Targeting Epithelial-to-Mesenchymal Transition with Met Inhibitors Reverts Chemoresistance in Small Cell Lung Cancer

Israel Cañadas1,4, Federico Rojo1,7, Álvaro Taus4, Oriol Arpí1,4, Montserrat Arumi-Uria5,6, Lara Pijuan6, Silvia Menéndez1,4, Sandra Zazo7, Manuel Domíne6, Marta Salido5, Sergi Mojal8, Antonio García de Herreros2,6, Ana Rovira1,4, Joan Albanell1,4,6, and Edurne Arriola1,4

Abstract

Purpose: Met receptor phosphorylation is associated with poor prognosis in human small cell lung cancer (SCLC). The aim of our work was to investigate the effects of hepatocyte growth factor (HGF)/Met–mediated epithelial-to-mesenchymal transition (EMT) in SCLC and to evaluate the role of Met inhibition in mesenchymal/chemoresistant SCLC models.

Experimental Design: SCLC models of HGF-induced EMT were evaluated in vitro and in vivo (subcutaneous xenografts in BALB/c nude mice) for chemosensitivity and response to Met inhibition with PF-2341066 (crizotinib). Human SCLC samples at diagnosis (N = 87) and relapse (N = 5) were evaluated by immunohistochemistry and immunofluorescence for EMT markers and Met status and these were correlated with patient outcome.

Results: We identified that the activation of the Met receptor through HGF induced expression of mesenchymal markers, an aggressive phenotype, and chemoresistance. Blockade of this process with the Met inhibitor resensitized cells to chemotherapy in vitro and in vivo. Moreover, mesenchymal markers in human SCLC specimens were associated with Met activation, predicted worse survival, and were upregulated in chemoresistant disease.

Conclusion: These results provide novel evidence on an important role of Met-dependent EMT in the adverse clinical behavior of SCLC and support clinical trials of Met inhibitors and chemotherapy in this fatal disease. Clin Cancer Res; 20(4); 938–50. ©2013 AACR.

Introduction

Small cell lung cancer (SCLC) accounts for approximately 15% of lung cancers. Its tight association to tobacco carcinogens’ exposure explains the high number of genetic alterations described in this disease (1, 2). First-line treatment with platinum plus etoposide results in a high percentage of responses. Despite this, early chemorefractory relapse causes short survival of patients with SCLC. So far, no trial evaluating targeted therapies in SCLC has shown benefit when compared with standard chemotherapy. Our group has previously described an association between the Met pathway and outcome in patients with SCLC (3), supporting a potential therapeutic interest of Met inhibitors in this disease.

Epithelial-to-mesenchymal transition (EMT) is a cellular process characterized by loss of epithelial markers and acquisition of a mesenchymal phenotype, which enables cancer cells to invade surrounding tissues and generate distant metastasis (4). Repression of E-cadherin defines the main hallmark of EMT. This process is initiated by Snail1 transcriptional factor, a key inducer of this conversion. Although Snail1 is induced at the early phases of EMT and is necessary for this process, its expression is not maintained in most mesenchymal cells; instead, E-cadherin silencing is dependent on other transcriptional repressors induced by Snail1, such as Zeb1 and 2 (5). Mesenchymal features are associated with poor prognosis and chemoresistance in different tumor models (6–8). EMT can be induced by growth factors; among them, hepatocyte growth factor (HGF), the natural ligand of Met tyrosine kinase receptor, has been reported to be a potent inducer of EMT (9, 10).

PF-2341066 (crizotinib) is a small molecule that specifically inhibits Met and Alk kinases acting as an ATP

Cancer Therapy: Preclinical

Cancer Therapy: Preclinical

Cancer Therapy: Preclinical

Cancer Therapy: Preclinical

Cancer Therapy: Preclinical

Cancer Therapy: Preclinical

Cancer Therapy: Preclinical

Cancer Therapy: Preclinical

Cancer Therapy: Preclinical

Cancer Therapy: Preclinical

Cancer Therapy: Preclinical

Cancer Therapy: Preclinical

Cancer Therapy: Preclinical

Cancer Therapy: Preclinical

Cancer Therapy: Preclinical

Cancer Therapy: Preclinical

Cancer Therapy: Preclinical

Cancer Therapy: Preclinical

Cancer Therapy: Preclinical

Cancer Therapy: Preclinical

Cancer Therapy: Preclinical

Cancer Therapy: Preclinical

Cancer Therapy: Preclinical

Cancer Therapy: Preclinical

Cancer Therapy: Preclinical

Cancer Therapy: Preclinical

Cancer Therapy: Preclinical

Cancer Therapy: Preclinical

Cancer Therapy: Preclinical

Cancer Therapy: Preclinical

Cancer Therapy: Preclinical

Cancer Therapy: Preclinical

Cancer Therapy: Preclinical

Cancer Therapy: Preclinical

Cancer Therapy: Preclinical

Cancer Therapy: Preclinical

Cancer Therapy: Preclinical

Cancer Therapy: Preclinical

Cancer Therapy: Preclinical

Cancer Therapy: Preclinical

Cancer Therapy: Preclinical

Cancer Therapy: Preclinical

Cancer Therapy: Preclinical

Cancer Therapy: Preclinical

Cancer Therapy: Preclinical

Cancer Therapy: Preclinical

Cancer Therapy: Preclinical

Cancer Therapy: Preclinical
Small cell lung cancer (SCLC) is a highly lethal disease due to its chemorefractory nature after initial treatment. No novel therapeutic strategy has been able to improve outcome to date. Our work demonstrates the existence of a group of patients with SCLC showing activated Met and mesenchymal features that present worse prognosis. Moreover, we observe upregulation of these features in relapsed disease. Our preclinical data show that Met mediates epithelial-to-mesenchymal transition, leading to chemoresistance and that Met inhibition reverses chemoresistance. These findings strongly support the design of clinical trials with Met inhibitors plus chemotherapy in patients with SCLC with Met activation and/or mesenchymal phenotype.

