의학석사 학위논문

MET amplification, protein expression, and mutations in pulmonary adenocarcinoma

면역조직화학염색을 이용한 *MET*조절장애 폐선암 환자의 선별

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***MET* amplification, protein expression, and mutations in pulmonary adenocarcinoma**

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ABSTRACT

Introduction: The proper target of a MET inhibitor has not been demonstrated in lung cancer. *MET* amplification, protein expression, and splice mutations at exon 14 are known to cause dysregulation of the MET/HGF pathway. Our study aimed to establish the strategy for finding target population of MET inhibitor by confirming the relationship among *MET* amplification, protein expression, and mutations in pulmonary adenocarcinoma.

Methods: MET protein expression by immunohistochemistry (IHC) and *MET* amplification by fluorescence *in situ* hybridization (FISH) were evaluated in 316 surgically resected lung adenocarcinomas. The IHC score was defined by the modified criteria used in the clinical trial for the MET inhibitor, and the score of 2 or 3 was defined as positivity. *MET* gene copy number (GCN) and amplification was defined by University of Coloradeo Cancer Center criteria. Patients were divided into 4 groups (IHC-negative/FISH-negative, IHC-negative/FISH-positive, IHC-positive/FISH-negative, and IHC-positive/FISH-positive), and 15–20 tumors in each group were randomly selected for mutation analyses to find splice mutations at exon 14.

Results: An IHC score of 0, 1, 2, and 3 was found in 168 (53.2%), 71 (22.5%), 59 (18.7%), and 18 (5.7%) tumors, respectively. The mean GCN was 3.56 (standard deviation 1.5); *MET* FISH positivity was detected in 123 (38.9%) samples, and 26 (8.2%) of them were gene amplifications. *MET*

amplification were significantly associated with the IHC score (P<0.001, χ^2 test), and the positive predictive value of the IHC score of 3 for predicting amplification was 44.4%. Splice mutations were identified in only 2 (2.9%) of 70 cases. One had a MET IHC score of 2 and negative FISH without amplification; The other had a MET IHC score of 0 and positive FISH without amplification. MET IHC or FISH results were not prognostic indicators of overall survival in multivariate analysis.

Conclusions: There is a significant relationship between *MET* amplification and protein expression, and selection of tumors with amplification using IHC was effective. However, because of its rarity, a selection strategy for mutated tumors is implausible using IHC or FISH.

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Keywords: MET; Gene Amplification; Gene Expression; Mutation; Non-Small-Cell Lung Carcinoma; Adenocarcinoma

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LIST OF ABBREVIATIONS

ALK	Anaplastic lymphoma kinase
ANOVA	Analysis of variance
CI	Confidence interval
EGFR	Epidermal growth factor receptor
FISH	Fluorescence in situ hybridization
GCN	Gene copy number
HGF	Hepatocyte growth factor
HR	Hazard ratio
IHC	Immunohistochemistry
TKI	Tyrosine kinase inhibitor
NSCLC	Non-small cell lung cancer
OS	Overall survival
PFS	Progression-free survival
RT-PCR	Reverse transcription polymerase chain reaction
UCCC	University of Colorado Cancer Center

INTRODUCTION

Because the epidermal growth factor receptor (EGFR) and anaplastic lymphoma kinase (ALK) tyrosine kinase inhibitors (TKIs) have shown benefits, other potential therapeutic target genes like *MET*, *ROS1*, *BRAF* and *HER2* have being actively investigated in non-small cell lung cancer (NSCLC) (1). MET is a heterodimeric receptor tyrosine kinase composed of extracellular, transmembrane, juxtamembrane, and kinase domains (2, 3). Binding of hepatocyte growth factor (HGF) to MET induces phosphorylation of the docking site and stimulates downstream signal pathways such as the extracellular signal-regulated kinase (ERK)/mitogen-activated protein kinase (MAPK) and phosphatidylinositol 3-kinase(PI3K)-Akt/protein kinase B pathways (3). These pathways are known to involve cell growth, migration, angiogenesis, and survival (4).

Overexpression of HGF or MET, amplification, or mutation of *MET* has been identified as a cause of MET pathway dysregulation. In addition to NSCLC, breast cancer, colon cancer, kidney cancer, and stomach cancer have demonstrated overexpression of MET (5-8). *MET* amplification has been discovered in colon cancer, esophageal cancer, and stomach cancer (9).

One of the activating mechanisms of *MET* is gene amplification or increased gene copy number (GCN). In an *in vitro* study, the level of tyrosine phosphorylation was greater in a *MET*-amplified cell line than in a non-amplified one, and the knockdown of *MET* in the amplified cell line caused

growth inhibition, cell cycle arrest, and apoptosis (10). The prevalence of high *MET* GCN and amplification were 10.6% to 20.8% and 2.1% to 4.4% in previous studies, and they were associated with poor prognosis in NSCLC patients (11-16). In addition to this *de novo* mechanism, *MET* amplification has been identified as the mechanism resulting in EGFR-TKI resistance in about 20% of resistant tumors (17, 18).

