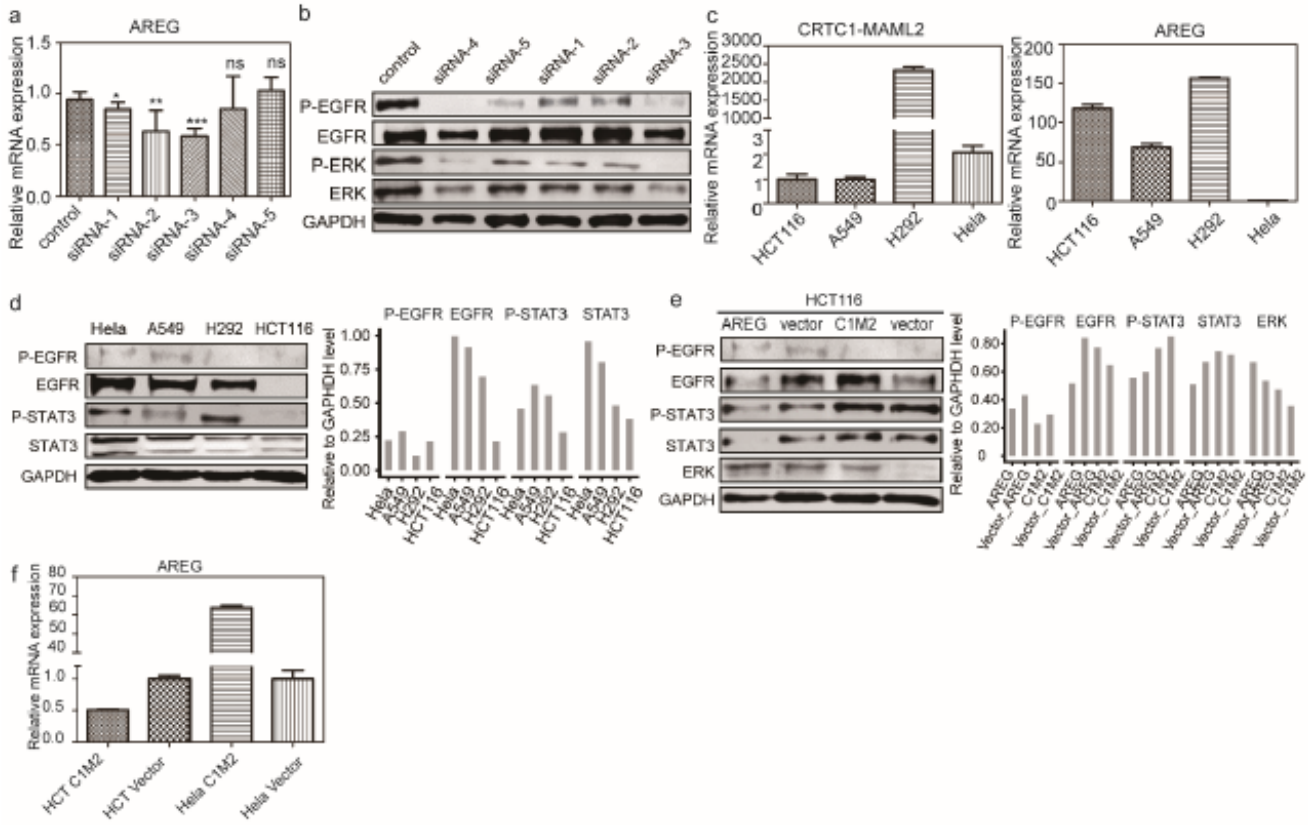

Fig. (3). CRTC1-MAML2 activation of JAK-STAT and EGFR pathway activation could be uncoupled from AREG expression. (a) The expression of AREG transcript was detected through Quantitative Real-time PCR (qRT-PCR) in H292 cells transient-transfected siRNA-1, siRNA-2, siRNA-3, siRNA-4 and siRNA-5 for 48h respectively. (b)The expression level of EGFR pathways related protein, EGFR, MAPK (p42/44), P-EGFR and P-MAPK (p42/44) of H292 cells treated by gefitinib in the presence of five siRNAs, were investigated by western blotting analysis. (c)The expression of AREG and C1-M2 transcript was detected through Quantitative Real-time PCR (qRT-PCR) in HCT116, A549, H292 and Hela cells respectively. (d)The expression level of EGFR pathways related protein, that EGFR, STAT3, P-EGFR and P-STAT3 of these cells were investigated by western blotting analysis. (e)The expression level of protein EGFR, STAT3, P-EGFR, P-STAT3 and MAPK (p42/44) in overexpression AREG and C1-M2 of HCT116 cells were investigated by western blotting analysis. (f)The expression of AREG transcript was detected through Quantitative Real-time PCR (qRT-PCR) in the samples in Fig. 3e.

