Molecular Diagnostic Assays and Clinicopathologic Implications of *MET* Exon 14 Skipping Mutation in Non–small-cell Lung Cancer

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Abstract

***MET* exon 14 skipping (*MET*ex14) has been reported as a biomarker that predicts the response to MET inhibitors. However, *MET*ex14 alterations exhibit a highly diverse sequence composition, posing a challenge for diagnostic testing in clinics. The present study showed that next-generation sequencing can be the first assay of choice as a multiplex testing and real-time quantitative reverse transcription polymerase chain reaction can be appropriate as a single gene testing. *MET*ex14 also had characteristic clinicopathologic features.**

Background: Recent studies revealed *MET* exon 14 skipping (*MET*ex14) as a biomarker that predicts the response to MET inhibitors in non–small-cell lung cancer (NSCLC). However, *MET*ex14 genomic alterations exhibit a highly diverse sequence composition, posing a challenge for clinical diagnostic testing. This study aimed to find a reasonable diagnostic assay for *MET*ex14 and identify its clinicopathologic implications. **Materials and Methods:** We performed a comprehensive analysis of *MET*ex14 in 414 *EGFR/KRAS/ALK/ROS1*-negative (quadruple negative) surgically resected NSCLCs. We used real-time quantitative reverse transcription polymerase chain reaction (qRT-PCR) and Sanger sequencing for the first assay, followed by next-generation sequencing (NGS; hybrid-capture targeted DNA/RNA sequencing). Clinicopathologic implications of the *MET*ex14 group were analyzed in a total of 880 NSCLCs. **Results:** *MET*ex14 was confirmed in 13 (3.1%) patients by DNA- and RNA-NGS. After comparison of assay results, qRT-PCR and NGS demonstrated the highest concordance rate. The mean variant allele frequency was 10.5% and 49% in DNA- and RNA-NGS, respectively. DNA-NGS revealed various lengths of indel and substitutions around and in exon 14. Moreover, *MET*ex14 was associated with adenocarcinoma (4.8%; 11/230) or sarcomatoid carcinoma (9.5%; 2/21), old age, never-smokers, and early stage of disease. **Conclusions:** *MET*ex14 occurs in about 3% of NSCLCs and has characteristic clinicopathologic features. NGS should be the first assay of choice as a multiplex testing. Sanger sequencing can detect *MET*ex14, but sensitivity can be hampered by large deletions or low allele frequency. qRT-PCR, an mRNA-based method, is sensitive and specific and can be appropriate for screening *MET*ex14 as a single gene testing.

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Introduction

Lung cancer is the leading cause of cancer-related deaths.[1] Approximately 85% to 90% of lung cancers are characterized as non–small-cell lung cancers (NSCLCs), and a considerable number present as locally advanced or metastatic disease. Molecularly targeted therapies have improved treatment for patients whose tumors harbor somatically activated oncogenes such as *EGFR* mutation, or *ALK* or *ROS1* rearrangement.[2,3] However, the various molecular drivers of 24% to 36% of lung adenocarcinomas (ADCs) and 37% to 60% of squamous cell carcinomas (SQCs) remain unknown.[4-11] Therefore, the identification of new driver mutations is critically important.

The hepatocyte growth factor receptor, encoded by the *MET* gene (*MET* proto-oncogene, located at chromosome 7q21-q31), is a receptor tyrosine kinase that plays a fundamental role in regulating development and cell growth. Pathologic activation of *MET*, through both gene amplification and point mutation, is a well-characterized driver of oncogenesis that occurs in several types of tumors. In cancer, *MET* activation promotes tumor proliferation, invasive growth, metastatic spread, and anti-apoptosis.[12,13] MET overexpression is observed in 25% to 70% of NSCLCs and *MET* amplification in 2% to 11% of ADCs.[4] *MET* amplification has been implicated as one of the mechanisms of acquired resistance to anti-epidermal growth factor receptor (EGFR) therapy.[14,15] Several studies have shown that an increased *MET* copy number is an independent negative prognostic factor in surgically resected NSCLCs.[16,17] Several clinical trials of MET-targeting agents for patients with NSCLC with these features have revealed favorable results.[18-21]

Previous studies reported that mutations of RNA splice acceptor and donor sites involving *MET* exon 14 could lead to exon skipping, resulting in an in-frame deletion of the juxtamembrane domain, which normally is a negative regulator of the kinase catalytic activities.[22-24] More importantly, this type of mutation was mutually exclusive with other oncogenic drivers and showed partial responses to MET-targeted therapies.[22,23] Somatic mutations affecting splice sites of *MET* exon 14 in primary lung cancer specimens and in a lung cancer cell line were published in the early 2000s.[24-26] Tyrosine 1003 (Y1003) in the juxtamembrane domain of MET is a binding site for c-Cbl, a ubiquitin protein ligase (E3) that causes ubiquitination, receptor endocytosis, and degradation of MET. Loss of *MET* exon 14 maintains the reading frame and leads to increased MET stability and prolonged signaling upon hepatocyte growth factor stimulation, leading to increased oncogenic potential.[27,28]

MET exon 14 skipping (*MET*ex14) alterations have been shown to occur in 3% to 4% of lung ADCs.[4,6,7,22,23,29] This frequency is comparable to that of *ALK* rearrangements; thus, identification of *MET*ex14 is important to apply appropriate target therapies. Additionally, *MET*ex14 has been reported as a biomarker that predicts the response to MET inhibitors.[22,23] However, *MET*ex14 alterations exhibit a highly diverse sequence composition, posing a challenge for diagnostic testing in clinics.[23]

This study aimed to find a reasonable diagnostic assay for *MET*ex14 and identify its clinicopathologic implications and relationship with other genetic alterations in East Asian patients with NSCLC.