MET protein is expressed in 22.2% to 74.6% of NSCLC, and it has been associated with poor prognosis in several studies (19-21). Some of those studies also reported that MET expression is more common in adenocarcinoma than in other histologic types (19, 22). Increased MET protein expression is associated with phosphor-MET expression, and this suggests that MET overexpression may be related to activation of the MET pathway (21). The prognostic value of *MET* GCN and protein expression is controversial, although one meta-analysis has documented that both of them are significantly associated with poor overall survival (OS) in surgically resected NSCLC (23).

The semaphorin domain and juxtamembrane domain are the key sites of *MET* mutations in NSCLC. *MET* can have splice mutations in the juxtamembrane region, which is the binding site of Cbl E3-ligase, and these mutations lead to exon 14 deletion. These somatic mutations are associated with ligand-mediated proliferation and tumor growth by decreased ubiquitination and delayed down-regulation of receptors, and are known to be important activating mechanisms of the MET pathway (24, 25).

Among the many kinds of alterations, GCN or amplification, protein

expression, and splice mutations at the juxtamembrane domain of *MET* have been extensively studied in NSCLC. However, the proper target of a MET inhibitor has not been established. The randomized phase II trial of the MET inhibitor, onartuzumab, in combination with erlotinib, has reported a benefit for OS and progression-free survival (PFS) in MET immunohistochemistry (IHC)-positive patients (26). However, the phase III trial using the same criteria for IHC did not confirm the efficacy of onartuzumab (27). A phase I trial for another MET inhibitor, crizotinib, used *MET* amplification as a target, and reported promising results in the interim analysis (28).

In order to define an adequate target population and selection strategy for treatment with MET inhibitors, it is essential to first understand the associations of *MET* alterations. The purpose of our study was to determine the relationship among *MET* GCN, protein expression, and mutations in pulmonary adenocarcinoma.

MATERIALS AND METHODS

1. Patient selection

The records of patients who underwent pulmonary resection between 2004 and 2011 at the Seoul National University Hospital were reviewed, and patients with adenocarcinoma whose surgical tissues were available for evaluation were included in the analysis. To perform our study in a homogeneous setting, histologic types other than adenocarcinoma, and patients who had received chemotherapy or TKI treatment before surgery were excluded. A total of 316 patients were enrolled, and clinical data were collected from the medical records. Survival data of the enrolled patients were obtained through the Korean civil registry. The median follow-up time was 73 months (range 2–153 months), and 104 patients (32.8%) died during the follow-up period. The study was approved by the Institutional Review Board at the Seoul National University Hospital (IRB No. H-1407-142-597).

2. Immunohistochemistry and fluorescence in Situ hybridization

A core tissue of 2 mm in diameter was taken from each representative tissue block, and tissue microarrays were created for evaluation. Sections with 4-μm thickness from each tissue microarray were cut for IHC and fluorescence *in situ* hybridization (FISH) analysis. MET protein expression was evaluated by IHC using a rabbit monoclonal antibody against c-MET (SP44, catalog 7904430, Ventana Medical Systems, Tucson, AZ, USA) and the Benchmark

XT autostainer from Ventana Medical Systems. IHC score was defined by the modified criteria used in the clinical trial for the MET inhibitor as follows: 0, absence of staining or any intensity staining in less than 50% of tumor cells; 1, weak to moderate intensity staining in more than 50% of tumor cells; 2, moderate to strong intensity staining (comparable to that in bronchial epithelium) in more than 50% of tumor cells; 3, strong intensity staining in more than 50% of tumor cells (26). An IHC score of 2 or 3 was defined as positivity.

MET GCN and amplification was estimated using and LSI MET SpectrumRed/CEP7SpectrumGreen probe (Abbott Molecular, Des Plaines, IL, USA), and was counted in at least 100 tumor nuclei. Gene amplification (*MET* to CEP7 ratio ≥2; >15 copies of the *MET* signals in >10% of the tumor cells; small gene cluster [4–10 copies] or innumerable tight gene cluster in >10% of the tumor cells) and high polysomy (≥40% of cells displaying ≥4 copies of the *MET* signal) were defined as FISH positivity according to University of Colorado Cancer Center (UCCC) criteria (12).

3. Reverse transcription polymerase chain reaction and direct sequencing

Patients were divided into four groups (IHC-negative/FISH-negative, IHC-negative/FISH-positive, IHC-positive/FISH-negative, and IHC-positive/FISH-positive). Then, 15 to 20 patients were randomly selected from each group, and reverse transcription polymerase chain reaction (RT-PCR) was performed to detect splice mutations in the juxtamembrane domain. Direct sequencing

was also performed at exon-intron 13 and 14 to identify the kinds of mutations.

For the selected cases, a pathologist reviewed representative hematoxylin and eosin-stained slides and manually microdissected tumor regions from consecutive formalin-fixed, paraffin-embedded sections. After deparaffinization, genomic RNA was extracted, and RT-PCR for detection of a c-*MET* exon 14 deletion was performed using a qualitative kit (catalog MET-001, Custom Diagnostics, Irvine, CA, USA). The protocol for the RT-PCR was one cycle of 45℃ for 30 min, 95℃ for 10 min, and 40 cycles of 95℃ for 15 s and 60℃ for 30 s. DNA was also extracted and subjected to nested-PCR amplification of *MET* exon-intron 13 and 14. PCR products were visualized on a 2% agarose gel, purified, and subsequently subjected to direct Sanger sequencing using an ABI-PRISM 3100 DNA Analyzer (Applied Biosystems, Vernon Hills, IL, USA).

