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Inhibition of epidermal growth factor receptor suppresses parathyroid hormone-related protein expression in tumours and ameliorates cancer-associated cachexia

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Abstract

Background Lung cancer is the primary cause of cancer deaths worldwide. Activation of epidermal growth factor receptor (EGFR) leads to lung cancer progression and poor prognosis while involuntary weight loss remains a major problem. Tumour-derived parathyroid hormone-related protein (PTHrP) emerged as a potential mediator of cachexia. Here, we investigated the modulatory role of EGFR signalling in PTHrP (encoded by *Pthlh*) gene expression and the impact of this relationship on cancer cachexia.

Methods Global gene expression profiles of Lewis lung carcinoma (LLC) cells were analysed. *Pthlh* mRNA levels were measured by qRT-PCR in LLC cells treated with EGFR ligands and tyrosine kinase inhibitors (TKIs). LLC tumour-bearing mice received EGFR TKI erlotinib for 7 days via intraperitoneal injection or oral gavage. Tumour *Pthlh* mRNA, weight of fat/muscle tissue, and grip strength were assessed. RNA-seq data from The Cancer Genome Atlas and gene expression analysis tools were used to characterize expression profiles of *PTHLH* and *EGFR* along with correlation analysis of *PTHLH* with *EGFR* and transforming growth factor alpha (*TGFA*) in human lung cancer and head and neck squamous carcinoma (HNSC). Survival of lung squamous cell carcinoma (LUSC) and lung adenocarcinoma (LUAD) patients with EGFR gene alterations was analysed in regard to *PTHLH* expression.

Results Expression of EGFR ligands, EGFR itself, and PTHrP co-clusters in LLC cells. Activation of EGFR signalling with its ligands significantly increases (3.8-fold, $P < 0.0005$) while EGFR TKIs significantly decrease (90%, $P < 0.0005$) *Pthlh* mRNA levels in LLC cells. *Pthlh* mRNA drops 65–75% ($P < 0.0005$) in tumours upon treatment of LLC tumour-bearing mice with erlotinib while their muscle mass and grip strength increase (9.2% $P < 0.05$, 23% $P < 0.005$, respectively) compared with tumour-bearing control mice. *PTHLH* is overexpressed in tumours of LUSC (45.8-fold, $P < 0.05$) and HNSC (17.5-fold, $P < 0.05$) compared with normal tissue. *PTHLH* expression correlates with *EGFR* and its ligand *TGFA* in both cancers (LUSC: $n = 745$, $R = 0.32$, $P < 0.0001$ and $R = 0.51$, $P < 0.0001$; HNSC: $n = 545$, $R = 0.34$, $P < 0.001$ and $R = 0.50$, $P < 0.001$, respectively). High *PTHLH* mRNA associates with poor overall survival in LUAD patients with activating EGFR mutations ($n = 40$, log-rank test, $P = 0.0451$).

Conclusions Epidermal growth factor receptor signalling regulates expression of cachexia mediator PTHrP. EGFR inhibition reduces PTHrP expression in LLC tumours and ameliorates cachexia in LLC tumour-bearing mice.

Keywords Epidermal growth factor receptor (EGFR); EGFR inhibitors; Parathyroid hormone-related protein (PTHrP); Lung cancer; Cancer-associated cachexia

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Introduction

Lung cancer is the most common malignant disease and world's leading cause of cancer deaths in both men and women.[1] Lung cancer is histologically divided into two main types: small cell lung cancer and non-small cell lung cancer (NSCLC). NSCLC comprises the vast majority (85%) of lung cancers with poor survival rates depending on the stages.[2] NSCLC has been classified into three main subtypes: adenocarcinoma (AD), squamous cell carcinoma (SCC), and large cell carcinoma, which represent 40%, 25% to 30%, and 5% to 10% of lung cancers, respectively.[3] Most lung cancers are detected at an advanced stage and inoperable with limited therapeutic options, including radiation therapy, chemotherapy, and targeted therapy.[3] However, due to the severe side effects of radiotherapy and chemotherapy, novel targeted therapy models became extremely essential for the treatment of lung cancer.[4]

Targeting the epidermal growth factor receptor (EGFR) has played a main role in advancing NSCLC treatment and patient outcome over the last several years.[5] Activation of EGFR tyrosine kinase (TK) downstream signalling promotes a malignant phenotype by promoting critical oncogenic pathways implicated in cell survival, proliferation, invasion, and metastasis.[6] In many tumour cells, TK activity of EGFR is dysregulated by various oncogenic mechanisms, including EGFR gene mutation, increased gene copy number, and EGFR protein overexpression.[7] EGFR overexpression observed approximately in 60% of patients with all types of lung cancers, with highest rates seen in squamous tumours (89%).[5,8] EGFR expression frequently correlates with a more aggressive phenotype and worse prognosis.[7] Therefore, the use of novel anti-cancer agents targeting EGFR such as EGFR-tyrosine kinase inhibitors (TKI), erlotinib and gefitinib, or EGFR-neutralizing antibodies, cetuximab and bevacizumab, has attenuated lung cancer.[4,9] For patients whose tumours exhibit classical activating EGFR mutations, the response rate to erlotinib and gefitinib is approximately 75%, proving that cancer cells are highly dependent on EGFR oncogenic pathways for survival.[7,9]

Despite the improvements in diagnosis and therapy, the prognosis and survival of patients with lung cancer is still unsatisfactory.[3] One of the most common problem facing patients with advanced lung cancer is cancer cachexia associated with reduced treatment tolerance, poor prognosis and survival.[10] Approximately 60% of lung cancer patients show significant weight loss at the time of diagnosis, and more than 10% of patients die with or from cancer cachexia itself.[10] Cachexia causes atrophy of fat and skeletal muscle, leading to dramatic weight loss. Cachectic patients have a combination of reduced food intake, abnormal metabolism, and chronic inflammation inducing a state of negative energy balance that cannot be fully reversed by nutritional supplementation.[11] The molecular basis of cachexia is poorly understood, and a fulfilling treatment method is still not available.[11] One hallmark of cachexia is increased resting energy expenditure that has been linked to elevated levels of thermogenesis in brown fat tissue. This process generates heat instead of chemical energy, thus plays a role in the negative energy balance.[12,13] Using Lewis lung carcinoma (LLC) model of cancer cachexia, previously we identified that tumour-derived parathyroid hormone-related protein (PTHrP) triggers cachexia, through driving the expression of thermogenesis genes in adipose tissue and resultant hypermetabolism. Neutralization of PTHrP in tumour-bearing mice blocked adipose tissue browning, wasting of fat and muscle tissues as well as muscle weakness. Hence, PTHrP is considered to be a potential therapeutic target to ameliorate cancer cachexia.[12–14]

