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Role of cMET expression in non-small-cell lung cancer patients treated with EGFR tyrosine kinase inhibitors

P. A. Zucali[1], M. G. Ruiz[2], E. Giovannetti[2], A. Destro[3], M. Varella-Garcia[4], K. Floor[2], G. L. Ceresoli[1], J. A. Rodriguez[2], I. Garassino[1], P. Comoglio[5], M. Roncalli[3], A. Santoro[1] & G. Giaccone[2]*

[1]*Department of Medical Oncology and Hematology, Istituto Clinico Humanitas, Rozzano, Milan, Italy;* [2]*Department of Medical Oncology, VU University Medical Center, Amsterdam, The Netherlands;* [3]*Department of Human Pathology, Istituto Clinico Humanitas, Rozzano, Milan and University of Milan Medical School, Milan, Italy;* [4]*Department of Medicine/Medical Oncology, University of Colorado Cancer Center, Aurora, CO, USA;* [5]*Division of Molecular Oncology, Institute for Cancer Research and Treatment, University of Torino Medical School, Candiolo, Turin, Italy*

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Background: Approximately 10% of unselected non-small-cell lung cancer (NSCLC) patients responded to the epidermal growth factor receptor tyrosine kinase inhibitors (EGFR-TKIs) treatment. However, resistance mechanisms are not well understood. We evaluated several potential biological markers of intrinsic EGFR-TKIs-resistance in NSCLC.

Materials and methods: pAKT, pERK, cSRC, E-cadherin, cMET[pY1003], cMET[pY1230/1234/1235], and cMET[pY1349] immunohistochemistry, cMET FISH analysis, and *EGFR*-, *KRAS*-, and *cMET* mutation analysis were carried out on tumor samples from 51 gefitinib-treated NSCLC patients. Biological parameters and survival end points were compared by univariate and multivariate analyses. cMET expression was also investigated in two additional series of patients. The *in vitro* antiproliferative activity of gefitinib alone or in combination with hepatocyte growth factor and the cMET antibody DN-30 was assessed in NSCLC cells.

Results: *EGFR19* deletion and pAKT expression were significantly associated with response ($P < 0.0001$) and longer time to progression (TTP) ($P = 0.007$), respectively. Strong cMET[pY1003] membrane immunoreactivity was expressed in 6% of 149 tumors analyzed and was significantly associated with progressive disease ($P = 0.019$) and shorter TTP ($P = 0.041$). *In vitro*, the DN-30 combination synergistically (CI < 1) enhanced gefitinib-induced growth inhibition in all cMET[pY1003]-expressing cell lines studied.

Conclusions: Activated cMET[pY1003] appears to be a marker of primary gefitinib resistance in NSCLC patients. cMET may be a target in treatment of NSCLC.

Key words: activated cMET, gefitinib resistance, multitarget therapy, NSCLC

introduction

The epidermal growth factor tyrosine kinase inhibitors (EGFR-TKIs), gefitinib (Iressa, AstraZeneca, Macclesfield, UK) and erlotinib (Tarceva; F. Hoffmann-La Roche, Basel, Switzerland), have demonstrated activity in ~10% of unselected non-small-cell lung cancer (NSCLC) patients. Several clinical and biological features have been shown to be associated to benefit from EGFR-TKIs and some biological factors have been associated with resistance. The *EGFR*20-T790M mutation is mainly associated with secondary resistance to EGFR-TKIs in NSCLC [1–3], whereas *KRAS* mutations are associated with primary resistance [4, 5]. Persistent concomitant activity of the ERK and AKT kinase pathways also contributes to resistance to EGFR-TKIs in NSCLC cells [6, 7]. Furthermore, SRC activation has been shown to induce gefitinib resistance by modulation of both AKT and ERK signaling pathways in human gallbladder adenocarcinoma cells [8], and oncogenic SRC and RAS proteins may induce the epithelial–mesenchymal transition (EMT) that is associated with loss of E-cadherin complexes at cell–cell adhesions and, consequently, with an increased invasive and metastatic potential [9]. A restored E-cadherin expression increased EGFR-TKIs sensitivity in lung cancer cells [10], while NSCLC patients with a strong E-cadherin staining had a significantly longer time to progression (TTP) and a trend toward longer survival with erlotinib/chemotherapy treatment versus chemotherapy alone [11].

