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Increased *MET* Gene Copy Number but Not mRNA Level Predicts Postoperative Recurrence in Patients with Non–Small Cell Lung Cancer[1]

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Abstract

The aim of the present study was to investigate the relationship of *MET* copy number (CN) and *MET* mRNA expression to other molecular alterations, clinicopathologic characteristics, and survival of patients with resected non–small cell lung cancer. One hundred fifty-one paired surgical samples of tumor and tumor-distant normal lung tissues were analyzed by comparative quantitative polymerase chain reaction (PCR) methods with commercially available assays and the CopyCaller software v. 1.0 for post-PCR data processing (downloadable from www.appliedbiosystems.com). *MET* copy gain (set as more than 3.0 copies per cell) was found in 18.5% of the samples and occurred more frequently in the adenocarcinomas (ADCs) with an increased epidermal growth factor receptor (*EGFR*) or human epidermal growth factor receptor 2 (*HER2*) CN (P = .001 and .030 for *EGFR* and *HER2*, respectively) and in the ADCs with *EGFR* activating mutations (P = .051) but did not correlate with *KRAS* dosage or mutational status. *MET* mRNA level was 1.76-fold higher [95% confidence interval (CI), 1.29-2.40] in the tumor compared to unaffected lung tissue and associated significantly with *MET* CN (beta coefficient, 1.51; 95% CI, 1.22-1.87; $P < .001$). In the multivariable analysis, patients diagnosed with ADC with increased *MET* CN had a significantly higher risk of disease recurrence (hazard ratio, 1.76; 95% CI, 1.20-2.57; P = .004). An increased *MET* CN in combination with histologic type appears to be a prognostic factor in patients with ADC after a curative surgery.

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Introduction

High mortality rate of non–small cell lung cancer (NSCLC) patients after a curative surgery [1] suggests that the tumor-node-metastasis (TNM) staging system is insufficient for patient's prognosis and therapeutic decisions and that new prognostic factors are needed [2]. Aberrations of *MET* proto-oncogene, frequently observed in cancer [3,4], are one of the molecular factors with a possible prognostic potential [5]. An association between *MET* copy gains and a worse prognosis in patients with NSCLC has been found previously [6–9], but the data are limited and inconsistent. Recently, an increase in *MET* copy number (CN) has been demonstrated to be responsible for about 20% cases of the acquired resistance to EGFR tyrosine kinase inhibitors (TKIs) in patients with NSCLC [10,11], suggesting that, as a pre-existing condition occurring before treatment, it may provide a primary lack of response [12], although a number of researchers deny that possibility [10,13]. The rate of *MET* copy gain in NSCLC reported thus far ranges significantly from 3% to 21% depending on the detection technique used [6,7,14–17] and patient cohort differences [15]. Moreover, although a few studies examined the association between *MET* CN

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alterations and protein level in cancers [16–18], no data regarding *MET* mRNA expression in lung cancer are available.

The aim of the present study was to evaluate *MET* CN and mRNA expression level in stage I to IIIA NSCLC tumor samples and to assess their associations with clinicopathologic characteristics of the patients including the postoperative outcome. In addition, the relations between the mutational status of epidermal growth factor receptor (*EGFR*), human epidermal growth factor receptor 2 (*HER2*), and *KRAS* genes and *MET* CN alterations were analyzed.

Materials and Methods

Study Subjects and Tissue Samples

The study was performed on pairs of freshly frozen cancerous and unaffected lung tissue specimens obtained from patients with NSCLC stage I to IIIA (pTNM, 7th edition, 2009) who underwent a curative surgery at the Bialystok Medical University Hospital between 2003 and May 2010 and were followed-up for at least 3 years. None of the patients received chemotherapy or radiotherapy before the surgery.

Tissue samples were collected intraoperatively and processed immediately after surgical resection: After the macroscopic visual assessment, the tumors were divided into two sections. One of them was fixed in formalin followed by paraffin embedding and the other, as well as the unaffected lung tissue specimen from the same lobe or lung of the patient, was frozen in liquid nitrogen followed by storage at −80°C. Routine hematoxylin-eosin and immunohistochemical examination of formalin-fixed paraffin-embedded tumor samples, including p63, cytokeratin 5/6 (CK5/6), thyroid transcription factor 1 (TTF1), and chromogranin detection, was performed to determine tumor histologic type. Before nucleic acid extraction, the cryosections of frozen tissue specimens were stained with hematoxylin-eosin and evaluated for tumor cell content. Only the tumor samples that contained at least 50% of tumor cells on a microscopic section were used for further processing. Consequently, 151 pairs of cancerous and matched unaffected lung tissues were selected for the study.

Clinicopathologic data and previously detected *EGFR*, *KRAS*, and *HER2* gene mutational status were available for all the patients. For survival analysis, the overall survival (OS) was estimated as the time from the date of the surgery to the date of death due to lung cancer recurrence or metastases (event) or to the date of the last control visit (censoring). The disease-free survival (DFS) was defined as the time from the date of the surgery to the date of disease relapse or death, whichever occurred first (events), or to the date of the last visit (censoring).

The study was approved by the Ethics Committee of the University, and written informed consent for specimen collection was obtained from each patient before the surgery.