4. Statistical analysis

To analyze the relationship between clinicopathological factors and IHC or FISH groups, a χ^2 test or Fisher's exact probability test was used. The mean *MET* GCN was compared among IHC score groups with an analysis of variance (ANOVA) test, and the trend of GCN was identified with the Jonckheere-Terpstra test. A Cox proportional hazards model was used for survival analysis. A *P*-value less than 0.05 was considered significant. Analyses were performed using SPSS for Windows, version 20.0 (IBM Corporation, Armonk, NY, USA).

RESULTS

1. Clinicopathological features

Of the 316 patients, 155 (49.1%) were men, and the median age was 63 years (range 23–86 years). Most patients had moderately (69.6%) or poorly differentiated (20.9%) adenocarcinomas, stage I (27.5%) or II (50.9%) adenocarcinomas, and were nonsmokers (63.6%). *EGFR* mutations were identified in 136 (43.0%) patients, and ALK was positive in 16 (5.1%) patients. The detailed clinicopathological features are described in Table 1.

	No. (%)	IHC score			FISH		
		0 or 1	2 or 3	*P*	Negative	Positive	*P*
Age				0.311			0.007
<65yrs	173 (54.7)	127 (73.4)	46 (26.6)		94 (54.3)	79 (45.7)	
≥65yrs	143 (45.3)	112 (78.3)	31 (21.7)		99 (69.2)	44 (30.8)	
Sex				0.130			0.281
Male	155 (49.1)	123 (79.4)	32 (20.6)		90 (58.1)	65 (41.9)	
Female	161 (50.9)	116 (72.0)	45 (28.0)		103 (64.0)	58 (36.0)	
Differentiation				<0.001			0.425
Well	30 (9.5)	13 (43.3)	17 (56.7)		21 (70.0)	9 (30.0)	
Moderate	220 (69.6)	179 (81.4)	41 (18.6)		135 (61.4)	85 (38.6)	
Poor	66 (20.9)	47 (71.2)	19 (28.8)		37 (56.1)	29 (43.9)	
Nodal involvement				0.795			0.009
Negative	222 (70.3)	167 (75.2)	55 (24.8)		146 (65.8)	76 (34.2)	
positive	94 (29.7)	72 (76.6)	22 (23.4)		47 (50.0)	47 (50.0)	
Pathologic stage				0.246			0.043
1	87 (27.5)	60 (69.0)	27 (31.0)		58 (66.7)	29 (33.3)	
2	161 (50.9)	124 (77.0)	37 (23.0)		101 (62.7)	60 (37.3)	
3	65 (20.6)	53 (81.5)	12 (18.5)		34 (52.3)	31 (47.7)	
4	3 (0.9)	2 (66.7)	1 (33.3)		0 (0.0)	3 (100.0)	
Smoking				0.582			0.135
No	201 (63.6)	150 (74.6)	51 (25.4)		129 (64.2)	72 (35.8)	
Yes	115 (36.4)	89 (77.4)	26 (22.6)		64 (55.7)	51 (44.3)	
EGFR				0.945			<0.001
Wild-type	85 (26.9)	59 (69.4)	26 (30.6)		65 (76.5)	20 (23.5)	
Mutation	136 (43.0)	95 (69.9)	41 (30.1)		70 (51.5)	66 (48.5)	
Unknown	95 (30.1)	-	-		-	-	
ALK				0.769			0.116
Negative	295 (93.3)	222 (75.3)	73 (24.7)		177 (60.0)	118 (40.0)	
Positive	16 (5.1)	13 (81.2)	3 (18.8)		13 (81.2)	3 (18.8)	
Unknown	5 (1.6)	-	-		-	-	

Table 1. Clinicopathological characteristics associated with MET immunohistochemistry and FISH results.

IHC, immunohistochemistry; FISH, fluorescence in situ hybridization; EGFR, epidermal growth factor receptor; ALK, anaplastic lymphoma kinase.

2. IHC and FISH

On IHC, a score of 0, 1, 2, and 3 was shown in 168 (53.2%), 71 (22.5%), 59 (18.7%), and 18 (5.7%) tumors, respectively (Figure 1A). The IHC-positive group showed a larger proportion of well-differentiated tumors than the IHC-negative group ($P < 0.001$). No other significant differences were detected between the positive and negative groups.

The mean GCN was 3.6 (standard deviation [SD] 1.5), ranging from 1.4 to 10.5 (Figure 1B). The mean MET/CEP7 ratio was 1.1 (SD 0.4, range 0.4-6.2), and only 6 (1.9%) tumors have 2 or more ratios. *MET* FISH positivity was detected in 123 (38.9%), and 26 (8.2%) of them were gene amplifications according to UCCC criteria. FISH positivity was more common in patients aged less than 65 years ($P = 0.007$) and in those with advanced stage tumors ($P = 0.043$). The proportion of nodal involvement was larger in the FISH-positive group than in the FISH-negative group ($P = 0.009$). FISH positivity was more common in *EGFR*-mutated tumors than wild-type tumors ($P < 0.001$). The other features of the IHC and FISH groups are shown in Table 1. The representative images of IHC and FISH are shown in Figure 2A-D and Figure 3A-C.

