ORIGINAL ARTICLE

Sensitivity and Resistance of MET Exon 14 Mutations in Lung Cancer to Eight MET Tyrosine Kinase Inhibitors In Vitro

Toshio Fujino, MD, Yoshihisa Kobayashi, MD, Kenichi Suda, MD, Takamasa Koga, MD, Masaya Nishino, MD, Shuta Ohara, MD, Masato Chiba, MD, Masaki Shimoji, MD, Kenji Tomizawa, MD, Toshiki Takemoto, MD, Tetsuya Mitsudomi, MD*

Department of Thoracic Surgery, Kindai University Faculty of Medicine, Osaka-Sayama, Japan

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ABSTRACT

Background: MNNG HOS transforming gene (*MET*) exon 14 mutations in lung cancer, including exon 14 skipping and point mutations, have been attracting the attention of thoracic oncologists as new therapeutic targets. Tumors with these mutations almost always acquire resistance, which also occurs in other oncogene-addicted lung cancers. However, the resistance mechanisms and treatment strategies are not fully understood.

Methods: We generated Ba/F3 cells expressing *MET* exon 14 mutations by retroviral gene transfer. The sensitivities of these cells to eight MET-tyrosine kinase inhibitors (TKIs) were determined using a colorimetric assay. In addition, using N-ethyl-N-nitrosourea mutagenesis, we generated resistant clones, searched for secondary *MET* mutations, and then examined the sensitivities of these resistant cells to different TKIs.

Results: Ba/F3 cells transfected with *MET* mutations grew in the absence of interleukin-3, indicating their oncogenic activity. These cells were sensitive to all MET-TKIs except tivantinib. We identified a variety of secondary mutations. D1228 and Y1230 were common sites for resistance mutations for type I TKIs, which bind the active form of MET, whereas L1195 and F1200 were common sites for type II TKIs, which bind the inactive form. In general, resistance mutations against type I were sensitive to type II, and vice versa.

Conclusions: MET-TKIs inhibited the growth of cells with *MET* exon 14 mutations. We also identified mutation sites specific for TKI types as resistance mechanisms and complementary activities between type I and type II inhibitors against those mutations. These finding should provide relevant clinical implication for treating patients with lung cancer harboring *MET* exon 14 mutations.

Keywords: Lung cancer; Drug resistance; Mutation; MET proto-oncogene; Receptor tyrosine kinase

Introduction

The *MET* gene was originally identified as a part of the oncogenic protein TPR-MET, which was isolated from an osteosarcoma cell line treated with a chemical carcinogen.[1] Later, MET was found to be identical to a receptor for hepatocyte growth factor (HGF) or scatter factor.[2] It is well known that papillary renal carcinoma often harbors an *MET* mutation, mainly in its kinase domain.[2] In the case of lung cancer, several *MET*

*Corresponding author.

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Address for correspondence: Tetsuya Mitsudomi, MD, Department of Thoracic Surgery, Kindai University Faculty of Medicine, 377-2 Ohno-Higashi, Osaka-Sayama, 589-8511, Japan. E-mail: mitsudom@med.kindai.ac.jp

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mutations have been described. Among them, somatic mutations that disrupt the splicing consensus are relatively common in NSCLC and result in the elimination of exon 14 from the *MET* gene, which was first identified in 2005.[3] Because exon 14 of the *MET* gene contains Y1003, a binding site for CBL, an E3-ubiquitin ligase, exon 14 skipping results in protection from protein degradation by a proteasome, and thus, continuous activation of downstream signaling pathways.[4,5]

MET exon 14 skipping mutations were reported to be present in approximately 3.0% of lung adenocarcinomas.[6-11] In addition, *MET* exon 14 point mutations (Y1003C/F/N/S and D1010H/N/Y) have been reported in lung cancer at a lower frequency.[6,11,12] Of them, Y1003F is known to interrupt CBL binding.[13] D1010 is located at the boundary of exon 14 and 15, which is also an *MET* breakpoint in the case of TPR-MET. However, the consequence of the D1010X mutation is unknown. We previously reported that there are various mechanisms of disruption of splicing consensus at the genome level, although all of these genomic alterations result in MET exon 14 skipping. We also found that *MET* exon 14 skipping is mutually exclusive with EGFR/KRAS/HER2 mutations, strongly suggesting that *MET* exon 14 skipping is another oncogenic driver.[14]

Shrinkage of NSCLCs harboring *MET* exon14 skipping upon treatment with crizotinib, an MET/ALK/ROS1 inhibitor, was initially reported in 2015.[15,16] Currently, several other MET-tyrosine kinase inhibitors (TKIs) are under clinical development. MET-TKIs are generally classified into three types depending on the region of MET that interacts with the TKI in question. Type I inhibitors represents adenosine triphosphate (ATP) competitors that bind to the ATP-binding pocket of the active form (DFG-in). Type I is further subdivided into Ia and Ib, according to their interaction with the solvent front residue G1163. Crizotinib belongs to type Ia, whereas capmatinib, tepotinib and savolitinib belong to type Ib. Type II TKIs represents ATP competitors that bind to the inactive state (DFG-out) and contain cabozantinib, merestinib and glesatinib.[17] Tivantinib is a type III inhibitor, which is an allosteric inhibitor. However, there has been no systematic cross-comparison of these drugs for *MET* exon 14 skipping because in vitro model systems have been limited.

Although MET inhibitors show initial efficacy, almost all patients inevitably acquire resistance to these drugs, similar to what occurs when TKI treatments are used against known driver oncogene-addicted NSCLCs, such as EGFR, ALK, and ROS1.[18-20] There have been several anecdotal reports describing the mechanisms of crizotinib resistance, such as secondary *MET* mutations or activation of a bypass signaling pathway, such as *KRAS* mutation and amplification.[21-24] However, resistance mechanisms to drugs other than crizotinib are poorly understood.

In this study, using Ba/F3 cells harboring *MET* exon 14 mutations, we evaluated the activities of eight MET-TKIs belonging to three types of MET-TKIs. Subsequently, we induced mutations to cause acquired resistance using the mutagen N-ethyl-N-nitrosourea (ENU), searched for secondary *MET* mutations, and evaluated the sensitivities of these cell lines to other MET-TKIs.

Materials and Methods

Cell Lines

The interleukin-3 (IL-3) dependent murine pro-B cell line (Ba/F3) and myelomonocytic, macrophage-like, BALB/c mouse leukemia cells (WEHI-3) were provided by the RIKEN Bio Resource Center (Tsukuba, Japan). WEHI-3 secretes IL-3 into the medium and was thus used as a source of IL-3 for Ba/F3. The Hs746t human gastric cancer cell line, which is known to harbor an *MET* exon 14 skipping mutation, was purchased from American Type Culture Collection (Manassas, Virginia). Gp2-293 packaging cells were purchased from Takara (Kusatsu, Japan).

