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Abnormality of the hepatocyte growth factor/MET pathway in pulmonary adenocarcinogenesis

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ABSTRACT

Background: Signaling mediated by hepatocyte growth factor (HGF)/MET promotes multiple biological activities, including cell proliferation, motility, invasion, angiogenesis, and morphogenesis. Overexpression of HGF and MET and an increase of the *MET* gene copy number have recently been found in various cancers that had a poor outcome. Here we investigated the copy number of the *MET* gene and expression of MET and HGF in small pulmonary adenocarcinomas.
Methods: Tumor tissues were obtained from 106 pulmonary small adenocarcinomas 2 cm or less in diameter. *MET* gene copy number, and the expression of MET and HGF, were analyzed using fluorescence *in situ* hybridization (FISH) and immunohistochemistry, respectively.
Results: MET FISH-positive signals were observed in 11 (10.4%) of 106 cases. One case (0.9%) showed gene amplification and 10 (9.4%) exhibited high polysomy. High immunoreactivity for MET and HGF in tumor cells was found in 30 (28.3%) and 19 cases (17.9%), respectively. HGF was also expressed in stromal cells in 32 cases (30.2%). No cases of non-invasive adenocarcinoma (adenocarcinoma *in situ*, localized bronchioloalveolar carcinoma) showed *MET* FISH-positive signals or high expression of HGF in the tumor cells. Expression of both MET and stromal HGF was stronger in invasive than in non-invasive adenocarcinoma. *MET* FISH-positive signals and high immunoreactivity for MET and HGF in tumor cells were associated with factors indicative of poor prognosis such as pleural invasion, vascular invasion, lymphatic permeation, lymph node metastasis, and nuclear grading. Univariate and multivariate analyses that included these factors showed that all statuses except for MET and HGF immunoreactivity were significantly associated with an increased risk of death. However, multivariate analysis revealed no independent factors related to poor prognosis.
Conclusion: Our results suggest that abnormality of the HGF/MET pathway occurs during the course of progression from non-invasive to invasive pulmonary adenocarcinoma. An increased *MET* gene copy number is indicative of a poor outcome in patients with small pulmonary adenocarcinomas.

1. Introduction

Lung carcinoma is the leading cause of cancer death worldwide [1], and one of the most common histologic types is adenocarcinoma, which has been showing a continuous increase in incidence in both Japan and Western countries. In particular, more cases of small peripheral adenocarcinoma are now being found as a result of technological advances in computed tomography [2]. Surgical resection remains the standard treatment for non-small cell lung carcinoma (NSCLC), including small peripheral adenocarcinomas. Noguchi et al. [3] examined a number of surgically resected small adenocarcinomas of the lung, and demonstrated that there are definite cases showing a very favorable outcome. According to their criteria, localized bronchioloalveolar carcinoma (LBAC, type A) and LBAC with alveolar collapse (type B) are defined as non-invasive adenocarcinoma and show a 100% 5-year survival rate. Therefore, type A and B tumors can be candidates for reduction surgery. On the other hand, LBAC with foci of active fibroblastic proliferation (type C) is a type of adenocarcinoma showing lepidic growth, but it represents a large category containing both minimally invasive and invasive adenocarcinomas. Some patients with type C tumors who have no detectable metastasis at the time of surgery die of their disease because type C tumors include adenocarcinomas with various prognoses. Therefore, there is a definite need for

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prognostic markers that can be used to determine which patients can be treated with reduction surgery or those who require the standard operation.

In the 2004 World Health Organization (WHO) classification [1], major adenocarcinoma was divided into five subtypes, including bronchioloalveolar carcinoma (BAC), acinar, papillary, solid with mucin production, and mixed subtypes. However, more than 90% of adenocarcinomas fall into the mixed subtype. In addition, although the term BAC is defined as non-invasive adenocarcinoma, it is used loosely for a broad spectrum of tumors showing BAC-type spread. In order to resolve these issues, a multidisciplinary adenocarcinoma classification has been recently proposed by the International Association for the Study of Lung Cancer in 2011 [4]. In this new classification, adenocarcinomas were fundamentally classified into three categories based on progression: (1) preinvasive lesions including atypical adenomatous hyperplasia (AAH) and adenocarcinoma *in situ* (AIS), (2) minimally invasive adenocarcinoma (MIA), and (3) invasive adenocarcinoma including lepidic-predominant, acinar-predominant, papillary-predominant, micropapillary-predominant, and solid-predominant with mucin production. AIS is considered to be synonymous with BAC, according to the 2004 WHO classification, and with type A and B tumors in the Noguchi classification. MIA is a small, solitary adenocarcinoma with a predominantly lepidic pattern and showing less than 5 mm of invasion in greatest dimension. Therefore, like AIS, MIA is expected to show an extremely favorable outcome. MIA is thought to include a proportion of adenocarcinomas of mixed subtype with BAC (the 2004 WHO classification) and also type C tumors (Noguchi classification). On the other hand, adenocarcinoma of mixed subtype with BAC (2004 WHO classification) includes any adenocarcioma subtype that includes a lepidic pattern (2011 proposed new classification) and type C adenocarcinoma (Noguchi classification).