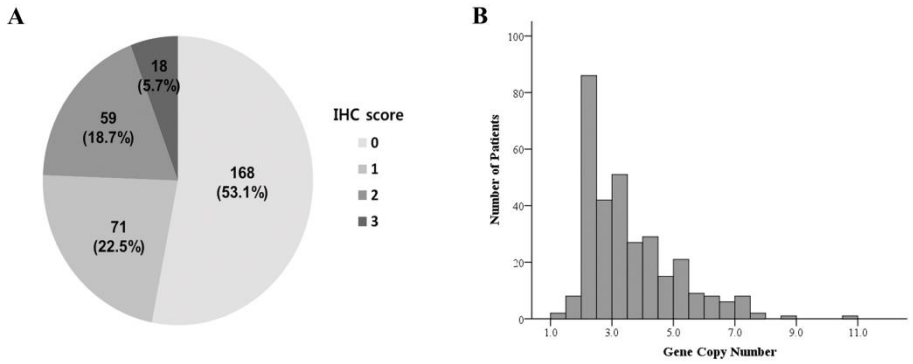

Figure 1. (A) Pie diagram showing the number and percentage of patients with each IHC score. (B) Histogram showing the number of patients in each Met gene copy number category.

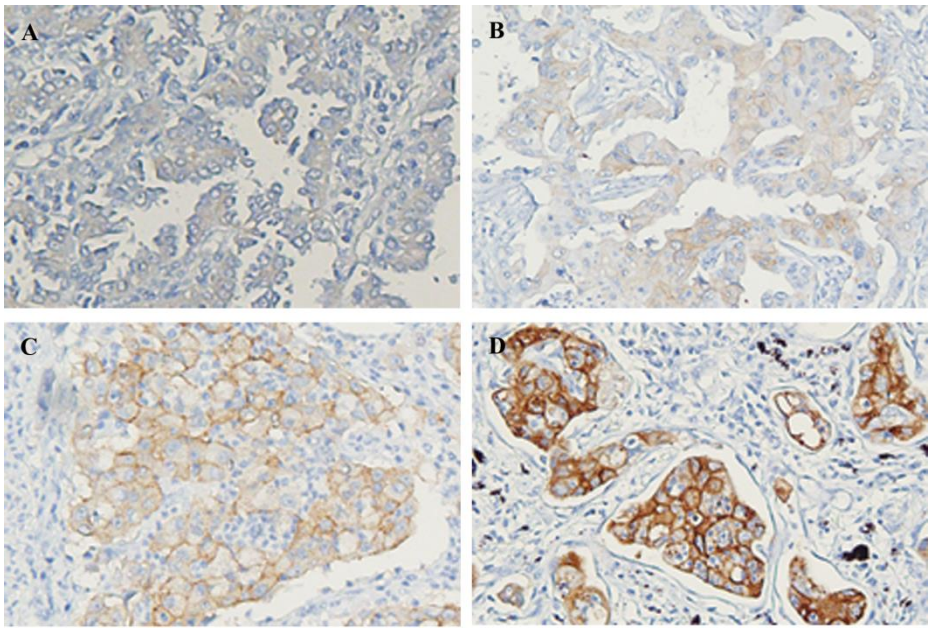

Figure 2. Representative images of Met protein expression by immunohistochemistry. (A) score 0, (B) score 1, (C) score 2, and (D) score 3.

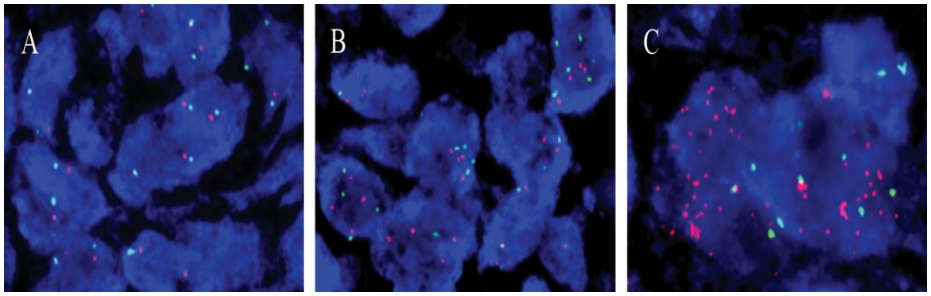

Figure 3. Representative images of Met expression on FISH. (A) negative FISH, (B) positive FISH with a GCN of 4.84 and negative gene amplification, and (C) positive FISH with a GCN of 5.25 and positive gene amplification.
FISH, fluorescence in situ hybridization; GCN, gene copy number.

3. Relationship between IHC and FISH

MET FISH positivity (P<0.001, χ^2 test) and amplification (P<0.001, χ^2 test) were significantly associated with the IHC score. As the IHC score increased, mean GCN and MET/CEP7 ratio have shown an increasing tendency (P < 0.001, Jonckheere-Terpstra test). The number of positive FISH and amplification were 51 (30.4%) and 10 (6.0%) for an IHC score of 0, 26 (36.6%) and 4 (5.6%) for an IHC score of 1, 34 (57.6%) and 4 (6.8%) for and IHC score of 2, and 12 (66.7%) and 8 (44.4%) for and IHC score of 3. *MET* FISH positivity was gradually increased according to the IHC score, and amplification was highly prevalent, especially in an IHC score of 3. The positive predictive values of IHC positivity for FISH positivity and amplification were 59.7% and 15.6%, and the negative predictive values were 67.8% and 95.3%. And the positive predictive value of the IHC score of 3 for predicting amplification was 44.4%. Other FISH results of *MET* in each immunohistochemical category are shown in Table 2 and Figure 4.