P-MAPK (p42/44), P-STAT1, and P-JAK2 were not (Fig. **4a**, Supplementary Fig. **4A**). Meanwhile, the total protein levels of EGFR, STAT1 and JAK2 were repressed by gefitinib treatment as well (Fig. **4a**, Supplementary Fig. **4A**). In a different bench of the experiment, we found that gefitinib-reduced EGFR expression was maintained (Supplementary Fig. **4B**). A slight reduction in P-EGFR was observed at 0.1 μM, which was not further decreased at 10 μM of the inhibitor treatment. Interestingly, Both isoforms of P-ERK were robustly reduced by 0.1 μM inhibitor, and the total protein level of larger ERK isoform was greatly reduced by 0.1 μM inhibitor as well. In this set of experiments, the levels of P-AKT, P-JAK2, JAK2, P-STAT3, and STAT3 were reduced at 20 μM inhibitor (Supplementary Fig. **4B**).

We then analyzed the combinatory effects of siC1-M2 and gefitinib on modulating EGFR, JAK, STAT and ERK signaling by adding siRNA treatments. In the set of the experiment shown in Fig. (**4a-c**), the results showed that the presence of siRNA-3 and siRNA-4 sensitized P-EGFR and EGFR, P-ERK and ERK, as well as STAT1 to gefitinib treatment, while four siRNAs could sensitize P-STAT3 to the gefitinib treatment (Fig. **4b-c**). The combinatory effects of siC1-M2 and gefitinib in the other experimental set are shown in Supplementary Fig. (**4b-c**), which demonstrated that siC1-M2 could sensitize P-AKT and P-STAT1 to the gefitinib treatment.

To further explore the relationship between C1-M2 fusion and gefitinib-inhibited EGFR signaling, we analyzed the phosphorylation state of EGFR and STAT3 after the ectopic expression of C1-M2 in HCT116. It was shown that gefitinib effectively reduced both P-EGFR and P-STAT3 in the control HCT116 cells at 10 μM, while enforced the expression of C1-M2 but did not enforce this reduction (Fig. **4d**).

We then examined whether the phosphorylation repression exerted by gefitinib is exerted by repressing C1-M2 transcription. We unexpectedly found that gefitinib increased the transcript levels of C1-M2 fusion, as well as CRTC1 and MAML2. Such an increase was not removed by siRNAs against C1-M2 (Fig. **5**). In contrast, gefitinib did not affect AREG expression in the absence of siC1-M2, and effectively repressed AREG expression when C1-M2 was knock-down by siRNAs (Supplementary Fig. **4D**). These results suggested that gefitinib successfully down-regulated AREG expression when the C1-M2 fusion is repressed. Gefitinib-sensitized C1-M2 expression suggested that C1-M2 counteracts gefitinib repression of kinase phosphorylation in H292 MEC cells.