Interestingly, our previous data demonstrated increased expression of EGFR and various EGFR ligands including betacellulin (BTC), heparin-binding EGF like growth factor (HB-EGF), and epiregulin (EREG) along with PTHrP in the microarray analysis of the thermogenesis-inducing LLC cell subclones.[13] Co-clustering of PTHrP with EGFR and EGFR ligands implicated that EGFR signalling pathway is involved in the regulation of PTHrP (encoded by *Pthlh*) gene expression. Therefore, in the present study, we aimed to understand the role of EGFR signalling in PTHrP gene regulation *in vitro* and the impact of this relationship on cancer cachexia using the murine Lewis lung carcinoma (LLC) model of cachexia *in vivo*. Our findings revealed that, variety of EGFR ligands induces *Pthlh* mRNA level, while different EGFR inhibitors and mitogen-activated protein kinase (MEK) inhibitors reduces it in LLC cells. We also identified that *Pthlh* expression significantly diminishes in tumours upon treatment of LLC tumour-bearing mice with EGFR-TKI erlotinib. Furthermore, erlotinib treatment attenuates loss of fat and muscle mass, and it enhances muscle strength, indicating a significant improvement in LLC tumour-induced cancer cachexia. We showed that *PTHLH* expression is significantly increased and correlates with expression of *EGFR* and its ligand transforming growth factor alpha (*TGFA*) in human lung squamous cell carcinoma (LUSC) patients. Moreover, tumours of human head and neck squamous carcinoma (HNSC) with high prevalence to cachexia also express very high levels of *PTHLH* and *PTHLH* expression correlates with *EGFR* and *TGFA*. Importantly, our data also revealed that high *PTHLH* expression associates with worse overall survival in lung adenocarcinoma (LUAD) patients harbouring classical activating EGFR mutations. Additionally, high *PTHLH* expression appears to be related with poor overall survival in LUSC patients with EGFR gene amplification. These findings argue that EGFR and PTHrP expression could be used as clinical biomarkers to identify lung cancer patients likely to benefit from anti-EGFR therapy not only to reduce tumour burden but also to ameliorate cancer cachexia.

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Materials and methods

Mice

C57BL/6 mice were housed in 12 h light/dark cycles and given ad libitum access to standard rodent chow diet and water; 8- to 12-week-old male mice were used in all animal experiments. Mice were kept in the Koc University Animal Research Facility in accordance with institutional policies and animal care ethics guidelines. Whole-body energy metabolism was evaluated using Phenomaster Metabolic Phenotyping chambers (TSE systems, 8 units). CO_2 and O_2 data were collected every 27 minutes for each mouse and were normalized to total body weight. Data on food intake and physical activity were measured continuously. Blood was collected into heparin coated tubes. Plasma calcium levels were measured using VetTest calcium slides and VetTest Chemistry Analyser. All animal protocols were approved by the Institutional Animal Care and Use Committee of Koc University.

Lewis lung carcinoma tumor inoculation and erlotinib administration in mice

Mice were divided into groups randomly while satisfying the criteria that the average body weight in each group is similar. LLC cells (5×10^6 per mouse) were injected subcutaneously over the flank. Nine days after LLC cell injection, mice received 10 mg/kg/day erlotinib (Cayman and Selleckchem) via intraperitoneal injections (i.p.) or 100 mg/kg/day erlotinib via oral gavage for 7 days. Non-treated group received vehicle [captisol or cremophor EL (i.p.) and hydroxypropyl methylcellulose (gavage)]. Mice were housed individually in all tumour inoculation experiments. Sixteen days after tumour inoculation, mice were sacrificed. Epididymal, inguinal and interscapular fat depots, gastrocnemious muscle and tumours were dissected and weighed using an analytical balance.

Lewis lung carcinoma cell culture and gene expression analysis

Lewis lung carcinoma cells were maintained in Dulbecco's modified Eagle medium (DMEM) with 10% fetal bovine serum (FBS), and penicillin/streptomycin. Cells were treated with EGFR ligands or kinase inhibitors for 24 h at the indicated doses. dChip software was used in the analysis of global gene expression profiles of the LLC cell subclones as described previously[13] (GSE57797).

RNA isolation and quantitative reverse transcription PCR

Total RNA from cultured cells or frozen tumour samples were extracted using TRIzol reagent (Invitrogen), purified with RNeasy minicolumns (Qiagen). Complementary DNA synthesis was carried out with High-Capacity cDNA Reverse Transcription kit (Applied Biosystems). The resultant cDNA was analysed by qRT-PCR using SYBR GreenER qPCR SuperMix (Invitrogen) according to the manufacturer's instructions. The following primers were used: *Pthlh* F: 5′-CGCTGATTC CTACACAAGTCC-3′, R: 5′-GACACTCCACTGCTGAACCA-3′; *Cyclo* F: 5′-GGAGATGGCACAGGAGGAA-3′, R: 5′-GCCCGTAGTGCT TCAGCTT-3. *Egfr* F: 5′-AACTGTACCTATGGATGTGCTG-3′, R: 5′-AGAGGGGAGTCAGAGATGGC-3′; *Actb* F: 5′-TTCTTGGGTA TGGAATCCTGTGG-3′, R: 5′-TTTACGGATGTCAACGTCACAC-3′; *Bim* F: 5′- CTTACACAAGGAGGGTGTTTGC-3′, R: 5′-GGGGTT CTCCCAGTCTGAAC-3′; *Bax* F: 5′-TGCTAGCAAACTGGTGCTCA-3′, R: 5′-TAGGAGAGGAGGCCTTCCCAG-3′; *Bcl2* F: 5′-GGATAACGGAGGCTGGGATG-3′, R: 5′-GCTGAGCAGGGT CTTCAGAG-3′. Reactions were run on CFX96 Real Time PCR detection system (Bio-Rad). Relative mRNA levels were calculated by the ΔΔCt method and normalized to cyclophilin mRNA.

Western blotting

Cells were homogenized in a lysis buffer containing 50 mM Tris (pH 7.4), 150 mM NaCl, 1% Triton X-100, 5 mM EDTA and 1 mM PMSF supplemented with protease and phosphatase inhibitor cocktails (Roche). A similar lysis buffer was used for tissue samples where 1% NP40 was used as the detergent and 10% glycerol was used. The homogenates were centrifuged at 13 000 rpm for 10 min and the supernatants were used as lysates. Protein concentration was determined by Bio-Rad Protein assay and 30 μg of protein lysate was used in each SDS-PAGE run. Nitrocellulose membrane was blotted with primary antibodies in TBS containing 0.05% Tween and 5% BSA (Cell Signaling, p-ERK #9101, total-ERK #9102 and abcam UCP1 ab10983). For secondary antibody incubation, TBS-T containing 5% milk was used (Cell Signaling anti-rabbit #7074). WesternBright blotting substrates from Advansta were used for visualization of the results on a Chemidoc imaging system (Bio-Rad). Image quantification was performed using Image Lab Software (Bio-Rad).

Grip strength

Forelimb grip strength was measured on the same day as sacrifice. Each mouse was allowed to grab a bar attached to a

force transducer while the mouse was steadily pulled by the tail along the sensor axle until grip is released (Ugo Basile grip strength meter). The maximum strength produced before releasing the grid was registered from five repetitions with a 30 s pause between each were averaged to determine the grip strength of each mouse.