Nucleic Acid Extraction

DNA and RNA were isolated simultaneously using a magnetic extraction method. Briefly, about 40 to 50 mg of tissue was disrupted in lysis buffer (Biomerieux, Marcy l'Etoile, France) with TissueRupter (Qiagen, Hilden, Germany) and incubated with Proteinase K for 2 hours at 56°C. Nucleic acids from deproteinated cell lysates were extracted automatically on the EasyMag machine (bioMérieux) according to the producer's protocol. Both DNA and RNA were present in the 100-μl resulting extracts. Nucleic acid quality was assessed electrophoretically.

For gene expression analysis, RNA was transcripted into cDNA in a reaction with High Capacity RNA-to-cDNA Master Mix (Applied Biosystems, Foster City, CA) according to the producer's recommendations.

MET CN

MET CN was analyzed by a quantitative real-time duplex polymerase chain reaction (qPCR) on an ABI PRISM 7900HT Sequence Detection System (Applied Biosystems) with a commercially available predesigned *MET* TaqMan Copy Number Assay (Hs0143282_cn) and a Reference RNase P Assay (PN4412907), both from Applied Biosystems. The qPCR was done in a 20-μl reaction mixture containing 10 μl of Applied Biosystems TaqMan Universal PCR Master Mix with UNG, 1 μl of the CN assay solution, 1 μl of the reference assay solution, and 5 μl of DNA solution according to the following cyclic conditions: 50°C for 2 minutes followed by holding for 10 minutes at 95°C and 40 cycles of 95°C for 15 seconds and 60°C for 60 seconds. Each sample was analyzed in quadruplicate. The raw post-PCR data were used for *MET* CN calculation by the relative quantification method using the CopyCaller v.1.0 software (PN4412907) downloadable from www.appliedbiosystems.com. *MET* copy gain was defined as more than three copies per cell.

MET mRNA Expression

MET mRNA expression level in the tumor and unaffected lung tissues was evaluated with the comparative real-time reverse transcription–PCR method. *Ribosomal 18S RNA* (*18SrRNA*) gene with a relatively low level of the expression variability in lung tissue [19,20] was used to normalize for the differences in the input cDNA concentration. The amplification was performed in a 20-μl mixture containing 10 μl of TaqMan Universal PCR Master Mix with UNG, 1 μl of the *MET* (Hs01565584_m1) or 18S rRNA (Hs99999901_s1) TaqMan Gene Expression Assay (all reagents from Applied Biosystems), and 5 μl of cDNA solution. Each sample was analyzed in triplicate on an ABI PRISM 7900HT Sequence Detection System equipped with the SDS v.2.4 software for baseline and Ct calculations. *MET* expression was inversely proportional to the difference between Ct for *MET* and Ct for *18S rRNA* gene (ΔCt = Ct*MET* − Ct*18S rRNA*). Fold changes (FCs) in *MET* expression between the tumor and paired normal lung tissues from the same patient were calculated as FC = 2 − $\Delta\Delta$Ct, where $\Delta\Delta$Ct equaled *MET* expression in tumor (ΔCtT) calibrated by its expression in the corresponding nonmalignant tissue (ΔCtN) as follows: $\Delta\Delta Ct = \Delta CtT - \Delta CtN$.

EGFR, HER2, and KRAS Alterations

EGFR and *KRAS* activating mutations were detected with direct sequencing of the PCR-amplified *EGFR* exons 19 and 21 and *KRAS* 2 exons. *EGFR*, *HER2*, and *KRAS* CNs were analyzed like *MET* CN with the corresponding TaqMan Copy Number Assays from Applied Biosystems (Hs014326560_cn, Hs00159103_cn, and Hs02802859_cn for *EGFR*, *HER2*, and *KRAS*, respectively). Gene copy gain was defined as more than three copies per cell.

Statistical Analysis

The nonparametric Mann-Whitney test, Kruskal-Wallis test, or Pearson chi-squared test was used to analyze the associations between clinicopathologic characteristics and *MET* CN. The differences in *MET* expression between the tumor and unaffected lung tissues were analyzed with paired *t* test. The linear regression model was used to estimate the relation between *MET* CN and the expression level. The associations between *MET* gene copy number (CNG) and *EGFR*, *HER2*, and *KRAS* gene status were analyzed with Pearson chi-squared test. OS and DFS were calculated and plotted with

Kaplan-Meier method with the log-rank test for the *comparison* between the groups. Cox proportional hazard model was used to evaluate the effect of clinicopathologic and molecular variables on OS and DFS. *P* values less than .05 were considered as significant. All the statistical analyses in this study were performed using STATA/SE 11.1 software.

Results

Patient Characteristics

A total of 151 patients with NSCLC aged from 39 to 82 years (median age, 63.0 years) was included in the study. The majority of the patients were males (78.8%) and current or former smokers (90.7%). According to the TNM classification, pathologic staging were given as follows: stage I in 58 (38.4%) patients, stage II in 62 (47.0%) patients, and stage IIIA in 22 (14.6%) patients. About 40% of the patients had mediastinal lymph node metastases at the time of surgery, classified as stage N1 and stage N2 in 43 (28.5%) and 18 (11.9%) patients, respectively. The study comprised 64 cases of adenocarcinoma (ADC), 35 cases of large cell carcinoma (LCC), and 52 cases of squamous cell carcinoma (SCC) of the lung (Table 1).