Hepatocyte growth factor (HGF), also known as scatter factor, was originally and independently isolated as a hepatic regeneration factor [5]. HGF is a pleiotropic cytokine, whose biological effects are mediated by activation of the *MET* proto-oncogene tyrosine kinase receptor [6]. HGF is currently considered to be both an autocrine and a paracrine mediator produced by mesenchymal cells, including fibroblasts [7–9]. The *MET* oncogene was originally isolated from a human osteogenic sarcoma cell line that had been subjected to chemical mutagenesis *in vitro* [10]. *MET*-receptor tyrosine kinase is activated by its cognate ligand HGF, and receptor phosphorylation activates the downstream pathways [11]. Signaling mediated by HGF/MET promotes multiple biological activities, including cell proliferation, motility, invasion, angiogenesis, and morphogenesis in a wide variety of normal and neoplastic cells [12–15].

Alteration of the *MET* gene, including amplification, overexpression, and mutation, has been described in various cancers [16,17]. Expression of MET and HGF proteins assessed by immunohistochemistry has been reported to predict poor outcome in patients with resected lung cancer [18–21]. Recently, *MET* amplification has been identified as one of the mechanisms of acquired resistance to epidermal growth factor receptor (EGFR)-tyrosine kinase inhibitor (TKI) [22,23], and has been found not only in tumors with acquired resistance to EGFR-TKI but also in primary untreated NSCLC [23]. Several studies have demonstrated that an increase in the *MET* gene copy number is associated with poor outcome in patients with NSCLC [24–27]. However, all of the patients analyzed in these studies had undergone resection of advanced NSCLC, and no previous investigations have focused on HGF/MET in small adenocarcinomas, including both non-invasive and invasive cases.

Besides HGF and *MET* abnormalities, a number of studies have demonstrated various gene abnormalities during the progression of adenocarcinoma [28–34]. These include mutation analyses of the *EGFR*, V-Ki-ras2 Kirsten rat sarcoma viral oncogene homolog (*KRAS*), and *p53*, and copy number amplifications of genes including *EGFR*, thyroid transcription factor (*TTF-1*), *MYC*, and *KRAS*. Some chromosomal alterations and deletions have also been examined. These abnormalities were shown to be added during the course of malignant progression, although extremely early-stage tumors (AIS, MIA, etc.) were not examined.

In the present study, we examined both the copy number of the *MET* gene by fluorescence *in situ* hybridization (FISH) and the expression of MET and HGF using immunohistochemistry in small adenocarcinomas. We demonstrated that an increased *MET* gene copy number and high immunoreactivity for MET and HGF in tumor cells were significantly associated with factors indicative of a poor prognosis, such as lymphatic permeation and nuclear grading. An increased *MET* gene copy number was particularly associated with a poor outcome. Our results also suggest that abnormality of the HGF/MET pathway occurs during the course of progression from non-invasive to invasive adenocarcinoma.

2. Materials and methods

2.1. Patients

We examined 106 consecutive small adenocarcinomas of the lung (20 mm or less in diameter) that were surgically resected at Tsukuba University Hospital (Ibaraki, Japan) between 2001 and 2008. None of the patients selected had received neoadjuvant or adjuvant chemotherapy or radiotherapy before or after surgery. The ethics committee of our institution approved this study, and informed consent for specimen collection was obtained from all patients.

In this study, we focused on genetic abnormalities of adenocarcinoma at a very early stage, and examined 32 cases of type A and B tumors (pure BAC, AIS). More than twice the number of cases (74 cases) of small but invasive adenocarcinomas (types C, D, E, and F) resected during the same period were also selected from the pathology archives.

2.2. Tissue specimens and pathologic information

The resected specimens were fixed with 10–15% neutral buffered formalin, and then embedded in paraffin for histologic examination. All of the sections (4 μm thick), including the largest cut surface of the tumor, were stained with hematoxylin and eosin as well as elastica van Gieson, followed by light microscopy examination. Diagnosis was performed by three pathologists (KT, YM, MN). If two or more opinions coincided, the diagnosis was considered to be firm. Tumors were classified according to the criteria of the 2004 WHO classification [1], the new adenocarcinoma classification [4], and the Noguchi classification [3]. In the latter system, type A, B, and C tumors show lepidic growth of the pulmonary alveolar structure, whereas type D (poorly differentiated solid growth pattern), type E (tubular growth pattern), and type F (papillary growth pattern) tumors show non-lepidic growth. We also employed the nuclear grading criteria proposed by Nakazato et al. [35], and this resulted in two groups (positive and negative). The pathological staging was evaluated according to the UICC TNM Classification of Malignant Tumors, 7th ed. [36].