**Translational Relevance**

Small cell lung cancer (SCLC) is a highly lethal disease due to its chemorefractory nature after initial treatment. No novel therapeutic strategy has been able to improve outcome to date. Our work demonstrates the existence of a group of patients with SCLC showing activated Met and mesenchymal features that present worse prognosis. Moreover, we observe upregulation of these features in relapsed disease. Our preclinical data show that Met mediates epithelial-to-mesenchymal transition, leading to chemoresistance and that Met inhibition reverses chemoresistance. These findings strongly support the design of clinical trials with Met inhibitors plus chemotherapy in patients with SCLC with Met activation and/or mesenchymal phenotype.

**Materials and Methods**

**HGF-induced EMT**

We seeded 6.7 x 10^5 cells in 60-mm^2 dishes with culture medium containing 10% FBS. After 24 hours, HGF was added at 40 ng/mL. Fresh culture medium containing HGF was added every 48 hours during 14 days. A morphologic study by light microscopy and a molecular study by Western blotting were performed at days 3, 6, and 10 to study if continuous exposure to HGF was able to induce EMT in H69 cells.

**Subcutaneous tumorigenesis**

Five-week-old male BALB/c nude mice were purchased from Charles River Laboratories and hosted in the pathogen-free animal facility at the Barcelona Biomedical Research Park (PRBB). Animal treatments were done according to institution-approved protocols. Cells were resuspended in sterile PBS with 50% Matrigel (BD Biorics) and subcutaneously injected into the flank of mice. Tumor volume was determined from caliper measurements of tumor length (L) and width (W) according to the formula L x W^2/2 three times a week. Tumors were allowed to grow until the volume reached approximately 200 to 300 mm^3. Mice were randomized to four groups with 10 mice in each group. Treatment groups consisted of control (vehicle), etoposide (12 mg/kg), PF-2341066 (100 mg/kg), and combination. Etoposide was diluted in 200 μL 0.9% NaCl and administered at a dose of 12 mg/kg in one daily intraperitoneal injection on days 1 to 3 of treatment. PF-2341066 was diluted in water and administered daily by oral gavage at 100 mg/kg for 14 days. This dose was selected on the basis of previous literature (16), suggesting that at this dose, Met is inhibited >90%, which potentially translates in tumor responses in a Met-dependent model. Control animals received 0.9% NaCl and water as vehicles. Mice were euthanized and tumors measured with calipers. Part of the tumors were fixed in 4% formalin and embedded in paraffin for immunohistochemical studies.

**Human samples and immunohistochemistry**

The following antibodies were used for immunohistochemical studies: Met (SP44) mouse mAb (monoclonal antibody; Ventana-Roche), p-Met Y1234/35 (130H2) rabbit mAb, p-Met Y1234/35 (D26) XP rabbit mAb (Cell Signaling Technology), E-cadherin (NCH-38) mouse mAb (Dako), NCAM (123C3) mouse mAb (Dako), Snail1 (EC3) mouse mAb, vimentin (V9) mouse mAb (Dako), and CD31 (SP38) rabbit mAb (Spring Bioscience).

The study population consisted of 87 patients diagnosed with SCLC at any stage from whom we had clinical and follow-up information. Specimens were retrospectively retrieved from Parc de Salut Mar Biobank (MARBiobanc, Barcelona, Spain) and Fundacion Jimenez Diaz Biobank (Madrid, Spain). This study was approved by the institutional review board of each participating center. Three-μm tissue sections from formalin-fixed and paraffin-embedded samples were obtained, mounted onto charged slides, and then deparaffinized in xylene and hydrated.

After heat antigen retrieval was performed at a high-pH solution using PT Link platform (Dako), slides for immunohistochemistry were incubated with primary antibody for 1 hour at a dilution of 1:1 for Met mAb, 1:20 for p-Met Y1349, and 1:50 for p-Met Y1234/35, 1:5 for Snail1, 1:100 for E-cadherin, 1:100 for NCAM (neuronal adhesion molecule), 1:100 for vimentin, and 1:200 for CD31. Then, sections were incubated with the specific polymer EnVision FLEX+ (Dako) and revealed with 3,3′-diaminobenzidine (DAB) as chromogen. In situ hybridization was done by deproteinization using proteinase K digestion for 5 minutes at room temperature and incubation with a set of specific digoxigenin-labeled probes for mRNA SPARC (agaaactgggagaggggagaaaagccatggagagggagagggagaga).
Clinical Cancer Research

Cañadas et al.

uted by two investigators (F. Rojo and S. Zazo) using a Cri
performed using a Dako Autostainer. Staining was evalu-
fluorescence assays were
diamidino-2-phenylindole dihydrochloride (DAPI; Abbott
anti-rabbit EnVision FLEX+
þ
digoxigenin rabbit mAb (9H27L19; Life Technologies),
(immunoglobulin G; X0943; Dako) or normal rabbit Ig
markers at various time points. Western blot analysis
induced EMT is a dynamic process, we tested for EMT
10 days. At day 6, HGF-treated cells grew as single cells
prevented by PF-2341066
HGF-induced EMT in SCLC cells is reverted and
Results
Ca
nadas et al.