MET CN

The median *MET* CN in tumor tissue was 2.05 (ranged from 0.50 to 7.40) and was not significantly affected by analyzed clinicopathologic variables. With 3.0 copies used as a cutoff in *MET* CN evaluation, gene copy gain was observed in 28 (18.5%) tumor samples, including 15 cases with 3.0 to 3.99 *MET* copies per cell and the remaining 13 samples containing from 4.0 to 7.7 copies (Table 1).

In our cohort of patients with NSCLC, *MET* CNG was observed approximately 2.7- and 2.0-fold more frequently in the tumors with increased *EGFR* and *HER2* CN compared to the tumors without the increase (*P* = .002 and .049 for *EGRF* and *HER2*, respectively) and about 2.4-fold more frequently in tumors harboring *EGFR* mutations compared to tumors with wild-type *EGFR* (*P* = .071). However, subgroup analysis for particular tumor histologic types revealed that statistically significant associations between *MET* CNG and *EGFR* or *HER2* gene alterations occurred only in the ADC group but not in the LCC or SCC group. No associations between *MET* CN and *KRAS* gene mutations or copy gain were found in particular histologic types of cancer (Table 2).

MET mRNA Expression

We were unable to determine *MET* cDNA in 16 analyzed tumor and/or normal lung tissue specimens and these paired samples were excluded from the assay. The *MET* mRNA level was significantly higher in tumor tissue as compared to unaffected tissue (relative quantity (RQ) geometric mean, 1.76; 95% confidence interval (CI), 1.29-2.40; $P < .001$). However, with respect to tumor histologic types, a statistically significant alteration was obtained only in ADCs (RQ geometric mean, 2.14; 95% CI, 1.33-3.45; $P < .001$). No significant associations between *MET* mRNA expression and patients' characteristics were found (Table 1).

Table 1. Relationship between *MET* CN, *MET* mRNA Level, and Patients' Clinicopathologic Characteristics

Variable	Calculated *MET* CN in Tumor Tissue			Patient Number with Calculated *MET* CN			*MET* mRNA Level in Tumor Tissue (ΔCt Value)			Differences in *MET* mRNA Levels between Tumor and Unaffected Lung Tissues				
	n (%)	Median	*P* Value	<3.0	≥3.0	*P* Value	*n*	Mean ± SD	*P* Value	Log_2(FC)			FC	
				n (%)	*n* (%)					*n*	Mean ± SD	*P* Value	Mean *	95% CI †
Total	151	2.05		123 (81.5)	28 (18.5)		142	15.63 ± 2.26		135	0.82 ± 2.63		1.76	1.29-2.40
Age (years)														
<63	75	2.09		59 (78.7)	16 (21.3)		71	15.60 ± 2.12		67	0.95 ± 2.33		1.93	1.30-2.86
≥63	76	1.98	.208 ‡	64 (84.2)	12 (15.8)	.381 §	71	15.67 ± 2.41	.858 ¶	68	0.69 ± 2.92	.567 ¶	1.61	0.99-2.63
Gender														
Female	32 (21.2)	2.04		27 (84.4)	5 (15.6)		30	15.11 ± 2.32		30	0.81 ± 2.93		1.75	0.82-3.73
Male	119 (78.8)	2.05	.859 ‡	96 (80.7)	23 (19.3)	.632 §	112	15.77 ± 2.23	.142 ¶	105	0.82 ± 2.56	.979 ¶	1.77	1.25-2.49
Smoking														
Never	14 (9.3)	2.08		12 (85.7)	2 (14.3)		13	15.23 ± 2.70		12	0.45 ± 3.81		1.36	0.26-7.30
Ever	137 (90.7)	2.04	.974 ‡	111 (81.0)	26 (19.0)	.667 §	129	15.67 ± 2.22	.466 ¶	123	0.85 ± 2.51	.725 ¶	1.81	1.32-2.46
Histology														
ADC	64 (42.4)	1.98		51 (79.7)	13 (20.3)		60	15.38 ± 2.15		59	1.10 ± 2.62		2.14	1.33-3.45
LCC	35 (23.2)	2.05		27 (77.1)	8 (22.9)		33	16.24 ± 2.59		32	0.47 ± 2.36		1.39	0.77-2.51
SCC	52 (34.4)	1.94	.779 #	45 (86.5	7 (13.5)	.484 §	49	15.54 ± 2.13	.205 **	44	0.69 ± 2.84	.516 **	1.61	0.88-2.93
pTNM														
I	58 (38.4)	2.04		48 (84.5)	9 (15.5)		57	15.64 ± 2.50		54	0.95 ± 2.31		1.93	1.25-3.00
II	71 (47.0)	2.05		58 (81.7)	13 (18.3)		66	15.61 ± 2.23		62	0.66 ± 3.03		1.91	0.93-2.70
IIIA	22 (14.6)	1.94	.759 #	26 (81.2)	6 (18.8)	.894 §	22	15.69 ± 1.65	.989 **	19	0.93 ± 2.14	.824 **	1.91	0.93-3.91
Lymph node metastases														
No	90 (59.6)	2.05		74 (82.2)	16 (17.8)		85	15.81 ± 2.35		81	0.77 ± 2.72		1.70	1.12-2.58
N1 to N2	61 (40.4)	2.04	.462 ‡	49 (80.3)	12 (19.7)	.769 §	57	15.38 ± 2.12	.267 ¶	54	0.89 ± 2.52	.790 ¶	1.85	1.15-2.99
Lymph node pathologic status														
N0	90 (59.6)	2.05		74 (82.2)	16 (17.8)			85		81	0.77 ± 2.72		1.70	1.12-2.58
N1	43 (28.5)	2.04		36 (83.7)	7 (16.3)			41		38	0.75 ± 2.68		1.68	0.91-3.10
N2	18 (11.9)	1.94	.759 #	13 (72.2)	5 (27.8)	.550 §		16	.527 **	16	1.22 ± 2.12	.808 **	2.33	1.07-5.11