Ba/F3 cells were maintained in Roswell Park Memorial Institute (RPMI)–1640 (Wako, Osaka, Japan) medium supplemented with 10% fetal bovine serum (FBS) (Sigma-Aldrich, St. Louis, Missouri) at 37°C in a humidified incubator with 5% CO_2. Gp2-293 cells and Hs746t cells were maintained in Dulbecco's modified Eagle medium (DMEM) with 10% FBS at 37°C in a humidified incubator with 5% and 10% CO_2, respectively.

Establishment of Ba/F3 Cells Expressing MET Mutations

The pBABE-puro TPR-MET retroviral vector was purchased from Addgene (Watertown, Massachusetts) (plasmid #10902). In addition to *MET* exon 14 skipping, we introduced Y1003F, D1010Y, and Y1230C mutations because Y1003F/N and D1010H/N/Y have been reported to occur in lung cancer (Fig. 1*A*). Y1230C in the *MET* TK domain is reported to be an oncogenic mutation in papillary renal cell carcinoma.[25]

The wild-type (WT) *MET* coding sequence, including the Kozak sequence, was subcloned from pDONR223-MET (Addgene, plasmid #23889) into the multicloning site of pRetroX IRES-ZsGreen1 retroviral vector (Clontech, Fremont, California) using the In-Fusion HD Cloning kit (Takara) according to the manufacturer's instructions. In addition, the TPR-MET retrovirus, which had been shown to cause the IL-3–independent

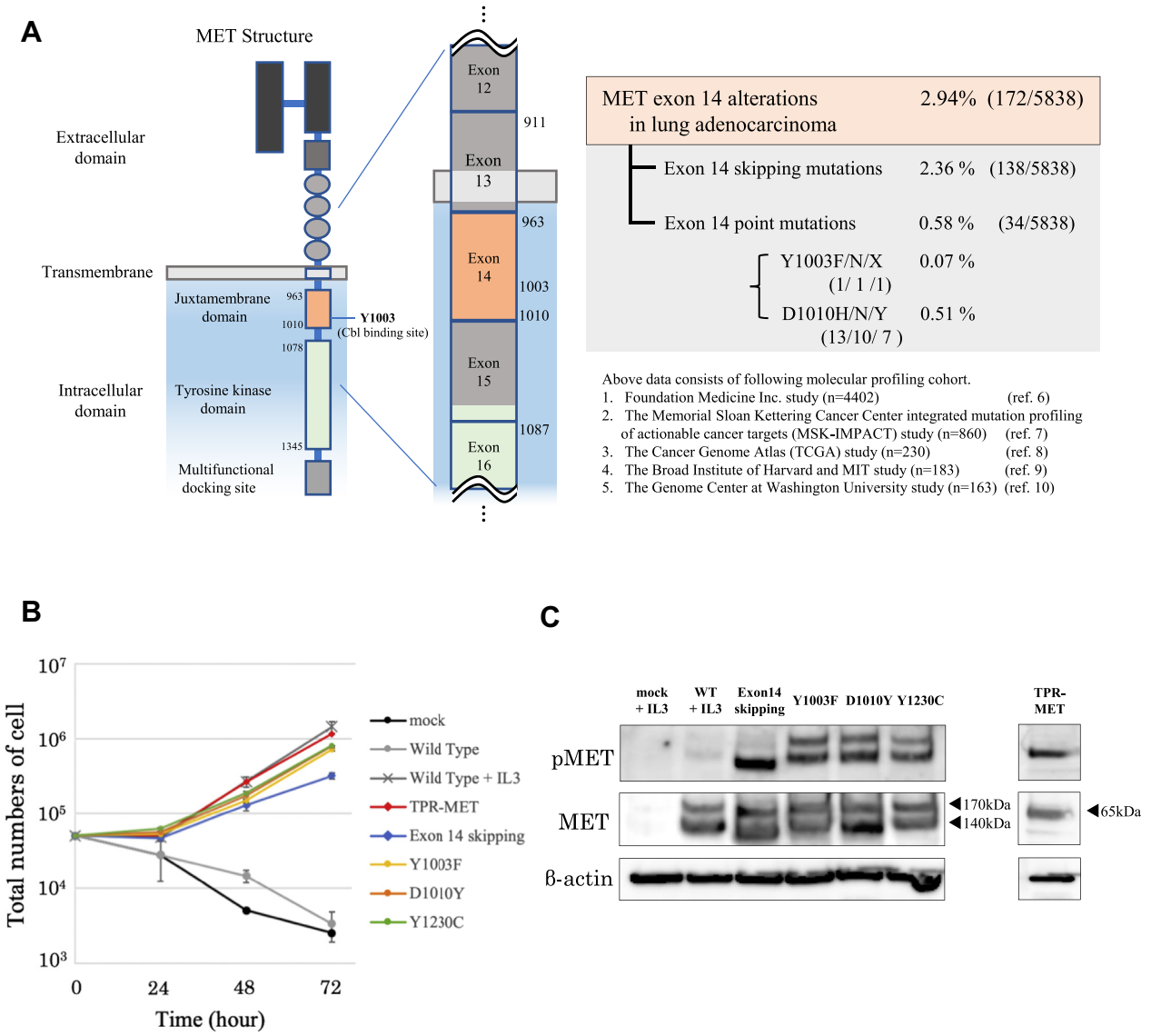
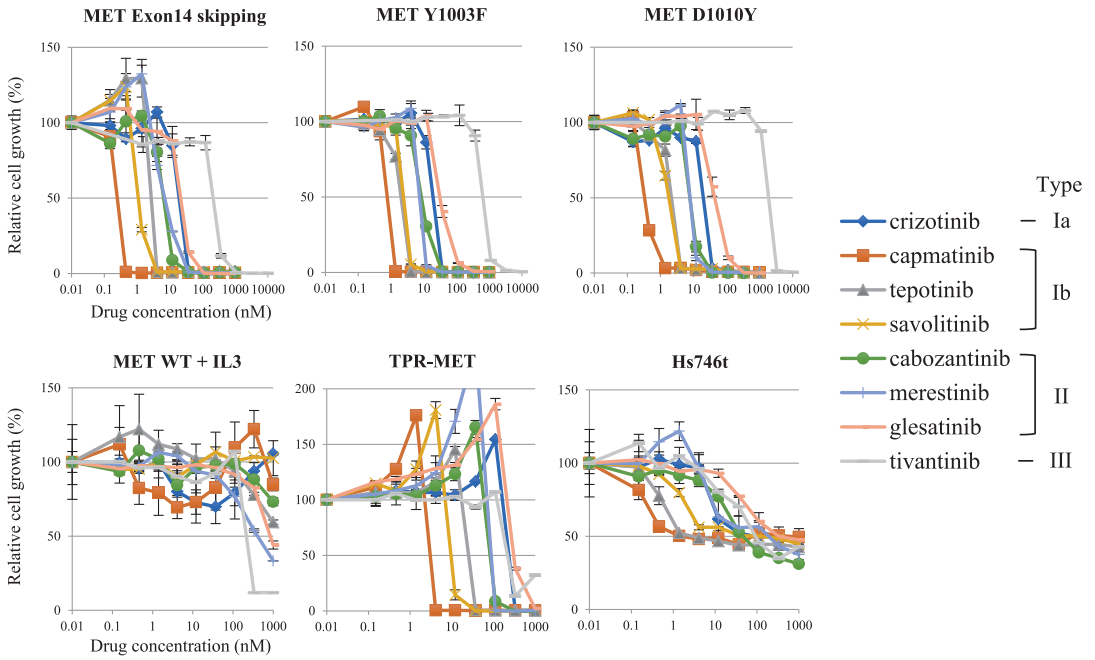


