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Novel MET inhibitor's efficacious effect in clinically relevant MET mutations

MET 억제제의 mutation별 효능 평가

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서 민 지

ABSTRACT

Novel MET inhibitor's efficacious effect in clinically relevant MET mutations

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Hepatocyte Growth Factor Receptor (HGFR) is also known as Mesenchymal-epithelial transition factor (MET). HGF is the only known ligand for *MET*. *MET* is required for wound healing, hematopoiesis, and liver regeneration. Activation of *MET*, which is achieved by binding of HGF, induces cell invasion and metastasis. *MET* expresses the oncogenic characteristics in several ways; *MET* amplification, over-expression, and mutations lead to constitutive activation of *MET*. Those alterations activate downstream signaling responsible for cell proliferation, motility, transformation, and survival. Across all the cancer types, *MET* alternation is frequently reported. Global pharmaceutical companies are currently developing MET targeting drugs. However, currently, there is no FDA approved MET inhibitors. Because of this medical unmet need, ABN401, a novel ATP competitive MET inhibitor was designed. In this study, we will show ABN401's specificity in

MET and its inhibitory effect in known clinically relevant *MET* mutations. To find out the *MET* specificity *in vitro*, *MET* amplified cell lines and non-*MET* expressing cell lines were chosen then treated with ABN401. *In vitro, in silico*, and recombinant protein study result suggest that ABN401 selectively inhibits *MET*. In addition, ABN401 has efficacy in clinically relevant *MET* mutations. For mutations, which ABN401 has an effect on, phosphorylation of *MET* was blocked by ABN401 dose dependently. As a result, this study suggests that ABN401 is the potent MET inhibitor that has a therapeutic effect on patients with *MET* alterations. Also, ABN401's effect on mutation suggests which patients should be included or excluded from clinical trials.

Keywords : MET, Mutation, Hepatocyte growth factor (HGF), ABN401, Patient Selection

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LIST OF ABBREVIATIONS

MET	**Mesenchymal epithelial transition factor**
HGF	**Hepatocyte Growth Factor**
HGFR	**Hepatocyte Growth Factor Receptor**
IC50	**50% inhibitory concentration**
PDX	**Patient-derived xenograft**
PRCC	**Papillary Renal Cell Carcinoma**
ccRCC	**clear cell Renal Cell Carcinoma**
nccRCC	**non-clear cell Renal Cell Carcinoma**

INTRODUCTION

MET, which is also called as HGFR, is a receptor for HGF. It is located on 7q21-q31, composed of 21 exons and 20 introns. HGF is the only known ligand for *MET*. *MET* plays a role in wound healing, liver regeneration, and hematopoiesis. Binding of HGF to MET sequentially phosphorylates tyrosine residue 1234/1235, 1349/1356, and 1003. While phosphorylating those sites, MET internalizes and recruit downstream effectors such as Gab1, SOS, and Grb2 etc. MET signaling regulates cell proliferation, survival, scattering, apoptosis, motility, invasion, and angiogenesis [9].

MET is a proto-oncogene and its oncogenic characteristics are caused by constitutive activation of MET. *MET* amplification, over-expression, and existence of activating mutant have a role in constitutive activation of MET. *MET* mutation is observed across all cancer types. For example, for NSCLC patients, *MET* exon 14 deletion is observed in 4% of total patients while 11% of head and neck cancer patients exhibits *MET* mutations. Also, it is known that 15% of PRCC patients harbor *MET* mutations. D1246H, M1149T, G1182T, V1110I, M1268T, and H1112R mutations were clinically observed in PRCC patients and some of the

mutations are known as activating mutations [34].