Materials and Methods

Clinical Samples

The study cohort was retrospectively retrieved from pathology archives. Formalin-fixed paraffin-embedded (FFPE) tumor tissues were obtained from patients with NSCLC who underwent surgical resection from January 2005 to January 2013 at our institution. They were reviewed according to the 2015 World Health Organization classification[30] and staged according to the seventh edition of the American Joint Committee on Cancer staging system.[31,32] Demographic data and clinicopathologic characteristics were obtained from medical records. This retrospective study was approved by the institutional review board and was conducted in accordance with ethical guidelines and the Declaration of Helsinki.

The study cohort consisted of 414 *EGFR/KRAS/ALK/ROS1*-negative NSCLCs. Primarily, real-time quantitative reverse transcription polymerase chain reaction (qRT-PCR) and Sanger sequencing were performed. The primers used for each test are as shown in Supplemental Table 1 (in the online version). We performed next-generation sequencing (NGS) for cases that showed positive in any tests. Clinicopathologic implications of the *MET*ex14 group were analyzed in a total of 880 NSCLCs.

For smoking status, the patients were categorized as "never-smokers" (had a lifetime exposure of < 100 cigarettes), "former smokers" (have quit smoking for > 12 months), and "current smokers" (currently smoking or have quit during the past 12 months).[33] Follow-up duration was calculated from the surgical operation date to the last follow-up date (cutoff on May 31, 2017) or death. Overall survival (OS) was defined from the time of surgery to the time of cancer-related death. Meanwhile, disease-free survival (DFS) was defined from the time of curative surgery to the time of radiologic or pathologic evidence of tumor relapse or progression.

DNA and RNA Preparations

FFPE tissue blocks were cut into 5 sections with 5-μm thickness using a microtome. Genomic DNA extraction was performed using the ReliaPrep FFPE gDNA Miniprep System (Promega, Madison, WI), whereas total RNA was isolated with the ReliaPrep FFPE Total RNA Miniprep System (Promega). The concentrations of genomic DNA and total RNA were measured using NanoDrop 1000 (Thermo-Scientific Inc, Wilmington, DE).

Real-time qRT-PCR

To identify *MET*ex14, qRT-PCR was performed using the following primers: forward primer for exon 13 of MET, 5′-TGGGTTTTTCCTGTGGCTGAA-3′; reverse primer for exon 15 of MET, 5′-GCATGAACCGTTCTGAGATGAATT-3′; and probes overlapping an exon 13-exon 15 junction, 5′-AAG-CAAATTAAAGATCAGTTTCC-3′.[34] TaqMan probes were labeled with the reporter dye molecule FAM (6-carboxyfluorescein) at the 5′ end and with TaqMan zip nucleic acid-4 black hole quencher-1 (ZNA-4-BHQ-1) probe at the 3′ end. The *GAPDH* gene (NM_001289746.1) was used as an endogenous control. We designed a forward primer (5′-CCTGCACCACCAACTGCTTAG-3′), a reverse primer (5′-TGAGTCCTTCCACGATACCAA-3′), and probes (5′-6-FAM-CCCT GGCCAAGGTCATCCATGA-BHQ-1-3′). With the SensiFAST Probe Lo-ROX One-Step Kit

(Bioline, London, UK), 10-μM primers (forward and reverse) each at 0.5 μL, a 10-μM TaqMan probe at 0.25 μL, and 50 ng RNA were mixed in a total reaction volume of 10 μL. PCR conditions were 45°C for 20 minutes and 95°C for 10 minutes, followed by 40 cycles of amplification at 95°C for 15 seconds and 60°C for 30 seconds on the ABI 7500 Real-Time PCR Detection System (Applied Biosystems). Threshold cycle (Ct) values of < 34 were considered as *MET*ex14-positive, and ≥ 34 as *MET*ex14-negative. All experiments were performed in triplicate for consistency.

Sanger Sequencing

For Sanger sequencing, we amplified 100 ng of genomic DNA and created several probes to sequence exon 14 and its vicinity as fully as possible, as shown in Supplementary Table 1 (in the online version at). The PCR products were analyzed by electrophoresis and purified using the QIAquick PCR Purification Kit (Qiagen, Valencia, CA). Each PCR product was sequenced with each forward or reverse primer. The PCR cycling parameters used were as follows: 94°C for 2 minutes; 35 cycles of (94°C for 20 seconds, 59°C for 10 seconds, and 72°C for 30 seconds); 72°C for 5 minutes; and 4°C hold. PCR products were checked on a 2% agarose gel.

NGS — Hybrid-capture Targeted DNA/RNA Sequencing

A customized hybrid-capture-based assay encompassing 46 genes for DNA and 17 genes for RNA was used (see Supplementary Table 2 in the online version). Synthetic DNA and RNA probes were designed to capture all protein-coding exons of target genes. We used the Qiagen AllPrep DNA/RNA FFPE Kit (Qiagen) following the manufacturer's instructions to extract FFPE DNA and RNA. The input amounts of extracted DNA and RNA were 100 to 1000 ng/50 μL and 50 to 100 ng/5 μL, respectively. Genomic DNA was fragmented to 150 to 250 bp using a Covaris E-Series instrument (Covaris, Woburn, MA). Libraries were constructed using the NEBNext Ultra II DNA/RNA Library Prep Kit for Illumina (New England Biolabs, Ipswich, MA), through a series of enzymatic steps including end repair, adaptor ligation, size selection, and PCR enrichment. Hybrid-capture selection was performed using Dynabeads M-270 Streptavidin (Life Technologies, Carlsbad, CA) and KAPA HiFi HotStart ReadyMix (Kapa Biosystems, Wilmington, MA). Sequencing of the libraries was carried out on a MiSeq (Illumina, San Diego, CA) according to the manufacturer's protocol ("Preparing libraries for sequencing on the MiSeq," October 2013). A final library concentration ranging from 8 to 10 pM was used to carry out cluster generation and sequencing on a standard flow cell and 300-cycle MiSeq Reagent Kit v3 (150 × 150). Tumor samples were sequenced to a target depth of coverage more than 300× (DNA) and 1000× (RNA). Detailed sequence analysis information is described in the Supplemental Materials and Methods (in the online version at).