* Geometric mean.
† 95% CI for geometric mean.
‡ Wilcoxon rank-sum test.
§ Pearson chi-squared test.
¶ Two-sample *t* test with Welch's correction for unequal variances.
\# Kruskal-Wallis rank test.
** One-way analysis of variance test with Bonferroni multiple comparison.

Table 2. Associations between *MET* CN and *EGFR*, *HER2*, and *KRAS* Gene Status (Pearson Chi-Squared Test)

Gene Status	All Patients				Patients with ADC				Patients with LCC				Patients with SCC			
	Total	*MET* CN			Total	*MET* CN			Total	*MET* CN			Total	*MET* CN		
		<3.0	≥3.0	*P* Value		<3.0	≥3.0	*P* Value		<3.0	≥3.0	*P* Value		<3.0	≥3.0	*P* Value
	N (%)	*n* (%)	*n* (%)		*N* (%)	*n* (%)	*n* (%)		*N* (%)	*n* (%)	*n* (%)		*N* (%)	*n* (%)	*n* (%)	
EGFR CN																
<3.0	119 (78.8)	103 (86.6)	16 (13.4)		48 (75.0)	43 (89.6)	5 (10.4)		24 (68.6)	20 (83.3)	4 (16.7)		47 (90.4)	40 (85.1)	7 (14.9)	
≥3.0	32 (21.2)	20 (62.5)	12 (37.5)	.002	16 (25.0)	8 (50.0)	8 (50.0)	.001	11 (31.4)	7 (63.6)	4 (36.4)	.198	5 (9.6)	5 (100.0)	0 (0.0)	.354
EGFR mutations																
No	141 (93.4)	117 (83.0)	24 (17.0)		55 (85.9)	46 (83.4)	9 (16.6)		34 (97.1)	26 (76.5)	8 (23.5)		52 (100.0)	45 (86.5)	7 (13.6)	
Yes	10 (6.6)	6 (60.0)	4 (40.0)	.071	9 (14.1)	5 (55.6)	4 (44.4)	.052	1 (2.9)	1 (100.0)	0 (0.0)	.581	0 (0.0)	0 (0.0)	0 (0.0)	
EGFR alterations (copy gain and/or mutation)																
No	113 (74.8)	97 (85.8)	16 (14.2)		43 (67.2)	38 (88.4)	5 (11.6)		23 (65.7)	19 (82.6)	4 (17.4)		47 (90.4)	40 (85.1)	7 (14.9)	
Yes	38 (21.8)	26 (68.4)	12 (31.6)	.017	21 (32.8)	13 (61.9)	8 (38.1)	.013	12 (34.3)	8 (66.7)	4 (33.3)	.286	5 (9.6)	5 (100.0)	0 (0.0)	.354
HER2 CN																
<3.0	118 (78.2)	100 (84.7)	23 (15.3)		49 (76.6)	42 (85.7)	7 (14.3)		26 (74.3)	21 (80.8)	5 (19.2)		43 (82.7)	37 (86.1)	6 (13.9)	
≥3.0	33 (21.8)	18 (69.7)	10 (30.3)	.049	15 (23.4)	9 (60.0)	6 (40.0)	.030	9 (25.7)	6 (66.7)	3 (33.3)	.385	9 (17.3)	8 (88.9)	1 (11.1)	.820
KRAS CN																
<3.0	106 (70.7)	87 (82.1)	19 (17.9)		47 (74.6)	37 (78.7)	10 (21.3)		25 (71.4)	19 (76.0)	6 (24.0)		34 (65.4)	31 (91.2)	3 (8.8)	
≥3.0	44 (29.3)	35 (79.6)	9 (20.4)	.717	16 (25.4|)	13 (81.4)	3 (18.7)	.829	10 (28.6)	8 (80.0)	2 (20.0)	.799	18 (34.6)	14 (77.8)	4 (22.2)	.178
KRAS mutations																
No	136 (90.1)	109 (80.2)	27 (19.8)		51 (79.7)	39 (76.5)	12 (23.5)		33 (94.3)	25 (75.8)	8 (24.4)		52 (100.0)	45 (86.5)	7 (13.5)	
Yes	15 (9.9)	14 (93.3)	1 (6.7)	.212	13 (20.30	12 (92.3)	1 (7.7)	.205	2 (5.7)	2 (100.0)	0 (0.0)	.428	0 (0.0)			

The Association between MET CN and MET mRNA Expression

Linear regression model revealed a statistically significant link between *MET* CN and mRNA expression in lung tumor tissue (Figure 1). Gain of an additional gene copy resulted in 1.51-fold increase in the expression level (95% CI, 1.22-1.87; *P* < .001).