PRCC is a type of non-clear cell renal cell carcinoma accounts up to 15% of renal cell carcinoma. Type 1 PRCC exhibits MET alterations while type 2 PRCC has different mechanisms such as FH mutation for tumorigenesis [2]. MET-driven PRCC is caused by chromosome 7 copy gain, *MET* or *HGF* gene amplification, or *MET* mutation [5]. Currently, there is no FDA approved drug for PRCC. Therefore, PRCC patients are treated with ccRCC drugs. However, ccRCC drugs on PRCC patients have limited effect such as decreased response rate and shorter response duration. There are several clinical trials ongoing for PRCC with drugs for different target; mTOR, VEGFR2, and PDGFR target drug are given to patients. Since there is no approved drug for PRCC, Sunitinib (dual inhibitor for VEGFR2 and PDGFR) is prescribed to patients. Several clinical studies show the comparative advantage of Sunitinib over Everolimus, which is a mTOR inhibitor (ESPN study) in nccRCC [3]. They concluded that Sunitinib has slightly better efficacy than Everolimus. However, another phase II nccRCC study with Sunitinib showed that Sunitinib has no major responses to PRCC patients [4]. No significant effect of Sunitinib aware the necessity to use other drugs for PRCC patients. Savolitinib, which is a MET selective TKI, showed anti-cancer effect on PRCC patients with *MET* positive [5]. This result gives an idea that for *MET* driven PRCC patients, MET inhibitor can be a possible drug.

ABN401 is a novel, selective MET TKI, which already showed inhibitory effect on *MET* amplified NSCLC and Gastric cancer cell lines. ABN401 is a type I, ATP

competitive inhibitor that binds to an active form of MET. Also, ABN401 showed inhibitory effect on number of *MET* mutation forms. The assay for ABN401 on *MET* mutation forms was performed on the recombinant protein. However, recombinant protein may not represent the mammalian protein due to absence of post-translational modification and chaperon protein. This concern leads to the idea to establish multiple *MET* mutated cell lines to find the efficacy of ABN401 in each mutation form.

There are two purposes for this study. One is to show the specificity and efficacy of ABN401 on *MET* expressing cell and the other is to identify the efficacy of the ABN401 on each mutated form of *MET*. To find out the specificity and efficacy of ABN401 on cell lines, three cell lines, two cell lines expressing *MET* and one cell line which does not expressing *MET* were chosen.

To identify the mutations that ABN401 works on, each clinically relevant *MET* mutated form was transfected into the Ba/F3 cell line. The Ba/F3 cell line was used because of its ability to transform caused by transfection of *MET.* We expected that when the cell is oncogenically addicted to *MET*, IL-3 dependent Ba/F3 cell line can proliferate without IL-3 [7]. Mouse fibroblast cell line, NIH-3T3 was also used to generate *MET* mutant cell lines. Western blot and FACS were used to check the expression and monoclonality. After the drug treatment, Ba/F3 and NIH-3T3 cells were harvested and their expression level of phosphorylated MET and their downstream effectors were confirmed by Western blot. For the Ba/F3 cell line, cell proliferation assay was conducted. Cell viability assay was used to find out

ABN401's cytotoxicity effect and cell migration assay was conducted to figure out the clinically known mutations' characteristics for NIH-3T3 cell line.

MATERIALS AND METHODS

Chemical

ABN401 was manufactured from Olon/Ricerca (USA). For the experiment, ABN401 was reconstituted in 100% DMSO to final concentration of 10mM and stored at -20°C.

Cell culture

Murine B cell Ba/F3 cell line was purchased from RIKEN. Ba/F3 cell was propagated in RPMI 1640 (Hyclone, USA), 10% Fetal Bovine Serum (Hyclone, USA), 1% penicillin/streptomycin solution (Hyclone, USA), and 10ng/ml IL-3 (R&D system). After transfection, 500ug/ml G-418 (Enzo Life Science) contained Ba/F3 medium was added to Ba/F3 cell lines. OE33 cell line was cultured in RPMI 1640 (Hyclone, USA) with 2mM L-Glutamine with 10% FBS. H1993 cell line was cultured in RPMI 1640 (Hyclone, USA) with 10% FBS. TOV-112D cell line was cultured in 50% MCDB 105 medium with 1.5g/L sodium bicarbonate and 50% Medium 199 with 2.2g/L sodium bicarbonate. NIH-3T3 was cultured in DMEM (Hyclone, USA), 10% Fetal Bovine Serum (Hyclone, USA), and 1% penicillin/streptomycin solution (Hyclone, USA). Transfected NIH-3T3 cell line

was cultured in completed medium with 500ug/ml of G-418 (Enzo Life Science). All the cell lines were cultured at 37°C in 5% CO_2