2.3. FISH analysis

Briefly, 4-μm-thick serial sections from each tissue block were subjected to dual-color FISH using a *MET/SE7* probe cocktail (Kreatech Diagnostics, Amsterdam, The Netherlands). After deparaffinization and dehydration, slides were immersed in 0.2 N HCl, incubated in 1 M NaSCN for 30 min at 80 °C, and immersed in pepsin solution for 15–45 min at 37 °C. A DNA probe set was applied

to the slides, and initial incubation was performed on a hot plate at 80 °C for 5 min to codenature the target DNA and probe, followed by incubation at 37 °C for 16 h to achieve hybridization. After the post-hybridization washing, the slides were counterstained with 4,6-diamidino-2-phenylindole. Using an epifluorescence microscope with single interference filter sets for green (GFP), red (TRITC), and blue (DAPI), as well as dual (red/green) and triple (blue, red, green) band-pass filters, FISH signals were enumerated in at least 100 non-overlapping tumor cell nuclei. *MET* gene copy number was classified into six categories (disomy, low and high trisomy, low and high polysomy, and gene amplification) according to the number of copies of the *MET* gene, and chromosome 7 centromere [37] *MET* gene status was further classified into two groups: *MET* FISH-negative (disomy, low and high trisomy, and low polysomy) and *MET* FISH-positive (high polysomy and gene amplification). For each case, the mean copy number of the *MET* gene per cell was also recorded.

2.4. Immunohistochemical staining

We used rabbit polyclonal antibodies against MET and HGF-α (IBL, Gunma, Japan) at 1:50 and 1:20 dilution, respectively. Immunohistochemical staining was carried out on formalin-fixed paraffin-embedded tissue sections of lung adenocarcinoma specimens with microwave antigen retrieval in 0.01 M citrate buffer (pH 6.0). The sections were reacted with the primary antibodies for 1 h at room temperature. We used EnVision/HRP Polymer Reagent (Dako, Glostrup, Denmark) and DAB (3,3′-diaminobenzidine tetrahydrochloride) Liquid (Dako, Glostrup, Denmark) for detection. For negative controls, the primary antibodies were omitted. Immunoreactivity was evaluated independently by two investigators (KT, MN). Whenever their evaluations were discordant, they jointly reviewed the specimen through a multiheaded microscope and reached a consensus.

2.5. Evaluation of immunohistochemical results

Immunoreactivity for MET was evaluated as follows in accordance with the method of Nakamura et al. [18] with slight modifications: MET-low, complete absence of staining or weak to moderate staining in less than 40% of cancer cells; MET-high, weak to moderate staining in at least 40% of tumor cells or strong staining in at least 10% of tumor cells. Weak to moderate staining was defined as staining similar to, or weaker than the staining of normal bronchial epithelium, and strong staining was defined as staining that was clearly more intense than this. The cases were then divided into two groups – either MET-low or -high – when correlations with other parameters were analyzed.

Immunoreactivity for HGF in tumor cells was evaluated as the percentage of tumor cells with positive cytoplasmic and/or membranous staining (0–100%), and the intensity of positive staining was evaluated on a scale from 0 to 3+ (0, complete absence of staining; 1+, staining weaker than that of normal bronchial epithelium; 2+, staining similar to that of normal bronchial epithelium; 3+, staining clearly more intense than that of normal bronchial epithelium). The percentage and intensity were multiplied to give a scoring index ranging from 0 to 300, according to the method of Turke et al. [38] with slight modifications. The cases were then divided into two groups, either HGF-low (0–150) or HGF-high (151–300), for analysis of correlations with other parameters. In addition, a sample was classified as stromal HGF-positive when ≥50% of the stromal cells in a specimen were positively stained for HGF, and as stromal HGF-negative when <50% of the stromal cells were stained [39].

2.6. Statistical analysis

Analysis of the correlation between clinicopathologic features and the results of FISH and immunohistochemistry was performed using the χ^2 test and Fisher's exact test. Survival curves were generated using the Kaplan–Meier method, and the log-rank test was used to assess the statistical significance of differences between the groups. The Cox proportional hazards model was used to identify the statistical significance of differences in survival, and for estimating the hazard ratios and 95% confidence intervals. Prognostic variables identified by univariate analysis were further analyzed using the multivariate Cox model. Statistical significance was defined as $p < 0.05$. Analyses were performed using SPSS version 12.0 (SPSS Inc., Chicago, IL, USA, 2003).

3. Results

3.1. Clinical and histological findings

A total of 106 consecutive adenocarcinoma samples were examined. The overall gender composition was 51 (48%) males and 55 (52%) females. Fifty-one (48%) patients had never smoked and 55 (52%) were smokers. The median age, follow-up period, and tumor size were 64 years (range, 31–89 years), 50 months (range, 5–99 months), and 15 mm (range, 4–20 mm), respectively. All adenocarcinomas were evaluable according to the Noguchi classification [3], the 2004 WHO classification [1], and the new adenocarcinoma classification [4]. According to the Noguchi classification, they included 15 type A tumors (pure BAC, AIS), 17 type B (pure BAC, AIS), 51 type C, 15 type D, 3 type E, and 5 type F. On the other hand, by the 2004 WHO classification, they included 32 BACs, 55 mixed subtype, 3 acinar, 3 papillary and 13 solid types, and by the new adenocarcinoma classification, 32 AISs, 5 MIAs, 31 lepidic-predominant, 10 acinar-predominant, 10 papillary-predominant, and 18 solid-predominant types. Pathologically, 96 cases were stage I, 6 were stage II, and 4 were stage III.