The cMET tyrosine kinase receptor, normally expressed by epithelial cells, is overexpressed and amplified in a variety of

*Correspondence to: Dr G. Giaccone, Clinical Cancer Research, National Cancer Institute, National Institutes of Health, 10 Center Drive Building 10, Room 12 N226, Bethesda, MD 20892-1906, USA. Tel: +1-301-4023415; Fax: +1-301-4020172; E-mail: giacconeg@mail.nih.gov

human tumors, including NSCLC [12–15]. *cMET* stimulation induces specific phosphorylation of several tyrosine residues (Y1003-Y1313-Y1230/1234/1235-Y1349-Y1365), which, in turn, activate multiple downstream signaling pathways, including the RAS/ERK, PI3K/AKT, and cSRC kinase pathways [16]. Through SRC activation, cMET is considered an EMT promoter [16]. High levels of hepatocyte growth factor (HGF) (the cMET ligand) and intratumoral cMET expression have been associated with a more aggressive biology and a worse prognosis in NSCLC [17]. HGF/cMET pathway alterations may confer a substantial growth advantage and invasive potential to NSCLC cells. In addition, recent studies have demonstrated that targeted cMET inhibition by different therapeutic strategies, including small interfering RNA, small molecules [15], and specific antibodies [18], leads to decreased NSCLC cell growth and viability. Engelman et al. [19] reported that cMET amplification induced resistance to gefitinib in a gefitinib-sensitive lung cancer cell line. Moreover, cMET inhibition with a cMET tyrosine kinase inhibitor (PHA-665,752) restored gefitinib sensitivity.

The aim of this study was to investigate tumor samples of EGFR-TKI-treated NSCLC patients for potential biological markers of intrinsic EGFR-TKI resistance, especially related to EMT. Given the significant association observed between strong cMET[pY1003] membrane staining and progressive disease (PD) and shorter TTP, we also evaluated the effect of the specific cMET antibody DN-30 combined with gefitinib in a panel of NSCLC cells.

materials and methods

patient populations

We evaluated 51 NSCLC patients treated within the expanded access study of gefitinib at Humanitas Clinical Institute (Rozzano, Milan, Italy). The protocol was approved by the institutional Medical Ethical Committee. Written informed consent was obtained from each patient. Patients received gefitinib 250 mg/day orally and were evaluated for response every 2 months according to the response evaluation criteria in solid tumors criteria [20]. To better assess the incidence of strong cMET[pY1003] expression in a larger NSCLC population, we also evaluated the cMET[pY1003] expression in tumors from two additional series treated at the Vrije Universiteit Medical Center (Amsterdam, The Netherlands): 27 advanced NSCLC patients treated with gefitinib or erlotinib and 71 radically resected NSCLC patients.

tissue microarray construction

Paraffin-embedded tumor specimens of the first series of 51 patients were collected. A pathologist selected areas for 1-mm^2 punches from tumor cores, tumor borders, and normal tissue to include in recipient tissue array block using a specific instrument (Beecher Instruments, Sun Prairie, WI).

immunohistochemical staining

Tissue microarray sections were stained with antibodies against pAKT (Ser473; Cell Signaling Technology, Danvers, MA, dilution 1 : 150), pERK (P-p44/42Mapk-Thr202/Tyr204; Cell Signaling Technology, dilution 1 : 100), E-cadherin (DAKO, Glostrup, Denmark, dilution 1 : 200), and SRC (gift from AstraZeneca, Clone 28, dilution 1 : 600) according to the manufacturers' protocols. cMET activation was determined with three different cMET antibodies ([pY1003], dilution 1 : 1000; [pY1230/1234/1235], dilution 1 : 1000; [pY1349], dilution 1 : 1500; BioSource International, Inc., Camarillo, CA) evaluating the phosphoepitopes expression that characterizes NSCLC-activated cMET [15]. At present, there are no validated scoring systems for interpreting immunohistochemical staining for pAKT, pERK, E-cadherin, SRC, or pMET. Since activation of AKT by phosphorylation results in its translocation from the cytoplasm to the nucleus, samples were considered pAKT positive if nuclear staining was present [21, 22]; for pERK, we used a system on the basis of the staining intensity as previously described [23]; the same scoring system was carried out for cytoplasm SRC expression, while for E-cadherin and pMET, we used a scoring system on the basis of the membrane staining intensity [11, 15]; samples were considered pMET positive if membrane staining was strong (score 3+) (Figure 1).

FISH analysis

A homebrew MET FISH probe developed using the bronchioloalveolar carcinoma (BAC) clone RP11-95I20 (CHORI, Oakland, CA) was used for this study. DNA from single colonies from the BAC clone was confirmed to encompass the MET gene sequences by PCR with two sets of primers for better specificity (*MET*5′-end: forward-CCAGTCAGGCCGCGTTGTTTATTT, reverse-AGTTATCATTTCCCAAGTGCCAGG; *MET*3′-end: forward-TGTCCCGGATATCAGCGATCTTCT, reverse-AAGCCTCTGGTTCTGATGCTCTGT).