HGF-induced EMT in SCLC cells is reverted and
prevented by PF-2341066
We exposed H69 SCLC cells to HGF (40 ng/mL) for
10 days. At day 6, HGF-treated cells grew as single cells
partially attached to the plate (Fig. 1A, left). Because HGF-
induced EMT is a dynamic process, we tested for EMT
markers at various time points. Western blot analysis
revealed that HGF promoted a partial downregulation of
Met protein, stimulation of phosphorylated Met and
ERK1/2, increase in Snail1 at day 6, and downregulation of
E-cadherin at day 10 (Fig. 1A, right, and Supplementary
Fig. S1A), but not in NCAM (data not shown).
We assessed whether PF-2341066 prevented HGF-
induced effects. PF-2341066 was added to fresh medium
upfront and every 48 hours along with HGF. As observed
in Fig. 1A, the inhibitor prevented the disruption of cell
clusters induced by HGF, as well as Met protein down-
modulation, ERK1/2 activation, Snail1 increase, or E-cad-
herin downregulation. We performed the experiment
adding PF-2341066 at day 6, once the EMT phenotype
was detectable. The Met inhibitor reversed the HGF-
induced morphologic (Fig. 1B, left) and molecular changes
(Fig. 1B, right).
To further demonstrate that the observed effect was a
result of Met inhibition, we performed the same experi-
ments with PHA-665752, which inhibits Met but not Alk,
obtaining similar results (Supplementary Fig. S1B–S1D;
ref. 18). Furthermore, we screened the cell lines used in
this study for ALK translocation by FISH and all were
negative (data not shown).
These data confirm that HGF, via Met activation, in-
duced morphologic and molecular changes in H69 con-
sistent with EMT, which are reversed or prevented by Met
inhibition.
We stably knocked down the endogenous expression of
Snail1 in H69 cells (shShSnailH69). We observed an almost
complete downregulation of the protein compared with
cells transfected with the control short hairpin RNA
(shRNA; shctH69; Fig. 1C, left). We then exposed shctH69
and shShSnailH69 to HGF. We observed scattering of the
shctH69 cells that grew partially attached to the plate
(Fig. 1C, right) on day 6. In contrast, in shShSnailH69 cells,
no morphologic changes were observed under HGF expo-
sure for 10 days. Detailed kinetic analysis showed that
HGF induces Snail1 expression at day 3 in shctH69 but
decayed at longer time points (day 6; Fig. 1C, left).
A mesenchymal cell type is generated in vitro from SCLC
cells treated with HGF
Upon prolonged treatment with HGF, we observed a
small pool of H69 cells with a mesenchymal (spindle-
shaped) morphology that grew attached to the plates
(Fig. 2A). This phenomenon was reproduced in six inde-
pendent experiments. We isolated this population
(H69-mesenchymal, H69M) and compared it with the
parental H69 cells. H69M cells were phenotypically sta-
ble, maintaining the same mesenchymal morphology in
the absence of HGF stimulation. A study of the karyotype
showed the same chromosomal characteristics than
H69 cells ruling out a potential contamination by another
cell line (Supplementary Fig. S2A). As shown in Fig. 2B,
left, H69M cells showed Met basal activation shown by
phosphorylation of Tyr1349, and mesenchymal features
characterized by the upregulation of fibronectin and
downregulation of NCAM and E-cadherin. We then trea-
ted the H69M cells with HGF for 15 minutes to assess if

aagtccacctggactac at; ctgtgacctggacaatgaca; Cenbimo) at
62°C for 1 hour. Reaction was revealed using an anti-
digoxigenin rabbit mAb (9H27L19; Life Technologies),
anti-rabbit EnVision FLEX+, and DAB. Slides were counter-
tstained with hematoxylin. Sections from same speci-
mens above were incubated with normal mouse IgG2
(Ca
nadas et al.
aagtccacctggactac at; ctgtgacctggacaatgaca; Cenbimo) at
62°C for 1 hour. Reaction was revealed using an anti-
digoxigenin rabbit mAb (9H27L19; Life Technologies),
anti-rabbit EnVision FLEX+, and DAB. Slides were counter-
tstained with hematoxylin. Sections from same speci-
mens above were incubated with normal mouse IgG2
(Ca
nadas et al.

Published OnlineFirst November 27, 2013; DOI: 10.1158/1078-0432.CCR-13-1330
they were still sensitive to HGF stimulation. We observed a higher increase in Met phosphorylation when compared with the increase observed in H69, and in the downstream molecules GAB1 (pTyr 307) and ERK1/2, indicating that these cells were more sensitive to exogenous HGF stimulation than parental H69.