Figure 1. *A*, The structure of MET and frequency of *MET* exon 14 alterations in lung adenocarcinoma. *B*, Establishment of Ba/F3 cells expressing MET mutations and assessment of interleukin 3 (IL-3)-independent growth. Cell number was counted every 24 hours in triplicate. *C*, Western blot of total and phosphorylated MET of transfected Ba/F3 cells. The size of MET with exon 14 skipping was approximately 6 kDa smaller than that of wild-type (WT) MET, as expected.

growth of Ba/F3 cells, was used as a positive control.[26] Using pRetroX IRES-ZsGreen1 carrying WT *MET* as a template, the retroviral vector constructs encoding *MET* exon 14 skipping and the Y1003F, D1010Y, and Y1230C mutations were generated by the Prime STAR Mutagenesis Basal Kit (Takara). All mutations were confirmed by direct sequencing. These viral vectors and a pVSV-G vector (Clontech) were co-transfected to Gp2-293 cells using the Xfect transfection reagent (Clontech). After 48 hours of transfection, the culture medium, including viral particles, was collected, mixed with RetroX concentrator (Clontech) and incubated overnight at 4°C. The viral particles were concentrated by centrifugation at 1500 xg for 45 minutes, and the viral pellet was re-suspended in DMEM and added to Ba/F3 cells. Ba/F3 cells infected with mock (pRetroX), WT *MET*, exon 14 skipping, Y1003F, D1010Y, or Y1230C were collected by green fluorescent protein–based fluorescence-activated cell sorting using the BD fluorescence-activated cell sorting Aria Cell Sorter Special Order Research Product (BD Biosciences, Franklin Lakes, New Jersey). The sequencing chromatograms of each transfected Ba/F3 cell are shown in Supplementary Figure 1. There were no other mutations except for the established mutation in the juxtamembrane and TK domain.

A



B

Origin	Mutation	Type Ia	Type Ib			Type II			Type III
		crizotinib	capmatinib	tepotinib	savolitinib	cabozantinib	merestinib	glesatinib	tivantinib
Ba/F3	Wild type + IL3	> 1000	> 1000	> 1000	> 1000	> 1000	408	842	715
	Exon 14 skipping	22	0.6	3.0	2.1	7.8	8.1	21	206
	Y1003F	19	0.6	2.4	2.5	8.6	6.9	26	633
	D1010Y	20	0.4	1.3	2.0	7.5	6.4	18	609
	TPR-MET	68	2.2	24	8.8	86	28	189	736
Human gastric cancer	Hs746t (Exon 14 skipping)	47	1.6	2.2	119	46	191	331	92
C_{max} (nM)		1123	9068	2621	10150	2810	539	906	7465
C_{trough} (nM)		540	N/A	N/A	135	N/A	N/A	N/A	N/A

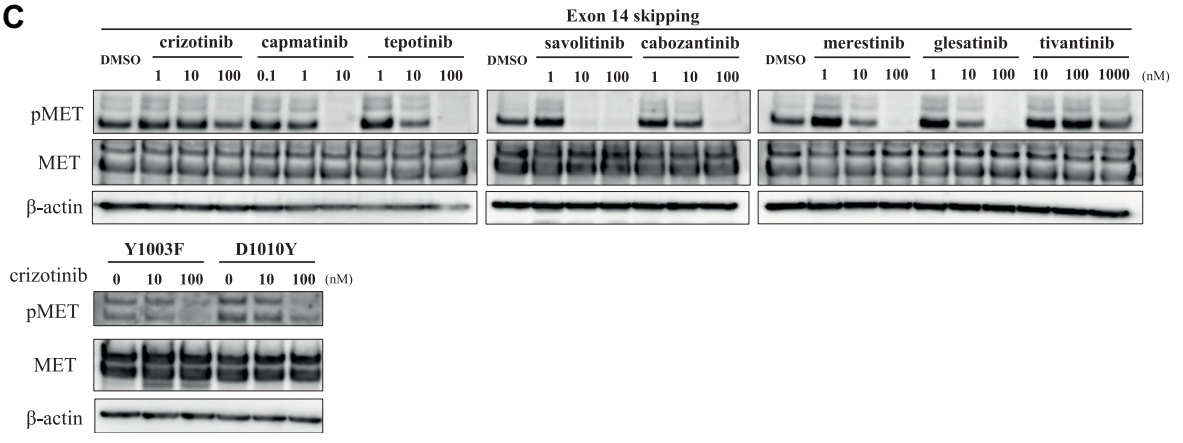
$IC_{50} \leqq 50nM$

$50 < IC_{50} < 200nM$

$IC_{50} \geqq 200nM$

N/A : Not Available

C



Reagents

Eight MET-TKIs (type Ia, crizotinib; Ib, capmatinib, tepotinib, and savolitinib; type II, cabozantinib, glesatinib, and merestinib; type III, tivantinib) were evaluated in this study. Savolitinib was purchased from MedChem Express (Monmouth Junction, New Jersey). All other drugs were purchased from Selleck Chemical (Houston, Texas). These drugs were dissolved in dimethyl sulfoxide (DMSO) (Sigma-Aldrich) and stored at -20°C until use.

IL-3–Independent Cell Growth Assay

Fifty thousand Ba/F3 cells infected with each virus were seeded in 6-well plates and grown in the absence of IL-3. The total number of cells in each well was counted every 24 hours in triplicate using OneCell Counter (Bio Medical Science, Tokyo, Japan).

Cell Growth-Inhibition Assay

A total of 3 to 5 $\times$ 10^3 cells were seeded in each well of 96-well plates. After 24 hours, MET-TKIs were added at the indicated concentrations. After a 72-hour-incubation, 10 μL of the Cell Counting Kit-8 solution (Dojindo Laboratories, Kumamoto, Japan) was added to each well, and the plates were incubated for an additional 3 hours. The absorbance at 450 nm was read using a multiplate reader (Tecan, Mannedorf, Switzerland). The percentage of viable cells was evaluated and compared with those of DMSO-treated controls.