The MET-DNA was labeled with SpectrumRed-conjugated deoxi-uridin-triphosphates by nick translation using the Vysis Nick Translation Kit (Abbott Molecular, Des Plaines, IL) and ethanol precipitated using herring sperm DNA (1 : 50) as carrier and human Cot-1 DNA for blocking of repetitive sequences. The DNA pellet was diluted in hybridization mix (50% formamide/10% dextran sulphate/2X SSC). The final MET/CEP7 probe set was prepared with the homebrew SR-MET and CEP7 labeled in SpectrumGreen (Abbott Molecular).

The specimens were classified according to the number of signals in the majority of cells in one of four FISH ranks [rank 1: low number of MET signals per cell (one to three copies); rank 2: intermediate number of MET signals per cell (two to four copies); rank 3: high number of

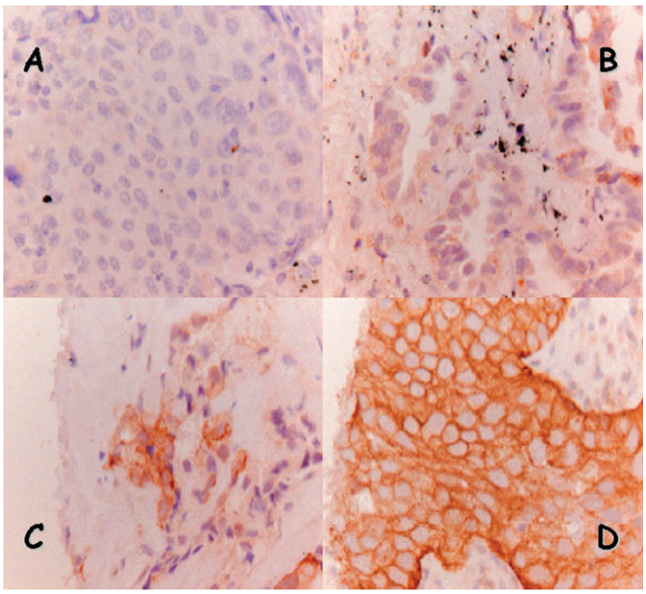

Figure 1. Representative immunohistochemistry for cMET[pY1003]. Digital pictures were taken at ×40 magnification (Leika Digital Microscope DM 4000B). (A) Negative membrane expression of cMET[pY1003]; (B) weak expression (1+); (C) moderate expression (2+); and (D) strong expression (3+).

MET signals per cell (two to six or seven copies); rank 4: gene amplification (small cluster of MET signals)]. FISH was considered positive at ranks 3 and 4.

mutation analysis

Total genomic DNA was isolated using the QIAamp DNA extraction kit (Qiagen, Venlo, The Netherlands). Nested PCR to amplify *EGFR* (exons 18–21) and *KRAS* (exons 1, 2) were carried out as previously described [4, 8]. *cMET* mutation analysis was carried out by direct sequencing, using the following primer sets for *cMET* exons 14 and 15 (juxtamembrane domain): hu-cMET-ex14F, 5′-CTTTAACAAGCTCTTTCTTTCT-3′; hu-cMET-ex14R 5′-TGTATAGGTATTTCTCAGAA-3′; hu-cMET-ex15F 5′-TTGTTCTTTAATAATTTTC-3′; hu-cMET-ex15F 5′-GAGTCGAAAAACAATTTATGCT-3′. Sequencing of PCR products was carried out as previously described [24].

lung cancer cell lines and culture conditions

Cell lines were purchased from the American Type Culture Collection (Manassas, VA) and maintained as previously described [23]. The human NSCLC cell lines NCI-H460 (H460), NCI-H1703 (H1703), NCI-H292 (H292), NCI-H1650 (H1650), NCI-H322 (H322), NCI-H3255 (H3255) and NCI-H1975 (H1975) and the SCLC cell line NCI-H69 (H69) were cultured in RPMI-1640, while A549, SW1573, Calu-1, and Calu-6 were cultured in DMEM as previously described [23].

western blot

In all, 1×10^6 cells were plated, grown, and stimulated with 40 ng/ml HGF (Sigma Chemical Co., Zwijndrecht, The Netherlands) as previously described [15]. The whole-cell protein extracts were transferred to nitrocellulose membranes that were incubated overnight at 4°C with specific primary antibodies (anti-cMETc-12—Santa Cruz Biotechnology—1 : 1000 dilution; anti-cMET[pY1003]—BioSource International—1 : 1000 dilution; anti-α-tubulin—Sigma—1 : 200 dilution) and then with the appropriate horseradish peroxidase-conjugated secondary antibodies (Cell Signaling Technologies) (1 : 2000 dilution) and detection was carried out [23].

cytotoxicity assay and pharmacological interaction study

Gefitinib was a generous gift from AstraZeneca. DN-30 is a mAb against the cMET extracellular domain, which behaves as a partial agonist, inducing receptor phosphorylation but being unable to trigger the whole set of downstream biological effects [24].