Cell Viability assay

For OE33, H1993, and TOV-112D cell lines, $5x10^3$ Cells were seeded in 96well plate for 12 hours and treated with 10-fold increase of ABN40l from 1nM to 10uM. After 72hours, cells were treated with WST reagent (DoGen, Korea) for 2hours. For NIH-3T3 cell line, $1x10^3$ cells were seeded with or without HGF and 48 hours after seeding, ABN401 was treated 10-fold increase from 1nM to 1uM for 72 hours. The viability of cells was observed by Multi-reader (Tecan, Switzerland) at 450nm. Only DMSO treated cells were used as control (100% viability). The graph was generated by GraphPad prism (GraphPad Software, USA).

Transfection and mutagenesis

pcDNA 3.1 vector was used to express *MET* gene. Whole *MET* gene was purchased from Bionics (Korea). H1112Y, H1112L, and Y1235D mutations were generated from Enzynomics (Korea). V1110I, exon 14deletion, D1246H, D1246N, and M1268T mutations were generated by over-lap PCR method using 2x PCR master mix (BIOFACT, Korea). The following primers were used to generate mutations. V1110I forward 5'-CACTTCGGCTGTATATACCACGGCACTC-3',

V1110I reverse 5'- GAGTGCCGTGGTATATACAGCCGAAGTG-3', Exon 14 deletion forward 5'-GGCTCAAGAAAAGAAAGCAGATTAAGGATCAGTTT CCCAATAGTTCTCAGAATGGATC-3', Exon 14 deletion reverse 5'-GATCCATTCTGAGAACTATTGGGAAACTGATCCTTAATCTGCTTTCTTTTC TTGAGCC-3', D1246H forward 5'-TTCGGCCTGGCCAGGCACATGTATGA TAAGGAG-3', D1246H reverse 5'- CTCCTTATCATACATGTGCCTGGCCAGG CCGAA-3', D1246N forward 5'-TTCGGCCTGGCCAGGAACATGTATGATA AGGAG-3', D1246N reverse 5'- CTCCTTATCATACATGTtCCTGGCCAGGCC GAA-3', M1268T forward 5'- CCGTGAAATGGACGGCCCTGGAATC-3', M1268T reverse 5'-GATTCCAGGGCCGTCCATTTCACGG-3'. pcDNA3.1 was transfected with FuGene HD reagent (Promega). Two days after transfection, selection medium was used for Ba/F3 and NIH-3T3 cell lines. Every 2-3 day, cells were subcultured with 500ug/ml G-418 containing media. For Ba/F3 cell lines, 14days after selection, single cell was seeded to 96well plate (Corning, USA, #3044). Each well's picture was captured by Celligo at day 0 and day 14 (Nexcelom Bioscience LLC, USA). For NIH-3T3 cell lines, single cells were seeded to 96well plate and after colony is formed, it was trypsinized and scaled up.

Flow cytometry analysis

Flow cytometry was used to check the monoclonality of the transfected cell lines. Cells were harvested and $3x10^5$ counted by using hemocytometer. Then cell was

fixed with fixation buffer (e-Bioscience, USA) for 30 minutes. Then cells were washed with FACS buffer (0.1% FBS in DPBS, Filtered) twice then adjusted concentration of MET antibody (Abcam, USA) was added to cell and incubated for an hour. Flow Cytometric Analysis was performed with BD Calibur.