One interesting observation is that differences in the intensity of baseline phosphorylation of Tyr1234/35 and Tyr1349 and in response to HGF were found in these experiments. This could be due to the performance of antibodies under our experimental conditions and/or to the different role of each phosphorylation site in response to HGF. Upon HGF binding, Met receptor is activated through dimerization and phosphorylation of Tyr1234/35 on the catalytic domain. Subsequently, phosphorylation of docking site Tyr1349 occurs with recruitment of downstream molecules as previously reported (19). Despite this, both were consistently upregulated upon HGF stimulation and expression decreased by Met inhibitors.

A global transcriptome analysis of H69 and H69M showed clear differences in mRNA expression. We found 9,712 genes that were differentially expressed (GEO accession number: GSE45120; Supplementary Fig. S2B). According to Ingenuity Pathway Analysis, the top altered functions in the comparison were “Cell movement,” “Cell morphology,” “Cellular growth and proliferation,” and “Cell...
Figure 2. HGF-induced EMT results in the appearance of a mesenchymal subpopulation. A, cell morphology by light microscopy of H69 cells treated with HGF during 14 days showing the appearance of an adherent cell subpopulation. Scale bars, 100 μm. B, Western blot analysis of H69 and H69M cells treated with PF-2341066 (200 nmol/L) for 3 hours and stimulated with HGF (40 ng/mL) for 15 minutes (left) and heat map of altered genes in H69M cells compared with H69 (right). Horizontal rows, individual genes; vertical columns, individual samples. Color intensity means degree of gene expression modulation. Blue, upregulation; yellow, downregulation. Black, no change. C, qRT-PCR of selected genes. Expression values were normalized to the levels of a control RNA (RPLP0). Relative mRNA levels were normalized to the expression of the control sample (left); HGF production in H69 and H69M culture supernatants measured by ELISA (right). D, cell growth of H69 and H69M cells treated with PF-2341066 (200 nmol/L) during 10 days. Cell number was assessed by Scepter Automated Cell Counter. The number of viable cells in each treatment was plotted as percentage of control (left); invasion assay of H69 and H69M cells treated with PF-2341066 (100 nmol/L) during 24 hours. Each data point represents the mean ± SD percentage cell invasion of three independent experiments, compared with controls at 100% (middle); clonogenic assay of H69M cells cultured with or without PF-2341066 at 50 or 200 nmol/L during 14 days. Each data point represents the mean ± SD percentage clonogenic growth of three independent experiments, compared with controls at 100% (right).
Signaling.” Within the “top-ten” genes that were overexpressed in H69M we found important players in EMT such as SPARC, caveolin, integrin α-5 (fibronectin receptor), fibronectin 1, and vimentin (20, 21). HGF/MET pathway genes such as HGF, GRB2, or Paxillin were also overexpressed in H69M. Figure 2B, right, shows differentially expressed genes involved in EMT, DNA repair and survival, and those associated with the MET pathway. The upregulation in SPARC, fibronectin, vimentin, and HGF was confirmed by quantitative real-time PCR (qRT-PCR; Fig. 2C, left). HGF overexpression levels in H69M cells were further confirmed at the protein level by ELISA for secreted HGF in the culture medium (Fig. 2C, right). These results confirm the mesenchymal nature of H69M and suggest that upregulation of EMT markers in these cells could be caused by an autocrine loop responsible for Met basal activation. Met expression in each H69M replicate was, however, variable. This observation may be related to small differences in Met expression in the original cell cultures, which possibly influence RNA levels. Having showed a distinct EMT phenotype between these two cell lines and the variable Met expression, we then addressed the role of MET in EMT phenotype in further experiments.

To elucidate if the molecular changes observed in H69M affected cell behavior, we compared growth kinetics and invasion capabilities between H69 and H69M. H69M showed a slightly but nonsignificant increased proliferation rate, and higher invasiveness (Fig. 2D, left and middle) when compared with H69. Similarly to H69, H69M was sensitive to the addition of PF-2341066 that blocked Met phosphorylation at basal and HGF-stimulated conditions (Fig. 2B, left). This inhibition translated in a significant decrease in proliferation, invasion, and clonogenic growth. The reduction on clonogenic growth was statistically significant in a dose-dependent manner, achieving a magnitude of almost 50% (Fig. 2D). Interestingly, PF-2341066 did not reverse the spindle-like morphology of H69M at light microscopy in the clonogenic assay (data not shown).

HGF-mediated EMT enhances tumorigenesis in SCLC cells

H69 and H69M cells were injected in the flanks of immunodeficient mice (n = 10) to establish tumor xenografts. We observed that tumors generated by H69M were larger (median volume, 1.5 × 10³ mm³) compared with the tumors generated by H69 (median volume, 0.57 × 10³ mm³; Fig. 3A). Moreover, H69M were more invasive locally (surrounding soft tissue infiltration), showing foci of infiltrating cells in 9 of 10 mice compared with 1 of 10 mice for H69 cells (Fig. 3B). To study whether the results occurred in other subpopulations, we performed an in vivo experiment with a different mesenchymal subpopulation derived from H69 obtained by prolonged exposure to HGF (H69M2). This cell line presented a mesenchymal phenotype by Western blotting and secreted higher levels of HGF compared with H69. We confirmed the increased tumorigenic capacity of this mesenchymal subpopulation (Supplementary Fig. S3).