IHC score	n	Mean GCN	95% CI	Mean MET/CEP 7 ratio	95% CI	No. of positive FISH (%)	No. of amplification (%)
0	168	3.30	3.08- 3.51	1.02	0.99- 1.06	51 (30.4)	10 (6.0)
1	71	3.40	3.10- 3.71	1.10	1.05- 1.15	26 (36.6)	4 (5.6)
2	59	4.05	3.65- 4.45	1.15	0.97- 1.34	34 (57.6)	4 (6.8)
3	18	5.04	4.18- 5.90	1.46	1.14- 1.78	12 (66.7)	8 (44.4)

Table 2. Results of *MET* FISH in each immunohistochemical category
FISH, fluorescence in situ hybridization; IHC, immunohistochemistry; GCN, gene copy number; CI, confidence interval.

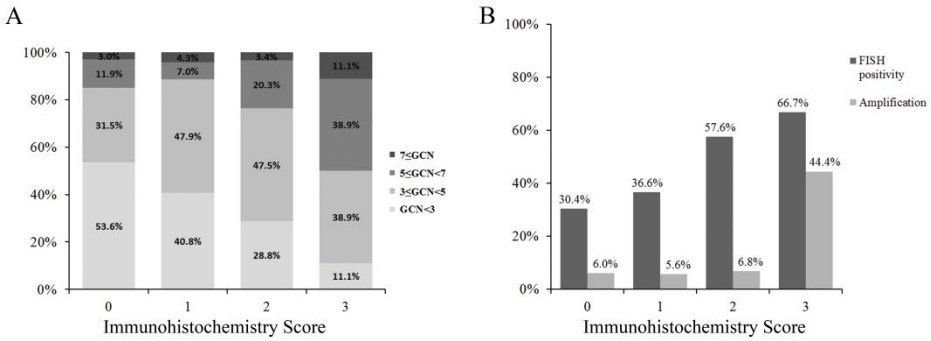

Figure 4. (A) Composition of gene copy number categories in each immunohistochemical score. (B) Prevalence of *MET* FISH positivity and amplification in each immunohistochemical score.

4. Mutation analysis

To find splice mutations at exon 14, RT-PCR was performed for a total of 70 patients. Age more or less than 65 years, sex, smoking status, MET IHC positivity, and *MET* FISH positivity were equally distributed in the tested population. Numerous patients had wild-type *EGFR* (48.6%) and negative ALK staining on IHC (98.6%), moderately (65.7%) or poorly differentiated (24.3%) tumors, and early pathologic stages (stage I, 28.6%; stage II 57.1%). Only 2 (2.9%) tumors were positive for *MET* mutations on RT-PCR. One was a tumor with a MET IHC score of 2, GCN of 2.14, negative gene amplification, and negative *MET* FISH. This tumor carried a point mutation at the 5′ splice site (c.3215 G>A), which is a known mechanism for exon 14 deletion of the *MET* gene transcript. The other one was a tumor with a MET IHC score of 0, GCN of 4.60, negative gene amplification, and positive FISH. Sequencing of this tumor's DNA failed. The sequencing results of the other patients are shown in Table 3.