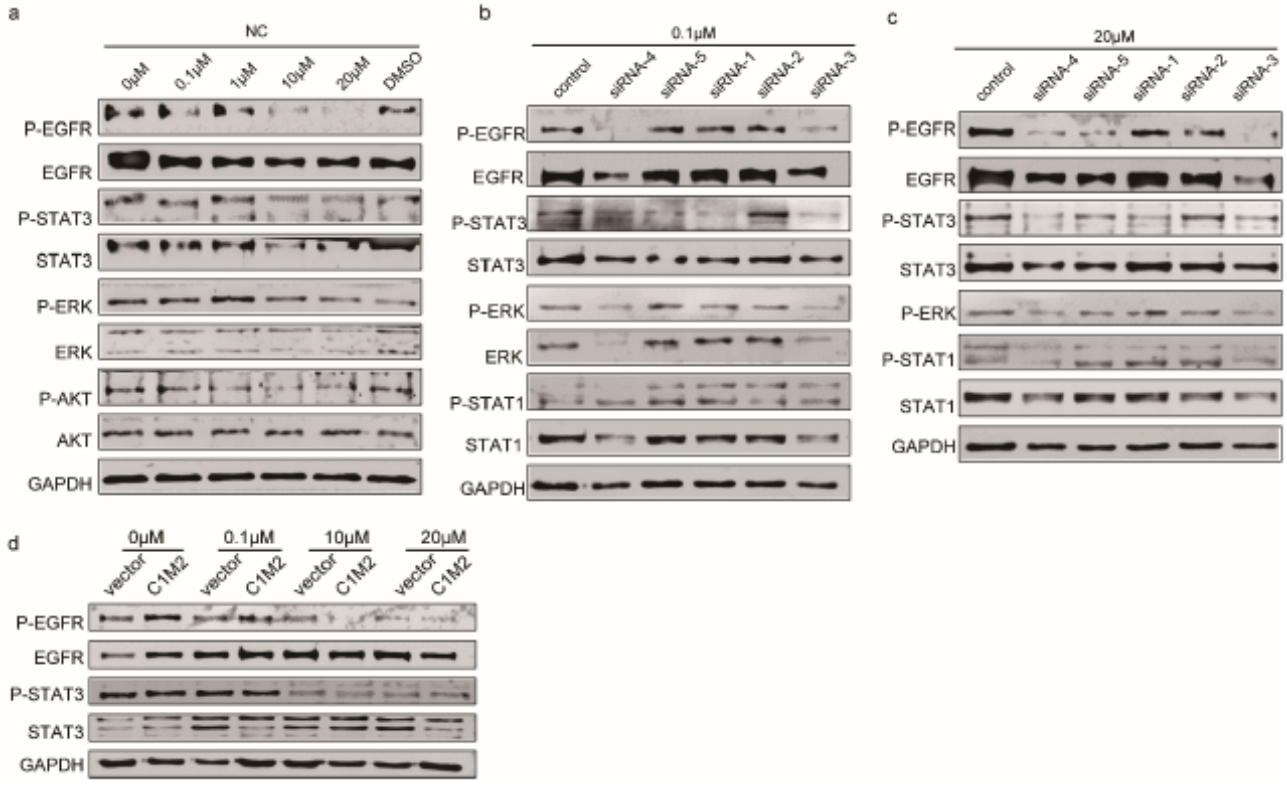

Fig. (4). The combinatory effect of gefitinib and siC1-M2 fusion on inhibition of EGFR/JAK-STAT/ERK/AKT signaling. (a-c) The expression level of EGFR pathways related protein, EGFR, MAPK (p42/44), STAT3, STAT1 AKT, P-EGFR, P-MAPK (p42/44), P-STAT3 , P-STAT1 and P-AKT of H292 cells treated by gefitinib at different concentration (a) and in the presence of different siRNA at 0.1 μM (b) and 20 μM (c) gefitinib concentration. The concentration of DMSO in DMSO-0 μM control (a) was set for 10 and 20 μM of gefitinib. (d) The expression level of protein P-EGFR and P-STAT3 in overexpression C1-M2 of HCT116 cells treated by gefitinib at different concentration were investigated by western blotting analysis.

3.5. Gefitinib Treatment Leads to an Elevated Expression of C1-M2 Fusion, CRTC1 and MAML2, Suggesting a Feedback Regulation

To assess whether gefitinib treatment could reduce the expression of CRTC1-MAML2, we used qPCR analysis after H292 cells were treated by the inhibitor. To our surprise, we found that the expression of C1-M2 fusion was increased upon gefitinib treatment as low as 0.1 μM concentration (Fig. **5a**). This result could be explained by a feedback regulation that resists gefitinib repression of C1-M2 fusion activity. We then used three siRNAs to treat H292 cells prior to gefitinib treatment. The results showed that the inhibitor-increased C1-M2 expression did not disappear (Fig. **5d-f**), indicating an effect which might not be specific to C1-M2 fusion. We then analyzed the effects of gefitinib treatment on the expression of CRTC1 and MAML2 genes, demonstrating that the inhibitor could increase the expression of these two genes as well (Fig. **5b-c**). These results indicated that H292 cell, and probably other cancer cells, encode a feedback regulation mechanism that antagonizes gefitinib inhibitory effect.