Establishment of MET-TKI-Resistant Clones Through ENU Mutagenesis and Identification of MET Secondary Mutations

MET-TKI–resistant clones were established by ENU (Sigma-Aldrich) mutagenesis, as previously described.[27] Briefly, Ba/F3 cells expressing MET exon 14 skipping were exposed to 100 μg/mL ENU for 24 hours. The cells were washed and cultured in RPMI with 10% FBS for 24 to 36 hours. Subsequently, 1 $\times$ 10^4 to 1 $\times$ 10^5 cells were plated in 96-well plates in the presence of each MET-TKI. Although it may be desirable to use clinically achievable concentrations of each MET-TKI, the C_{trough} values of most of these drugs were not available. Therefore, we began by adding each MET-TKI at a concentration that inhibits 50% (IC_{50}), obtained in precedent experiments. As a result, the drug concentrations for resistance selection were 1.5- to 30-fold higher than the IC_{50} values. The medium containing each MET-TKI was changed twice weekly for 3 weeks. When the cell growth was observed macroscopically, the cells were transferred to 24-well plates and incubated until they reached confluence in the absence of drug. Using the RNeasy Plus Mini Kit (Qiagen, Hilden, Germany), the total RNA was isolated from resistant clones. Then, RNA was converted to cDNA using ReverTra Ace (TOYOBO, Osaka, Japan). The *MET* gene encompassing from exon 13 to 21 were sequenced using a Genetic Analyzer 3130 or 3500XL (Applied Biosystems, Waltham, Massachusetts).

Western Blot Analysis

Ba/F3 cells with *MET* mutations were treated with MET-TKIs at the indicated concentrations for 2 hours. The cells were then washed twice with phosphate-buffered saline and re-suspended in lysis buffer. Lysates were quantified using a BCA protein assay (Bio-Rad, Hercules, California), and lysates containing 9 μg of protein were electrophoresed and transferred to polyvinylidene difluoride membranes. Immunoblotting was performed according to the antibody manufacturers' instructions. Blocking buffer, antibody dilutions, and incubation conditions were obtained from Takara. Antibodies against pMET (#3126), total MET (#8198), and β-actin (#4970) and secondary anti-rabbit immunoglobulin G, horseradish peroxidase (HRP)–linked antibody (#7074) were purchased from Cell Signaling Technology (Danvers, Massachusetts). Immunoblots were scanned using an Amersham Imager 680 (GE Healthcare, Chicago, Illinois).

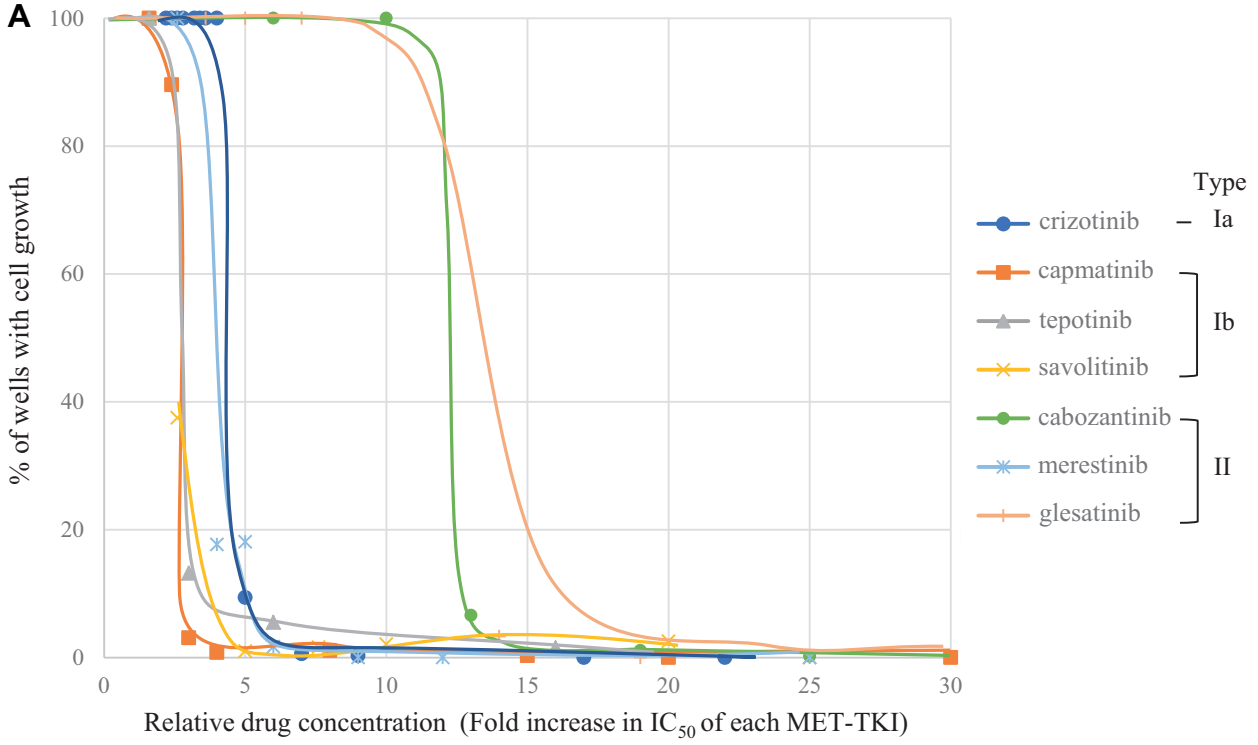
Results

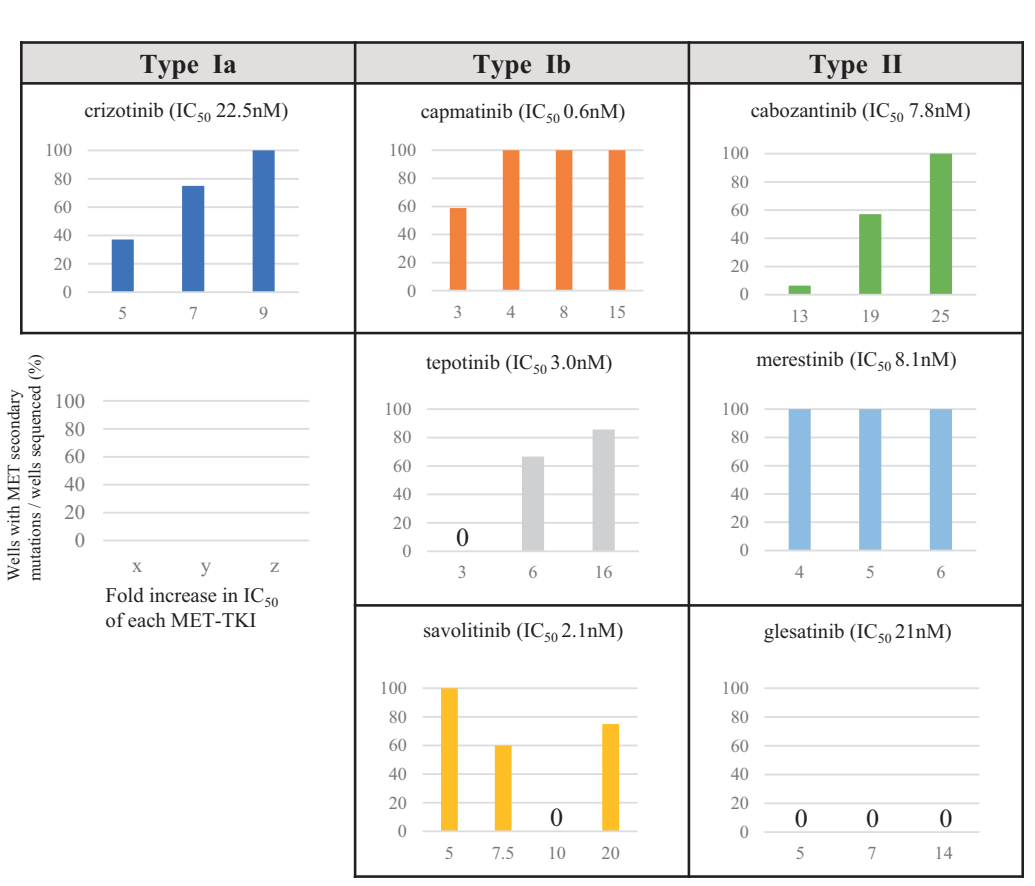
Ba/F3 Cells Expressing MET Exon 14 Mutations Became IL-3 Independent