The drugs were diluted in fresh medium before each experiment. A549, SW1573, H460, H292, and H1650 cells were plated and treated with gefitinib (0.01–100 μM) with and without HGF (40 ng/ml) and DN-30 (80 μg/ml) and their combinations, as indicated, as previously described [23]. Each experiment was carried out in two replicate wells for each drug concentration and repeated at least three times. After drug exposure, an MTT assay [MTT, 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium-bromide, Sigma] was carried out as previously described [23]. DN-30 (80–1500 μg/ml) cytotoxicity was also evaluated by MTT. Absorbance values were expressed as the percentage of the untreated controls, and the 50% inhibitory concentration of cell growth (IC_{50}) relative to untreated cultures was calculated. Drug interaction between gefitinib and DN-30 was assessed, at a nonconstant concentration ratio, using the combination index (CI) method [25].

statistical analysis

Patient groups were compared with the Fisher's exact or χ^2 tests when appropriate. Correlations were considered significant at the <0.05 level (two tailed). Survival curves were constructed using the Kaplan–Meier method, and differences between groups were analyzed using the log-rank test. Multivariate analysis was carried out using the Cox proportional hazards model. Only variables significant at the $P < 0.05$ level in the univariate analyses were entered into the Cox regression analysis. We carried out manual backward elimination using likelihood ratio tests with a significance level of 0.05. All statistical analyses were carried out using SPSS software (version 14.0; SPSS Inc., Chicago, IL).

results

Tables 1 and 2 summarize the outcome according to clinical and biological characteristics of the 51 advanced NSCLC patients treated with gefitinib. No statistically significant association between clinical and biological characteristics was observed.

Table 1. Outcome of study population according to clinical characteristics

Characteristic	Total (*N*)	Response rate (*N*)	Median TTP (months)	Median survival (months)
Overall population	51	3	3.0	6.6
PR	3	–	9.0	–
SD + PD	48	–	2.8	6.6
			***P* = 0.045**	*P* = 0.099
PD	24	–	1.5	3.4
PR + SD	27	–	5.7	10.4
			***P* < 0.001**	***P* < 0.001**
Sex	51	3	3.0	6.6
Male	34	0	2.8	5.0
Female	17	3	3.2	9.6
		***P* = 0.033**	*P* = 0.260	*P* = 0.139
Histology	51	3	3.0	6.6
Adenocarcinoma	38	3	3.2	6.8
Other histology	13	0	1.9	3.4
		P = 0.561	***P* = 0.009**	*P* = 0.486
BAC	7	2	9.0	28.6
Other histology	44	1	2.4	5.0
		***P* = 0.046**	***P* = 0.0029**	***P* = 0.0092**
WHO PS	51	3	3.0	6.6
PS 0–1	41	3	3.2	7.6
PS 2	10	0	1.5	2.1
		P = 1.0	***P* = 0.004**	***P* = 0.0067**
Lines of previous chemotherapy	51	3	3.0	6.6
0–1	31	3	3.2	7.1
≥2	20	0	1.9	4.0
		P = 0.271	***P* = 0.012**	*P* = 0.148
Stage	51	3	3.0	6.6
III	3	0	1.7	2.5
IV	48	3	3.1	6.8
		P = 1.0	***P* = 0.029**	*P* = 0.432
Smoker status	50	3	2.8	6.6
Never smoker	9	3	5.7	7.1
Current/former smoker	41	0	2.4	6.5
		***P* = 0.004**	*P* = 0.065	*P* = 0.183

The numbers in bold means statistically significant.
TTP, time to progression; PR, partial response; SD, stable disease; PD, progressive disease; BAC, bronchioloalveolar carcinoma; WHO, World Health Organization; PS, performance status.