Western blot assay

Each *MET* mutant transfected Ba/F3 cell lines, H1993, and OE33 were incubated with ABN401 from 1nM to 1uM for 72hours then harvested. For NIH-3T3 cell lines, cells with HGF was incubated with HGF for 48hours then treated with ABN401 with HGF for 24hours. NIH-3T3 cell lines without HGF were treated with ABN401 for 24hours then harvested. Harvested cells were lysed with RIPA buffer (Biosesang, Korea) containing phosphatase inhibitor (Roche, USA), and protease inhibitor (Roche, USA). BCA kit (Thermo Fisher, USA) was used to measure the protein concentration. 5X loading buffer (Biosesang, Korea) was added into the protein and boiled at 100°C for 10minutes, then, 1X loading buffer (Biosesang, Korea) was used to adjust the concentration of protein to 1ug/ul. Total 20ug proteins were loaded onto the 8% SDS-polyacrylamide gel for separation and proteins were transferred to PVDF membrane (Millipore, USA). After transfer, membrane was blocked with 5% skim milk (Sigma, USA) with 0.1% Tween-20. First antibodies, *MET* antibody (Cell signaling, USA, #8198), phosphorylated MET Y1234/1235 (Cell signaling, USA, #3077), phosphorylated MET Y1349 (Cell signaling, USA, #3133), p44/42 MAPK (Cell signaling, USA, #4695),

phospho-p44/42 MAPK (Cell signaling, #4370), PARP (Cell signaling, USAS, #9532), STAT3 (Cell signaling, USA, #30835), phospho-STAT3 (Cell signaling, USA, #9138), AKT (Cell signaling, USA, #4691), and phospho-AKT (Cell signaling, USA, #4695) were diluted in TBS-T with 1% skim milk. House keeping gene antibody, GAPDH (Santa Cruz Biotechnology, sc-47724) was also diluted in TBS-T with 1% skim milk. Secondary Goat anti-rabbit IgG and Goat anti-Mouse IgG antibodies (Thermo Fisher, USA, #31460,#31430) were diluted in 1% skim milk in TBS-T. EZ Western Lumi Plus (DoGen, Korea) was used to detect the absorbance.

Migration assay

After harvesting the cells, cells were spun down at 3000rpm, 5min then resuspended with 0.5% FBS contained medium. Then, cells were counted and 50ul of $2x10^3$ cells were seeded on the top of the polycarbonate trans-well with pore size of 8.0uM (Corning, USA). 12 hours after, trans-well was transferred to other well which contains 600 μL 10% FBS. The medium for bottom chamber contained either ABN401 (100nM) or DMSO as a control. For the top chamber, 50ul of either ABN401 (200nM) or DMSO as a control were added. 6 hours after incubation, membrane was stained with hematoxylin (Sigma, USA) for 30 minutes then washed with tap water. Picture of membrane was taken.

Wound healing assay

$3x10^5$ cells were seeded on 6 well plate for 12 hours then scratched with tip. 500nM ABN401 was treated to cells for 48 hours. Then, cells were stained with hematoxylin for 30 minutes for detection.

Cell proliferation assay

Ba/F3 cells were deprived from FBS for 24 hours (0.5% FBS). Then $5x10^3$ cells were counted. One group was cultured in 10% FBS with IL-3 and the other group was cultured in 10% FBS without IL-3. 72hours after incubation, cells were collected and number of live cells were counted.

Recombinant protein assay

Recombinant protein assay was conducted from Reaction biology corp, USA. 10-doses of ABN401 was tested by 3-fold serial dilution starting at 1uM. Staurosporine was used as control compound and 10-dose by 4-fold serial dilution starting at 20uM was used for experiment. 10uM ATP was added to each well.