3.2. MET FISH

The copy numbers of the *MET* gene were determined using FISH and the data were analyzed according to the criteria described previously (Fig. 1). Eleven cases (10.4%) were considered to be *MET* FISH-positive. Specifically, 1 case (0.9%) showed gene amplification and 10 (9.4%) showed high polysomy. The *MET* FISH-positive patients are listed in Table 1. Relationships between *MET* FISH and clinicopathological features are shown in Table 2. As both Tables 1 and 2 show, type A and B tumors (pure BAC, AIS) did not exhibit *MET* FISH-positive signals. As well as the Noguchi classification, the presence of *MET* FISH-positive signals was significantly associated with positive nuclear grading, pleural invasion, lymphatic permeation, and lymph node metastasis.

3.3. Immunohistochemical analysis for MET and HGF

Immunoreactivity for MET was found in the cytoplasm of tumor cells (Fig. 2), and was positive in 30 cases (28.3%). Relationships between MET immunoreactivity and clinicopathological features are shown in Table 2. High immunoreactivity for MET was significantly associated with Noguchi type C and D–F tumors, non-lepidic-predominant, positive nuclear grading, vascular invasion, and lymphatic permeation. Relationships between *MET* FISH and MET immunoreactivity are shown in Table 3. Eight (73%) of the cases showing *MET* FISH-positive signals also showed high immunoreactivity for MET. This frequency was significantly higher than that for *MET* FISH-negativity (22 cases; 23%) ($p = 0.002$).

Table 1
Clinicopathological features of *MET* FISH-positive patients.

Case number	*MET* FISH	*MET* GCN (per cell)	Age (year)	Sex	Smoking status	Tumor size (mm)	Histology[a] (type)	Stage[b]	Prognosis (months)
1	Amp	Amp	64	M	S	19	C	IB	26:NED
2	HP	4.8	58	M	S	18	F	IIIA	31:DOD
3	HP	4.0	55	M	S	20	C	IIIA	50:DOD
4	HP	3.9	56	F	NS	18	C	IIA	59:NED
5	HP	3.9	80	M	S	15	C	IA	24:NED
6	HP	3.8	59	M	S	13	C	IB	40:NED
7	HP	3.6	72	F	NS	17	C	IA	58:NED
8	HP	3.4	51	F	S	20	C	IB	84:NED
9	HP	3.4	57	M	S	18	C	IIIA	44:DOD
10	HP	3.4	62	F	S	12	C	IA	90:NED
11	HP	3.4	72	F	S	20	D	IIB	27:NED

FISH, fluorescence *in situ* hybridization; GCN, gene copy number; IHC, immunohistochemistry; Amp, amplification; HP, high polysomy; M, male; F, female; S, smoker; NS, never-smoker; NED, no evidence of disease; DOD, dead of disease.

[a] Noguchi classification, see Ref. [3].

[b] Pathological stage.

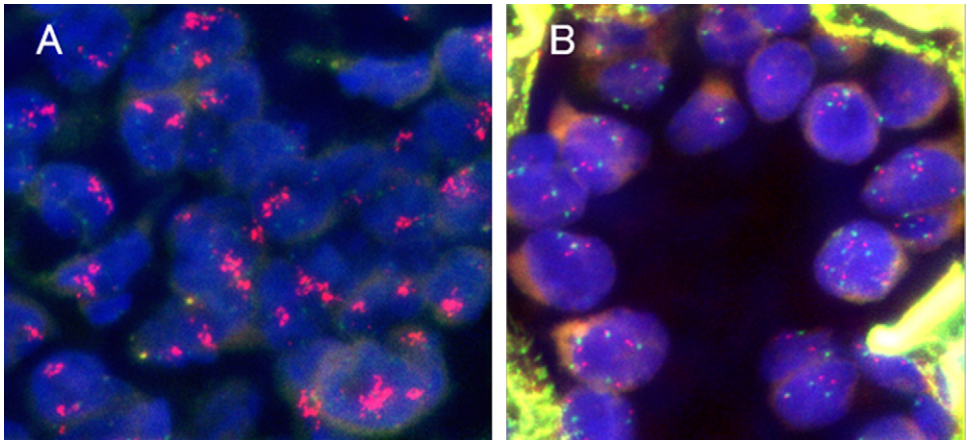

Fig. 1. FISH images from *MET* FISH-positive patients. (A) A case with *MET* amplification showing many tight *MET* gene (red signal) clusters in more than 10% of the tumor cells. (B) A case with *MET* high polysomy showing more than four copies of the *MET* gene in more than 40% of the tumor cells. The green signal shows the chromosome 7 satellite enumeration. FISH, fluorescence *in situ* hybridization. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of the article.)