Construction of Tissue Microarray

The tumor area on the FFPE tissue block was marked on the hematoxylin and eosin-stained section. Three representative 2-mm cores were obtained from each tumor and were sectioned by 4-μm thickness for silver in situ hybridization (SISH), fluorescence in situ hybridization (FISH), and immunohistochemistry (IHC).

SISH

MET gene copy number was analyzed using bright-field microscopy and SISH technology. Probing was carried out using both MET-specific and centromere 7 (CEP7)-specific probes according to the manufacturer's protocols (Ventana Medical Systems). The assessment of gene copy number was performed independently and blinded from IHC. The scoring was carried out in 50 nonoverlapping nuclei per core in regions identified by optical analysis of tissue sections as having a higher gene copy number. The following data were recorded for each sample: mean *MET* gene and mean *CEP7* copy number per cell, and *MET/CEP7* ratio. The core with the highest average *MET* gene copy number per cell was selected. Small clusters were scored as 6 signals, and big clusters as 12 signals, similar to HER2 breast cancer practice.[35]

Molecular Analyses of EGFR, KRAS, ALK, and ROS1 Genetic Alterations

The peptide nucleic acid-locked nucleic acid PCR (PNA-LNA PCR) PNAClamp EGFR Mutation Detection Kit (Panagene Inc, Daejeon, Korea)[36,37] was used to detect *EGFR* mutations in real-time PCR. *KRAS* mutation was assessed by Sanger sequencing. After IHC screening, FISH was performed to identify *ALK* and *ROS1* rearrangements. A break-apart *ALK* or *ROS1* probe (Vysis LSI Dual Color, Break Apart Rearrangement Probe; Abbott Molecular, Abbot Park, IL) was used according to the manufacturer's instructions. Detailed testing methods are described in the Supplemental Materials and Methods (in the online version).

Statistical Analysis

Statistical analysis was performed to analyze associations of mutation, protein expression, and gene copy number with clinical characteristics using the Pearson χ^2 test and the Fisher exact test. The Kaplan-Meier method was used to estimate the survival rates of the different groups. The equivalences of the survival curves were tested by log-rank statistics. The Cox proportional hazards model was employed for univariate and multivariate survival analyses. Statistically significant variables found in the univariate survival analysis were evaluated in the multivariate survival analysis. All results with a 2-sided *P*-value of $< .05$ were considered significant. Statistical calculations were performed using the statistical package SPSS 21.0 (IBM Corp, Armonk, NY).

Results

Diagnostic Assay for METex14

We conducted qRT-PCR and Sanger sequencing on 414 cases and 102 of 414 cases, respectively. We found 14 qRT-PCR-positive and 8 sequencing-positive cases. Among them, 13 were confirmed as *MET*ex14-positive by NGS (Table 1 and Figure 1). Additionally, NGS was performed in 37 cases that were randomly selected from qRT-PCR- and Sanger sequencing-negative cases. A total of 51 cases were tested by all 3 assays. qRT-PCR showed 100% sensitivity, and Sanger sequencing showed 100% specificity (Table 2, Figure 1, and Supplementary Figure 1 [in the online version]). One PCR-positive/NGS-negative case revealed *KRAS* mutation (Q61H) through NGS. Five Sanger sequencing-negative cases were positive by qRT-PCR and NGS. Among them, 3 cases had large deletions (237, 335,

Table 1 Summary of *MET*ex14 Alterations (Chr7; NM_000245.2)

Case No.	*MET*ex14 Alterations (gDNA; cDNA)	Type of Alteration	VAF, %		qRT-PCR	Sanger Sequencing
			DNA	RNA		
1	g.116411891_116411901del11; c.2888-12_2888-3delCTCTGTTTTAAinsT	Indel splice acceptor	17.5	53.9	Deletion	Deletion
2	g.116412044G>T; c.3028+1G>T	Base substitution splice donor	15.2	40.5	Deletion	Substitution
3	g.116412043G>A; c.3028G>A	Base substitution splice donor	14.9	66.1	Deletion	Substitution
4	g.116411875_116411897del23; c.2888-28_2888-6delCAAGCTCTTTCTTTCTCTCTGTTinsA	Indel splice acceptor	15.0	73.9	Deletion	Deletion
5	g.116411889_116411905del17; c.2888-14_2900delCTCTCTGTTTTAAGATC	Indel splice acceptor	5.5	47.8	Deletion	Deletion
6	g.116412033_116412045del13; c.3018_3028+2delTTTTCCAGAAGGT	Indel splice donor	6.2	21.3	Deletion	Wild
7	g.116411885_116411897del13; c.2888-19_2888-8delTCTTTCTCTCTGTinsG	Indel splice acceptor	18.5	60.2	Deletion	Deletion
8	g.116411748_116412484del737; c.2887+40_3028+441del	Whole exon 14 deletion	2.3	24	Deletion	Wild
9	g.116411876_116411887del12; c.2888-27_2888-16delAAGCTCTTTCTT	Indel splice acceptor	1.0	27.7	Deletion	Wild
10	g.116411894_116411896delinsGG; c.2888-9_2888-7delinsGG	Indel/base substitution splice acceptor	11.3	58.1	Deletion	Deletion
11	g.116411758_116412092del335; c.2887+50_3028+49del	Whole exon 14 deletion	1.0	14.1	Deletion	Wild
12	g.116411885_116411903del19; c.2888-18_2888-1delCTTTCTCTCTGTTTTAAG	Indel splice acceptor	3.9	72.4	Deletion	Deletion
13	g.116411953_116412189del237; c.2938_3028+146del	Indel splice donor	26.8	76.7	Deletion	Wild

Abbreviations: cDNA = Complementary DNA; gDNA = genomic DNA; *MET*ex14 = MET exon 14 skipping; qRT-PCR, real-time quantitative reverse transcription polymerase chain reaction; VAF = variant allele frequency.

and 737 bp), and others showed low DNA variant allele frequency (VAF; 1% and 6%). Most mutations were deletions, which occurred at a splice donor or acceptor, and 2 cases caused a whole exon 14 deletion. The mean VAF of DNA and RNA sequencing in NGS were 10.5% and 49.0%, respectively.