Patient Derived Xenograft (PDX) model

PDX model service was provided from CrownBio, Inc. The BALB/c nude mouse with age 7-9 weeks, body weight between 17.4 gram to 22.3 gram mouse

were used for PDX model experiment. Human derived tumor was inoculated to mice and treatment began when tumor volume reached approximately 170mm^3. PDX model number was LU2503 and this model's *MET* amplification was confirmed PCR based assay. ABN401 was prepared as 2.5mg/0.5mL PEG400 stock solution for 10mg/kg dose and 7.5mg/0.5mL PEG400 stock solution for 30mg/kg dose. Both solutions were diluted with 2mL of 0.1M acetate buffer pH4.0. Mouse were treated with ABN401 for three weeks and tumor volume was observed for two weeks after treatment was ended. The mouse with mean tumor size over 1500mm^3 was euthanized prior to the termination.

RESULTS

Expression of *MET* on cell lines

Western blot assay showed that OE33 and H1993 express *MET* and both cell lines constitutively expresses *MET*. TOV-112D, NIH-3T3, and Ba/F3 cell lines did not express *MET*. Because of this characteristic, NIH-3T3 and Ba/F3 cell lines were chosen to transfect *MET* mutants (Figure 2).

Selectivity of ABN401 on MET

To investigate the ABN401's selectivity on MET, recombinant protein based tyrosine kinase selectivity assay was performed. The result shows that ABN401 specifically inhibits enzymatic effect of MET (Data not shown). *In silico* data also suggests that ABN401 binds strongly to MET with forming salt bridge and hydrogen bonding (Figure 1). To find out ABN401's efficacy and specificity *in vitro*, three cell lines, OE33, H1993, and TOV-112D, were selected. OE33 is *MET* amplified cell line which expresses *ERBB2* [25,26,27]. H1993 cell line also exhibits *MET* amplification and it has known MET resistance mechanisms which are *c-MYC* expression and constitutive *EGFR* activation [23]. TOV-112D is a cell

line that does not express endogenous *MET* [28,29] (Table 1, Figure 2). ABN401 showed inhibitory effect on OE33 and H1993 with IC50 between 10nM and 100nM. ABN401's IC50 for H1993 was at higher concentration than OE33. ABN401 does not have cytotoxicity effect on TOV-112D (Figure 3A). Phosphorylation of *MET* was decreased for both OE33 and H1993 cell lines. Even though phosphorylation of *MET* was reduced, phosphorylation of ERK1/2 was not reduced for H1993 cell line while phosphorylation of AKT was decreased for the cell line. For OE33 cell line, phosphorylation of AKT was not decreased while phosphorylation of ERK1/2 was decreased. This data suggests that ABN401 successfully inhibits phosphorylation of *MET* (Figure 3B).

ABN401's inhibitory effect on *MET* mutations

MET mutations on recombinant protein based assay showed that ABN401 has effect on MET P1009S, T1010I, V1110I, T1191I, Y1253D, and M1268T mutations. For the known type I MET inhibitor's resistance mutations, such as Y1248A, Y1248C, Y1248D, Y1248H, D1246H, and D1246N, ABN401 did not have effect on those mutations (Figure 4B) [12]. *MET* mutation transfected Ba/F3 cell line's western blot analysis shows similar inhibitory effect with recombinant protein assay. However, inhibition of phosphorylation was observed at much lower concentration of ABN401 on cell based assay (Figure 5, Figure 8). *In silico* data also suggests that ABN401 can be classified as Type Ia inhibitor and it forms pi-stacking with Y1248 (Figure 1). Which leads to the idea that mutation on Y1248

site might decrease the binding affinity of ABN401 on MET. It is known that formation of salt bridge with D1246 site and V1110 stabilize the Y1248 tyrosine residue [12]. As a result, mutations in those sites hinder ABN401's binding to MET.