We transduced several mutant forms of the *MET* genes to Ba/F3 cells to determine whether they could sustain growth in the absence of IL-3. Whereas Ba/F3 cells transfected with WT MET did not proliferate under this condition, those transfected either with MET exon 14 skipping and point mutations (Y1003F, D1010Y, and Y1230C) each proliferated without IL-3, suggesting their oncogenic activity (Fig. 1*B*).

Western blot experiments showed that Ba/F3 cells with *MET* exon 14 skipping resulted in strong MET phosphorylation (Fig. 1*C*). Ba/F3 cells transfected with WT *MET* expressed total MET to levels similar to those with *MET* mutations; however, the cells expressing WT *MET* did not express phosphorylated MET even in the presence of IL-3.

Figure 2. *A*, Growth inhibition curves of Ba/F3 cells harboring MET mutations and Hs746t cells treated for each MET-tyrosine kinase inhibitor (TKI). The assay was performed in triplicate, and the mean concentration that inhibits 50% (IC_{50}) value was calculated. *B*, The IC_{50} values and plasma drug concentrations obtained from the literature are presented. The IC_{50} values are expressed according to the indicated colors. *C*, Western blot analyses of transfected Ba/F3 cells in the presence of MET-TKIs at the indicated drug concentrations for 2 hours.


A
% of wells with cell growth
Relative drug concentration (Fold increase in IC50 of each MET-TKI)
Type
crizotinib
capmatinib
tepotinib
savolitinib
cabozantinib
merestinib
glesatinib
Ia
Ib
II


B
Type Ia
Type Ib
Type II
crizotinib (IC50 22.5nM)
capmatinib (IC50 0.6nM)
cabozantinib (IC50 7.8nM)
tepotinib (IC50 3.0nM)
merestinib (IC50 8.1nM)
savolitinib (IC50 2.1nM)
glesatinib (IC50 21nM)
Wells with MET secondary mutations / wells sequenced (%)
Fold increase in IC50 of each MET-TKI

Type Ia/b and II MET-TKIs Had Potent Activity Against Ba/F3 Cells With MET Exon 14 Mutations

Next, we evaluated the sensitivities of these cells to various MET-TKIs. The growth inhibition curves and IC_{50} values are shown in Figures 2*A* and 2*B*, respectively.

The IC_{50} values for all type Ia/b and II MET-TKIs were less than 30 nM, suggesting a high efficacy of these drugs. However, the IC_{50} of tivantinib was greater than 200 nM. In general, there were no differential sensitivities among the various MET exon 14 mutations to a given TKI. We also noted that capmatinib had the lowest IC_{50} values for all MET mutants, including TPR-MET and Hs746t. We then evaluated MET phosphorylation upon MET-TKI treatment (Fig. 2*C*). Inhibition of MET phosphorylation was in accordance with the growth inhibition assay.

ENU Mutagenesis Revealed a Difference in Likelihood to Develop Resistance According to Drugs

To understand the mechanisms of acquired resistance for MET-driven tumors, we derived resistant clones using ENU mutagenesis. Because it was anticipated that the emergence of resistant clones would be affected by MET-TKI concentrations, we evaluated several different conditions. Figure 3*A* shows the relationship between the percentage of wells with resistant cells relative to all tested wells and drug concentrations, expressed as fold-IC_{50}. In general, the proportion of wells with the emergence of resistant clones decreased as the drug concentration increased. One hundred percent of wells from the 96-well plates were positive for resistant clones when cabozantinib or glesatinib was present, even at concentrations as high as 10 times the IC_{50} concentration (Fig. 3*A*). In contrast, less than 10% of wells were positive in the presence of capmatinib, savolitinib, or tepotinib at five times the IC_{50} concentration. A drug whose curve is closer to lower left corner of the graph is considered to be more unlikely to develop resistance. In this sense, capmatinib, tepotinib, and savolitinib, followed by crizotinib and merestinib, were considered to be unlikely to do so.

Each MET-TKI Had Distinct Characteristics for the Proportion of Secondary MET Mutations as Well as Mutation Spectrums

We then sequenced the *MET* TK domain of 201 resistant clones to search for secondary mutations. Generally speaking, as the drug concentration increased, and the incidence of acquiring secondary mutations became greater (Fig. 3*B*). Differences were also evident based on the drugs used. For capmatinib and merestinib, secondary mutations were always present at four-fold the IC_{50} value or higher. On the other hand, we could not identify any secondary mutations in the case of glesatinib, although we generated clones resistant to glesatinib four times using ENU mutagenesis and sequenced 15 clones.

We identified 80 secondary *MET* mutations in 201 resistant clones. Of them, 26 were different missense mutations occurring at 12 codons (Fig. 4 and Supplementary Table 1). D1228 and Y1230 were common sites after exposure to type Ia/b inhibitors, whereas L1195 and F1200 were common for type II inhibitors. The G1163 mutation was only shared by crizotinib (Ia) and tepotinb (Ib). G1090 and V1092 mutations were shared by all type I inhibitors, except for tepotinib.

Activities of MET Inhibitors Against Each Secondary Mutation

We next evaluated the sensitivities of the above-mentioned resistant clones to other MET-TKIs (Fig. 5). Ba/F3 cells harboring Y1230C/D/S/H/N or D1228E/G/H/N in an activation loop were resistant to type I but sensitive to type II inhibitors. However, D1228A/Y showed weak to moderate resistance to any type II inhibitor. Conversely, L1195F/V and F1200I/L were resistant to type II TKIs but sensitive to type Ib inhibitors. For other secondary mutations, including G1163E/R, capmatinib and merestinib were more active ($IC_{50} < 10$ nM) than crizotinib or cabozantinib.