Table 2. Outcome of the study population according to biological characteristics

Characteristic	Total (*N*)	Response rate (*N*)	PD versus SD + PR (*N*)	Median TTP (months)	Median survival (months)
Overall population	49	3	23	3.0	6.5
*EGFR*19 del	49	3	23	3.0	6.5
Wt	45	0	22	2.8	6.5
Mut+	4	3	1	4.5	5.1
		***P* < 0.0001**	*P* = 0.612	*P* = 0.067	*P* = 0.180
KRAS mutation	49	3	23	3.0	6.5
Wt	34	3	17	2.8	5.0
Mut+	15	0	6	4.9	8.2
		P = 0.543	*P* = 0.552	*P* = 0.164	*P* = 0.518
cMET(FISH)	42	3	17	3.2	7.7
Negative	21	2	6	4.9	10.4
Positive	21	1	11	2.8	5.0
		P = 1.0	*P* = 0.208	*P* = 0.421	*P* = 0.332
cMET[pY1003]	43	3	17	3.2	7.1
0, 1+, 2+	39	3	13	3.2	7.4
3+	4	0	4	1.2	1.2
		P = 1.0	***P* = 0.019**	***P* = 0.042**	*P* = 0.164
cMET[pY1230/1234/1235]	42	3	17	3.2	7.4
0, 1+, 2+	40	3	15	3.2	7.6
3+	2	0	2	1.2	1.2
		P = 1.0	*P* = 0.16	*P* = 0.23	*P* = 0.058
cMET[pY1349]	47	3	20	3.2	7.1
0, 1+, 2+	45	3	18	3.2	7.4
3+	2	0	2	1.2	1.2
		P = 1.0	*P* = 0.176	*P* = 0.24	*P* = 0.068
E-cadherin	46	3	19	3.2	7.1
0, 1+	7	0	3	3.2	10.4
2+, 3+	39	3	16	3.2	6.5
		P = 1.0	*P* = 1.0	*P* = 0.762	*P* = 0.424
cSRC	43	3	17	3.2	7.1
0, 1+	3	1	0	6.6	10.4
2+, 3+	40	2	17	3.1	7.1
		P = 0.199	*P* = 0.256	*P* = 0.086	*P* = 0.367
pERK	42	3	17	3.2	7.1
0, 1+	17	0	8	3.2	6.8
2+, 3+	25	3	9	3.2	7.6
		P = 0.260	*P* = 0.534	*P* = 0.820	*P* = 0.495
pAKT	41	3	16	3.2	7.1
Negative	19	0	9	2.2	4.0
Positive	22	3	7	4.1	7.7
		P = 0.235	*P* = 0.352	***P* = 0.007**	*P* = 0.154

Immunohistochemical staining and mutation analysis were carried out on 49 evaluable tumor samples. Two tumors were not evaluable because of extensive necrosis. Additionally, some samples were not evaluable for certain markers because of tissue loss in the tissue microarray during the immunostaining procedure.

PD, progressive disease; SD, stable disease; PR, partial response; TTP, time to progression; del, deletion; wt, wild type; mut+: mutation.

immunohistochemistry

Among 43 evaluable tumors, four (9.3%) had strong cMET[pY1003] membrane staining (3+), 12 (27.9%) had weak or moderate expression (1+: five patients; 2+: seven patients), and 27 (62.8%) were negative. No significant association was observed with response ($P = 1.0$), PD ($P = 0.72$), TTP ($p = 0.45$), and overall survival (OS) ($P = 0.82$) in patients with combined moderate and strong (2+ and 3+) cMET[pY1003] membrane staining. The four patients with cMET[pY1003] overexpression had PD to the treatment (four of four patients; $P = 0.019$) and a shorter TTP ($P = 0.0416$). Of 27 additional advanced/metastatic NSCLC patients treated with erlotinib or gefitinib, two (7.5%) had cMET[pY1003] overexpression and both had PD. Among 71 NSCLC patients treated with radical surgery, only three (4%) had cMET[pY1003] overexpression. The overall frequency of cMET[pY1003] overexpression was 6% (nine of 149 patients). Table 3 summarizes the clinical and biological characteristics of these nine patients.

Table 3. Clinical and biological characteristics of cMET[pY1003] positive patients in three different series of NSCLC patients

	Pt 13	Pt 15	Pt 46	Pt 50	Pt a	Pt b	Pt R1	Pt R2	Pt R3
Sex	Male	Female	Male	Male	Male	Male	Male	Female	Male
Histology	ADC	SCC	ADC	ADC	ADC	Other	Other	SCC	SCC
Stage	IV	IV	IV	IV	ND	ND	I	I	I
Smoker	Former	Current	Former	Former	Former	Current	Current	Current	Former
PS	0	1	0	1	1	1	ND	0	0
Response	PD	PD	PD	PD	PD	PD	UT	UT	UT
*EGFR*19	wt	wt	wt	wt	wt	wt	wt	wt	wt
KRAS	wt	wt	wt	MUT	MUT	MUT	wt	wt	wt
cMET[pY1003] memb	+	+	+	+	+	+	+	+	+
pAKT	+	+	+	+	−	+	−	+	+
pERK	+	+	+	+	−/+	+	−	−	+
cSRC	+	+	+	+	ND	ND	ND	ND	ND
E-cadherin	−	−	−	+	ND	ND	+	+	−

Pt + number, patient from the first series of 51 patients treated with gefitinib; Pt + letter, patient from the second series of 27 patients treated with gefitinib or erlotinib; Pt R, patient from the resected group; ADC, adenocarcinoma; SCC, squamous cell carcinoma; ND, not done; PD, progressive disease; UT, untreated; wt, wild type; MUT, mutated.