4. DISCUSSION

Development of siRNAs, specifically reducing the expression of the fusion CRTC1-MAML2 (C1-M2), was important for further studying the biological function and molecular target of C1-M2 fusion, but also for deciphering the contribution of this fusion transcript during gefitinib or other TKI inhibitor treatment and even for developing the anti-CRTC1-MAML2 molecular therapies. With the development of a set of siRNAs specifically targeting C1-M2 fusion, we have applied unbiasedly transcriptome analysis of the gefitinib targets and C1-M2 fusion targets in a MEC cell line, which successfully leads to a couple of novel findings validated by phosphorylation analysis.

4.1. Gefitinib Targets Multiple Tyrosine Kinases

The success of several clinical responses of MEC patients to gefitinib encourages the studies of the mechanism of this TKI inhibitor [11-14]. A few previous reports have indicated that gefitinib targets EGFR in MEC cells [15, 40 17]. Interestingly, our unbiased transcriptome analysis and the followed validation showed that gefitinib specifically repressed the transcription of genes in JAK-STAT signaling pathway, MAPK (ERK) pathway and TNF signaling pathway at 4 h of treatment.

Consistent with the transcriptome results, gefitinib represses the phosphorylation of JAKs, STATs, MAPK (ERK) and AKT signaling proteins, and the repression is uncoupled from the repression of EGFR activation. These findings are consistent with a hypothesis that this TKI inhibitor can simultaneously target multiple tyrosine kinases, which are