We then analyzed H69M xenografts compared with those generated by H69 cells. Histologic characteristics of both tumor xenografts were similar, suggesting that H69M were able to recapitulate the original round-small cell morphology of the parental H69 (Fig. 3C, hematoxylin and eosin).

Immunohistochemical studies revealed that H69M cells presented increased Met phosphorylation, expression of Snail1, vimentin, and SPARC and decreased expression of E-cadherin and NCAM. We also found increased vascularization of H69M-derived tumors (Fig. 3C). Multiplexing assays with Snail1 and p-Met antibodies demonstrated that Snail1 was expressed in cells with upregulated Met activity (Fig. 3D).

We repeated these assays using a lower number of cells, 100 (N = 5) and 500 (N = 5). For 100 cells, we observed tumor formation in 5 of 5 mice with H69M injection and in 3 of 5 mice for H69 (median tumor volume, 165 vs. 79 mm³, respectively). When injecting 500 cells, 5 of 5 mice with H69M formed tumors, whereas 3 of 5 mice did it for H69 cells (median tumor volume, 384 vs. 82 mm³, respectively).

In addition, we performed an in vivo assay, injecting 10⁶ H69M cells in the flank of mice and treating with vehicle and PF-2341066 100 mg/kg from day 0 (N = 5 per condition). The Met inhibitor significantly decreased tumor growth (final median volume, 1,047 mm³ for untreated vs. 550 mm³ for treated mice; P < 0.05; Supplementary Fig. S4A). We also found a significant decrease in local invasion in the treated mice. Immunohistochemical analysis of the xenograft confirmed that treatment with PF-2341066 significantly decreased p-Met, mesenchymal marker expression, and angiogenesis and increased epithelial marker expression (Supplementary Fig. S4B).

These results suggest that Met activation by HGF induces a mesenchymal transformation that translates in a more tumorigenic and aggressive behavior that can be blocked with a Met inhibitor.

HGF-mediated EMT in SCLC induces chemoresistance in vitro and in vivo and MET inhibition sensitizes cells to chemotherapy

Because EMT has been associated with apoptosis resistance in other cellular systems (22), we investigated if the mesenchymal phenotype caused increased chemoresistance in our model. We treated H69 and H69M with etoposide, a standard chemotherapeutic agent used in SCLC. MTS assays demonstrated that H69M were chemoresistant, compared with H69 (Supplementary Fig. S5A).

To elucidate if Met inhibition could revert chemoresistance, we performed a clonogenic assay with H69M cells with etoposide, PF-2341066, and the combination of both. We observed the highest inhibition rate with the combination of etoposide and PF-2341066, suggesting that Met inhibition by PF-2341066 conferred chemosensitivity (Supplementary Fig. S5B, left).

We confirmed this hypothesis in mouse heterotopic tumors. We xenografted H69M cells and treated mice with...
etoposide, PF-2341066, or the combination. We did not observe a significant effect on tumor growth by any of the two drugs alone; however, the combination produced a significant decrease in tumor growth compared with either agent alone ($P < 0.05$, Fig. 4A).

Of note, inhibition of p-Met by PF-2341066 of mesenchymal cells did not affect the expression of EMT markers in short term assays performed following 3 hours of drug exposure (Western blotting; Fig. 2B and Supplementary Fig. S3A). However, longer time exposure experiments in vitro (i.e., cell growth 8 days, invasive capacity 24 hours, and clonogenic growth lasting 14 days) demonstrated the cellular effects of Met inhibition (Fig. 2D). These effects and their correlation with molecular changes were more robustly demonstrated in vivo (>20-day experiments), suggesting that HGF-induced EMT is a dynamic process and inactivation of p-Met does not elicit phenotypic EMT changes until later time points (Fig. 4C).

We then evaluated effects of the different treatment strategies in H69-derived xenografts. Tumors originated from H69 were sensitive to etoposide; addition of PF did not significantly modify the response (Fig. 4B).

To further confirm the capacity of PF-2341066 of conferring chemosensitivity, we used the same experimental approach with another SCLC cell line, H841. This cell line was sensitive to HGF stimulation showing increased expression of p-MET (Supplementary Fig. S5C, left), and a mesenchymal phenotype. It secreted higher levels of HGF when compared with H69 (Supplementary Fig. S5C, top, right). Sequencing and FISH studies revealed no mutations.