Patient Survival

During the follow-up period, 34.4% of the patients showed disease recurrence and most of them (31.8%) died. The median OS was 30 months (ranged from 2 to 86 months), and the DFS was 33 months (ranged from 2 to 85 months). In Kaplan-Meier curve analysis, neither *MET* CN alterations nor *MET* mRNA expression level influenced patients' OS or DFS (Figure 2, *A* and *B*). However, when the analysis was restricted to patients with ADC histology, both DFS and OS were shorter in the cases with an increased *MET CN*, although only DFS difference was statistically significant (log-rank test, *P* = .044 and *P* = .071 for DFS and OS, respectively; Figure 2, *C* and *D*). In contrast, in patients with SCC, *MET* copy gain was associated with a better outcome in terms of both DFS and OS in Kaplan-Meier analysis (log-rank test, *P* = .03 and *P* = .05 for DFS and OS, respectively; Figure 2, *E* and *F*). In patients with LCC, no effect of *MET* CNG on DFS or OS was found (data not shown).

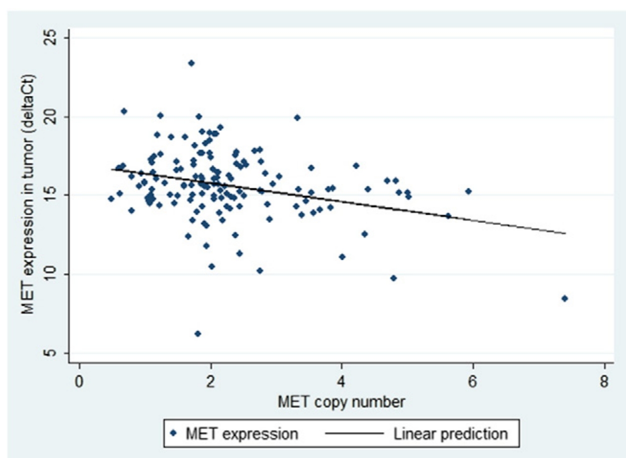

Figure 1. Association between *MET* CN and *MET* mRNA expression in lung tumor tissue of the 135 patients with NSCLC (with a model of linear regression).

For the whole cohort of the patients, in both univariate and multivariate proportional hazards models including patients' age, gender, smoking habit, TNM stage of the disease (I *vs* II + IIIA), lymph node metastases, *MET* CN, *MET* mRNA level in tumor, and tumor-associated alteration in *MET* mRNA, only the disease stage was an independent prognostic factor in terms of OS and DFS [hazard ratio (HR), 12.95 and 2.66; 95% CI, 4.36-38.46 and 1.13-6.23; *P* < .001 and *P* = .024 for OS and DFS, respectively; Table 3]. However, in the univariate model, patients with ADC harboring increased *MET* CN had a 1.58-fold higher risk of disease relapse than those without a CNG (HR, 1.58; 95% CI, 1.10-2.27; *P* = .013). The significance also remained in the simplified multivariate model after age, TNM stage, lymph node metastases, *MET* mRNA level in tumor, and tumor-associated alteration in *MET* mRNA removal (HR, 1.76; 95% CI, 1.20-2.57; *P* = .004; Table 4). No effect of analyzed parameters on DFS or OS in patients with LCC or SCC was found (Tables 4 and 5).

Discussion

In the current study, we showed a gain in *MET* CN in 18.5% of the analyzed tumors and a 1.76-fold tumor-associated increase in *MET* mRNA expression level. The observed proportion of *MET* copy gain was about two-fold higher than those in most previously reported studies, possibly due to different methods and scoring criteria used. In most investigations, the fluorescence *in situ* hybridization (FISH) or a similar (like silver or bright-field *in situ* hybridization) method was used and about 10% of NSCLCs were defined as *MET* FISH-