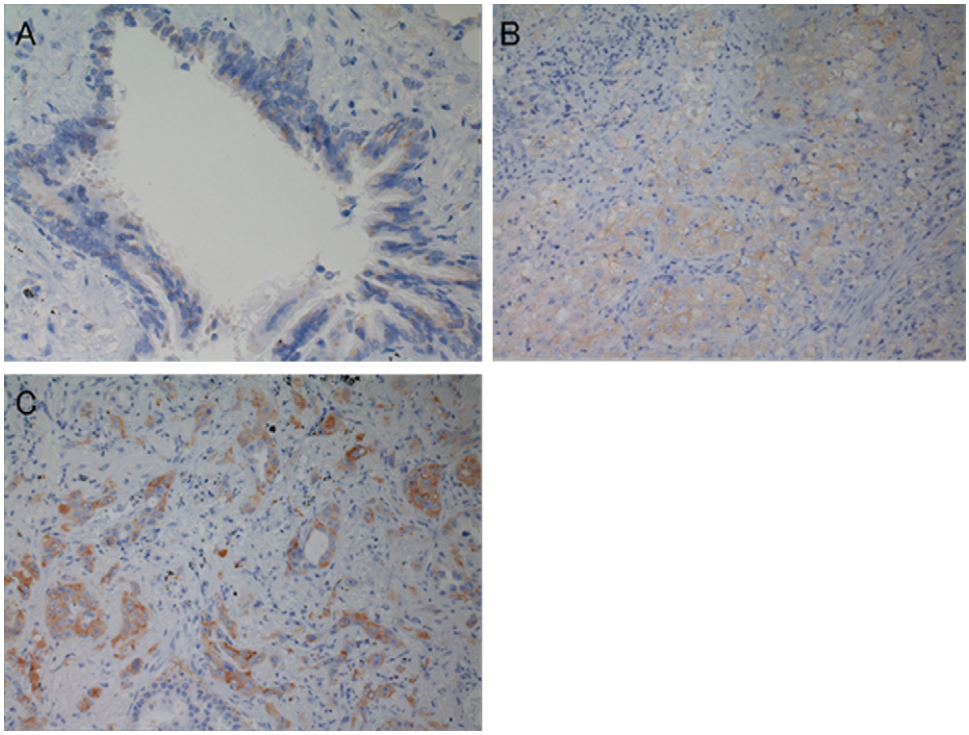

Fig. 2. Representative images of MET expressed in tissue sections of lung adenocarcinoma by immunohistochemistry. (A) MET expression in normal bronchiolar epithelium (400×). (B) Weak to moderate MET staining in tumor cells (200×). (C) Strong MET staining in tumor cells (200×).

Table 2
Clinicopathological features related to *MET* FISH positivity and MET immunoreactivity.

	Total	*MET* FISH		*p* value	MET IHC		*p* value
	n = 106	Negative	Positive		Low	High	
Noguchi classification[a]							
Types A and B	32	32	0		29	3	
Type C	51	42	9	0.011[b]	35	16	0.030[b]
Types D, E and F	23	21	2	0.170[c]	12	11	0.002[c]
The new adenocarcinoma classification[d]							
Lepidic predominant[e]	68	64	4	0.053	56	12	0.001
Non-lepidic predominant	38	31	7		20	18	
Nuclear grading[f]							
Negative	71	69	2	0.001	59	12	<0.001
Positive	35	26	9		17	18	
Pleural invasion							
Negative (pl0)	88	82	6	0.020	64	24	0.603
Positive (pl1, 2, 3)	18	13	5		12	6	
Vascular invasion							
Negative	87	80	7	0.107	67	20	0.009
Positive	19	15	4		9	10	
Lymphatic permeation							
Negative	88	82	6	0.020	67	21	0.025
Positive	18	13	5		9	9	
Lymph node metastasis							
Negative	98	91	7	0.004	72	26	0.218
Positive	8	4	4		4	4	

FISH, fluorescence *in situ* hybridization; IHC, immunohistochemistry.
[a] See Ref. [3].
[b] Types A and B versus type C.
[c] Types A and B versus types D, E and F.
[d] See Ref. [4].
[e] Including adenocarcinoma *in situ* and minimally invasive adenocarcinoma.
[f] See Ref. [35].

Overall, there was a good correlation between *MET* FISH data and MET immunoreactivity.

Immunoreactivity for HGF was found in the cytoplasm of tumor cells and stromal cells (Fig. 3). HGF was positive (1+, 2+, or 3+) in tumor cells in 77 cases (72.6%). The HGF scoring index of tumor cells was recorded in all cases, and was 0–50 in 59 cases (55.7%), 51–100 in 16 cases (15.1%), 101–150 in 12 cases (11.3%), 151–200 in 11 cases (10.4%), 201–250 in 6 cases (5.7%), and 251–300 in 2 cases (1.9%). High immunoreactivity (scoring index 151–300) for HGF in tumor cells was found in 19 cases (17.9%). On the other hand, HGF was also positive in the stromal cells in 32 cases (30.2%). Relationships between HGF immunoreactivity and clinicopathological features are shown in Table 4. High immunoreactivity for HGF in tumor cells was significantly associated with non-lepidic-predominant, positive nuclear grading, and lymphatic permeation. Among type A and B tumors (pure BAC, AIS; 32 tumors), none showed high immunoreactivity for HGF in the tumor cells, but HGF was weakly stained in the matrix of the collapsed areas in 5 type B tumors. In contrast, high immunoreactivity for HGF in the tumor cells and HGF positivity in stromal cells were found in 12 of 51 (23.5%) and 23 of 51 (45.1%) type C tumors, respectively. Immunoreactivity for HGF (pure BAC, AIS) in tumor and stromal cells in type C tumors was stronger than in type A and B tumors ($p = 0.003$ and 0.006, Table 4).

Table 3
Relationship between *MET* FISH and MET IHC.