Human gene expression and genomics analysis

For human gene expression analysis, The Cancer Genome Atlas (TCGA) gene expression quantification and the Genotype-Tissue Expression (GTEx) data for HNSC, LUSC and LUAD were downloaded from Genomic Data Commons Data Portal on 6 April 2021. We selected all samples with open access; 202 samples from Clinical Proteomic Tumour Analysis Consortium 3 (CPTAC-3) and 543 samples from TCGA-LUSC project, 203 samples from CPTAC-3 and 547 samples from TCGA-LUAD project and 546 samples from TCGA-HNSC project. RNA sequencing data were analysed using R and the R studio interface 1.4.1106. Each expression value was log2-transformed before visualization. Significant correlations between *PTHLH* and *EGFR or TGFA* mRNA expression levels were evaluated using the Pearson correlation. *PTHLH* and *EGFR* gene expression levels in normal tissue and tumours of LUSC, LUAD, and HNSC cancer datasets were compared using Gene Expression Profiling Interactive Analysis (GEPIA) web server with default parameters.[15] EGFR genetic and copy number alterations in TCGA-LUAD and TCGA-LUSC patients were examined using cBioPortal for Cancer Genomics.[16] Kaplan–Meier survival curves were assessed to determine the association between EGFR gene alterations and overall survival in *PTHLH* high and low expressing LUAD and LUSC patients.

Statistical analysis

The values are expressed as mean ± SEM (standard error of mean) shown for all results derived from biological replicates. Kaplan–Meier analysis utilizing log-rank test was used to estimate overall survival. Correlation of gene expression was determined by Pearson correlation test. One-way ANOVA was used for box-plot data analysis acquired using GEPIA tool. Two-way ANOVA with repeated measures was used for the analysis of metabolic data such as VO_2, VCO_2, physical activity and food intake. An unpaired two-tailed Student's *t*-test was used for all other statistical analyses. In all *t*-test analyses, statistical significance of one independent variable in two different groups was tested. Statistical significance was indicated using a *P* value of 0.05 or less. Analyses were performed using GraphPad Prism version 8.1.1.

Results

Epidermal growth factor receptor signalling regulates parathyroid hormone-related protein gene expression in Lewis lung carcinoma cells

Previously, PTHrP was associated with thermogenesis-inducing activity derived from the secretion of LLC cells.[13] Briefly, expression of Uncoupling Protein 1 (UCP1) was found to be elevated in mouse primary adipocytes treated with media conditioned by LLC cells. We generated multiple single-cell clones from heterogeneous LLC cells and the media conditioned with the subclones were used to induce thermogenesis/UCP1 expression in adipocytes. Subclones producing the highest and lowest levels of thermogenesis-inducing activity were selected for global gene expression profile analysis using microarrays, which revealed PTHrP as the browning factor present in the LLC cell secretion that promotes UCP1 expression in adipocytes.[13] Our further analysis of gene expression profiles of the subclones has also shown that various EGFR ligands; BTC, EREG, HB-EGF, and amphiregulin (AREG) co-cluster with PTHrP gene expression in highly thermogenic clones compared with less thermogenic clones and EGFR expression also parallels this cluster group (*Figure* 1A), suggesting that activation of EGFR signalling pathway is potentially associated with PTHrP gene expression. To assess whether EGFR signalling modulates PTHrP gene expression, we treated LLC cells with all seven ligands that can bind to and activate EGFR. *Pthlh* mRNA expression is significantly increased in all conditions, with highest activation attained in TGFα (3.5-fold) and HB-EGF (3.8-fold) treatment groups (*Figure* 1B). To investigate whether EGFR activity is required for *Pthlh* mRNA expression, we utilized pharmacological EGFR inhibition. We treated LLC cells with increasing concentrations of EGFR-TKI erlotinib. Inhibition of EGFR phosphorylation by erlotinib caused a dose dependent significant decrease in *Pthlh* mRNA compared with control without altering expression of *Egfr* or *β-Actin* (*Actb*) (*Figure* 1C) and apoptosis-related genes *Bim*, *Bax*, and *Bcl2* (Supporting Information, *Figure* S1a). A similar effect was also observed in C26 colon carcinoma cells (*Figure* S1b). To show that this effect is not only restricted to erlotinib, we tested other EGFR-TKIs, including lapatinib, gefitinib, and afatinib, in low (1 μM) and high doses (10 μM) in LLC cells. All EGFR-TKIs appear to reduce *Pthlh* mRNA with the highest suppression (around 90%) attained in afatinib and erlotinib treatment groups (*Figure* 1D). These data indicate that PTHrP gene expression is modulated by the activation and disruption of EGFR signalling. To determine the downstream players involved in *Pthlh* regulation, we also treated LLC cells with inhibitors of kinases that are related to EGFR signalling. While Janus Kinase 2 (JAK2) inhibitor ruxolitinib, phosphoinositide 3-kinase (PI3K) inhibitor wortmannin and mammalian target of rapamycin (mTOR) inhibitor Torin failed to suppress