Discussion

Using the Ba/F3 system, we showed that MET exon 14 skipping mutations, as well as Y1003F and D1010Y, could confer IL-3–independent growth in Ba/F3 cells, and these cells became sensitive to both type I and type II MET inhibitors, but not to type III. Although efficacy of MET-TKIs for exon 14 skipping has been indicated by clinical observations, we were able to show that Y1003F and D1010Y were also sensitizing mutations.[15,16]

Previously, we reported an in vitro model for *MET* exon 14 skipping created using the CRISPR/Cas9

Figure 3. *A*, Relationship between the emergence of resistance and drug concentration. Ordinate: percentage of wells with growth relative to the all wells. Abscissa: each drug concentration relative to their concentration that inhibits 50% (IC_{50}) value. Because the number of cells seeded onto each well changed according to the number of positive wells, the data were standardized to the inoculum size of 1×10^4 cells/well. When we obtained 20 wells with cell growth in an experiment where 10^5 cells were seeded, it was scored as two positive wells. *B*, The relationship between the percentage of clones with secondary mutations relative to all resistant clones and drug concentration.

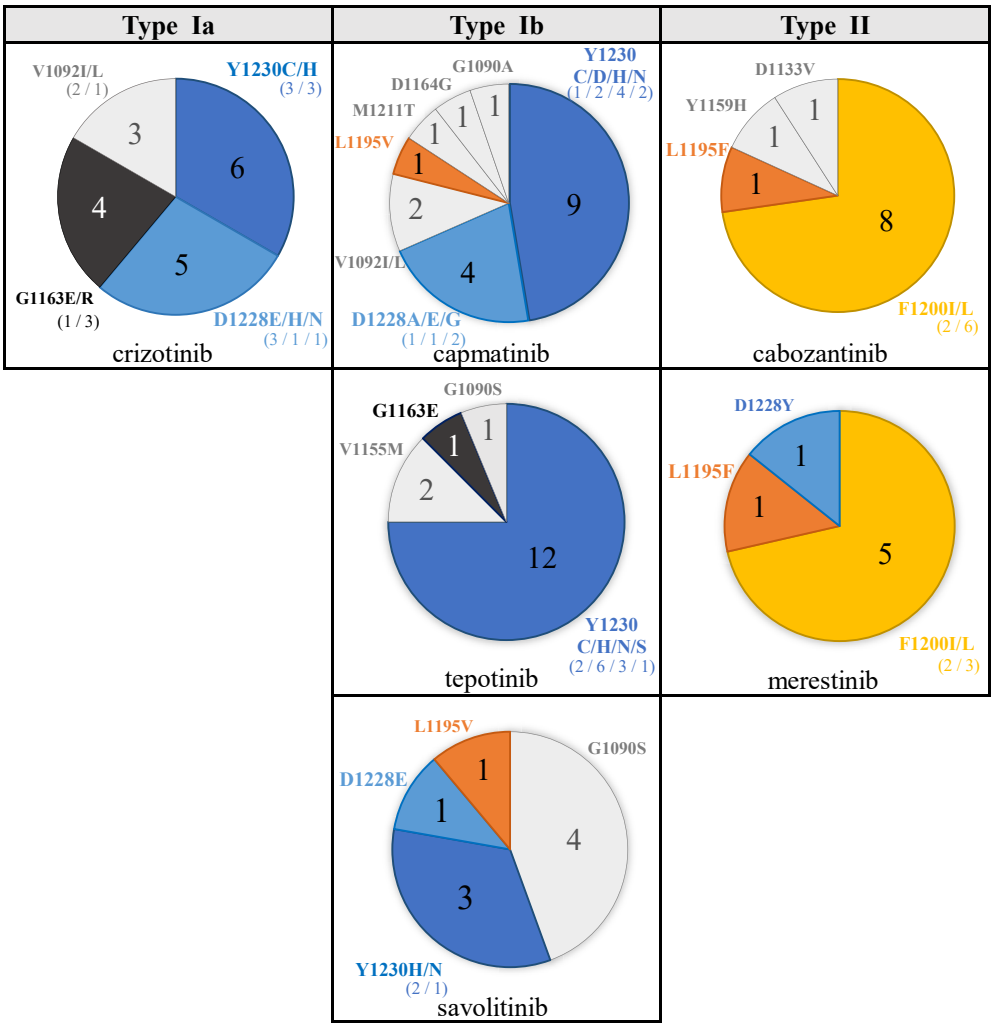


Figure 4. The breakdown of secondary mutations in each MET-tyrosine kinase inhibitor (TKI) obtained from the N-ethyl-nitrosoura mutagenesis experiments.

technology.[28] However, the degree of dependence of the *MET* gene was not very strong, probably because the parental HEK293 can survive without oncogene transduction, resulting in relatively low sensitivity to crizotinib, which hampered further experiments. Similarly, Lu et al.[29] used the CRISPR/Cas9 system and showed that endogenously expressed levels of MET exon 14 skipping transformed human epithelial lung cells in an HGF-dependent manner, which is not very suitable for the drug sensitivity assay described here. Engstrom et al.[30] created NIH3T3 cells expressing MET exon 14 skipping by retroviral gene transfer, but these cells are also HGF dependent. The NCI-H596 lung cancer cell line has been known to harbor *MET* exon 14 skipping. However, likely because of the co-existence of the PIK3CA mutation, this cell line is not sensitive to MET inhibitors.[31] Our in vitro model was suitable for the evaluation of various MET TKIs and other drugs as well as analyses of resistance mechanisms.

We showed that Ba/F3 cells upon transduction of mutated *MET* genes found in lung cancer became sensitive to all MET TKIs, except tivantinib. Capmatinib (type Ib) had the lowest IC_{50} for all MET mutations and also had a high C_{max} value (Fig. 2). This finding suggests a high efficacy of capmatinib in human subjects as well. According to recent data from phase II trials, the overall response rate for capmatinib (72%) was numerically higher than that for crizotinib or tepotinib (32% and 59%, respectively).[32-34]

To gain insights into acquired resistance against MET-TKIs, we used ENU mutagenesis. This method has been used for the efficient establishment of resistant clones in various situations.[35] ENU is reported to preferentially induce AT to TA transversions and GC to AT transitions.[36] Therefore, there is a concern that mutations induced by ENU are artificial and thus less relevant to those occurring in patients. However, according to Brammeld et al.,[37] of the more than 10,000 missense