FISH analysis

Out of a total of 153 cores (51 patients in triplicate), results were obtained in 121 cores (corresponding to 42 patients). Twenty-one patients exhibited at least one tumor core with FISH positivity (only one patient with *MET* gene amplification) (Table 2). Three of four tumors with cMET[pY1003] overexpression exhibited FISH positivity. No significant association was observed between cMET[pY1003] overexpression and FISH positivity ($P = 0.6$). The patient with *cMET* amplification had stable disease and harbored *KRAS* mutation. Two FISH-positive patients also harbored *EGFR* mutations: one had PD and the other partial response (PR).

mutation analysis

Four patients harbored an *EGFR* exon 19 deletion (Table 2) and 15 patients had *KRAS* mutations. No mutations in *EGFR* exons 18, 20, and 21 were found. No tumor with KRAS mutation harbored *EGFR19* mutations. None of the patients bearing *KRAS* mutations achieved PR after gefitinib treatment. No mutations in *cMET* exons 14 and 15 were found in the tumors overexpressing cMET[pY1003].

multivariate analysis

We carried out two different Cox regression analyses to evaluate the risk of disease progression and the risk of death entering only variables that were significantly associated with TTP in the univariate model in the first analysis and with OS in the second analysis (Table 4). Adenocarcinoma histology [hazard ratio (HR) = 0.217, 95% CI 0.087–0.542, $P = 0.001$] and pAKT positivity (HR = 0.461, 95% CI 0.298–0.713, $P = 0.001$) remained significantly associated with reduced risk of PD. Performance status (PS) of two or less (HR = 4.605, 95% CI 1.773–11.961, $P = 0.002$) and cMET[pY1003] (HR = 2.464, 95% CI 1.293–4.696, $P = 0.006$) were significantly associated with an increased risk of PD. PS two or less was significantly associated with an increased risk of death (HR = 2.398, 95% CI 1.079–5.330, $P = 0.032$) whereas BAC histology associated with a reduced risk of death (HR = 0.506, 95% CI 0.277–0.924, $P = 0.026$).

Table 4. Multivariate analysis

	Risk	HR	95% CI	Significativity
Risk of disease progression				
Adenocarcinoma	↓	0.217	0.087–0.542	*P* 0.001
pAKT	↓	0.461	0.298–0.713	*P* 0.001
PS ≥ 2	↑	4.605	1.773–11.961	*P* 0.002
cMET[pY1003]	↑	2.464	1.293–4.696	*P* 0.006
Risk of death				
BAC	↓	0.506	0.277–0.924	*P* 0.032
PS ≥ 2	↑	2.398	1.079–5.330	*P* 0.026

HR, hazard ratio; CI, confidence intervals; PS, performance status; BAC, bronchioloalveolar carcinoma.

in vitro studies

Characterization and gefitinib IC_{50}s of the cell lines used have been reported previously [6, 23]. Figure 2A shows the cMET protein expression. Gefitinib induced 50% growth inhibition in NSCLC cells at concentrations ranging from 0.1 to 24 μM in H1650 and H460 cells, respectively (Figure 2D). DN-30 treatment resulted in a modest inhibition of cell growth (Figure 2B). However, 80 μg/ml DN-30 was able to induce cMET down-regulation, as detected by western blot in SW1573 cells (Figure 2C). The activity of gefitinib was slightly reduced by HGF, while the addition of DN-30 enhanced the growth inhibition in H460, SW1573, A549, and H292 cells, with IC_{50}s ranging from 0.06 μM (H292) to 18 μM (H460). The CI calculation showed a synergistic effect in all these cells (CI < 1, Figure 2E). In contrast, the H1650 cells, negative for cMET[pY1003] expression, showed negligible cell growth