Concurrent Genetic Alterations Including MET Amplification

Other accompanying genetic alterations are described in Supplemental Table 3 (in the online version at). Two (15.4%) cases had *TP53* mutation, known to be likely oncogenic in NSCLC. No coexisting driver mutations or known oncogenic copy number alterations in NSCLC were observed. Concurrent *MET* amplification was also not observed.

The results of MET SISH showed no correlation with *MET*ex14 (see Supplemental Table 4 in the online version). *MET*-amplified NSCLCs were found in 3.8% (13/338) of cases. They were associated with higher stage ($P = .014$) and were relatively more frequent in sarcomatoid carcinomas and large cell carcinomas (LCCs) ($P = .028$) (see Supplemental Table 5 in the online version).

Clinicopathologic Features of METex14 NSCLCs

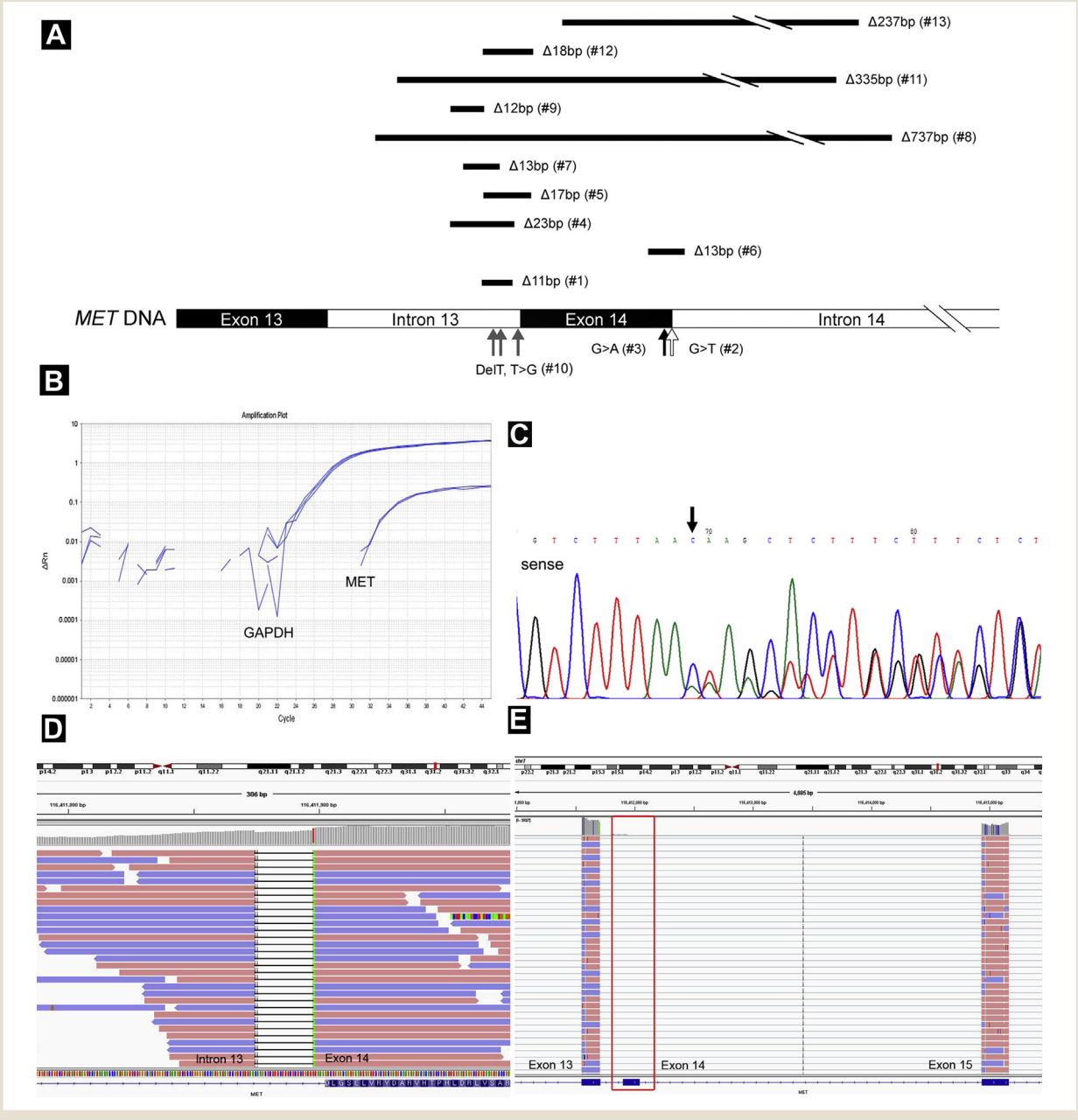
The study cohort consisted of 264 ADCs (including 34 mucinous ADCs), 105 SQCs, 21 sarcomatoid carcinomas, 15 LCCs, 2 adenosquamous carcinomas, and 7 carcinoid tumors. *MET*ex14 was found in 3.14% (13/414) of cases (see Supplemental Tables 6 and 7 in the online version at). The histologic type was ADC (11/13; 84.6%) and sarcomatoid carcinoma (2/13; 15.4%) (Figure 2). The incidence was 4.8% (11/230) in ADCs and 9.5% (2/21) in sarcomatoid carcinomas. The predominant subtype was acinar or lepidic in ADCs. Two sarcomatoid carcinomas consisted of spindle cells admixed with a solid adenocarcinomatous component. Moderate to marked lymphocytic infiltration with or without lymphoid follicle formation was found in 7 (53.8%) cases.