Mutations		Type Ia	Type Ib			Type II		
		crizotinib	capmatinib	tepotinib	savolitinib	cabozantinib	merestinib	glesatinib
Exon 14 skipping (parental)		22	0.6	3.0	2.1	7.8	8.1	21
1090	G1090A	176	7.3	145	69	0.3	0.8	1.7
	G1090S	41	3.0	42	24	0.7	1.3	6.7
1092	V1092I	292	2.8	2.6	2.9	16	13	5.7
	V1092L	223	2.5	2.3	13	1.8	10	6.5
1133	D1133V	30	0.9	2.6	7.3	88	29	62
1155	V1155M	89	3.4	23	16	17	5.6	22
1159	Y1159H	181	0.9	22	8.1	107	28	46
1163	G1163E	91	0.9	10	3.3	49	9.3	89
	G1163R	> 1000	2.5	70	8.5	62	14	66
1164	D1164G	213	7.2	74	28	25	9.7	24
1195	L1195F	23	0.3	2.6	1.8	> 1000	83	90
	L1195V	235	8.1	55	22	118	44	236
1200	F1200I	199	6.1	45	30	694	212	275
	F1200L	23	0.8	8.0	7.7	229	109	111
1211	M1211T	26	2.8	24	11	22	7.5	18
1228	D1228A	> 1000	> 1000	> 1000	> 1000	200	89	216
	D1228E	690	137	> 1000	573	37	19	30
	D1228G	319	697	> 1000	431	72	23	46
	D1228H	665	> 1000	> 1000	> 1000	79	25	38
	D1228N	> 1000	> 1000	> 1000	> 1000	36	26	22
	D1228Y	> 1000	477	> 1000	> 1000	539	149	74
1230	Y1230C	645	> 1000	> 1000	> 1000	8.4	7.4	12
	Y1230D	698	> 1000	> 1000	> 1000	16	5.5	11
	Y1230S	811	> 1000	> 1000	> 1000	23	12	14
	Y1230H	216	401	> 1000	> 1000	20	8.2	19
	Y1230N	> 1000	> 1000	> 1000	> 1000	19	4.1	14

$IC_{50} \leqq 50nM$	$50 < IC_{50} < 200nM$	$IC_{50} \geqq 200nM$

Figure 5. The concentration that inhibits 50% (IC_{50}) values of type I and type II MET-tyrosine kinase inhibitors (TKIs) against Ba/F3 cells expressing MET exon 14 skipping mutations plus each secondary mutation. Underlines indicate which MET-TKI induced the resistant mutation.

mutations induced by ENU, only 51% were either AT to TA transversions and GC to AT transitions. In our experiments, only 31% of mutations belonged to these base substitutions (Supplementary Fig. 2). Furthermore, most if not all of the resistant mutations found in ENU mutagenesis in the case of EGFR-TKI are also found in patients.[27]

We derived many resistant clones using ENU mutagenesis. Efficacy in the emergence of resistance inversely correlated with drug concentration, as expected. There was also a difference of likelihood of developing resistance based on the drug. Generally, type Ib inhibitors were more unlikely to develop resistance than type II inhibitors. We also noted that the percentages of secondary *MET* TK mutations were in general also proportional to the drug concentration. Previously, we reported that rare and weakly resistant secondary mutations tended to be present when resistant clones were selected at low concentrations of EGFR-TKI.[27] This result is likely because a high drug concentration requires stronger resistance mechanisms for cell survival.

We identified 26 unique *MET* secondary mutations occurring at 12 codons as possible resistance mechanisms. There were some common sites of secondary mutations according to the type of drug. Mutations at codons D1228 or Y1230 accounted for 64% of resistant mutations for type I TKIs, whereas mutations at codon F1200 accounted for 72% of resistant mutations for type II TKIs. In 26 mutations identified in this study, seven mutations (D1228A/N/H, Y1230C/H/S, and G1163R) have been previously reported as resistance mechanisms to crizotinib in NSCLCs harboring MET exon 14 skipping mutations (Supplementary Table 2).[22,30,38-40] Multiple mutations are detected in three of five patients. However, we did not obtain such multiple occurrences of secondary mutations in the present ENU mutagenesis system. We also identified mutations at codons V1155, Y1159, F1200, and M1211; these mutations have been previously reported as secondary mutations to NVP-BVU972 (type I) and AMG 458 (type II) in a preclinical model using Ba/F3 cells expressing TPR-MET, whereas G1090A/S, V1092I/L, and D1133V were novel mutations.[41]

When the amino acid sequence of the MET TK was aligned with those of ALK, ROS1, and EGFR according to their homology (Fig. 6*A*), four common codons (D1228, Y1230, L1195, and F1200) of the MET-resistant mutations did not have corresponding ALK/ROS1/EGFR-resistant mutations. In contrast, although EGFR T790M and ALK L1196M are frequent gatekeeper mutations responsible for acquired resistance, mutations at corresponding MET L1157 were not identified. ALK G1202 and ROS1 G2032 have been referred to as solvent front, and we found the corresponding MET G1163E/R as a resistance mechanism, especially for type Ia crizotinib, which interacts with MET G1163. The newly identified G1090A/S and V1092I/L are located in the P loop, where several resistant mutations have been reported in the cases of *ALK, EGFR* and *BCR-ABL* in chronic myeloid leukemia.[42-44]

To understand differential codon usage dependent on the type of MET-TKI, we evaluated the crystal structures of MET TK domain with crizotinib (Ia), savolitinib (Ib), and merestinib (II) (Fig 6*B* and Supplementary Fig. 3). Type I inhibitors bind to the active state of a kinase (DFG-in). Type Ia inhibitors interact with Y1230 in the MET activation loop, the hinge region, and the solvent front residue G1163, whereas type Ib inhibitors interact more strongly with Y1230 and the hinge region but have no interaction with G1163.[45] This structural difference provides a plausible explanation that mutations at Y1230 emerged for type Ia and Ib but G1163 almost exclusively emerged for type Ia inhibitors. In contrast, type II inhibitors bind to the inactive state of kinases (DFG-out), exploiting interactions inside the lipophilic pocket that emerge from flipping of the F residue of the DFG N-terminus of the activation loop.[17] In this structure, F1200 has a direct interaction with type II MET TKI, and thus, this codon was a frequent site of resistance mutation.

Reungwetwattana et al.[45] suggested switching from type I to type II inhibitors or vice versa for acquired resistance mutations to type I and II, respectively. Engstrom et al.[30] previously showed that *MET* mutations (e.g., D1228N and Y1230C/H) conferred resistance to type I inhibitors in MET-addicted SNU-638 gastric cancer cells without *MET* mutation/amplification. Engstrom et al.[30] also showed that cells with these mutations were also resistant to other type I MET-TKIs (capmatinib, savolitinib, and AMG-208) but were sensitive to glesatinib (type II) in enzymatic assays. However, the authors did not analyze cells resistant to type II MET TKI.[30] We were able to evaluate this hypothesis in a systematic manner. Crystal structure analysis also helped us to understand the complementary activities between type I and type II inhibitors for resistance mutations (Fig. 6*B*). Mutations at D1228 and Y1230 that emerged after type I inhibitor treatment and were not exposed on the surface of the ATP-binding pocket in the DFG-out conformation were generally sensitive to type II inhibitors, which interact with MET in the DFG-out conformation. In contrast, resistance mutations at L1195 and F1200 shared by type II inhibitors that were not exposed in the DFG-in conformation were sensitive to type I inhibitors. In addition, the G1163R solvent front mutation induced by type Ia crizotinib was sensitive to type Ib and type II MET inhibitors, especially to capmatinib, savolitinib, and merestinib. Other secondary mutations, including the newly identified G1090A/S, V1092I/L, and D1133V,