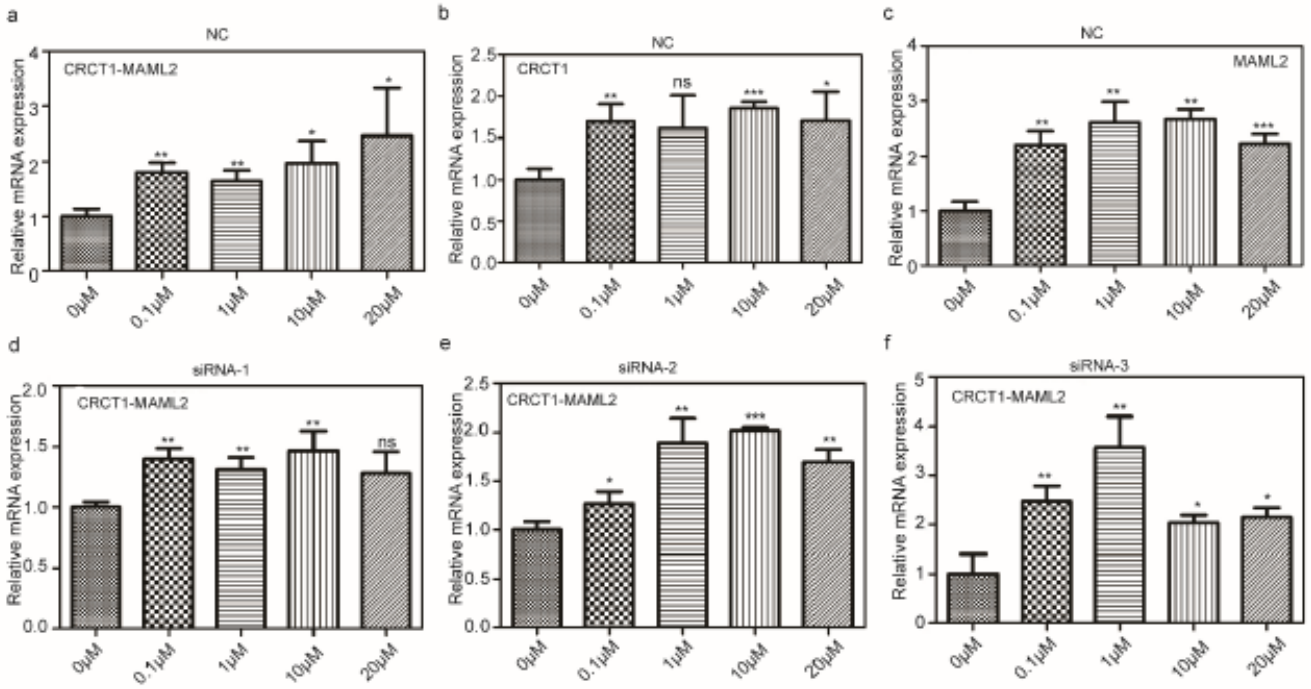

Fig. (5). Gefitinib treatment leads to an elevated expression of C1-M2 fusion, CRTC1 and MAML2, suggesting a feedback regulation. The H292 cells were treated with gefitinib of 0μM, 0.1μM, 1μM, 10μM, 20μM. (a-c) and CRTC1-MAML2, CRTC1, and MAML2 transcripts in H292 cells was detected through Quantitative Real-time PCR (qRT-PCR) (using #c, #a, and #b primers respectively).(d-f)The effectiveness of siRNAs-1, siRNAs-2, siRNAs-3 downregulated CRTC1-MAML2 transcripts in H292 cells was detected through Quantitative Real-time PCR (qRT-PCR).

normally activated in cancers. For example, we showed that C1-M2 fusion activated most of these signaling proteins. Moreover, a number of previous studies documented the aberrant activation of these and other kinases in MEC tissue or cell lines [23-26].

4.2. Combination of Gefitinib and other Inhibitors Against C1-M2 Fusion to Combat MEC

We have shown that C1-M2 fusion activates a number of signaling proteins, while gefitinib inhibits such an activation, indicating that gefitinib antagonizes C1-M2 function in modulating some of the signaling pathways. Moreover, we showed that siC1-M2 sensitizes expression of genes in TNF signaling, TGF-signaling, VEGF signaling, ERB signaling and JAK-STAT signaling in MEC cells to be targeted by gefitinib. Consistently, we showed that siC1-M2 sensitizes the phosphorylation of a number of signaling proteins to gefitinib treatment.

Gefitinib robustly repressed transcription of genes in JAK-STAT, MAPK and TNF pathways, while C1-M2 did not exert such effects, suggesting that gefitinib could target MEC independent of C1-M2 fusion. Therefore, we predict that gefitinib may also be effective in treating MEC patients without C1-M2 fusion. In addition, we showed that gefitinib could elevate the expression level of C1-M2 fusion, as well as CRTC1 and MAML2, suggesting the presence of a feedback loop in cancer cells to counteract the effect of gefitinib. The capability of siC1-M2 in enhancing the gefitinib treatment is consistent with the hypothesis.

CONCLUSION

These results together suggest that a combinatory control of MEC by gefitinib and other inhibitors against C1-M2 fusion transcript could be more powerful in combating MEC. Current known inhibitors targeting MAP kinase pathways could be included in developing therapeutics of MEC in the future [41].

ETHICS APPROVAL AND CONSENT TO PARTICIPATE

Not applicable.

HUMAN AND ANIMAL RIGHTS

No Animals/Humans were used for studies that are the basis of this research.

CONSENT FOR PUBLICATION

Not applicable.

CONFLICT OF INTEREST

The authors declare no conflict of interest, financial or otherwise.

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NOVELTY AND IMPACT

It remains largely elusive whether gefitinib has additional kinase targets other than EGFR. In this article, the authors present the first unbiased study of gefitinib targets in mucoepidermoid carcinoma cell, which has led to the novel finding of JAK-STAT signaling as the targets. The findings provide new insights into the efficacy of gefitinib in treating MEC and suggesting a combination of gefitinib and other inhibitors against C1-M2 fusion could be more effective.

SUPPLEMENTARY MATERIAL

Supplementary material is available on the publishers web site along with the published article.

REFERENCES

[1] Eck, M. J.; Hahn, W. C., EGFR in limbo. *Cell* **2012,** *149* (4), 735-7.

[2] Nyati, M.; Morgan, M.; Feng, F.; Lawrence, T., Integration of EGFR inhibitors with radiochemotherapy. *Nat. Rev. Cancer* **2006,** *6* (11), 876-85.

[3] Pao, W.; Miller, V. A., Epidermal growth factor receptor mutations, small-molecule kinase inhibitors, and non-small-cell lung cancer: current knowledge and future directions. *J Clin Oncol* **2005,** *23* (11), 2556-68.

[4] Sequist, L. V.; Bell, D. W.; Lynch, T. J.; Haber, D. A., Molecular predictors of response to epidermal growth factor receptor antagonists in non-small-cell lung cancer. *J Clin Oncol* **2007,** *25* (5), 587-95.