In the study cohort (see Supplemental Table 7 in the online version), *MET*ex14 appeared more frequently in never-smokers ($P = .019$), lepidic subtype ($P = .002$), and early stage ($P = .033$). When compared with other driver mutation-positive groups and all-negative groups (n = 880) (Table 3), the *MET*ex14 group revealed correlations with old age ($P = .022$), females ($P < .001$), never-smokers ($P < .001$), ADCs and sarcomatoid carcinomas ($P < .001$), lepidic subtype ($P < .001$), and early stage ($P < .001$).

Survival Analysis

*MET*ex14 showed a tendency of better OS and DFS. The *EGFR* mutation group revealed significantly better OS and DFS compared with the wild-type group ($P < .001$ and $P = .027$, respectively) (Figure 3). Meanwhile, *MET* amplification showed poorer OS ($P = .188$) and DFS ($P = .025$). In the univariate Cox proportional hazards models for OS (see Supplemental Table 8 in the online version), old age, males, smokers, histology (sarcomatoid carcinoma), subtype (solid and micropapillary), advanced stage, and wild type (non-*MET*ex14 and non-*EGFR* mutation) were associated with worse prognosis. Among them, old age, solid predominant pattern, and advanced stage were independent worse prognostic factors on

Figure 1 Genomic Position of *MET*ex14 Alterations (A, #; case number) and a Representative case Showing Consistent Results (B-D, case #4; 23-Bp Deletion). qRT-PCR Shows Amplification curves (B, Ct = 32.38). Sanger Sequencing Shows Deletion (C, arrow; starting point). DNA-NGS Demonstrated by the Integrative Genomics Viewer Shows Deletion on Intron 13 (D). RNA-NGS Result Shows Absence of Exon 14 (Red Rectangle) Between Exon 13 and Exon 15 (E)



Abbreviations: *MET*ex14 = MET exon 14 skipping; NGS = next-generation sequencing.

multivariate analysis. In the univariate Cox proportional hazards models for DFS (see Supplemental Table 9 in the online version at), histology (sarcomatoid carcinoma), non-lepidic subtype, advanced stage, and *MET* amplification were associated with worse prognosis. Among them, only advanced stage was an independent worse prognostic factor on multivariate analysis.

Discussion

In this study, we compared molecular diagnostic assays to arrive with the proper clinical method for detecting *MET*ex14 in NSCLCs. When NGS testing was used as the gold standard, qRT-PCR, an mRNA-based method, was highly sensitive and specific, and classic DNA-based sequencing showed low sensitivity because

Table 2 Comparison Between qRT-PCR, Sanger Sequencing, and NGS in Cases Tested by all 3 Assays (n = 51)

Methodology	NGS		Sensitivity, %	Specificity, %
	*MET*ex14	Wild Type		
qRT-PCR				
*MET*ex14	13	1	100.0	97.4
Wild type	0	37		
Sanger sequencing				
*MET*ex14	8	0	61.5	100.0
Wild type	5	38		

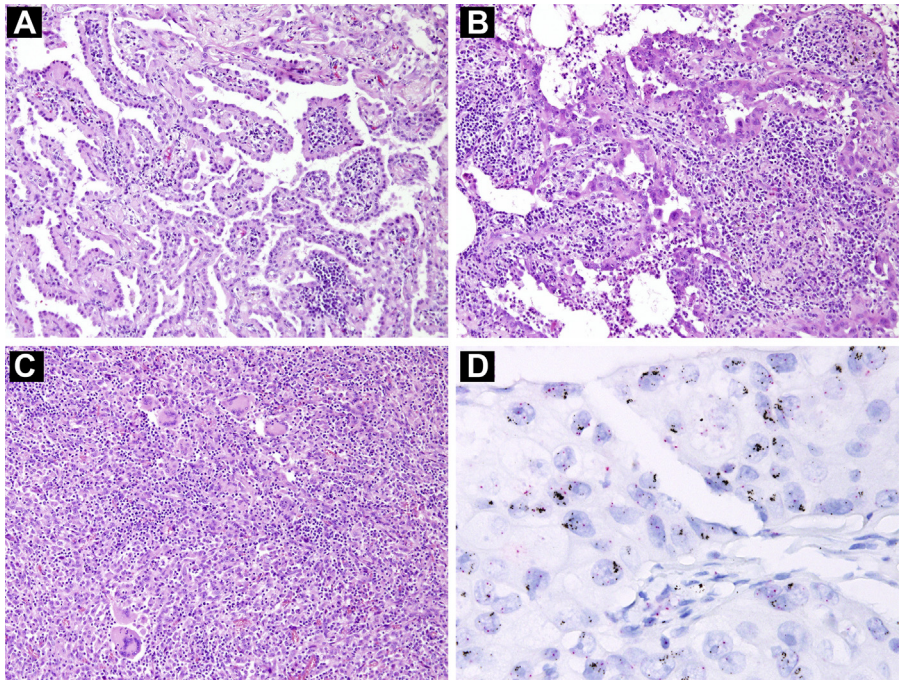
Abbreviations: *MET*ex14 = MET exon 14 skipping; NGS = next-generation sequencing; qRT-PCR, real-time quantitative reverse transcription polymerase chain reaction.

genomic alterations inducing exon 14 skipping are very diverse. We also found that NSCLCs harboring *MET*ex14 showed distinct clinicopathologic features compared with those of the *MET*ex14 wild type in surgically resected cases.