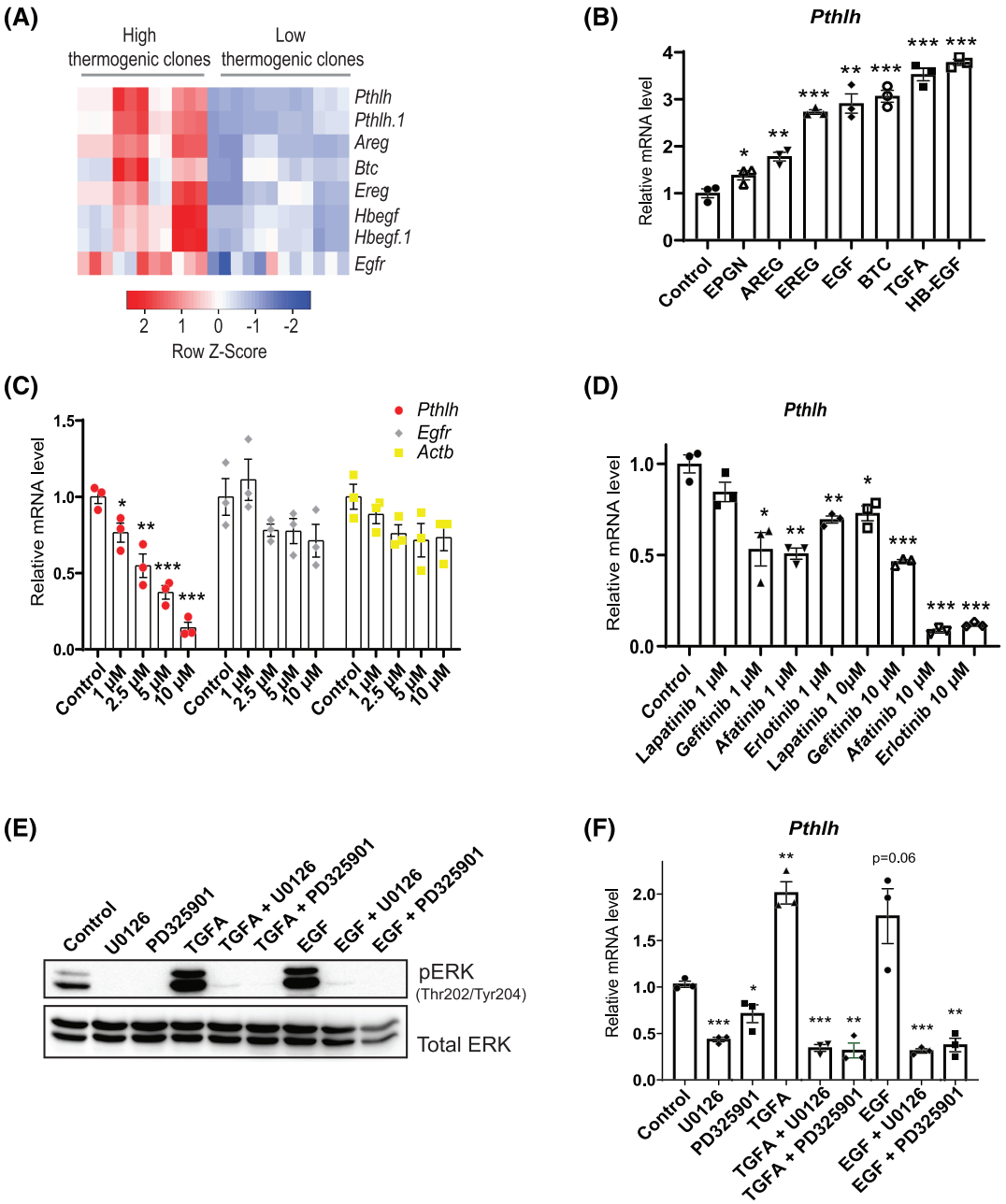

Figure 1 EGFR signalling regulates *Pthlh* mRNA levels in LLC cells. (*A*) Global gene expression profiles of LLC subclones with high and low thermogenic activities were obtained by analysing previously published microarray dataset (the GEO accession number: GSE57797). Heatmap shows clustering of higher *Pthlh* expression with EGFR ligands *Areg*, *Btc*, *Ereg*, and *Hbegf* in highly thermogenic LLC clones. *Egfr* expression correlates with this cluster. (*B*) Relative *Pthlh* mRNA levels were measured by qRT-PCR in LLC cells treated for 24 h with (100 ng/mL) EGFR ligands; EGF, EPGN, TGFA, AREG, EREG, BTC, HB-EGF. (*C*) Relative *Pthlh*, *Egfr*, *β-actin* (*Actb*) mRNA levels were measured by qRT-PCR in LLC cells treated for 24 h with different concentrations of EGFR tyrosine kinase inhibitor Erlotinib (1, 2.5, 5, and 10 μM). *Pthlh* mRNA level was detected by qRT-PCR in LLC cells treated for 24 h (*D*) with low (1 μM) and high (10 μM) concentrations of EGFR TKIs; Lapatinib, Gefitinib, Afatinib, and Erlotinib. (*E*) LLC cells were treated for 1 h with MEK inhibitors U0126 (10 μM) or PD325901 (50 nM) in combination with TGFα or EGF (500 ng/mL, 10 min). Total and phospho-ERK levels were determined by western blotting in LLC cell lysates. (*F*) Relative *Pthlh* mRNA levels were also detected by qRT-PCR in LLC cells treated for 24 h with MEK inhibitors U0126 (10 μM) and PD325901 (50 nM) in combination with TGFα or EGF (500 ng/mL). Values are mean ± SEM ($n = 3$). The statistical analysis was performed using two-tailed *t*-test compared with the control groups (*$P < 0.05$; **$P < 0.01$; ***$P < 0.001$) ($n = 3$). AREG, Amphiregulin; BTC, Betacellulin; EREG, Epiregulin; HB-EGF, heparin-binding EGF-like growth factor; EGF, epidermal growth factor; EPGN, Epigen; TGFA, transforming growth factor-α.

basal *Pthlh* expression, mitogen-activated protein kinase (MEK) inhibitors U0126 and PD325901 potently reduced *Pthlh* mRNA levels (*Figure* S1c). Furthermore, treatment of LLC cells with EGF or TGFα potently induced ERK phosphorylation which was completely blocked by MEK inhibitors (*Figure* 1E). MEK inhibition also blocked EGF or TGFα-induced *Pthlh* expression in LLC cells (*Figure* 1F), arguing that the MEK/ERK kinase signalling is likely involved in EGFR-dependent *Pthlh* regulation.

Inhibition of epidermal growth factor receptor reduces tumor parathyroid hormone-related protein gene expression and ameliorates cancer cachexia in Lewis lung carcinoma tumor-bearing mice

Next, we analysed the impact of EGFR inhibition on tumour PTHrP gene expression and cancer cachexia *in vivo*. We employed syngeneic LLC lung cancer model that is accompanied by cachexia in C57BL/6 mice. After formation of palpable LLC tumours, mice were administered with EGFR-TKI erlotinib for 7 days. We observed that *Pthlh* mRNA expression was reduced by 65% in tumour tissue after i.p. injection (*Figure* 2A) and 75% after oral gavage of erlotinib (*Figure* 2B). While i.p. treatment of erlotinib (10 mg/kg/day) had no effect on body weight of non-tumour-bearing mice (*Figure* 2C), oral gavage (100 mg/kg/day) was toxic and led to significant weight loss and therefore this group was not further analysed (data not shown). Sixteen days post tumour inoculation, mice lost significant body weight. However, erlotinib treatment group displayed significantly less weight loss upon tumour inoculation compared with tumour-bearing control group (*Figure* 2C). A significant difference in tumour mass was not seen while ERK1/2 phosphorylation was greatly reduced in tumour samples (*Figures* 2D and S2a). We analysed the weight of different fat depots; epididymal WAT (eWAT), inguinal white adipose tissue (iWAT), interscapular brown adipose tissue (BAT), and gastrocnemius muscle (gastroc) to assess wasting as an indicator of cancer cachexia. This analysis revealed that fat and muscle tissues showed significant losses in LLC tumour-bearing mice, which were preserved upon erlotinib administration (eWAT 49.7%; iWAT 74.4%; BAT 30%; gastroc 9.2%) (*Figure* 2E). Moreover, erlotinib treatment remarkably ameliorated (23%) the reduction in grip strength of LLC tumour-bearing mice (*Figure* 2F). We also investigated energy metabolism of these mice utilizing metabolic chambers. Erlotinib administration comparably decreased O_2 consumption (VO_2) and CO_2 production (VCO_2) in tumour-bearing mice (particularly during the light phase), while respiratory exchange ratio (RER) and food intake remained unchanged (*Figure* S2b–e). Physical activity of tumour-bearing mice improved upon erlotinib administration (*Figure* S2f). In line with these observations, UCP1 protein levels in brown adipose tissue was found to be reduced upon erlotinib administration in LLC tumour-bearing mice, arguing that reduced thermogenic activity may account for the decrease in energy expenditure (*Figure* 2G). Additionally, no significant change was detected in plasma calcium levels of these mice (*Figure* S2g). These findings demonstrate that pharmacological inhibition of EGFR disrupts tumour PTHrP gene expression and erlotinib administration to LLC tumour-bearing mice ameliorates wasting syndrome together with muscle strength.