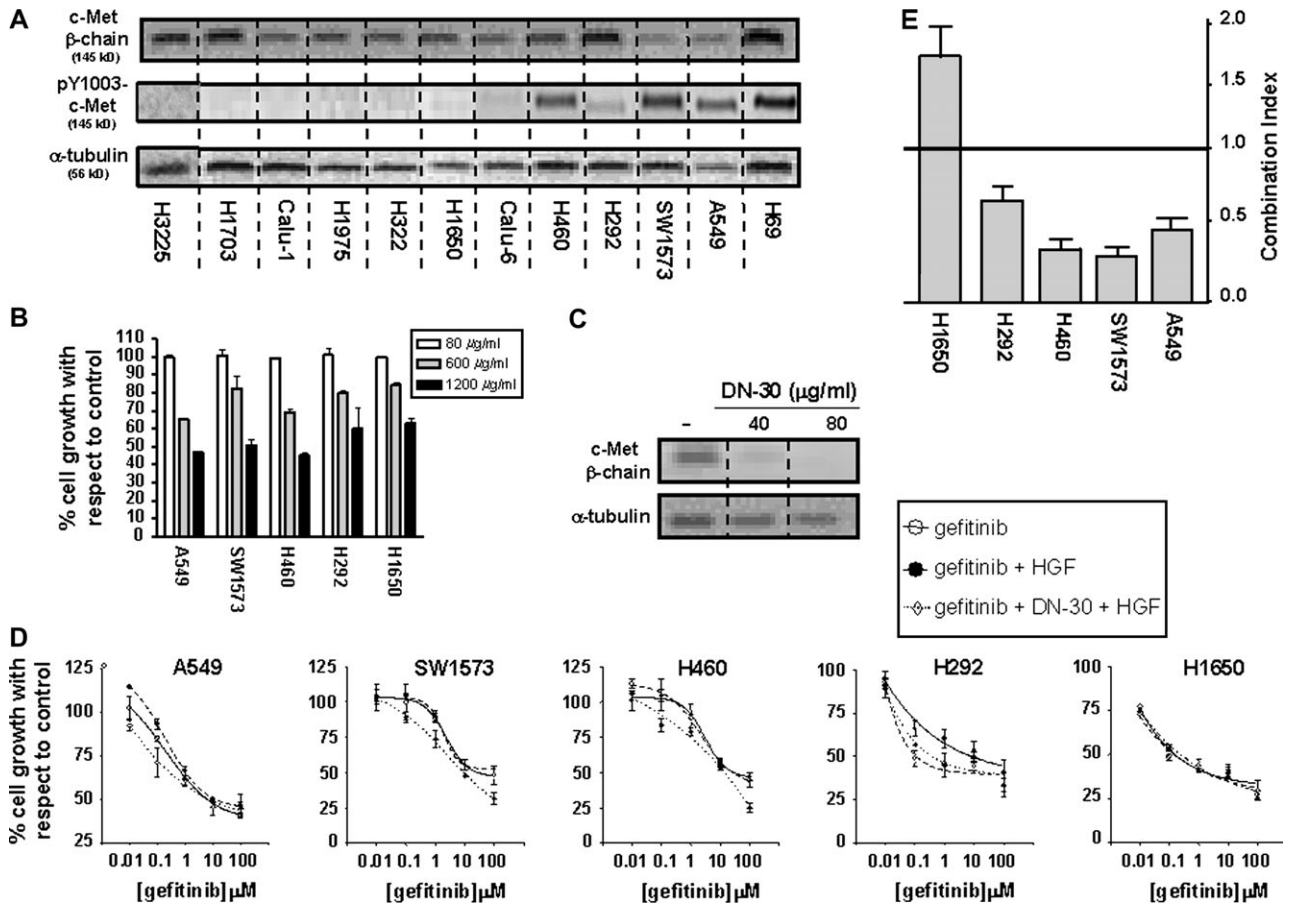

Figure 2. (A) Representative western blot of cMET expression (cMET β-subunit and phosphoY1003) in 11 NSCLC cell lines (and a positive control, H69 SCLC cell line). A 145-KDa protein band corresponding to the proteolitically processed (biologically active) form of cMET phosphorylated at Y1003 was detected in whole-cell protein extracts from H460, SW1573, H292, A549, and, much fainter, Calu-6 cells. (B) Growth curves of A549, SW1573, H460, H292, and H1650 cells treated with different concentrations of DN-30 (80–1500 μg/ml) for 72 h in 0.5% fetal calf serum medium. After drug exposure, an MTT assay [MTT,3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium-bromide, Sigma] was carried out as previously described [23]. Each data point represents the mean percentage values of proliferating cells with respect to untreated control and is the average of three independent experiments; bars, SE.
(C) Representative western blot of cMET expression after exposure to 40–80 μg/ml DN-30 for 72 h in SW1573 cells. DN-30 treatment resulted in a modest inhibition of cell growth (Figure 2B). However, 80 μg/ml DN30 was able to induce cMET down-regulation as detected by western blot in SW1573 cells.
(D) Growth curves of A549, SW1573, H460, H292, and H1650 cells treated with different concentrations of gefitinib (0.01–100 μM) with and without the combination of HGF (40 ng/ml) and DN-30 (80 μg/ml) for 72 h in 0.5% fetal calf serum medium as previously described [23]. Each experiment was carried out in two replicate wells for each drug concentration and repeated at least three times. Absorbance values were expressed as the percentage of the untreated controls, and the 50% inhibitory concentration of cell growth (IC_{50}) relative to untreated cultures was calculated. Gefitinib induced 50% growth inhibition at concentrations ranging from 0.1 to 24 μM in H1650 and H460 cells, respectively. The activity of gefitinib was slightly reduced by HGF, while the addition of DN-30 enhanced the growth inhibition in H460, SW1573, A549, and H292 cells, with IC_{50}s ranging from 0.06 μM (H292) to 18 μM (H460).
(E) Combination index (CI) values in HGF-stimulated A549, SW1573, H460, H292, and H1650 cells treated with gefitinib and DN-30, carried out by the CalcuSyn software (Biosoft, Oxford, UK). Drug interaction between gefitinib and DN-30 was assessed, at a nonconstant concentration ratio, using the CI, where CI < 1, CI = 1, and CI > 1 indicates synergistic, additive, and antagonistic effects, respectively [25]. The CI calculation showed a synergistic effect in A549, SW1573, H460, and H292 cells (CI < 1). In contrast, the H1650 cells, negative for cMET[pY1003] expression, showed negligible cell growth reduction by the addition of DN-30 to gefitinib, and the pharmacological interaction study demonstrated antagonism (CI > 1). Columns, mean values obtained from three independent experiments; bars, SE.