MET FISH	Total	MET IHC (%)	
	n = 106	Low	High
Amplification	1	0 (0)	1 (100)
High polysomy	10	3 (30)	7 (70)
Low polysomy	21	13 (62)	8 (38)
High trisomy	9	7 (78)	2 (22)
Low trisomy	45	35 (78)	10 (22)
Disomy	20	18 (90)	2 (10)

FISH, fluorescence *in situ* hybridization; IHC, immunohistochemistry.

3.4. Survival analysis and prognostic implications

We analyzed the outcome of patients according to *MET* FISH and the immunohistochemical status of MET and HGF. Patients with *MET* FISH-positive tumors showed significantly shorter survival than those with *MET* FISH-negative tumors (5-year survival rate, 58.3% versus 92.9%; $p = 0.011$; Fig. 4A). On the other hand, there were no significant differences in survival according to the immunoreactivity of MET and HGF in the tumor cells (5-year survival rate, 80.9% versus 94.0%; $p = 0.261$, 88.9% versus 89.0%; $p = 0.775$; Fig. 4B and C). Table 5 presents the results of univariate and multivariate survival analyses that included pleural invasion, vascular invasion, lymphatic permeation, nuclear grading, *MET* FISH, and immunoreactivity for MET and HGF. Univariate analysis showed that all statuses except for immunoreactivity for MET and HGF were significantly associated with an increased risk of death. Multivariate analysis revealed no independent prognostic factors.

4. Discussion

Various abnormalities in genes such as *EGFR*, *KRAS*, *MYC* and *TTF-1* during the course of adenocarcinoma progression have been examined and reported [30–34]. However, in those previous studies, cases of AIS and MIA received scant attention. In the present study, we focused on cases at a very early stage, including AIS and MIA, and examined them for abnormalities of HGF and *MET*.

An increase of the *MET* gene copy number (*MET* FISH-positive signals) was observed in 10.4% of cases (0.9% gene amplification and 9.4% high polysomy). *MET* amplification has been found in 20% of patients with NSCLC showing acquired resistance to EGFR-TKI [22,23]. However, in patients with untreated NSCLC, the frequency of *MET* amplification is low (1–7%) [22–26]. The *MET*

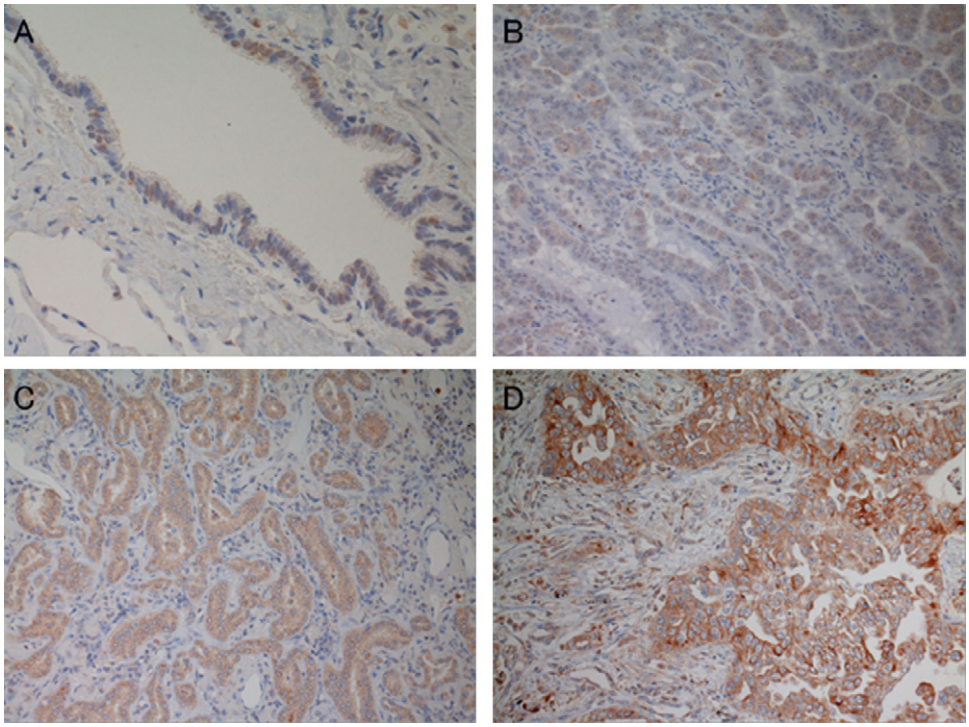

Fig. 3. Representative images of hepatocyte growth factor (HGF) expressed in tissue sections of lung adenocarcinoma by immunohistochemistry. (A) HGF expression in normal bronchiolar epithelium (400×). (B) Weak HGF expression in tumor cells (scored from 1+) (200×). (C) Moderate HGF expression in tumor cells (scored from 2+) (200×). (D) Strong HGF expression in tumor cells (scored from 3+) and in stromal cells (200×).

FISH-positivity rate of untreated NSCLC has been reported to be 10–17% [24,25]. Although our data showed good agreement with previously published results, the frequency of *MET* amplification was lower than in previous studies. This disagreement is thought to have arisen through sample selection, since here we focused on early-stage adenocarcinomas. Cappuzzo et al. [24] reported that an increase of the *MET* gene copy number (defined as a mean of ≥5 copies/cell) negatively affected the survival of NSCLC patients.