Parathyroid hormone-related protein mRNA is upregulated and correlates with epidermal growth factor receptor and transforming growth factor alpha expression in human non-small cell lung cancer and head and neck squamous cell carcinoma

To test whether our findings on the interaction between EGFR signalling and PTHrP expression is of clinical relevance, we analysed NSCLC datasets from TCGA. We used GEPIA to depicture the expression profiles of *PTHLH* and *EGFR* genes in LUAD and LUSC samples. This analysis demonstrated increases in *PTHLH* expression in the tumours of LUAD (1.61-fold) and LUSC (45.8-fold; $P < 0.05$) patients compared with normal tissue (*Figure* 3A). In the same samples, *EGFR* showed a trend for increased expression in the LUSC tumours compared with healthy tissue (*Figure* 3B). Analysis of 745 LUSC patient samples from TCGA database demonstrated a positive correlation between *EGFR* expression and *PTHLH* expression (Pearson correlation: 0.32, $P < 0.0001$) (*Figure* 3C). Among EGFR ligands, only *TGFA* had a significant correlation with *PTHLH* expression (Pearson correlation: 0.51, $P < 0.0001$), indicating that TGFα might be crucial for EGFR-mediated PTHrP induction (*Figure* 3D). In line with this finding, TGFα expression was recently reported to be associated with cachexia in LUSC and LUAD tumours.[17] On the other hand, analysis of 752 LUAD patient samples from TCGA gene expression dataset detected a positive correlation between *PTHLH* and *TGFA* (Pearson correlation: 0.21, $P < 0.0001$) expression and not with *EGFR* (Pearson correlation: -0.07, $P = 0.06$). *PTHLH* gene expression was previously shown to be elevated in HNSC.[18] Analysing TCGA RNA-seq dataset using GEPIA tool, we confirmed that *PTHLH* expression is the highest in HNSC tumours compared with all other tumour types (*Figure* S3). Compared with normal tissue, HNSC tumours have 17.5-fold higher *PTHLH* mRNA that is statistically significant ($P < 0.05$) (*Figure* 3A). Analysis of 545 TCGA HNSC samples detected a significant positive correlation between *PTHLH* expression and *EGFR* expression (Pearson correlation: 0.34, $P < 0.0001$) as well as *PTHLH* expression and *TGFA* expression (Pearson correlation: 0.50, $P < 0.0001$) (*Figure* 3E–F). These findings argue that PTHrP

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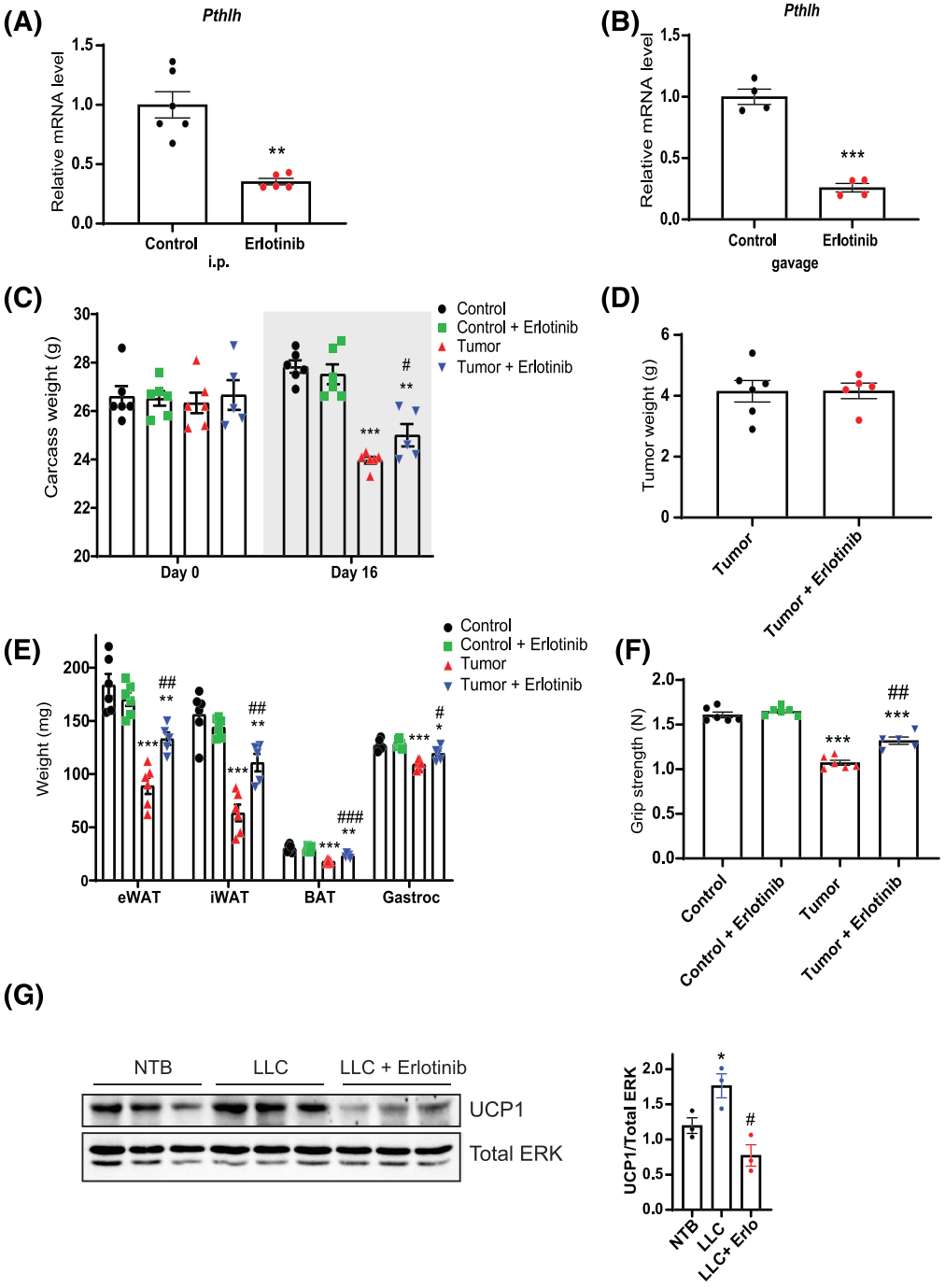

Figure 2 Inhibition of EGFR reduces *Pthlh* mRNA levels in LLC tumours and ameliorates cancer cachexia in LLC tumour-bearing mice. Mice were inoculated with 5 million LLC cells each and monitored up to 16 days. Relative *Pthlh* mRNA expression levels were measured by qRT-PCR in LLC tumours of mice treated with vehicle or erlotinib for 7 days starting 9 days post tumour inoculation. Control and LLC tumour-bearing mice received erlotinib (*A*) via i.p. injections (10 mg/kg/day; $n = 5–6$) or (*B*) via oral gavage (100 mg/kg/day; ($n = 4$). (*C*) Carcass weight (calculated by subtracting tumour weight from the total weight), (*D*) weight of tumours, (*E*) weight of fat and muscle tissues, and (*F*) grip strength of mice treated with i.p. injections of erlotinib (10 mg/kg/day; $n = 5–6$) were measured. (*G*) Western blot analysis was performed to detect UCP1 and total ERK protein levels in dissected BAT tissues of non-tumour-bearing (NTB) mice and LLC tumour-bearing treated with or without erlotinib. Blots were quantified for UCP1/Total ERK ratio for each sample. Values are mean ± SEM. The statistical analysis was performed using two-tailed *t*-test compared with the control groups. * compares differences between control versus tumour or control + erlotinib versus tumour + erlotinib groups while # compares differences between tumour versus tumour + erlotinib treatment groups. ($*P < 0.05$; $**P < 0.01$; $***P < 0.001$, $\#P < 0.05$, $\#\#P < 0.01$, $\#\#\#P < 0.001$). N, Newton; eWAT, epididymal white adipose tissue; iWAT, inguinal white adipose tissue; BAT, brown adipose tissue; Gastroc, gastrocnemius muscle; NTB, non-tumour bearing mice; LLC, Lewis lung carcinoma; Erlo, Erlotinib.