Group	Pt. No.	Direct sequencing				RT-PCR	GCN	AMP	IHC
		Exon13	Intron13	Exon14	Intron14				
IHC (-)	4	(-)	(-)	(-)	(-)	(-)	2.00	(-)	0
FISH (-)	9	NA	NA	NA	NA	(-)	2.00	(-)	0
	46	NA	(-)	NA	NA	(-)	2.04	(-)	0
	50	(-)	(-)	NA	NA	(-)	2.14	(-)	0
	51	(-)	(-)	NA	NA	(-)	2.18	(-)	0
	53	NA	NA	NA	NA	(-)	1.64	(-)	0
	63	(-)	(-)	(-)	(-)	(-)	2.00	(-)	0
	77	(-)	(-)	(-)	(-)	(-)	2.14	(-)	0
	88	(-)	NA	NA	NA	(-)	2.06	(-)	0
	95	(-)	(-)	(-)	(-)	(-)	2.16	(-)	0
	122	c.3049 G>A (p.W954*)	(-)	NA	NA	(-)	2.10	(-)	0
	132	c.3035 G>A (p.G950R)	c.3074+140 C>T	(-)	(-)	(-)	2.20	(-)	0
	152	NA	NA	NA	NA	(-)	1.72	(-)	1
	158	(-)	(-)	(-)	c.3215+25 G>A	(-)	2.00	(-)	0
	159	(-)	(-)	NA	NA	(-)	1.78	(-)	0
	174	(-)	(-)	(-)	(-)	(-)	2.08	(-)	0
	188	(-)	(-)	(-)	(-)	(-)	2.10	(-)	1
	199	(-)	(-)	(-)	(-)	(-)	2.20	(-)	0
	259	c.2973 C>T (p.Q936*) c.2997 G>A (p.G937D) c.3035 C>T (p.L949F) c.3065 C>T (p.Q960*)	(-)	(-)	(-)	(-)	2.02	(-)	0
	314	(-)	c.3074+138 G>A	NA	NA	(-)	2.05	(-)	0
IHC (-)	6	NA	NA	NA	NA	(+)	4.60	(-)	0
FISH (+)	8	(-)	(-)	(-)	(-)	(-)	5.32	(-)	0
	13	(-)	c.3074+55 C>T, c.3074+63 G>A	NA	NA	(-)	4.46	(-)	1
	21	(-)	(-)	NA	NA	(-)	5.00	(-)	1
	103	(-)	(-)	(-)	(-)	(-)	6.82	(+)	0
	118	NA	c.3074+35 C>T	NA	NA	(-)	4.60	(-)	0
	125	(-)	c.3074+36 C>T	NA	NA	(-)	5.14	(-)	0
	129	(-)	c.3074+4 C>T	(-)	(-)	(-)	7.16	(+)	0
	134	(-)	(-)	NA	NA	(-)	7.75	(+)	1
	135	NA	NA	NA	NA	(-)	4.34	(-)	1
	156	(-)	c.3074+140 C>T	NA	NA	(-)	4.48	(-)	0
	169	(-)	c.3074+158 C>T	(-)	(-)	(-)	4.94	(-)	0
	170	(-)	c.3074+50 C>T, c.3074+56 G>A	NA	NA	(-)	4.72	(-)	1
	177	c.3065 C>T (p.Q960*)	c.3074+12 C>T c.3074+ 35 C>T c.3074+95 C>T	NA	NA	(-)	5.26	(-)	0
	178	(-)	(-)	(-)	(-)	(-)	7.10	(+)	0
	190	c.3032 C>T (p.L949F)	c.3074+159 G>A c.3074+183 C>T	(-)	c.3215+31 C>T	(-)	6.74	(+)	0
	201	(-)	(-)	(-)	(-)	(-)	5.64	(-)	1
	244	(-)	(-)	(-)	(-)	(-)	4.88	(-)	0
	313	(-)	c.3074+15 C>T c.3074+140 C>T	(-)	(-)	(-)	7.46	(+)	0
IHC (+)	19	(-)	c.3074+57 G>A	NA	NA	(-)	3.48	(-)	2
FISH (-)	24	(-)	(-)	(-)	(-)	(-)	2.88	(-)	2
	43	NA	NA	NA	NA	(-)	2.48	(-)	2
	60	NA	(-)	(-)	(-)	(-)	3.42	(-)	3
	66	NA	NA	NA	NA	(-)	2.48	(-)	2
	72	(-)	(-)	(-)	(-)	(-)	3.50	(-)	3
	79	c.2967 C>T (p.P927L) c.2972 C>T (p.Q929*)	c.3074+185 C>T	(-)	(-)	(-)	2.87	(-)	2

	104	(-)	(-)	c.3177 C>T (p.S997L)	(-)	(-)	3.88	(-)	2
	108	(-)	c.3074+119 C>T	NA	NA	(-)	2.80	(-)	3
	207	(-)	(-)	c.3215 G>A (p.D1010N) †	c.3215+31 C>T	(+)	2.14	(-)	2
	219	(-)	(-)	(-)	NA	(-)	2.84	(-)	3
	223	(-)	(-)	(-)	(-)	(-)	3.12	(-)	2
	242	(-)	c.3074+11 G>A	(-)	(-)	(-)	2.38	(-)	2
	249	NA	NA	NA	NA	(-)	3.15	(-)	2
	276	(-)	(-)	(-)	(-)	(-)	2.87	(-)	2
	290	c.3043 C>T (p.F952F)	c.3074+128 G>A	(-)	(-)	(-)	2.68	(-)	2
IHC (+)	3	(-)	(-)	c.3122 C>T (p.H979Y)	(-)	(-)	6.24	(+)	3
FISH (+)	10	(-)	(-)	(-)	(-)	(-)	4.25	(-)	2
	85	NA	NA	NA	NA	(-)	4.18	(-)	2
	101	(-)	(-)	(-)	(-)	(-)	4.75	(-)	2
	128	c.3015 C>T (p.T943I)	c.3074+84 G>A c.3074+111 C>T c.3074+158 C>T c.3074+177 C>T	(-)	(-)	(-)	5.46	(-)	2
	136	NA	NA	NA	NA	(-)	8.70	(+)	3
	163	(-)	(-)	(-)	(-)	(-)	6.62	(+)	3
	165	(-)	(-)	c.3132 G>A (p.R982K)	(-)	(-)	5.82	(-)	2
	171	NA	NA	NA	NA	(-)	5.14	(+)	3
	247	(-)	c.3074+9 T>C	NA	NA	(-)	4.56	(-)	2
	253	(-)	(-)	(-)	(-)	(-)	4.18	(-)	2
	264	(-)	c.3074+119 C>T	NA	NA	(-)	4.88	(-)	3
	292	(-)	c.3074+129 G>A	(-)	(-)	(-)	4.86	(-)	2
	295	(-)	c.3074+170 G>A	NA	NA	(-)	5.23	(-)	2
	310	(-)	(-)	c.3162 C>T (p.T992I)	(-)	(-)	3.62	(-)	2

Table 3. Direct sequencing and RT-PCR results with FISH and IHC results of 70 patients.