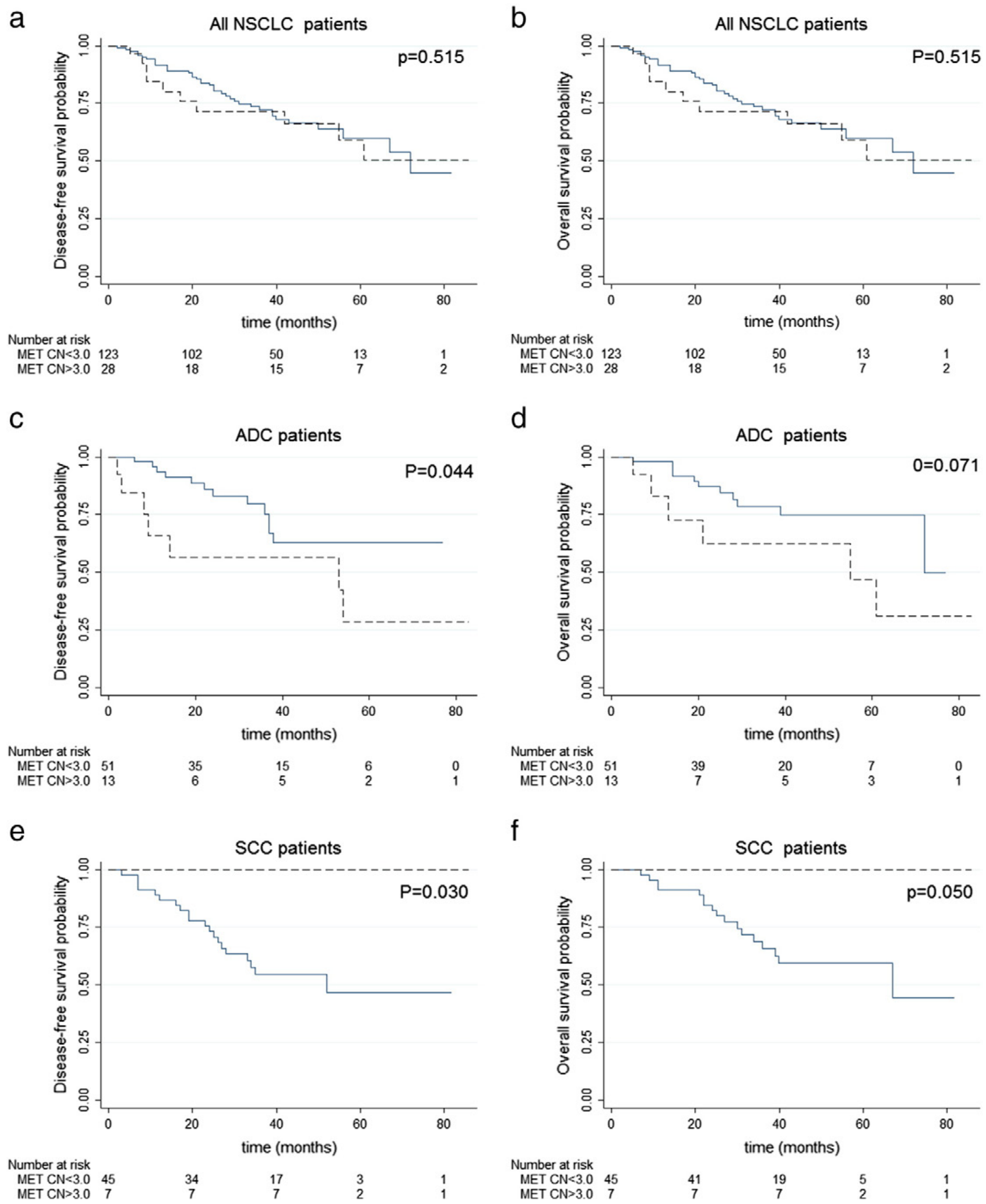

Figure 2. Kaplan-Meier survival curves of OS and DFS for the 151 patients with NSCLC in relation to *MET* CN (solid line in the case of *MET* CN < 3.0; dashed line in the case of *MET* CN ≥ 3.0) in lung tumor tissue: (a and b) for the overall NSCLC patient group, (c and d) for the ADC patients only, and (e and f) for the SCC patients only.

Table 3. Univariate and Multivariate Analyses of Prognostic Factors for DFS and OS of Patients with NSCLC (Cox Proportional Hazards Model)

Variable	DFS						OS					
	Univariate			Multivariate			Univariate			Multivariate		
	HR	95% CI *	*P* Value	HR	95% CI	*P* Value	HR	95% CI	*P* Value	HR	95% CI	*P* Value
Age	1.00	0.97-1.04	.999	0.99	0.95-1.02	.458	1.01	0.98-1.05	.536	0.98	0.99-1.02	.258
Gender (female/male)	0.82	0.43-1.57	.549	0.67	0.32-1.43	.303	1.35	0.63-2.88	.444	1.12	0.44-2.83	.817
Smoking (never/ever)	2.15	0.67-6.91	.199	1.96	0.50-7.74	.335	2.15	0.67-6.91	.199	1.02	0.25-4.18	.983
pTNM (I/II + IIIA)	2.41	1.30-4.46	.005	2.66	1.13-6.23	.024	9.24	3.58-23.82	<.001	12.95	4.36-38.46	<.001
Lymph node metastases (no/yes)	1.55	0.90-2.68	.115	1.14	0.53-2.43	.745	1.93	1.08-3.44	.027	0.75	0.36-1.56	.445
MET CN calculated	1.13	0.91-1.41	.279	1.21	0.92-1.59	.175	1.06	0.83-1.37	.625	1.03	0.76-1.40	.849
MET mRNA in tumor (ΔCt value)	0.98	0.88-1.10	.749	1.04	0.86-1.27	.687	0.97	0.87-1.12	.832	0.96	0.78-1.19	.731
Log_2(*MET* mRNA RQ)	1.01	0.91-1.13	.845	1.10	0.92-1.32	.309	1.04	0.92-1.17	.546	1.12	0.92-1.36	.260
MET mRNA RQ	1.00	0.99-1.01	.481	0.99	0.98-1.00	.204	1.00	0.99-1.01	.651	0.99	0.98-1.01	.330

* 95% CI for HR.