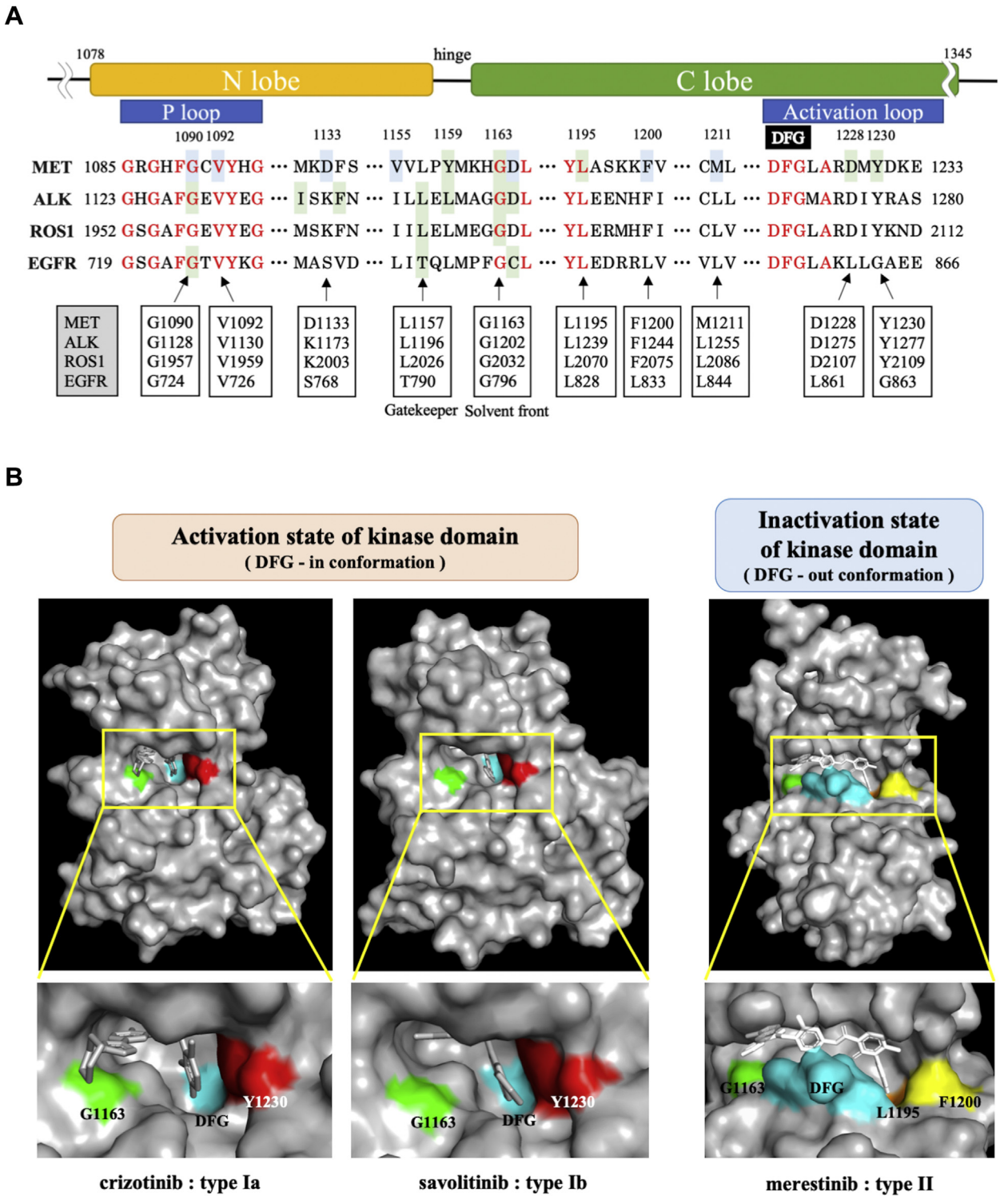


Figure 6. *A*, Secondary MET mutations were aligned with the ALK/ROS1/EGFR amino acid sequence. Amino acid residues preserved in all four kinases are shown in *red*. Amino acids for which resistant mutations have been reported in clinical samples are shown on a *green* background. Amino acids for which mutations are identified in the present study are shown on a *blue* background. *B*, Three-dimensional crystal structure of the binding model of the MET-tyrosine kinase inhibitor and MET tyrosine kinase domain. Crizotinib (type Ia) and savolitinib (type Ib) bind to active state (DFG-in) of the kinase. On the other hand, merestinib (type II) binds to inactive state (DFG-out). The common sites for resistant mutations for each drug (G1163 for type Ia, Y1230 for type Ia/b, and L1195 and F1200 for type II) are indicated by *green*, *red*, *cyan* and *yellow*, respectively. Because D1228 was located behind Y1230 in the active kinase form, it is not shown in the model of crizotinib and savolitinib. In the merestinib model, the structure of activation loop residues (1225 - 1243) was not resolved. The figures were created using the PyMOL software. The crystal structure information was obtained from Protein Data Bank, and the PDB identifier was as follows: crizotinib, 2WGJ; savolitinib, 3CCN; merestinib, 4EEV.

were still sensitive to other types of drugs different from the drug that induced the secondary mutation. In accordance with our results, mutations at D1228 and Y1230 were reported to be resistant to type I inhibitors but sensitive to type II inhibitors in vitro.[30,46,47] In addition, with regard to the Y1230H mutation, it was found that when an NSCLC patient with *MET* exon 14 skipping acquired resistance against crizotinib (type Ia), the patient could be successfully treated with glesatinib (type II).[30]

Switching type I to type II or the opposite may not always work for overcoming each resistance. Some secondary mutations such as D1228A/Y were resistant to both type I and type II MET-TKIs. Additionally, concurrent aberrations such as *KRAS* mutation or amplification, *MET* amplification and upregulation of HGF may also influence sensitivity to MET-TKIs.[23,24] Not all such mechanisms cannot be evaluated in our Ba/F3 system. Combination of MET-TKI with other agents such as MET/HGF antibodies and MET antibody drug conjugate, which are under clinical development, may be options for MET-TKI refractory lung cancer harboring *MET* exon 14 alterations.

Conclusions

Using the Ba/F3 system, we showed that *MET* exon 14 skipping and point mutations found in NSCLC conferred IL-3–independent growth and sensitized cells to MET-TKIs. These findings further underscore the importance of identifying these mutations as actionable driver gene alterations. We also identified mutation patterns specific for TKI types as resistance mechanisms and complementary activities between type I and type II inhibitors against these resistant mutations. These finding should provide relevant clinical implication for treating patients with lung cancer harboring *MET* mutations.

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Supplementary Data

Note: To access the supplementary material accompanying this article, visit the online version of the *Journal of Thoracic Oncology* at www.jto.org and at https://doi.org/10.1016/j.jtho.2019.06.023.

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