reduction by the addition of DN-30 to gefitinib, and the pharmacological interaction study demonstrated antagonism (CI > 1, Figure 2E).

discussion

We investigated several molecules as potential biologic markers of resistance to EGFR-TKIs in NSCLC. Strong cMET[pY1003] membrane staining was significantly correlated to primary gefitinib resistance and shorter TTP, and the multivariate analysis confirmed an increased risk of PD in this subset of patients. Specimens classified as 3+ had an impressive membrane staining compared with 1+ and 2+ tumors, suggesting a particular subset of NSCLC population. The Y1003 residue has an important role in binding to proteins, such as c-Cbl that modulates cMET internalization. When the receptor

is not internalized, it remains tyrosine phosphorylated inducing a potentially oncogenic overstimulation. Although we did not find a significant correlation between activated cMET, cSRC, and the E-cadherin, the majority of patients with cMET[pY1003]-positive tumors in the three different series of patients analyzed had positive cSRC expression and negative E-cadherin, consistent with the role of cMET activation in EMT. Interestingly, all these patients presented typical EGFR-TKIs nonresponder characteristics, such as smoker status, absence of *EGFR* mutations, and presence of *KRAS* mutations. Moreover, pAKT and pERK were both positive in six of nine patients, supporting the notion that persistent activity of both RAS/ERK and PI3K/AKT kinase pathways contributes to EGFR-TKIs resistance in NSCLC cells [6, 7, 9]. Recently, Engelman et al. [19] reported that ~20% of lung cancers with acquired resistance to gefitinib or erlotinib had *cMET* amplification and that cMET-driven gefitinib resistance was mediated through ERBB3/PI3K/Akt activation. In our study, one patient harboring *EGFR* mutation and exhibiting high *MET* copy numbers experienced PD. However, among the patients with cMET[pY1003] strong overexpression, three of four analyzed by FISH exhibited also high *MET* copy numbers and seven of nine (78%) had also pAKT-positive expression. None of these patients harbored *EGFR19* mutations or experienced some benefit from EGFR-TKIs when treated, suggesting that cMET activation may promote primary as well as acquired EGFR-TKI resistance. Given the small number of patients with cMET[pY1003], our findings will need to be reproduced in larger cohort of patients treated with EGFR-TKIs.

We observed cMET[pY1003] expression in 45% (five of 11) of NSCLC cell lines and cMET[pY1003]-positive NSCLC cells were associated with other biological features typical for EGFR-TKI resistance, such as presence of *KRAS* mutations. A549, SW1573, and H460 cells, with a strong cMET[pY1003] expression and the presence of *KRAS* mutation, showed gefitinib resistance.

Engelman et al. [19] reported that *cMET* amplification induced resistance to gefitinib in a gefitinib-sensitive lung cancer cell line and a cMET tyrosine kinase inhibitor (PHA-665,752) restored gefitinib sensitivity. In our study, the gefitinib–DN-30 combination demonstrated a synergistic effect in H460, SW1573, A549, and H292 cells, with IC_{50} ranging from 0.06 μM in H292 cells (wild-type KRAS), to 18 μM in H460 cells (mutant *KRAS*). Interestingly, the greater difference between cytotoxicity curves was recorded at gefitinib concentration between 0.1 and 1 μM, which are clinically achievable (1 μM). The gefitinib–DN-30 combination proved to be particularly effective in SW1573 cells, which have the highest cMET[pY1003] expression and carry a *KRAS* mutation [19, 26]. In contrast, in the H1650 cells, which are negative for cMET[pY1003] expression and carry an *EGFR*19 deletion, the addition of DN-30 to gefitinib led to an antagonistic effect.

Our data indicate that activated cMET, indicated by cMET[pY1003] strong overexpression, is a possible mechanism of gefitinib resistance in a small percentage of NSCLC patients and treatment with combinations of EGFR-TKIs and cMET inhibitors could be a promising approach to circumvent EGFR-TKIs resistance in this subset of patients.

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