Figure 3. H69M cells are more tumorigenic and maintain mesenchymal features in vivo. A, photograph of a representative mouse with H69 and H69M cells subcutaneously inoculated at right and left flanks, respectively. Photograph of the excised tumors from the same animal (left) and tumor volume measurement after 25 days of injection (right). Each data point represents mean ± SD tumor volumes. B, hematoxylin and eosin (H&E) stained sections from heterotopic H69M xenografts revealed infiltration of adjacent soft tissues by tumor cells compared with H69 tumors. Dashed black lines indicate the edge of the tumor in H69 and invasion of muscle and adipose tissues in H69M. Scale bar, 100 mm. Quantification of the number of invasive foci in complete H&E-stained section for each group (mean ± SEM) and significance are displayed. C, staining for H&E, p-Met, E-cadherin, NCAM, Snail, vimentin, SPARC, and CD31 in H69 and H69M tumors. Scale bars, 75 μm. Quantification of expression for each marker between H69 and H69M tumors (mean H-score ± SEM) and significance are displayed. D, representative image of immunofluorescence staining in H69M tumor for Snail (red), p-Met (green), and DAPI (blue). Snail is detected in the nuclei (pink, red + blue) and p-Met in the membrane and cytoplasm (green). Scale bars, 15 μm.
Figure 4. PF-2341066 increases sensitivity to etoposide (Et) in H69M-derived tumors. A and B, photograph of representative mice of each group of treatment with H69M (A) or H69 cells (B) subcutaneously inoculated at right flanks, and photograph of the excised tumors from the same animal. Graphs show changes in tumor volume of H69M (A) and H69 (B) xenograft treated with etoposide alone, PF-2341066 alone, or the combination of both treatments. Dose schedules were etoposide (12 mg/kg/i.p. on days 1 to 3 of treatment), PF-2341066 (100 mg/kg/oral gavage daily), and the combination of both. Each data point represents the mean ± SD tumor volume of each group of mice compared with control. C, p-Met, E-cadherin, NCAM, Snail, vimentin, SPARC, and CD31 staining in H69M tumors in control, PF 100 mg/kg, etoposide 12 mg/kg, and combination conditions of treatment. Met inhibition by PF alone or in combination with etoposide induces epithelial phenotype in tumor cells. Scale bars, 75 μm. Quantification of expression for each marker in the different treatment conditions (mean H-score ± SEM) and significance are displayed. *, P < 0.05 (PF arm compared with the combination). **, P < 0.05 (etoposide arm compared with the combination).
or amplification in *MET* (data not shown). In clonogenic assays, H841 and H69M cells presented a similar sensitivity to PF-2341066. It was also sensitive to etoposide, although the inhibitory effect was much greater when both drugs were combined (Supplementary Fig. S5C, bottom, right). Accordingly, PF-2341066 or etoposide alone did not significantly inhibit tumor xenograft growth when compared with the control group (Supplementary Fig. S5D). However, the combined therapy produced a significant decrease in tumor growth with respect to either group alone (*P* < 0.05; Supplementary Fig. S6A), suggesting that Met inhibition increased sensitivity of etoposide in chemoresistant H841-derived tumors (Supplementary Fig. SSD). The similar phenotypic response to chemotherapy and Met inhibition of H69M and H841 cell lines might be related to the presence of a mesenchymal phenotype in both cell lines in basal conditions as well as a common effect of exogenously added HGF in terms of induction of p-Met and downstream molecules p-GAB and p-ERK. However, differences in baseline phosphorylation of Tyr1234/35 and Tyr1349 do not seem to account for the effects observed in these mesenchymal-like cell lines (baseline phosphorylation is not present in H841). Furthermore, no common genetic alterations in the Met gene were detected. Of note, we tested another mesenchymal-like SCLC cell line, SHP77, which was chemoresistant, did not express Met or p-Met with HGF addition, and was unresponsive to PF-2341066 (data not shown), suggesting that the mesenchymal phenotype per se is not sufficient to predict response to anti-Met therapy.

Immunohistochemical studies of H841-derived tumors in the different treatment groups, confirmed previously observed downregulation of p-Met and mesenchymal markers in xenografts treated with PF-2341066 (Supplementary Fig. S6B). Finally, in the H841-derived xenograft experiment, in the combined treatment group, we continued the experiment with 6 mice for 12 additional days, withdrawing the drugs in three cases. When the treatment was discontinued, the tumors significantly regrew compared with those that were kept treated (Supplementary Fig. S6C).

**Mesenchymal phenotype in human SCLC is associated with a worse outcome**

We retrospectively analyzed 87 SCLC specimens obtained at diagnosis between 2002 and 2012 with sufficient material for biomarker analysis and available clinical information. This series did not include patients from our previous publication (3). Supplementary Table S1 shows the patients’ characteristics. All the patients received standard first-line chemotherapy with platinum and etoposide and concomitant radiotherapy in cases if indicated. Mean follow-up time was 14 months (0.2–105 months). We determined Snail1, vimentin, SPARC, NCAM, Met, p-Met, and E-cadherin expression in these specimens. Figure 5A and Supplementary Table S2 show the pattern of expression of each biomarker. As observed, most of the cases were negative (no expression) for Snail1, vimentin, SPARC, and p-Met. There was not a relationship among the magnitude (intensity) of the signal in the positive cases (Supplementary Table S3). As no cutoff with clinical validity has been established for these biomarkers, we used the median as an arbitrary cutoff point. p-Met was expressed in 34% of patients, confirming our previous results. As dichotomous variables, we found direct association of p-Met expression with Snail1, vimentin, and Met and inverse correlation with E-cadherin (all *P* values < 0.001; Supplementary Table S4). There was a significant direct association between Snail1, vimentin, and SPARC and an inverse correlation between each of them and E-cadherin (all *P* values < 0.001). We found no association between Met and p-Met with clinical characteristics (Performance Status, stage).

We then assessed coexpression of p-Met staining with Snail1 and vimentin in human samples. Figure 5B demonstrates that both markers are coexpressed and tumor cells with Snail1 and vimentin expression also presented Met phosphorylation. The percentage of tumor cells with coexpression for Snail1 and vimentin, Snail1 and p-MET, and p-MET and vimentin was estimated in a subset of specimens (*N* = 25) by double immunofluorescence. The mean of Snail1-positive cells coexpressing vimentin was 73% (±20% SD), Snail1-positive cells coexpressing p-MET was 74% (±17% SD), and p-MET–positive cells coexpressing vimentin was 80% (±13% SD).