To compare molecular diagnostic methods for detecting *MET*ex14, we first performed qRT-PCR and Sanger sequencing in our cohort. Subsequently, NGS was performed for positive cases and some negative cases. We defined a case as *MET*ex14-positive when DNA- and RNA-NGS showed genomic alterations around or in exon 14 and mRNA exon 14 skipping, respectively. *MET*ex14 was found in 13 cases. We constructed the PCR probes used in this study to cover a sequence connecting exons 13 and 15 directly, and it showed 100% sensitivity to *MET*ex14. There were discordant cases between the assays. One case was qRT-PCR-positive, but NGS revealed *KRAS* Q61H mutation and no genomic alterations in *MET*ex14.

Sanger sequencing had no false-positive result (100% specificity), but the false-negative rate was relatively high. Five cases without observed mutations were actually found to have *MET*ex14 by NGS. These cases had either large deletions or small numbers of supporting DNA reads, which thus could not be detected owing to the limitations of the Sanger sequencing test itself. Thus, considering sensitivity and specificity, qRT-PCR may be the most reasonable single gene testing for detecting *MET*ex14.

Figure 2 Representative Images of *MET*ex14 and *MET* Amplification. Lepidic Predominant Adenocarcinoma With Mild Lymphocytic Infiltration (A), Acinar Predominant Adenocarcinoma With Moderate Lymphocytic Infiltration (B), Sarcomatoid Carcinoma With Marked Lymphocytic Infiltration (C), and Sarcomatoid Carcinoma With *MET* Amplification Demonstrated by MET SISH (D, *MET/CEP7* Ratio = 7.24 and Average *MET* copy number = 15.68)



Abbreviations: *MET*ex14 = MET exon 14 skipping; SISH = silver in situ hybridization.

Table 3 Comparison of *MET*ex14 and Other Mutation-positive/Mutation-negative Groups

	Total	*MET*ex14	*EGFR*	*KRAS*	*ALK*	*ROS1*	All-negative	*P* Value
N (%)	880 (100)	13 (1.5)	382 (43.4)	31 (3.5)	29 (3.3)	8 (0.9)	417 (47.4)	
Age, y								**.022**
≤ 60	309 (35.1)	2 (15.4)	150 (39.3)	11 (35.5)	16 (55.2)	4 (50.0)	126 (30.2)	
> 60	571 (64.9)	11 (84.6)	232 (60.7)	20 (64.5)	13 (44.8)	4 (50.0)	291 (69.8)	
Gender								**<.001**
Male	487 (55.3)	7 (53.8)	144 (37.7)	20 (64.5)	14 (48.3)	3 (37.5)	299 (71.7)	
Female	393 (44.7)	6 (46.2)	238 (62.3)	11 (35.5)	15 (51.7)	5 (62.5)	118 (28.3)	
Smoking status								**<.001**
Never-smoker	439 (49.9)	9 (62.2)	271 (70.9)	12 (37.8)	15 (51.7)	6 (75.0)	126 (30.2)	
Ex-smoker	254 (28.9)	2 (15.4)	81 (21.2)	8 (25.8)	8 (27.6)	0 (0)	155 (37.2)	
Current smoker	187 (21.3)	2 (15.4)	30 (7.9)	11 (35.5)	6 (20.7)	2 (25.0)	136 (32.6)	
Histology								**<.001**
Adenocarcinoma	671 (76.3)	11 (84.6)	375 (98.2)	18 (58.1)	29 (100.0)	8 (100.0)	230 (55.2)	
Squamous cell ca	107 (12.2)	0 (0)	0 (0)	0 (0)	0 (0)	0 (0)	107 (25.7)	
Mucinous ca	46 (5.2)	0 (0)	0 (0)	11 (35.5)	0 (0)	0 (0)	35 (8.4)	
Sarcomatoid ca	24 (2.7)	2 (15.4)	1 (0.3)	1 (3.2)	0 (0)	0 (0)	20 (4.8)	
Large cell ca	18 (2.0)	0 (0)	1 (0.3)	1 (3.2)	0 (0)	0 (0)	16 (3.8)	
Adenosquamous ca	7 (0.8)	0 (0)	5 (1.3)	0 (0)	0 (0)	0 (0)	2 (0.5)	
Carcinoid tumor	7 (0.8)	0 (0)	0 (0)	0 (0)	0 (0)	0 (0)	7 (1.7)	
Subtype								**<.001**
Lepidic	96 (13.8)	5 (45.5)	61 (16.1)	7 (22.6)	0 (0)	0 (0)	23 (9.7)	
Acinar	380 (54.8)	6 (54.5)	219 (57.8)	14 (45.2)	18 (64.3)	5 (62.5)	118 (49.8)	
Papillary	86 (12.4)	0 (0)	52 (13.7)	0 (0)	3 (10.7)	1 (12.5)	30 (12.7)	
Solid	86 (12.4)	0 (0)	21 (5.5)	6 (19.4)	5 (17.9)	1 (12.5)	53 (22.4)	
Micropapillary	46 (6.6)	0 (0)	26 (6.9)	4 (12.9)	2 (7.1)	1 (12.5)	13 (5.5)	
AJCC stage								**<.001**
I	542 (61.6)	11 (84.6)	272 (71.2)	17 (54.9)	17 (58.6)	3 (37.5)	222 (53.2)	
II	158 (18.0)	2 (15.4)	44 (11.5)	9 (29.0)	3 (10.3)	1 (12.5)	99 (23.7)	
III	149 (16.9)	0 (0)	54 (14.1)	3 (9.7)	8 (27.6)	3 (37.5)	81 (19.4)	
IV	31 (3.5)	0 (0)	12 (3.1)	2 (6.5)	1 (3.4)	1 (12.5)	15 (3.6)	