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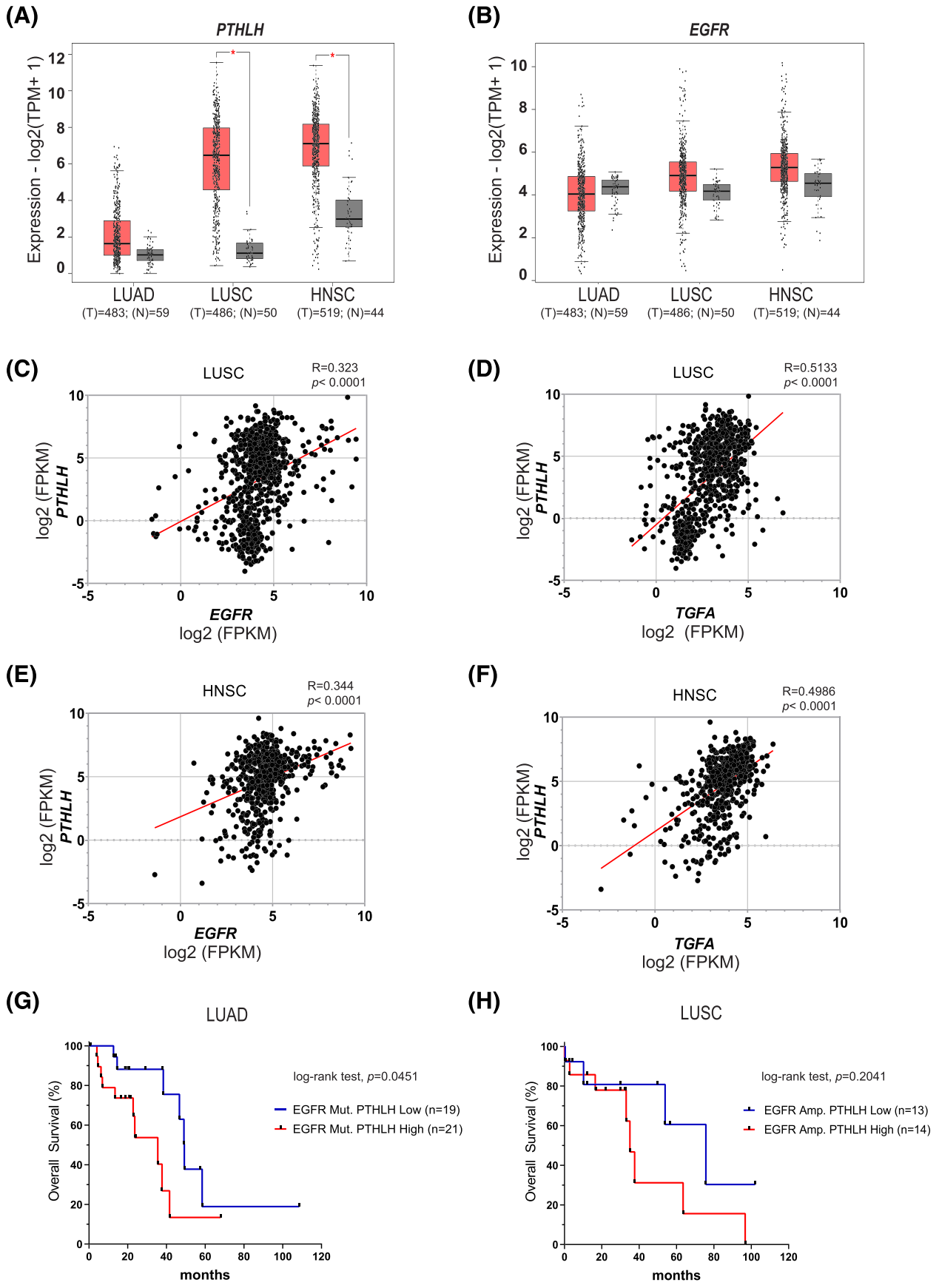
(A)
PTHLH
Expression - log2(TPM+ 1)
LUAD
(T)=483; (N)=59
LUSC
(T)=486; (N)=50
HNSC
(T)=519; (N)=44
(B)
EGFR
Expression - log2(TPM+ 1)
LUAD
(T)=483; (N)=59
LUSC
(T)=486; (N)=50
HNSC
(T)=519; (N)=44
(C)
LUSC
R=0.323
p< 0.0001
log2 (FPKM) PTHLH
EGFR
log2 (FPKM)
(D)
LUSC
R=0.5133
p< 0.0001
log2 (FPKM) PTHLH
TGFA
log2 (FPKM)
(E)
HNSC
R=0.344
p< 0.0001
log2 (FPKM) PTHLH
EGFR
log2 (FPKM)
(F)
HNSC
R=0.4986
p< 0.0001
log2 (FPKM) PTHLH
TGFA
log2 (FPKM)
(G)
LUAD
log-rank test, p=0.0451
EGFR Mut. PTHLH Low (n=19)
EGFR Mut. PTHLH High (n=21)
Overall Survival (%)
months
(H)
LUSC
log-rank test, p=0.2041
EGFR Amp. PTHLH Low (n=13)
EGFR Amp. PTHLH High (n=14)
Overall Survival (%)
months

Figure 3 PTHrP mRNA correlates with *EGFR* and *TGFA* expression in human NSCLC and HNSC and predicts poor survival. The box plot represents (*A*) *PTHLH* and (*B*) *EGFR* expression levels in tumour (T) tissue (red) of LUAD (n = 483), LUSC (n = 486) and HNSC (n = 519) patients compared with normal (N) tissue (grey) of LUAD (n = 59), LUSC (n = 50) and HNSC (n = 44). RNA-seq data analysis was obtained from TCGA database. Log-2-transformed RNA-seq read counts for respective genes are shown on the y-axis (*$P < 0.05$). Correlations between *PTHLH* expression and (*C*) *EGFR* expression or (*D*) *TGFA* expression were analysed using LUSC datasets, CPTAC-3 (n = 202) and TCGA-LUSC (n = 543). Correlations between *PTHLH* expression and (*E*) *EGFR* expression or (*F*) *TGFA* expression were analysed using TCGA-HNSC dataset (n = 545). Expression values are log2 transformed. Each dot represents a patient and red line depicts the linear best fit. Pearson correlation coefficient value and *P* values are shown at the top of each graph. (*G*) Kaplan–Meier overall survival plot of *PTHLH* expression (high; above median, low; below median) in TCGA-LUAD patients harbouring classical activating EGFR mutations (n = 40). (*H*) Kaplan–Meier overall survival plot of *PTHLH* expression (high; above median, low; below median) in TCGA-LUSC (n = 27). TPM, transcripts per million; FPKM, fragments per kilobase of transcript per million mapped reads, Mut., mutants; Amp., amplification.

expression may be regulated by EGFR signalling and potentially depend on TGFα activity in NSCLC and HNSC. Cancer-associated cachexia is highly prevalent among patients with these tumour types,[10,19,20] and therefore, elevated PTHrP may contribute to the development of the wasting syndrome.