For survival analysis, we first evaluated the association of classic prognostic factors for patients with SCLC. As expected, poor performance status (≥2) and stage IV disease were associated with worse outcome (*P* < 0.001). We first performed a univariate analysis of each biomarker with survival illustrated in Fig. 6A. All biomarkers were associated with outcome except for p-Met and E-cadherin that showed a trend toward statistical significance. In our previous work (3), p-Met was significantly (*P* < 0.001) associated with survival in univariate and multivariate analysis. In the present study, p-Met was of borderline significance in the univariate analysis and significantly associated with survival in multivariate analysis. The difference in the *P* values between the two studies by univariate analysis may be related to relatively small sample size (albeit large for a SCLC study), or minor differences in the cutoffs used for positivity due to intrinsic differences in both study populations. However, the independent significance observed in both studies by multivariate analysis strongly supports the role of p-Met expression in SCLC outcomes. In the multivariate analysis, Snail1, vimentin, and SPARC expression also correlated with poor prognosis (all *P* values < 0.05). In contrast, E-cadherin expression was a marker of better outcome (*P* < 0.05; Supplementary Table S5 and Supplementary Fig. S7).

**Mesenchymal features are enhanced in relapsed disease in human SCLC**

We prospectively obtained paired samples (biopsies or cytology block) from 11 patients before first-line treatment and at first relapse/progression. We sought to compare EMT markers and Met status in these samples to validate our
preclinical hypothesis. Only five paired cases were adequate for immunohistochemical analysis. Globally, we observed upregulation of mesenchymal markers and p-Met in the specimens obtained at relapse (Supplementary Table S6 and Fig. 6B).

Discussion

Here, we demonstrate using preclinical models that the activation of the HGF/Met pathway induces EMT phenotype in SCLC cells and generates a mesenchymal population, more tumorigenic and chemoresistant than the parental cells. More importantly, Met-specific inhibition, via modulation of these mesenchymal biomarkers reverses this mesenchymal transition and subsequently increases chemosensitivity in SCLC models. Of translational relevance, a mesenchymal phenotype is associated with Met activation in the same tumor cells. Importantly, these mesenchymal features and Met phosphorylation are predictive of poor survival in patients with SCLC and are upregulated in chemoresistant or relapsed tumors. Globally, these results suggest a potential therapeutic role for PF-2341066 in combination with chemotherapy in the treatment of Met-activated SCLC.

SCLC is a highly lethal disease. No improvements in the treatment outcome have occurred for many years, leaving “old agent” chemotherapy and radiotherapy as the backbone treatment for these patients. Moreover, resistance to available therapy is a typical feature of this tumor at relapse, a phenomenon that has been elusive to tackle, to date. We have previously observed that Met activation is an independent prognostic factor in SCLC (3), suggesting a potential role for Met-targeted therapies in this disease. HGF has been previously associated...
with poor prognosis and tumor burden in patients with SCLC (23, 24). Because Met is a well-known inducer of EMT in other models, we hypothesized that this transition, when induced by HGF, could be responsible for resistance to chemotherapy. We confirmed this hypothesis using H69 cells treated with HGF. Upon HGF-chronic exposure, we isolated a mesenchymal chemoresistant and highly tumorigenic subpopulation mimicking chemoresistant SCLC. Interestingly, these cells showed an increased basal Met phosphorylation when compared with the parental H69 along with upregulation of mesenchymal markers in vitro and in vivo and increased angiogenesis. The role of HGF as an inducer of EMT (10, 25, 26) and angiogenesis (27, 28) has been reported previously.

These cells endogenously expressed HGF, leading to an autocrine loop and constitutive activation of the pathway. This has been demonstrated in other tumor models (29, 30). The existence of autocrine loops has been shown to be common in mesenchymal cells participating in the stabilization of their phenotype (5).

PF-2341066, now crizotinib, is a dual Met/Alk inhibitor that has demonstrated a clear benefit in the treatment of patients with Alk-positive NSCLC (11). This drug is orally delivered and has a good safety profile. In our study, Met inhibition by PF-2341066 decreased the expression of Snail1, SPARC, and vimentin in SCLC xenografts. Although this was not sufficient to arrest tumor growth in vivo, there was a potent effect of PF-2341066 in resensitizing cells to chemotherapy, specifically to etoposide. In addition, in our

Figure 6. Met and EMT markers: association with survival in SCLC and changes after treatment. A, Kaplan–Meier curves for overall survival univariate analysis according to biomarker status. B, paired samples from SCLC patient #5 at diagnosis and relapse time points, stained for Met, p-Met, E-cadherin, Snail, vimentin (VIM), and SPARC. Upregulation of mesenchymal markers and downregulation in E-cadherin expression were detected in relapse sample. Scale bars, 75 μm.
patient population, there was a strong and independent correlation of this mesenchymal phenotype with Met activation and poor patient outcome. Without the data of untreated patients’ arm, it is not possible to specify whether these biomarkers are predictive or prognostic in this disease. However, 85% of patients in this series showed response to first-line treatment, and this was not associated with biomarker status (data not shown).