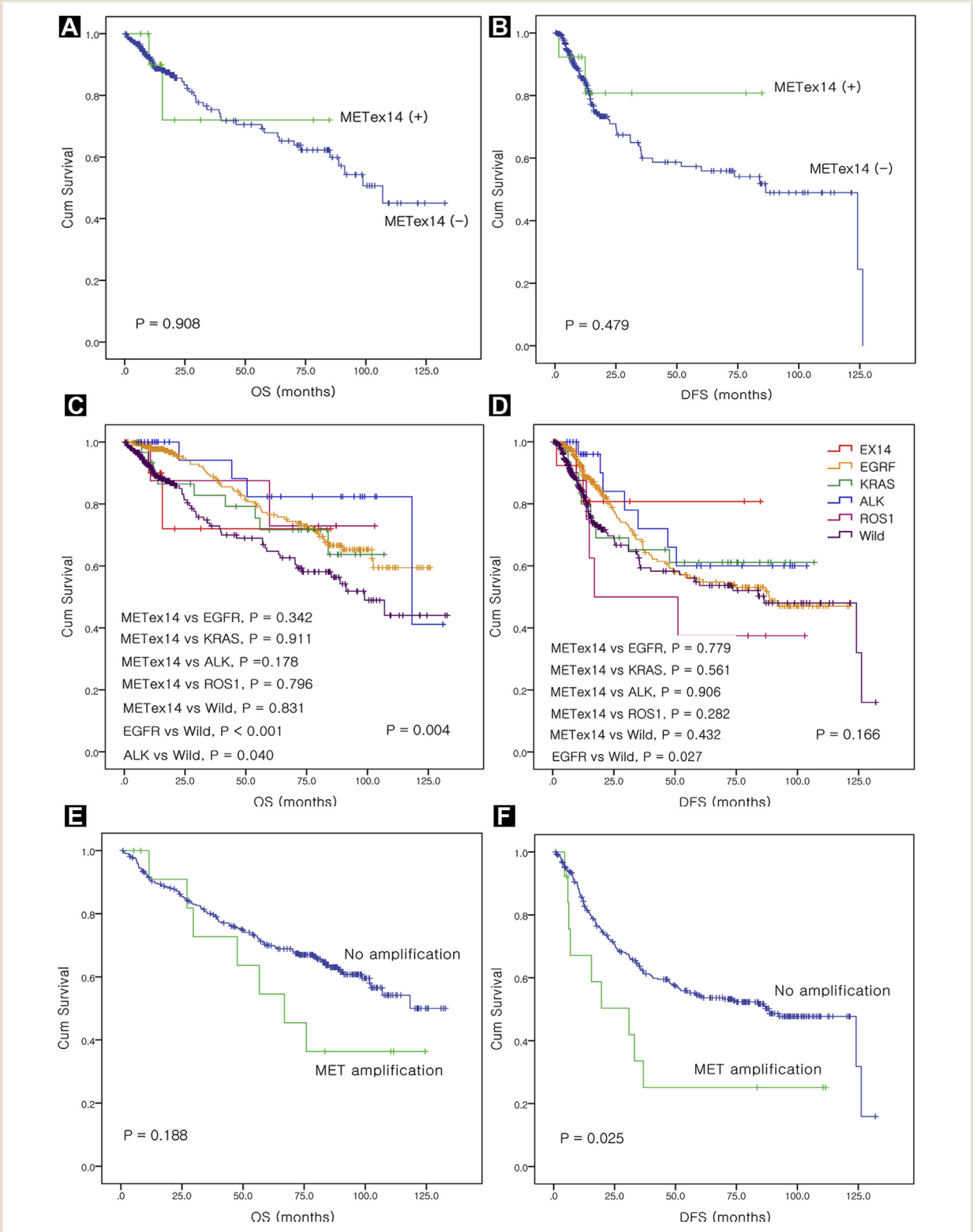
The bold *P*-values mean statistically significant (<.05).
Abbreviations: AJCC = American Joint Committee on Cancer; ca = carcinoma; *MET*ex14 = MET exon 14 skipping.

Previous studies have used diverse types of NGS, including whole-exome sequencing,[4,38] anchored multiplex PCR-based RNA sequencing,[39] and hybrid-capture NGS.[22,23,40] We used hybrid-capture targeted DNA and RNA sequencing methods. DNA sequencing revealed various lengths of indel and substitutions around and in exon 14. Because genomic alterations of *MET*ex14 are very diverse, proper probes (for the hybrid-capture method) or primers (for the amplicon-based method) targeting exon 14 and its surrounding introns should be employed in DNA-NGS. In our study, with RNA sequencing, it was much easier to find out *MET*ex14 using the Integrative Genomics Viewer, and the mean supporting read (%) was higher than with DNA sequencing. The higher supporting reads and higher VAF in RNA sequencing can be explained by the following. Generally, extracted DNAs come from various cells, including non-neoplastic cells (such as inflammatory cells and stromal cells) and neoplastic cells. Wild-type DNAs from normal cells reduce the proportion of mutant DNA. On the other hand, mRNAs are extracted only from cells expressing MET mRNA. Because not all normal cells undergo MET transcription, the proportion of spliced RNA increases compared with genomic DNA. Although RNA handling in FFPE tissue can be complicated and less manageable than using DNA, it is a good method for novel transcripts, fusions, or detection of allele-specific expression and identification of alternatively spliced genes.[41,42]

In recently updated molecular testing guideline for the selection of patients with lung cancer for treatment with targeted tyrosine kinase inhibitors, *MET* molecular testing was recommended as part of larger testing panels performed either initially or when routine *EGFR*, *ALK*, and *ROS1* testing is negative.[43] Given the scarcity of tissue and growing approval of targeted therapies, the role for single gene testing seems to be falling out of favor in clinical practice. Our study also demonstrated that NGS can be the first assay of choice as a multiplex testing. In addition, DNA- and RNA-NGS may be complementary in the detection of *MET*ex14 because of diverse genomic changes of *MET*ex14.