Table 4. Multivariate Analysis of Prognostic Factors for DFS by Histologic Type (Cox Proportional Hazards Model)

Variable	ADC			SCC			LCC		
	HR	95% CI	*P* Value	HR	95% CI	*P* Value	HR	95% CI	*P* Value
Age	0.97	0.92-1.02	.197	1.00	0.93-1.06	.898	1.08	0.96-1.22	.214
Gender (female/male)	0.24	0.06-0.89	.033	0.56	0.14-2.20	.407	0.24	0.03-2.14	.202
Smoking (never/ever) *	40.97	2.04-823.49	.015				0.59	0.09-3.75	.578
pTNM (I/II + IIIA)	3.64	0.68-19.48	.131	1.97	0.46-8.48	.361	11.63	1.13-119.33	.039
Lymph node metastases (no/yes)	0.65	0.13-3.15	.592	0.70	0.23-2.17	.540	2.42	0.45-12.88	.301
MET CN calculated	1.46	0.91-2.37	.118	0.58	0.29-1.19	.136	1.44	0.80-2.61	.225
MET mRNA in tumor (ΔCt value)	0.76	0.49-1.18	.218	1.17	0.87-1.58	.300	0.68	0.35-1.32	.255
Log_2(*MET* mRNA RQ)	0.88	0.59-1.31	.529	1.08	0.85-1.36	.538	0.83	0.44-1.57	.569
MET CN calculated †	1.76	1.20-2.57	.004						
Gender (female/male) †	0.29	0.09-0.94	.038						
Smoking (never/ever) †	19.62	2.03-189.77	.010						
MET CN calculated ‡							1.33	0.86-2.06	.199
pTNM (I/II + IIIA) ‡							5.77	1.46-22.90	.013
MET CN calculated §	1.58	1.10-2.27	.013	0.60	0.35-1.04	.068	1.42	0.91-2.21	.118

* There were no nonsmokers among patients with SCC included in the study.

† Simplified analysis after age, pTNM, lymph node metastases, *MET* mRNA level in tumor, and log_2(*MET* mRNA FC) removal from the model at the last step of the multivariable analysis.

‡ Simplified analysis after age, gender, smoking, lymph node metastases, *MET* mRNA level in tumor, and log_2(*MET* mRNA FC) removal from the model at the last step of the multivariable analysis.

§ Univariate analysis.

positive [6,8,9,16,18,20], although the results strongly depended on the cutoff criteria applied [8,9]. Very recently, Jin et al. found *MET* gene CNG by silver *in situ* hybridization in 24.1% of Korean NSCLC patients, although only stage I ADCs had been included in the study [17]. In our study, we used a qPCR method with a commercially available assay for *MET* CN evaluation and defined the cutoff value for copy gain as 3.0. Our results are similar to those obtained by Beau-Faller et al. [21] who also applied the qPCR technique. However, when we followed the cutoff definition by Beau-Faller as a mean CN in the corresponding normal lung tissues plus two SDs (equal to 3.99; data not shown), only 8.6% of the tumor samples analyzed in our study demonstrated an increased gene dosage, similar to the data reported by others [16,22].

According to our study, *MET* dosage status was not associated with the analyzed clinicopathologic features like age, gender, smoking history, histology, or pathologic stage. These results are in line with most previously reported data [8,15,16], although in a number of studies an increased *MET* CN was found to be more common in ADCs [18], women [7], smokers [7,17,22], and in larger [17] and poorly differentiated tumors [6,18]. A higher prevalence of *MET* amplification was also shown in advanced (pTNM III-IV) NSCLCs compared to early-stage (pTNM I-II) cases [6,9,22] and in stage IA ADCs compared to stage IB ones [17], as well as in lymph node stage 2 metastases compared to primary tumors [23].

We also found a statistically significant association between *MET* copy gain and an increase in *MET* mRNA level in tumor tissue. The association between *MET* dosage status and the expression at protein level by immunohistochemistry has been explored in a number of studies and a strong correlation has invariably been shown [7,16,17]. However, to our best knowledge, the present study is the first investigation where this association was demonstrated at mRNA level, suggesting that MET overexpression in the cells with an increased gene CN at least partly results from an enhanced transcription level.

According to the present study, the rate of *MET* copy gain was found to be higher in the tumors harboring increased *EGFR* or *HER2* CN and/or *EGFR* activating mutations as compared to the tumors without these alterations. However, these associations were statistically significant only in ADC cases (with the exception of the association with *EGFR* mutations that did not reach the statistical significance) but not in LCC or SCC tumors. However, no correlation between *MET* copy gain and *KRAS* dosage or mutational status was found. The association between *EGFR* and *MET* copy gains had been demonstrated

Table 5. Multivariate Analysis of Prognostic Factors for OS by Histologic Type (Cox Proportional Hazards Model)

	ADC (*N* = 64)			SCC (*N* = 52)			LCC (*N* = 35)		
	HR	95% CI	*P* Value	HR	95% CI	*P* Value	HR	95% CI	*P* Value
Age	0.96	0.92-1.00	.116	0.99	0.92-1.07	.775	1.14	0.94-1.38	.192
Gender (female/male)	1.04	0.21-5.24	.965	0.61	0.11-3.31	.565	0.04	0.01-0.68	.026
Smoking (never/ever) *	3.30	0.12-87.46	.475				0.17	0.01-2.57	.201
pTNM (I/II + IIIA)	12.37	1.95-78.50	.008	9.35	1.03-84.78	.047	605.46	10.50-34901.09	.002
Lymph node metastases (no/yes)	0.63	0.13-3.07	.567	0.50	0.16-1.57	.238	0.21	0.02-2.69	.235
MET CN calculated	1.00	0.57-1.75	.993	0.66	0.30-1.44	.293	1.99	0.84-4.70	.116
MET mRNA in tumor (ΔCt value)	0.80	0.50-1.29	.363	1.24	0.88-1.74	.214	0.40	0.11-1.41	.155
Log_2(*MET* mRNA RQ)	1.08	0.71-1.64	.708	1.13	0.88-1.46	.325	0.69	0.22-2.26	.550
MET CN calculated †	1.17	0.79-1.74	.429	0.65	0.35-1.20	.169			
pTNM (I/II + IIIA) †	6.44	1.81-22.99	.004	8.37	1.05-66.98	.045			
MET CN calculated ‡							1.52	0.97-2.39	.069
Gender (female/male) ‡							0.16	0.03-0.86	.032
pTNM (I/II + IIIA) ‡							38.67	45.04-370.22	.002
MET CN calculated §	1.39	0.92-2.11	.121	0.63	0.35-1.12	.117	1.20	0.79-1.82	.393