The mechanistic association between Met activation and mesenchymal features was supported by the coexpression of p-Met, Snail1, and vimentin in the same tumor cells and by the changes in EMT markers upon treatment with crizotinib. Upregulation of mesenchymal biomarkers and Met activation have both been associated separately with prognosis in several tumor types (6, 31–33) and coexistence of both markers with stemness (34). One could hypothesize that the presence of a subset of cells with these markers in SCLC at diagnosis could predict the enrichment of this chemoresistant population at progression after chemotherapy and, therefore, explain the poor prognosis of these patients in our study. This would be consistent with the greater antitumor effect observed when PF-2341066 is added upfront (Supplementary Fig. S4A) when compared with in vivo experiments using established H69M tumor xenografts (i.e., average tumor volume ~200 mm³). In the latter model, PF-2341066 alone did not significantly affect tumor growth albeit EMT markers and vasculogenesis were modified in the tumor specimens assayed at the time animals were sacrificed (Fig. 4). In contrast, PF-2341066 significantly reduced tumor growth and mesenchymal biomarkers when drug exposure was initiated before tumor xenografts were established (Supplementary Fig. S4). A possible explanation, beyond a potential greater effect of drug just based on lower tumor volume, is that a higher proportion of tumor cells at earlier time points is more mesenchymal than at later time points. Regardless of this, our data suggest that the inhibition of EMT and vasculogenesis markers plays a role in the sensitizing effects of PF-2341066 toward etoposide and we propose that the main role of Met inhibition would be to enhance chemotherapy activity instead of a role as a single-drug strategy.

However, the selection of patients who may benefit from this drug is crucial. Some researchers postulate that a selection according to Met expression by immunohistochemistry would be a good criterion for further evaluation of Met inhibitors (i.e., onartuzumab; ClinicalTrials.gov identifier: NCT01456325).

In our experience, Met levels (neither by mRNA or protein quantification) do not reflect Met activity and do not predict for Met inhibitor sensitivity; accordingly, Met phosphorylation would be a superior biomarker for pathway activation. However, p-Met antibodies have poor performance due to lack of sensitivity and specificity on routine clinical samples.

We previously reported that Met mutations or polymorphisms could be a marker of response to Met inhibitors. However, in the present work, we report that a Met-dependent cell line, H841, without any known Met mutation or polymorphism, but enriched with mesenchymal features, was also sensitive to Met inhibition. This novel observation suggests the potential benefit of Met inhibitors for patients with SCLC with mesenchymal features in the tumor.

We initially focused on Snail1 as it is the best-characterized inducer of EMT and its role in our SCLC models was confirmed by shRNA experiments. Because our ultimate goal was to explore an EMT phenotype of potential clinical interest, as assayed by robust epithelial and mesenchymal markers, but not to study EMT at the transcriptional level, we did not assay additional EMT transcriptional repressors such as Zeb1, Zeb2, Twist1, or Slug.

Our work suggests that the selection of patients according to mesenchymal biomarkers (SPARC, vimentin, and E-cadherin) in combination with Met expression is a good alternative for selecting patients with SCLC for clinical trials of Met inhibitors plus chemotherapy.

Disclosure of Potential Conflicts of Interest
E. Arriola is a consultant/advisory board member of Pfizer. Inc. No potential conflicts of interest were disclosed by the other authors.

Authors’ Contributions
Conception and design: I. Cañasadas, F. Rojo, M. Dómíne, A.G. de Herreros, E. Arriola
Acquisition of data (provided animals, acquired and managed patients, provided facilities, etc.): I. Cañasadas, F. Rojo, A. Taus, O. Arpi, M. Arumí-Uría, I. Pijuán, S. Menéndez, S. Zazo, M. Dómíne, E. Arriola
Writing, review, and/or revision of the manuscript: I. Cañasadas, F. Rojo, S. Zazo, M. Dómíne, A.G. de Herreros, A. Rovira, J. Albanell, E. Arriola
Administrative, technical, or material support (i.e., reporting or organizing data, constructing databases): I. Cañasadas, F. Rojo, A. Taus, O. Arpi, I. Pijuán, E. Arriola
Study supervision: I. Cañasadas, F. Rojo, M. Dómíne, A. Rovira, J. Albanell, E. Arriola

Acknowledgments
The authors thank Lara Nonell and Elulala Puigdecanet for the support in the performance of microarray experiments, Raul Peña and Rosa Vinas for experimental support, and Xavier Villanueva for his kind collaboration in sample management. The authors also thank Pfizer for generously providing PHA-665752 and PF-2341066.

Grant Support
This work was supported by RD12/0036/0051, RD09/0076/0036, RD09/0076/0101, RD06/0020/0040, PI12/00680, PI12/01552, PI09/01594, SAE2010-16089, 2009 SGR 321, and the ’Xarxa de Banys de tumors’ sponsored by Pla Director d’Oncologia de Catalunya (XBTC) I. Albanell and F. Rojo are recipients of the intensification program ISICII/FEDER. The authors thank Fundació Cellex (Barcelona) for a generous donation to the Hospital del Mar Medical Oncology Service.

The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked advertisement in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.

Received May 21, 2013; revised November 6, 2013; accepted November 7, 2013; published OnlineFirst November 27, 2013.
Canadas et al.

References