Epidermal growth factor receptor gene alterations and high levels of parathyroid hormone-related protein mRNA associate with poor survival in lung adenocarcinoma and lung squamous cell carcinoma patients

A previous study reported an association between PTHrP overexpression in HNSC tumours and poor overall survival, suggesting that PTHrP is an independent negative prognosis factor in HNSC patients.[18] We investigated whether PTHrP gene expression correlates with the survival of lung cancer patients. Human EGFR is one of the major targets of precision therapy in NSCLC with activating EGFR mutations which are associated with sensitivity to gefitinib and erlotinib.[9,21] Activating EGFR mutations are common in LUAD tumours and less frequent in LUSC tumours. The presence of EGFR gene alterations causes worse overall survival in LUAD patients.[4,9,22] Analysing TCGA-LUAD dataset, we identified 40 patients (out of 585) with major activating EGFR mutations including nucleotide substitutions in exon 18 (G719C, G719S, G719A), in-frame deletion of exon 19 (ΔE746-A750) and L858R substitution in exon 21. High *PTHLH* expression (above median) was found to be associated with worse overall survival within this group (n = 40, log-rank test, P = 0.0451) (*Figure* 3G). In contrast, EGFR gene amplification is more common in LUSC tumours and higher EGFR expression is evident in LUSC compared with LUAD tumours (*Figure* 3B).[22] Analysis of TCGA-LUSC dataset identified 27 patients (out of 501) with EGFR gene amplification and we observed a trend for worse overall survival (n = 27, log-rank test, P = 0.2042) with high *PTHLH* expression (above median) (*Figure* 3H).

Discussion

Our findings demonstrated that expression of various EGFR ligands and EGFR itself co-cluster with PTHrP in highly thermogenic LLC clones, indicating a relation between EGFR signalling and PTHrP gene expression. We found that PTHrP mRNA is significantly altered upon activation or inhibition of EGFR signalling by EGFR ligands or TKIs in LLC cells. These findings are in line with previous studies which indicated that autocrine EGFR signalling regulates PTHrP gene expression in keratinocytes, breast and lung cancer cells.[23,24] Notably, it was reported that inhibition of EGFR with gefitinib reduces PTHrP gene expression, circulating PTHrP concentrations and total calcium concentrations in lung xenograft models of hypercalcemia.[24] Our results demonstrated that all EGFR ligands significantly induce PTHrP gene expression in murine LLC cells and this induction reaches to maximum levels with HB-EGF and TGFα. EGFR TKIs have been shown to provide clinical benefits over chemotherapy for lung cancer patients harbouring EGFR activating mutations.[25] Some of the first generation (gefitinib, erlotinib, lapatinib; reversible binding) and second generation (afatinib; irreversible binding) EGFR TKIs are clinically approved to treat NSCLC patients.[25] In our study, we tested the impact of these EGFR TKIs on PTHrP gene expression in LLC cells. Treatment with increasing concentrations of erlotinib demonstrated dose-dependent suppressive effects on PTHrP gene expression. Furthermore, all other EGFR TKIs decreased PTHrP gene expression in LLC cells with most prominent inhibitory effects seen with the treatment of afatinib and erlotinib. Similarly the inhibitors of MEK kinase repressed *Pthlh* mRNA and its induction by EGFR ligands arguing a role for the MEK/ERK pathway in PTHrP gene regulation at the downstream of EGFR signalling (*Figure* 4). However, it should be noted that activation of the MEK/ERK pathway may also induce PTHrP expression independently of EGFR signalling.

We also demonstrated that erlotinib significantly decreases PTHrP gene expression in tumours of LLC tumour-bearing mice when administered via either i.p. (10 mg/kg/day) or oral gavage (100 mg/kg/day). Administration of erlotinib via oral gavage caused significant weight loss in non-tumour-bearing mice and failed to improve cachexia-associated tissue loss (data not shown). This observation is consistent with previous studies describing toxic effects of

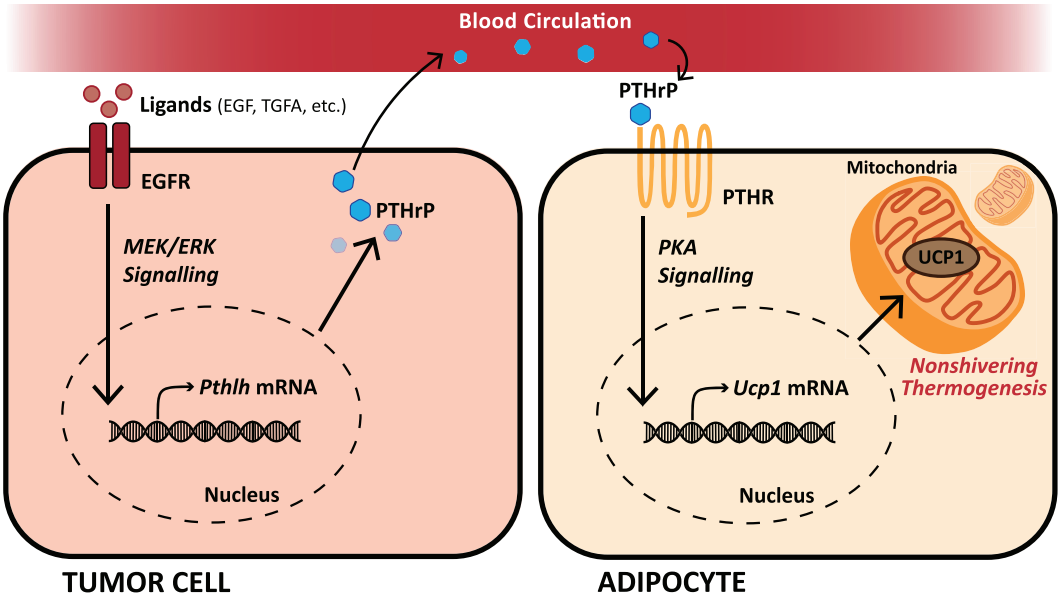

Figure 4 EGFR-dependent regulation of PTHrP in tumour cells can influence thermogenic activity in adipocytes. EGFR activation induces the MEK/ERK signalling and PTHrP expression in tumour cells. Circulating PTHrP binds to PTHR in adipocytes and activates PKA signalling and UCP1-mediated non-shivering thermogenesis.

erlotinib at daily doses of 50 and 100 mg/kg.[26] Because oral gavage causes further weight loss in cachectic mice, we continued with i.p. injection of erlotinib at a dose of 10 mg per kg body weight which is closer to but still higher than the doses used clinically. Erlotinib treatment (10 mg/kg/day) via i.p. ameliorated cancer-associated cachexia by reducing loss of carcass weight, fat and muscle mass as well as improving muscle strength without changing tumour mass. This modulatory role of EGFR TKI on cancer cachexia likely depends on suppression of cachexia mediator PTHrP, but further studies are needed to confirm this hypothesis. Erlotinib administration (10 mg/kg/day) for 7 days failed to affect the tumour size indicating that the improvement in cachexia is not due to the shrinking of the tumours. Inability of erlotinib to decrease tumour size is likely due to the short time period of erlotinib administration compared with other studies applying erlotinib more than a week.[26,27]