[5] Zhao, N. J.; Sun, Z.; Wang, Y.; Ning, X.; Jia, N.; Meng, C.; Wang, Y., Gefitinib-Integrated Regimen versus Chemotherapy Alone in Heavily Pretreated Patients with Epidermal Growth Factor Receptor-Mutated Lung Adenocarcinoma: A Case-Control Study. *Transl Oncol* **2014,** *7* (4), 508-12.

[6] Park, K.; Tan, E. H.; O'Byrne, K.; Zhang, L.; Boyer, M.; Mok, T.; Hirsh, V.; Yang, J. C.; Lee, K. H.; Lu, S.; Shi, Y.; Kim, S. W.; Laskin, J.; Kim, D. W.; Arvis, C. D.; Kolbeck, K.; Laurie, S. A.; Tsai, C. M.; Shahidi, M.; Kim, M.; Massey, D.; Zazulina, V.; Paz-Ares, L., Afatinib versus gefitinib as first-line treatment of patients with EGFR mutation-positive non-small-cell lung cancer (LUX-Lung 7): a phase 2B, open-label, randomised controlled trial. *Lancet Oncol* **2016,** *17* (5), 577-89.

[7] Lynch, T. J.; Bell, D. W.; Sordella, R.; Gurubhagavatula, S.; Okimoto, R. A.; Brannigan, B. W.; Harris, P. L.; Haserlat, S. M.; Supko, J. G.; Haluska, F. G., Activating mutations in the epidermal growth factor receptor underlying responsiveness of non-small-cell lung cancer to gefitinib. *New England Journal of Medicine* **2004,** *350* (350), 2129-39.

[8] Sordella, R.; Bell, D. W.; Haber, D. A.; Settleman, J., Gefitinib-Sensitizing EGFR Mutations in Lung Cancer Activate Anti-Apoptotic Pathways. *Science* **2004,** *305* (5687), 1163.

[9] Paez, J. G.; Janne, P. A.; Lee, J. C.; Tracy, S.; Greulich, H.; Gabriel, S.; Herman, P.; Kaye, F. J.; Lindeman, N.; Boggon, T. J.; Naoki, K.; Sasaki, H.; Fujii, Y.; Eck, M. J.; Sellers, W. R.; Johnson, B. E.; Meyerson, M., EGFR mutations in lung cancer: correlation with clinical response to gefitinib therapy. *Science* **2004,** *304* (5676), 1497-500.

[10] Kobayashi, S.; Boggon, T. J.; Dayaram, T.; Jänne, P. A.; Kocher, O.; Meyerson, M.; Johnson, B. E.; Eck, M. J.; Tenen, D. G.; Halmos, B., EGFR mutation and resistance of non-small-cell lung cancer to gefitinib. *New England Journal of Medicine* **2005,** *352* (8), 786.

[11] Han, S. W.; Kim, H. P.; Jeon, Y. K.; Oh, D. Y.; Lee, S. H.; Kim, D. W.; Im, S. A.; Chung, D. H.; Heo, D. S.; Bang, Y. J.; Kim, T. Y., Mucoepidermoid carcinoma of lung: potential target of EGFR-directed treatment. *Lung Cancer* **2008,** *61* (1), 30-4.

[12] Rossi, G.; Sartori, G.; Cavazza, A.; Tamberi, S., Mucoepidermoid carcinoma of the lung, response to EGFR inhibitors, EGFR and K-RAS mutations, and differential diagnosis. *Lung Cancer* **2009,** *63* (1), 159-60.

[13] Alsidawi, S.; Morris, J. C.; Wikenheiser-Brokamp, K. A.; Starnes, S. L.; Karim, N. A., Mucoepidermoid carcinoma of the lung: a case report and literature review. *Case reports in oncological medicine* **2013,** *2013*, 625243.

[14] Macarenco, R. S.; Uphoff, T. S.; Gilmer, H. F.; Jenkins, R. B.; Thibodeau, S. N.; Lewis, J. E.; Molina, J. R.; Yang, P.; Aubry, M.-C., Salivary gland-type lung carcinomas: an EGFR immunohistochemical, molecular genetic, and mutational analysis study. *Modern Pathology* **2008,** *21*, 1168.