Cell based assay suggested that ABN401 can inhibit viability of mutant *MET* transfected NIH-3T3 cell lines. Mutations' known characteristics were confirmed by cell viability assay. The mutations which are already known as constitutively activating mutations such as V1110I, H1112Y, and Y1253D, cell viability assay result suggested that HGF does not have role in viability of those mutations. For the mutations such as Exon 14 deletion and H1112L mutations, presence of HGF exhibited more decrease in cell viability than that without HGF. This assay also showed that ABN401 does not have effect on viability of H1112Y mutation (Figure 7). To find out ABN401's effect on *MET* mutation construct transfected NIH-3T3 cell lines in molecular level, western blot assay was performed. Level of *MET*, phosphorylated MET (1234/5), AKT, phosphorylated AKT, ERK1/2, and phosphorylated ERK1/2 were examined in presence or absence of HGF. For all the mutations, phosphorylation of *MET* was decreased while total *MET* level was not changed. For H1112Y mutation, when cell was cultured with 100ng/mL HGF, phosphorylation level of AKT and ERK1/2 was increased. However, HGF did not have effect on phosphorylation level of downstream effector for other mutations (Figure 8).

Western blot assay on *MET* mutant transfected Ba/F3 cell line was conducted to find out ABN401's ability to inhibit phosphorylation of MET. The result showed

similar inhibitory effect as *MET* mutant transfected NIH-3T3 cell lines. WT, V1110I, H1112Y, and M1268T cell line constructs were established on both NIH-3T3 and Ba/F3 cell lines. Decrease in phosphorylation of MET was observed in V1110I, H1112Y, and M1268T mutations for both cell lines. However, D1246N and D1246H mutations which are known as Type I inhibitor resistance mutations' phosphorylation level was not changed on Ba/F3 cell line (Figure 8).

ABN401 inhibits characteristics of *MET* mutation on NIH-3T3 cell lines

In treatment with 100nM of ABN401, migration ability of V1110I, H1112Y, and Y1253D mutation were decreased in wound trans-well migration assay (Figure 9). For all the mutations except for Y1253D, 48 hours after scratch, the wound was healed. This suggested that all mutations have ability to migrate except for Y1253D cell line. Also, other mutations inability to migration when 500nM of ABN401 was treated to *MET* mutated NIH-3T3 cell lines suggest that ABN401 have inhibitory effect on those mutations (Figure 10).

Ba/F3 cell line does not have ability to transform after transfection

To identify Ba/F3's transforming ability influenced by constitutively phosphorylation of *MET*, Ba/F3 cell line's proliferation assay in presence or absence of IL-3 was conducted. For all transfected Ba/F3 cell lines, without IL-3, they wouldn't be able to survive and proliferate (Figure 6).

ABN401 has efficacy on *MET* amplified and exon 14 deleted PDX model

LU2503 PDX model harbors *MET* amplification (copy number>15 based on whole exome sequencing) and *MET* exon 14 deletion. In addition to *MET* alterations, LU2503 model harbors ERBB2 P1170A mutation. However, from this PDX model study, therapeutic efficacy of ABN401 was observed even though known resistance mechanism, ERBB2 mutation, was present. On day 19, compare to mean tumor size of control was 1603.50mm^3, two doses of ABN401 showed tumor inhibitory effect. TGI value on day 19 were 108.3% and 110.9% respectively for 10 and 30mg/kg dosing. After treatment ended, tumor volume increased rapidly. This result suggests the possibility that PDX model developed resistance mechanism for ABN401 or remained tumor volume proliferate right after ABN401 treatment was ended (Figure 11).