* There were no nonsmokers among patients with SCC included in the study.

† Simplified analysis after age, gender, smoking, lymph node metastases, *MET* mRNA level in tumor, and log_2(*MET* mRNA FC) removal from the model at the last step of the multivariable analysis.

‡ Simplified analysis after age, smoking, lymph node metastases, *MET* mRNA level in tumor, and log_2(*MET* mRNA FC) removal from the model at the last step of the multivariable analysis.

§ Univariate analysis.

previously [6,9,20] and proposed to result from frequent chromosome 7 aneuploidy in cancer cells [6]. However, a concept of the functional cross talk between MET and EGFR family receptors in cancer cells has also be suggested [10,24,25].

The reported relations between increased *MET* CN and *EGFR* mutations are controversial. The alterations were found to be mutually exclusive in some studies [25,26], yet they coexisted but not correlated in others [7,17,21,22]. In the recent study of Jin et al., no association between *MET* CNG and three most common genetic alterations (*EGFR* and *KRAS* activating mutations and *ALK* rearrangements) in lung ADCs was found. Only stage I Korean patients had been included into the study resulting in much higher proportion of nonsmokers and women in the patients' cohort and higher incidence of EGFR mutations compared to our study [17].

The relations between *MET* and *EGFR* alterations are of a great clinical importance in the light of the hypothesis that increased *MET* dosage might lead to the primary resistance of NSCLCs with *EGFR* mutations to EGFR TKIs [12], as has been demonstrated for the acquired resistance in approximately 20% of patients with NSCLC [10,11]. Recent investigations on cell cultures and clinical studies revealed that only a high level of *MET* amplification developed under EGFR TKI treatment and very rarely found in untreated tumors could result in TKI resistance [10,13,27], rather contradicting the impact of *MET* gene dosage on the primary response [15]. Only a moderate increase in *MET* CN was found in our study. However, the mean gene CN value for all the cells of the sample is defined by qPCR, not excluding a high level of gene amplification in a subset of cells due to tumor heterogeneity, as has been recently demonstrated for *KRAS* [28]. A more detailed analysis of tumor samples with *MET* alterations established with FISH method should clarify the issue.

Another important aspect concerning *MET* status is its possible significance as a prognostic factor in NSCLC. Most of the studies reported thus far consistently indicated a negative impact of *MET* abnormalities on the survival of patients with NSCLC [6,8,17,22], although contradictory results have also been reported [16]. According to the present study, ADC patients with an increased *MET* CN had a significantly shorter DFS, and the effect was independent of other clinicopathologic variables in the multivariate analysis. Similar results had been obtained in a number of previous investigations where different methods for *MET* gene dosage evaluation were used [9,17,18,21]. To our surprise and in contrast to Beau-Faller results [21], an increased *MET* CN correlated significantly with a better outcome of our SCC patients in terms of both DFS and OS but was not an independent prognostic factor in the multivariate analysis. The prognostic impact of *MET* FISH status in patients with SCC had been reported previously by Go et al. [8], although in their study FISH positivity was associated with a poor survival of the patients. In the light of the current state of knowledge on the role of hepatocyte growth factor (HGF)/MET signaling in cell invasive growth and tumor progression, we are not able to explain the beneficial influence of an increased *MET* CN on SCC patients' outcome. Interestingly, the elevated *MET* CN correlated positively with a better prognosis in patients with NSCLC in the retrospective analysis by Kanteti et al. [29]. Further investigations on a larger patient cohort are needed to validate these observations.

We also demonstrated a lack of correlation between *MET* mRNA expression and the clinical outcome in the whole patient cohort as well as, respectively, to a particular histologic type of tumor. Contradictory results have been reported by others, although the prognostic implications of MET protein expression by immunohistochemistry (ICH) instead of gene transcription level have been examined [6,9,29]. However, no association between MET protein expression level and survival was found in Dziadziuszko investigation, which was performed on a similar cohort of Polish NSCLC patients [16].

Conclusion

In conclusion, the obtained results demonstrate an increase in *MET* CN in a subset of untreated stage I to IIIA NSCLCs that occurs more frequently in tumors with *EGFR* and/or *HER2* copy gain and *EGFR* activating mutations. An association between *MET* CN and *MET* mRNA expression level in tumor tissue also exists. An increased *MET* CN determined by qPCR with a commercially available assay might be a prognostic factor in patients with ADC after a curative surgery.

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