[15] Coxon, A.; Rozenblum, E.; Park, Y. S.; Joshi, N.; Tsurutani, J.; Dennis, P. A.; Kirsch, I. R.; Kaye, F. J., Mect1-Maml2 fusion oncogene linked to the aberrant activation of cyclic AMP/CREB regulated genes. *Cancer Res* **2005,** *65* (16), 7137-44.

[16] O'Neill, I. D., Gefitinib as targeted therapy for mucoepidermoid carcinoma of the lung: possible significance of CRTC1–MAML2 oncogene. *Lung Cancer* **2009,** *64* (1), 129-130.

[17] Chen, Z.; Chen, J.; Gu, Y.; Hu, C.; Li, J. L.; Lin, S.; Shen, H.; Cao, C.; Gao, R.; Li, J.; Ha, P. K.; Kaye, F. J.; Griffin, J. D.; Wu, L., Aberrantly activated AREG-EGFR signaling is required for the growth and survival of CRTC1-MAML2 fusion-positive mucoepidermoid carcinoma cells. *Oncogene* **2014,** *33* (29), 3869-77.

[18] Yonesaka, K.; Zejnullahu, K.; Lindeman, N.; Homes, A. J.; Jackman, D. M.; Zhao, F.; Rogers, A. M.; Johnson, B. E.; Janne, P. A., Autocrine production of amphiregulin predicts sensitivity to both gefitinib and cetuximab in EGFR wild-type cancers. *Clin Cancer Res* **2008,** *14* (21), 6963-73.

[19] Shan, Y.; Eastwood, M. P.; Zhang, X.; Kim, E. T.; Arkhipov, A.; Dror, R. O.; Jumper, J.; Kuriyan, J.; Shaw, D. E., Oncogenic mutations counteract intrinsic disorder in the EGFR kinase and promote receptor dimerization. *Cell* **2012,** *149* (4), 860-70.

[20] Sharma, S. V.; Bell, D. W.; Settleman, J.; Haber, D. A., Epidermal growth factor receptor mutations in lung cancer. *Nature reviews. Cancer* **2007,** *7* (3), 169-81.

[21] Speight, P.; Barrett, A., Salivary gland tumours. *Oral diseases* **2002,** *8* (5), 229-240.

[22] Liu, X.; Adams, A. L., Mucoepidermoid carcinoma of the bronchus: a review. *Archives of pathology & laboratory medicine* **2007,** *131* (9), 1400-1404.

[23] Press, M. F.; Pike, M. C.; Hung, G.; Zhou, J. Y.; Ma, Y.; George, J.; Dietz-Band, J.; James, W.; Slamon, D. J.; Batsakis, J. G., Amplification and overexpression of HER-2/neu in carcinomas of the salivary gland: correlation with poor prognosis. *Cancer Research* **1994,** *54* (21), 5675.

[24] Yoo, J.; Robinson, R. A., H‐ras gene mutations in salivary gland mucoepidermoid carcinomas. *Cancer* **2000,** *88* (3), 518-523.

[25] Handra-Luca, A.; Bilal, H.; Bertrand, J.-C.; Fouret, P., Extra-Cellular Signal-Regulated ERK-1/ERK-2 Pathway Activation in Human Salivary Gland Mucoepidermoid Carcinoma. *The American Journal of Pathology* **2003,** *163* (3), 957-967.

[26] Lujan, B.; Hakim, S.; Moyano, S.; Nadal, A.; Caballero, M.; Diaz, A.; Valera, A.; Carrera, M.; Cardesa, A.; Alos, L., Activation of the EGFR/ERK pathway in high-grade mucoepidermoid carcinomas of the salivary glands. *Br J Cancer* **2010,** *103* (4), 510-6.

[27] Nordkvist, A.; Gustafsson, H.; Juberg-Ode, M.; Stenman, G., Recurrent rearrangements of 11q14–22 in mucoepidermoid carcinoma. *Cancer genetics and cytogenetics* **1994,** *74* (2), 77-83.

[28] Behboudi, A.; Enlund, F.; Winnes, M.; Andren, Y.; Nordkvist, A.; Leivo, I.; Flaberg, E.; Szekely, L.; Makitie, A.; Grenman, R.; Mark, J.; Stenman, G., Molecular classification of mucoepidermoid carcinomas-prognostic significance of the MECT1-MAML2 fusion oncogene. *Genes Chromosomes Cancer* **2006,** *45* (5), 470-81.