Table 4
Clinicopathological features related to immunoreactivity of HGF in tumor and stromal cells.

	Total	HGF in tumor cells		*p* value	HGF in stromal cells		*p* value
	n = 106	Low	High		Negative	Positive	
Noguchi classification[a]							
Types A and B	32	32	0		27	5[b]	
Type C	51	39	12	0.003[c]	28	23	0.006[c]
Types D, E and F	23	16	7	0.001[d]	19	4	0.861[d]
The new adenocarcinoma classification[e]							
Lepidic predominant[f]	68	61	7	0.006	45	23	0.276
Non-lepidic predominant	38	26	12		29	9	
Nuclear grading[g]							
Negative	71	66	5	<0.001	53	18	0.122
Positive	35	21	14		21	14	
Pleural invasion							
Negative (pl0)	88	73	15	0.736	63	25	0.378
Positive (pl1, 2, 3)	18	14	4		11	7	
Vascular invasion							
Negative	87	73	14	0.326	59	28	0.338
Positive	19	14	5		15	4	
Lymphatic permeation							
Negative	88	76	12	0.018	63	25	0.378
Positive	18	11	7		11	7	
Lymph node metastasis							
Negative	98	82	16	0.152	71	27	0.052
Positive	8	5	3		3	5	

HGF, hepatocyte growth factor.
[a] See Ref. [3].
[b] Five cases were positive for HGF in the matrix of the collapsed area.
[c] Types A and B versus type C.
[d] Types A and B versus types D, E and F.
[e] See Ref. [4].
[f] Including adenocarcinoma *in situ* and minimally invasive adenocarcinoma.
[g] See Ref. [35].

Table 5
Univariate and multivariate overall survival analyses.

Variable (category)	Univariate		*p* value	Multivariate		*p* value
	HR	95% CI		HR	95% CI	
Pleural invasion (positive/negative)	5.405	1.350–21.644	0.017	1.088	0.234–5.049	0.914
Vascular invasion (positive/negative)	4.793	1.197–19.186	0.027	1.110	0.206–5.991	0.903
Lymphatic permeation (positive/negative)	6.630	1.639–26.813	0.008	2.064	0.443–9.618	0.356
Nuclear grading (positive/negative)	14.302	1.759–116.262	0.013	7.200	0.584–88.830	0.124
Lymph node metastasis (positive/negative)	8.209	1.921–35.090	0.005	1.658	0.248–11.098	0.602
MET FISH (positive/negative)	5.306	1.266–22.246	0.022	1.785	0.354–9.004	0.483
MET IHC (high/low)	2.173	0.542–8.703	0.273			
HGF IHC (high/low)	0.738	0.091–6.009	0.777			

HR, hazard ratio; CI, confidence interval; HGF, hepatocyte growth factor; IHC, immunohistochemistry; FISH, fluorescence *in situ* hybridization.

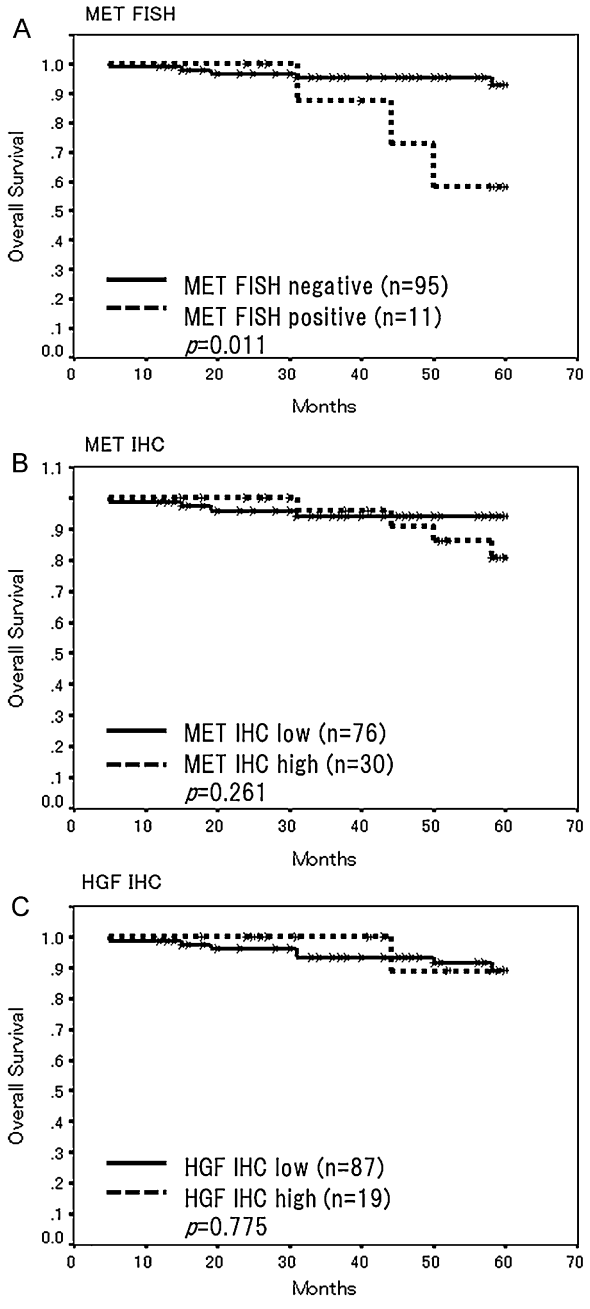

Fig. 4. Kaplan–Meier curves analyzed using the log-rank test showing the overall survival of 106 patients with small adenocarcinoma. (A) In relation to *MET* FISH status. (B) MET immunohistochemical status. (C) HGF immunohistochemical status. FISH, fluorescence *in situ* hybridization; HGF, hepatocyte growth factor.