We observed no correlation between *MET*ex14 and *MET* amplification in this cohort, which is composed primarily of early-stage disease. Many studies have reported that *MET* amplification

Figure 3 Kaplan-Meier Curves of OS and DFS. The *MET*ex14-positive and *MET*ex14-negative Groups Show no Differences in OS and DFS (A and B). The OS of *EGFR* Mutation and *ALK* Rearrangement (vs. wild Type Group) are Significantly Different (C and D). The *MET*-amplified Group Shows Poorer DFS than the non-amplified Group (E and F)



Abbreviations: DFS = Disease-free Survival; *MET*ex14 = MET Exon 14 Skipping; OS = overall Survival.

was associated with an advanced stage, undifferentiated pathologic grade, and shorter OS.[17,44] In this study, *MET* amplification was associated with advanced stage and LCCs or sarcomatoid carcinomas and was a poor prognostic factor on univariate analysis. Although concurrent *MET* amplification with *MET*ex14 was not detected by NGS or SISH in this study, this phenomenon has been identified in up to 30% of cases.[44,45] In these cases, poor histologic differentiation and relatively high mutational burden have been reported,[45] supporting that *MET*ex14 is an early event in lung tumorigenesis, followed by *MET* amplification, resulting in a more aggressive clinical feature.[40]

The Cancer Genome Atlas shows that *MET*ex14 was not accompanied by any remarkable driver mutations other than *TP53*, but *CDK4* and *MDM2* amplification was relatively not uncommon, similar to the findings of other studies.[22,45,46] In this study, *TP53* mutation was observed in 2 cases. Together with the results of recent researches, *MET*ex14 is likely to act as an oncogenic driver.[47]

Compared with the *EGFR*, *KRAS*, *ALK*, and *ROS1* positive group and all-negative group, the *MET*ex14 group showed characteristic clinicopathologic features. *MET*ex14 was associated with old age, lepidic predominant pattern, and early-stage disease. Compared with the *EGFR*, *ALK*, and *ROS1*-positive group, the *MET*ex14 group showed that *MET*ex14 was more frequent in males; however, compared with the *KRAS* mutant group and all-negative group, the *MET*ex14 group showed that *MET*ex14 was more common in females. *MET*ex14 was also more frequent with never-smokers, compared with the *KRAS* positive group and all-negative group. In our cohort, *MET*ex14 was found in ADC (4.8%; 11/230) and sarcomatoid carcinoma (9.5%; 2/21) histology only. In previous studies, *MET*ex14 was frequently found in sarcomatoid carcinomas, and our data showed similar results.[38,48-50] Ethnic differences in oncogenic mutations such as *EGFR* and *KRAS* are well-known. Comparing studies of Asian and Western cohorts, the frequency of *MET*ex14 was 3% to 4% and was not significantly different in the cohorts (see Supplemental Table 10 in the online version). Older age, early-stage disease, and slight female predominance were not significantly different in terms of geographic regions. The number of never-smokers tended to be slightly higher in Asians, whereas the number of ever-smokers was higher in Westerners. One study suggested that a relatively low mutational burden of *MET*ex14 might be associated with a higher proportion of never-smokers.[45]

We observed moderate to marked lymphocytic infiltration in 53.8% of *MET*ex14 NSCLCs as an additional histologic feature. A recent retrospective analysis of the immunophenotypes of *MET*ex14 NSCLCs revealed that programmed death-ligand 1 (PD-L1) expression ($\geq$50%) was observed in 44% (18/41) of cases. However, the overall response rate to immunotherapy was only 13%.[51] Thus, more investigations are needed to understand the interaction of *MET* with immune checkpoint pathways.

Each study has shown a difference in the prognostic relevance of *MET*ex14. We found that *MET*ex14 had a relatively good OS and a low hazard ratio in patients who underwent complete surgical resection. In advanced stage, *MET*ex14 and *MET* amplification are relatively good predictors of response to MET-targeting therapies, providing the basis for screening patients with *MET*ex14 — particularly for elderly patients with ADC or sarcomatoid carcinoma histology.

There are some limitations to our study. First, not all 414 samples underwent NGS testing or Sanger sequencing, whereas we performed qRT-PCR analysis on all samples. However, our data can suggest that Sanger sequencing is not an appropriate test for *MET*ex14. Second, our cohort consisted mainly of surgically resected early stage, which may not reflect the characteristics of *MET*ex14 in the advanced stage. Third, MET SISH may not reflect the status of the *MET* amplification in an entire tumor because of the use of tissue microarrays.

In summary, this comparative study of molecular diagnostic assays showed that qRT-PCR, an mRNA-based method, is a sensitive and specific test and can be a good single gene test to screen *MET*ex14. A classic DNA-based method is possible; however, sensitivity can be hampered by large deletions of the *MET* gene or low allele frequency. If NGS is available, NGS should be the first assay of choice as a multiplex gene testing. DNA- and RNA-NGS may be complementary in the detection of *MET*ex14. *MET*ex14 occurs in about 3% of NSCLCs and has characteristic clinicopathologic features in surgically resected cases.

Clinical Practice Points

- Recent studies revealed *MET*ex14 as a biomarker that predicts the response to MET inhibitors in NSCLC. However, *MET*ex14 genomic alterations exhibit a highly diverse sequence composition, posing a challenge for clinical diagnostic testing. This study aimed to find a reasonable diagnostic assay for *MET*ex14 and identify its clinicopathologic implications and relationship with other genetic alterations.
- Analysis of results revealed that *MET*ex14 occurs in approximately 3% of NSCLC and is associated with clinicopathologic features such as old age, no history of smoking, and early stage of disease. Furthermore, the study showed that next-generation sequencing can be the first assay of choice as a multiplex testing. It also showed that real-time qRT-PCR is a sensitive and specific test that can be used as a single gene test to screen *MET*ex14.

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Disclosure

The authors have stated that they have no conflicts of interest.

Supplemental Data

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