* Stop codon; † 5′ splice site mutation

RT-PCR, Reverse Transcription Polymerase Chain Reaction; GCN, gene copy number; AMP, amplification; IHC, immunohistochemistry; NA, not available.

5. Survival analysis

In the univariate analysis, the group with positive IHC had slightly better OS than the group with negative IHC (hazard ratio [HR] 0.53, 95% confidence interval [CI] 0.30-0.93, $P = 0.026$). However, the FISH-positive group did not have a different survival than the FISH-negative group (HR 1.32, 95% CI 0.89-1.95, $P = 0.164$). In multivariate analysis, both MET IHC (HR 0.65, 95% CI 0.36-1.20), $P = 0.171$) and FISH (HR 1.32, 95% CI 0.88-1.98, $P = 0.183$) were not independent prognostic factors for OS. Age more than 65 years (HR 2.13, 95% CI 1.43-3.19, $P < 0.001$) and use of EGFR-TKIs (HR 2.94, 95% CI 1.92-4.50, $P < 0.001$) were significantly poor prognostic markers (Table 4).

	Univariate		Multivariate			
	HR (95% CI)	*P*	aHR (95% CI)	*P*	aHR (95%CI)	*P*
Age						
≥65 / <65 years	1.90 (1.28- 2.81)	0.001	2.13 (1.43- 3.19)	<0.001	2.10 (1.40- 3.14)	<0.001
Differentiation						
Moderate-poor / well	1.60 (0.74- 3.44)	0.234	1.02 (0.45- 2.32)	0.962	1.09 (0.48- 2.48)	0.834
Nodal involvement						
Positive / negative	1.20 (0.80- 1.82)	0.378	0.87 (0.49- 1.54)	0.870	0.90 (0.50- 1.61)	0.718
Pathologic stage						
3 or 4 / 1 or 2	1.51 (0.99- 2.32)	0.057	1.29 (0.72- 2.32)	0.391	1.29 (0.71- 2.35)	0.398
Use of EGFR TKI						
No/ yes	3.10 (2.10- 4.59)	<0.001	2.94 (1.92- 4.50)	<0.001	3.06 (2.00- 4.68)	<0.001
MET IHC score						
2 or 3 / 0 or 1	0.53 (0.30- 0.93)	0.026	0.65 (0.36- 1.20)	0.171-	0.74 (0.40- 1.35)	0.325
***MET* FISH**						
Positive / negative	1.32 (0.89- 1.95)	0.164	1.32 (0.88- 1.98)	0.183	-	-
***MET* amplification**						
Yes / no	0.67 (0.29- 1.53	0.339	-	-	0.76 (0.33- 1.77)	0.526

Table 4. Univariate and multivariate analyses for overall survival by Cox proportional hazards model

aHR, adjusted hazard ratio; CI, confidence interval; EGFR, epidermal growth factor receptor; TKI, tyrosine kinase inhibitor; IHC, immunohistochemistry; FISH, fluorescence in situ hybridization.

DISCUSSION

Our study was conducted to identify the relationship among *MET* GCN, protein expression, and mutations in pulmonary adenocarcinoma.

In our study, positive FISH and amplification of *MET* occurred in 38.9% and 8.2%, showing higher prevalence than previous studies (16, 19, 22, 29). The difference may be owing to patient selection, methods of test or different criteria of positivity. *MET* FISH and IHC had a strong correlation with each other in our study. This result corresponds well with the earlier study by Dziadziuszko et al (15). They defined an H-score combining staining intensity (0–4) and percentage of positive cells (0–100), and reported the significant association between *MET* GCN and the H-score by parametric analysis. Two other studies also reported a significant relationship between MET protein expression and GCN using cross-tabulations and nonparametric analysis (20, 22). One of these studies used both University of Colorado Cancer Center criteria and Cappuzzo criteria (GCN of 5 as a cut-off value) to define the positivity of GCN increase, and the other study utilized a GCN of 3 as a cut-off value of positivity.

The criteria of the IHC score used in our study are identical with those used in a large recent clinical trials of a MET inhibitor (26, 27). The proportion of patients with more than 5 of *MET* GCN is dramatically increased in tumors with MET IHC scores 2 (23.7%) or 3 (50%) compared with those with IHC scores 0 (14.9%) or 1 (11.3%). Based on these results, a population with high

MET GCN can be selected according to the MET IHC scores. In addition to the MET GCN, our study demonstrated the correlation between *MET* amplification and IHC scores for the first time. Because the proportion of the amplified tumors is exceedingly prevalent in an IHC score of 3 compared with the IHC scores of 0 to 2, highly expressed MET protein can be a cue for recognizing amplification in the tumor (Table 2 and Figure 4).

A recent study has reported a correlation between non-lepidic predominant tumors and MET protein expression (30). In contrast, there was a significant relationship between well-differentiated tumors and MET protein expression in our study. This result correspond well with the earlier study which has documented higher IHC stain in well to moderately differentiated tumors than poorly differentiated tumors (31). Further studies are needed to clarify this relationship. *MET* FISH positivity was significantly associated with nodal involvement in our study, and this was consistent with previous studies (20, 30, 32). In one of these previous studies, invasion-related markers were investigated along with HGF/MET expression, and increase of cell motility has been considered the mechanism of lymph node invasion in MET-positive tumors (32). Contrary to nodal involvement, MET alteration has been found to have little association with tumor size in previous studies and in our study (data not shown) (19, 21).