However, in the cases we examined, only one case had a mean of ≥5 *MET* gene copies/cell (Table 1). Therefore, to evaluate the *MET* gene copy number, we adopted the Colorado Cancer Center criteria for EGFR [37], rather than the mean per cell method of Cappuzzo et al. [24]. In the present study, *MET* FISH-positive signals were significantly associated with factors indicative of poor prognosis (Table 2). Furthermore, patients with *MET* FISH-positive tumors showed markedly shorter overall survival than those with *MET* FISH-negative tumors (Fig. 4A). However, multivariate analysis showed that *MET* FISH-positivity was not an independent factor indicative of poor prognosis. We speculated that, as the cases examined in this study were limited to small and relatively early-stage adenocarcinomas, including many cases of AIS (type A and B tumors), *MET* FISH signals would be more clearly associated with poor prognosis in advanced cases than in relatively early-stage cases.

In the present study, immunoreactivity for HGF was found not only in tumor cells (72.6%, 17.9% of cases showing high-level expression) but also stromal areas (30.2%). This suggests that HGF is both an autocrine and a paracrine mediator, as has been reported previously [7–9]. Immunoreactivity for MET was observed in 30 (28.3%) of the present cases. High MET and HGF expression in tumor cells were significantly associated with other poor prognostic factors such as nuclear grading and lymphatic permeation (Tables 2 and 4). On the other hand, there were no significant differences in survival between patients whose tumor cells showed immunoreactivity for MET and HGF. Several clinical studies have demonstrated that overexpression of MET and/or HGF is associated with a poor survival rate in patients with NSCLC, suggesting that overexpression of these factors is prognostically significant [18–21]. Although our data did not support these reports, we speculate that the discrepancy may have arisen from the immunohistochemical methodology employed.

We have previously reported that peripheral adenocarcinoma of the lung undergoes sequential progression from AAH through LBAC (type A tumors, AIS) to small but advanced LBAC with fibroblastic proliferation (type C tumors, MIA and invasive adenocarcinoma with a lepidic pattern) [3,28,29]. Adenocarcinogenesis has been interpreted as a multistep process because of the accumulation of several sequential molecular abnormalities including *EGFR* mutation, *KRAS* mutation, and *p53* mutation, as well as *p16* inactivation and some allelic imbalances [28,29]. AAH progresses to non-invasive adenocarcinoma (type A and B tumors, AIS) as a result of genetic abnormalities such as *EGFR* and *KRAS* mutations and *p16* inactivation. Non-invasive adenocarcinoma (type A and B tumors, AIS) progresses to invasive adenocarcinoma (type C tumors, MIA and invasive adenocarcinoma with a lepidic pattern) through various additional genetic abnormalities such as *p53* mutation and allelic imbalances. In the present study, none of the type A or B tumors examined showed *MET* FISH-positive signals or high immunoreactivity for HGF, whereas these features were

evident in type C tumors. MET and stromal HGF were expressed more strongly in type C tumors than in types A and B. Our results suggest that abnormality of the HGF/MET pathway is very rare in non-invasive adenocarcinoma and may be one of the causes of its progression to invasive adenocarcinoma. On the other hand, using three-dimensional coculture of a ductal breast carcinoma *in situ* cell line and HGF-secreting fibroblasts, Jedeszko et al. [40] found that fibroblast-divided HGF promoted progression of the *in situ* ductal carcinoma to invasive carcinoma. In the present study, it was of considerable interest that high expression of HGF was found in type C tumors, but not in types A and B. We speculate that active fibroblasts in type C tumors might produce HGF, which may be one of the factors triggering transition from non-invasive to invasive adenocarcinoma. In the course of malignant transformation of pulmonary adenocarcinomas, it is also possible that stromal fibroblasts might promote a stepwise progression from non-invasive adenocarcinoma to invasive adenocarcinoma. We suggest that abnormality of the HGF/MET signaling pathway, including an increase of the *MET* gene copy number, expression of MET and HGF in tumor cells, and stromal HGF expression, is an important factor in multistep adenocarcinogenesis. Further research is needed to clarify the role of the HGF/MET signaling pathway in multistep adenocarcinogenesis.

In conclusion, abnormality of the HGF/MET pathway occurs during the course of progression from non-invasive to invasive adenocarcinoma. An increase of the *MET* gene copy number and overexpression of MET and HGF, particularly the former, are associated with factors indicative of poor prognosis in patients with small pulmonary adenocarcinomas.

Conflict of interest statement

None of the authors have a financial relationship with any commercial entity that has an interest in the area of study.

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