Parathyroid hormone-related protein was previously linked to the cachexia associated with hypercalcemia of malignancy.[28] In addition, our previous study unveiled that tumour-derived PTHrP induces adipose tissue thermogenesis and contribute to hypermetabolism associated with cachexia in the LLC model.[12,13] Binding of PTHrP to PTH receptor (PTHR) activates protein kinase A (PKA) signalling in adipocytes and upregulates UCP1-mediated uncoupled respiration.[13] Neutralization of PTHrP or the depletion of PTHR in tumour-bearing mice blocks loss of body weight, muscle wasting, and weakness suggesting PTHrP as a possible target molecule for the treatment of cancer cachexia.[12,13,28] Furthermore, LLC-derived extracellular vesicles were shown to induce adipose tissue browning and fat loss in mice implanted with LLC cells by delivering PTHrP, which can be abrogated by inhibition of extracellular vesicles release.[14] Investigation of a cohort of patients diagnosed with metastatic NSCLC or colorectal cancer showed that patients with higher serum PTHrP concentrations had significantly lower lean body mass and higher resting energy expenditure compared with patients lacking detectable levels of PTHrP.[13] Moreover, further studies analysing large groups of cancer patients also reported that serum PTHrP levels are positively associated with cancer-related weight loss independent of the presence of hypercalcemia, inflammation, tumour burden, and other comorbidities, arguing that PTHrP may not only be a mediator but also a powerful predictor of cachexia.[29,30] Therefore, it is conceivable that suppression of PTHrP expression in tumours by EGFR inhibition can be beneficial to cachectic cancer patients.

In this study, PTHrP gene expression was found to be significantly increased in the tumours of LUSC and HNSC patients. This is in agreement with previous studies describing PTHrP production by squamous cells.[31] Our gene expression analysis demonstrated that *PTHLH* mRNA correlates with mRNA levels of *EGFR* and *TGFA* in these tumours. Recently, TGFα expression was shown to correlate with cancer cachexia in LUAD and LUSC patients.[17] Additionally, the overexpression of EGFR and TGFα was shown to be frequent in resectable non-small cell lung cancer.[32] Among the EGFR ligands, TGFα, AREG, and EREG are more frequently expressed (65–93%), whereas EGF and HB-EGF are infrequently detected in lung tumours.[33] Our analysis detected a positive correlation between PTHrP expression and TGFα expression only, indicating

that TGFα may play a prominent role in PTHrP gene regulation. Indeed, our cell culture work also showed the highest level of induction in *Pthlh* mRNA level upon TGFα treatment. There have been a number of reports for high prevalence of cachexia (~50%) in all clinical stages of HNSC patients.[19,20] EGFR and TGFα are overexpressed in 80–90% of HNSCs which is associated with poor survival and serves as the most important prognostic factor in HNSCs.[32,34] Previously, an association between PTHrP overexpression and poor overall survival was reported in HNSC patients.[18] Collectively, these findings support the hypothesis that activated EGFR signalling may regulate PTHrP gene expression and contribute to cancer-associated cachexia in NSCLC and HNSC.

In tumour cells, the tyrosine kinase activity of EGFR is dysregulated by various oncogenic mechanisms, including EGFR gene mutation, increased gene copy number and EGFR protein overexpression.[9] In NSCLC, *EGFR* mutation status is considered as a poor prognostic factor, which is often associated with a more aggressive behaviour and decreased patient survival. The classical activating EGFR mutations account for deletions in exon 19 (45%) and single-nucleotide substitutions causing amino acid alterations; predominantly L858R point mutation (41%) and mutations in G719 (5%).[9] Treatment with EGFR TKI is very effective and provide significantly improved patient outcomes, particularly for LUAD patients with *EGFR* mutations.[21] EGFR has been shown to be overexpressed in both LUSC and LUAD, but a stronger correlation between EGFR overexpression and an adverse outcome was noted in LUSC.[32] *EGFR* expression has no impact on overall survival or progress-free survival in resectable LUAD and LUSC patients.[22] Additionally, we did not observe a direct association between *PTHLH* expression and overall survival of LUAD and LUSC patients (data not shown). However, survival analysis restricted to LUAD patients with activating EGFR mutations unveiled a significant association between high *PTHLH* expression and poor outcome. Overrepresentation of *PTHLH* expression in LUSC patients with EGFR gene amplification also correlated with a tendency for poor overall survival. Elevated serum levels of PTHrP were correlated with an increased frequency of bone metastases and shorter survival in lung carcinoma patients with hypercalcemia (n = 23).[35] Interestingly, carboxyl terminal PTHrP peptide was described as a positive prognostic indicator for survival in female patients with NSCLC while amino terminal PTHrP peptide, which can stimulate thermogenic gene expression and induce cachexia, was found to be a negative survival factor in lung adenocarcinoma.[13,36] While negative influence of high *PTHLH* mRNA on overall survival of the patients may depend on potential oncogenic effects driven by PTHrP protein,[37] it is possible that PTHrP-associated cancer cachexia may contribute to the worse outcome. Our findings argue that EGFR signalling regulates PTHrP expression in tumours, thus the cachectic effects of PTHrP may be pronounced in patients with EGFR active tumours. EGFR-induced and MEK/ERK signalling-driven PTHrP expression in tumour cells would lead to elevated levels of this polypeptide in the circulation eventually activating PTHR/PKA-driven and UCP1-mediated thermogenic activity in adipocytes (*Figure* 4). Therefore, we hypothesize that PTHrP could be a useful clinical biomarker to identify cachectic lung cancer patients which could benefit from anti-cachectic effects of an anti-EGFR therapy. However, further studies testing serum PTHrP and cachexia status in lung cancer patients receiving EGFR inhibitors is needed to validate the translational potential of the current study. It should also be noted that cachexia-driven by PTHrP overexpression in the absence of EGFR pathway activation would not be ameliorated by erlotinib administration. Further experiments combining forced PTHrP expression and EGFR inhibition in murine cachexia models must be utilized to establish the inter-dependency between these factors.

Clinical studies have strongly linked presence of cachexia to poor survival of patients within all stages of lung cancer.[10] Surprisingly, Shiono *et al*.[38] elucidated that the mean weight loss was not significantly changed in patients with activating EGFR mutations as well as by EGFR TKI therapy status. Because changes in total body weight loss alone cannot be a good predictor of cancer cachexia, measurements of muscle composition and function is needed to rule out any beneficial effects of EGFR TKI therapy. In fact, low skeletal muscle mass in cancer patients was shown to be independently associated with poor survival regardless of body mass index.[39] Taken together, our studies utilizing murine LLC cachexia model argue that EGFR inhibition can potentially improve cachexia associated with PTHrP overexpression in lung cancer and *in silico* analysis of human tumours indicates a negative influence of EGFR activation and PTHrP overexpression on survival of lung cancer patients. Clinical studies addressing the progression of cachexia and levels of serum PTHrP in lung, head, and neck cancer patients receiving anti-EGFR therapy is needed to demonstrate beneficial effects of suppression of the EGFR-PTHrP axis.

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Conflict of interest

The authors declare no conflict of interest.

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Online supplementary material

Additional supporting information may be found online in the Supporting Information section at the end of the article.

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