Positive FISH of EGFR is associated with positive FISH of *MET* (12, 22). However, there no study has demonstrated a significant relationship between FISH-positive *MET* and *EGFR* mutations in TKI-naïve NSCLC patients. In our study, *EGFR*-mutated tumors showed positive *MET* FISH more frequently

than tumors with wild-type *EGFR*, but there was no significant difference in PFS after the treatment with EGFR-TKIs between the *MET* FISH-positive and negative groups ($P = 0.665$, log-rank test). This result is different from the recent study reporting shorted PFS after gefitinib treatment in *MET* FISH-positive lung adenocarcinoma (33). The clinical importance of *de novo MET* amplification in EGFR-mutated tumors needs to be further investigated.

In an *in vivo* study of *MET* splice mutations at the juxtamembrane domain, increased expression of deleted forms of the receptor compared with wild-type MET receptor was confirmed by western blotting (24). This study raised the possibility of a correlation between MET protein expression and splice mutations. However, our study documented that splice mutation is a very rare event, and selection of mutated patients using IHC or FISH was impractical. Failure of the phase III clinical trial of a MET inhibitor indicated that targeting MET needs more delicate and strict criteria supported by sufficient evidence (27). Criteria for targeting *MET* can be prepared by using cut-off values of IHC or FISH, or a combination of both methods. The results of our study may help determine the proper inclusion and selection strategy in future clinical trials.

Limitations of this study include the small sample size to identify the exact relationship between *MET* splice mutations and other *MET* alterations, and possible selection bias related to sampling of 15- 20 tumors for mutation analysis in each IHC and FISH group. However, this approach is valuable for understanding the usefulness of IHC and FISH to find mutated tumors. Another limitation of this study is the retrospective collection of clinical data.

The result of our study suggests that there are definite correlations between MET protein expression and GCN. MET IHC can be helpful to for select MET FISH-positive or amplified tumors. However, *MET* splice mutation is a rare condition and is difficult to identify it by IHC or FISH results.

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국문 초록

서론: 폐암에서 MET 억제제의 적절한 표적은 아직 정립되지 않은 상태이다. MET 유전자 증폭, 단백질 발현, 그리고 14 번 엑손의 스플라이스 변이(splice mutation)는 MET/HGF 신호전달경로의 조절 장애 기전으로 알려져 있다. 본 연구의 목적은 MET 유전자 증폭, 단백질 발현, 그리고 변이의 관계를 탐구함으로써 MET 억제제의 치료 대상군을 찾는 전략을 수립함에 있다.

방법: 316 개의 수술적으로 절제된 폐선암 조직에 대해 면역조직화학(immunohistochemistry, IHC)검사를 시행하여 MET 단백질 발현을 검사하였고, 형광동소보합법(fluorescence in situ hybridization, FISH)을 이용하여 유전자 복제 수 및 증폭 여부를 검사하였다. IHC 결과는 MET 억제제에 대한 임상시험과 동일한 기준으로 판정하였고 2 또는 3 점일 경우 양성으로 정의하였다. 복제 수 및 증폭은 University of Colorado Cancer Center 기준에 따라 판정하였다. 이 후 IHC 음성/FISH 음성 군, IHC 음성/FISH 양성 군, IHC 양성/FISH 음성 군, 그리고 IHC 양성/FISH 양성 군으로 나누어 각 군에서 15 개에서 20 개의 조직을 무작위로 선별한 후 변이 검사를 시행하였다.

결과: MET IHC 점수가 0, 1, 2, 그리고 3 인 환자수는 각각 168 (53.2%), 71 (22.5%), 59 (18.7%), 그리고 18 (5.7%)명이었다.

평균 유전자 복제 수는 3.56(표준편차 1.5)이었고 MET FISH 양성 환자는 123 (38.9%)명이었으며 이 중 26 (8.2%)명이 유전자 증폭을 보였다. MET 유전자 증폭은 IHC 점수와 유의한 상관 관계 ($P<0.001$, χ^2 test)를 보였으며, MET IHC 점수 3 의 유전자 증폭에 대한 양성예측도는 44.4%였다. 스플라이스 변이는 검사를 시행한 70 명 중 2 (2.9%)명에서만 발견 되었는데, 그 중 한 명은 MET IHC 점수 2, FISH 와 증폭 음성 환자였고, 나머지 한 명은 MET IHC 점수 0, FISH 양성 그리고 증폭 음성이었다. 다변량 분석에서 MET IHC 또는 FISH 의 결과는 환자 전체 생존기간 (overall survival)에 대한 예후인자가 아니었다.

결론: MET 유전자 복제 수 또는 증폭과 단백질 발현 사이에는 유의한 상관관계가 있어, MET 복제 수가 높거나 증폭이 있는 환자를 선별할 때 IHC 가 도움이 된다. 하지만 스플라이스 변이는 드물어 IHC 나 FISH 를 이용하여 변이 환자를 찾아내기 어렵다.

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주요어 : MET, 유전자 복제 수, 유전자 증폭, 유전자 발현, 변이, 비소세포폐암, 폐선암

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