[29] O'Neill, I. D., t(11;19) translocation and CRTC1-MAML2 fusion oncogene in mucoepidermoid carcinoma. *Oral Oncol* **2009,** *45* (1), 2-9.

[30] Seethala, R. R.; Dacic, S.; Cieply, K.; Kelly, L. M.; Nikiforova, M. N., A reappraisal of the MECT1/MAML2 translocation in salivary mucoepidermoid carcinomas. *American Journal of Surgical Pathology* **2010,** *34* (8), 1106.

[31] Tonon, G.; Modi, S.; Wu, L.; Kubo, A.; Coxon, A. B.; Komiya, T.; O'Neil, K.; Stover, K.; El-Naggar, A.; Griffin, J. D.; Kirsch, I. R.; Kaye, F. J., t(11;19)(q21;p13) translocation in mucoepidermoid carcinoma creates a novel fusion product that disrupts a Notch signaling pathway. *Nat Genet* **2003,** *33* (2), 208-13.

[32] Komiya, T.; Park, Y.; Modi, S.; Coxon, A. B.; Oh, H.; Kaye, F. J., Sustained expression of Mect1-Maml2 is essential for tumor cell growth in salivary gland cancers carrying the t(11;19) translocation. *Oncogene* **2006,** *25* (45), 6128-32.

[33] Wu, L.; Liu, J.; Gao, P.; Nakamura, M.; Cao, Y.; Shen, H.; Griffin, J. D., Transforming activity of MECT1-MAML2 fusion oncoprotein is mediated by constitutive CREB activation. *Embo Journal* **2005,** *24* (13), 2391–2402.

[34] Canettieri, G.; Coni, S.; Della Guardia, M.; Nocerino, V.; Antonucci, L.; Di Magno, L.; Screaton, R.; Screpanti, I.; Giannini, G.; Gulino, A., The coactivator CRTC1 promotes cell proliferation and transformation via AP-1. *Proc Natl Acad Sci U S A* **2009,** *106* (5), 1445-50.

[35] Amelio, A. L.; Fallahi, M.; Schaub, F. X.; Zhang, M.; Lawani, M. B.; Alperstein, A. S.; Southern, M. R.; Young, B. M.; Wu, L.; Zajackaye, M., CRTC1/MAML2 gain-of-function interactions with MYC create a gene signature predictive of cancers with CREB-MYC involvement. *Proceedings of the National Academy of Sciences of the United States of America* **2014,** *111* (32), E3260.

[36] Kim, D.; Pertea, G.; Trapnell, C.; Pimentel, H.; Kelley, R.; Salzberg, S. L., TopHat2: accurate alignment of transcriptomes in the presence of insertions, deletions and gene fusions. *Genome Biol* **2013,** *14* (4), R36.

[37] Robinson, M. D.; McCarthy, D. J.; Smyth, G. K., edgeR: a Bioconductor package for differential expression analysis of digital gene expression data. *Bioinformatics* **2010,** *26* (1), 139-40.

[38] Wittrup, A.; Lieberman, J., Knocking down disease: a progress report on siRNA therapeutics. *Nat Rev Genet* **2015,** *16* (9), 543-52.

[39] Boettcher, M.; McManus, M. T., Choosing the Right Tool for the Job: RNAi, TALEN, or CRISPR. *Mol Cell* **2015,** *58* (4), 575-85.

[40] Favoni, R. E.; Pattarozzi, A.; Lo Casto, M.; Barbieri, F.; Gatti, M.; Paleari, L.; Bajetto, A.; Porcile, C.; Gaudino, G.; Mutti, L.; Corte, G.; Florio, T., Gefitinib targets EGFR dimerization and ERK1/2 phosphorylation to inhibit pleural mesothelioma cell proliferation. *Current cancer drug targets* **2010,** *10* (2), 176-91.

[41] Tolcher, A. W.; Peng, W.; Calvo, E., Rational Approaches for Combination Therapy Strategies Targeting the MAP Kinase Pathway in Solid Tumors. *Molecular Cancer Therapeutics* **2018,** *17* (1), 3-16.