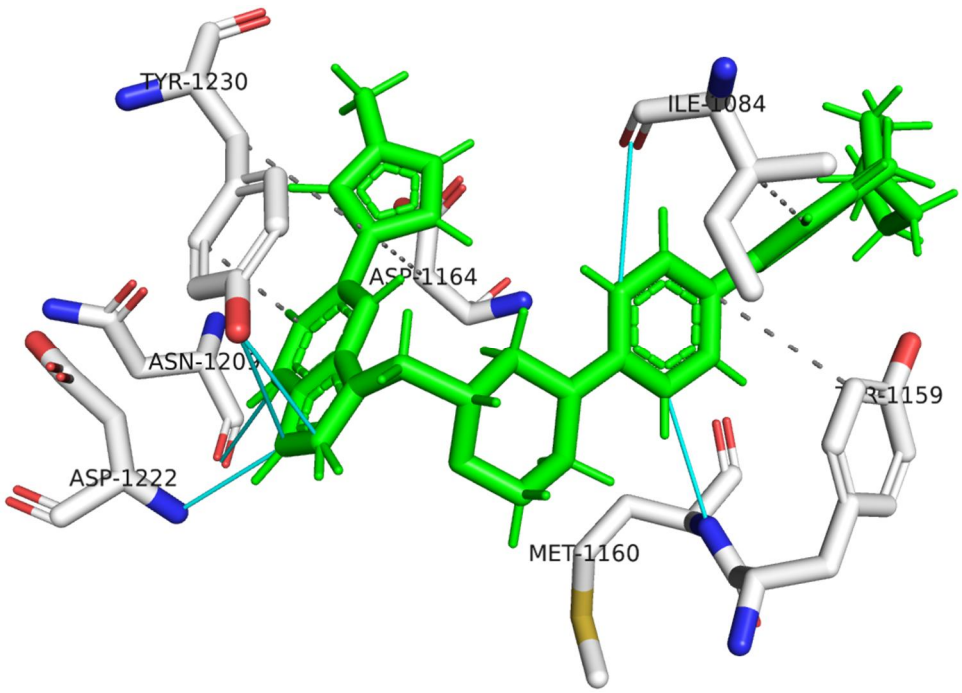

Figure 1. Binding configuration of ABN401 on MET. *In silico* study of ABN401 on MET protein. Green color represents ABN401, white color represents MET kinase domain, Gray color represents hydrophobic interactions between MET tyrosin kinase and ABN401, and cyan color represents hydrogen bond between MET tyrosine kinase and ABN401.

Table 1. Mutation status of cell lines

Cell line	Cell line characteristic	MET status	Other mutation status	Reference
Ba/F3	Murine B Cell	No expression		[7]
NIH-3T3	Mouse fibroblast cell	No expression	-Endogenous murine HGF	[24]
H1993	NSCLC	-Constitutive activation -amplification and overexpression	-MET dependent EGFR constitutive activation -c-MYC amplification	[23]
OE33	Esophageal cancer	-Amplification	-CBL loss -ERBB2 activation	[25] [26] [27]
TOV-112D	Ovarian cancer	-no endogenous MET activity	-No endogenous HGF expression -No/less EGFR, HER3, HER4 expression	[28] [29]

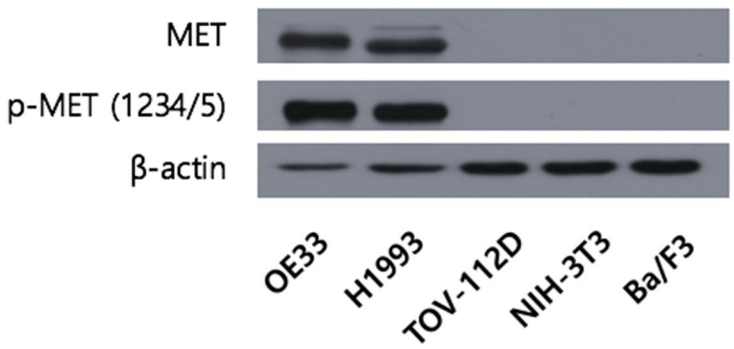

Figure 2. Expression of *MET* on each cell lines. Protein expression of MET and phosphorylated MET was analyzed using western blotting in OE33, H1993, TOV-112D, NIH-3T3, and Ba/F3 cell lines. β-actin was used as the control.

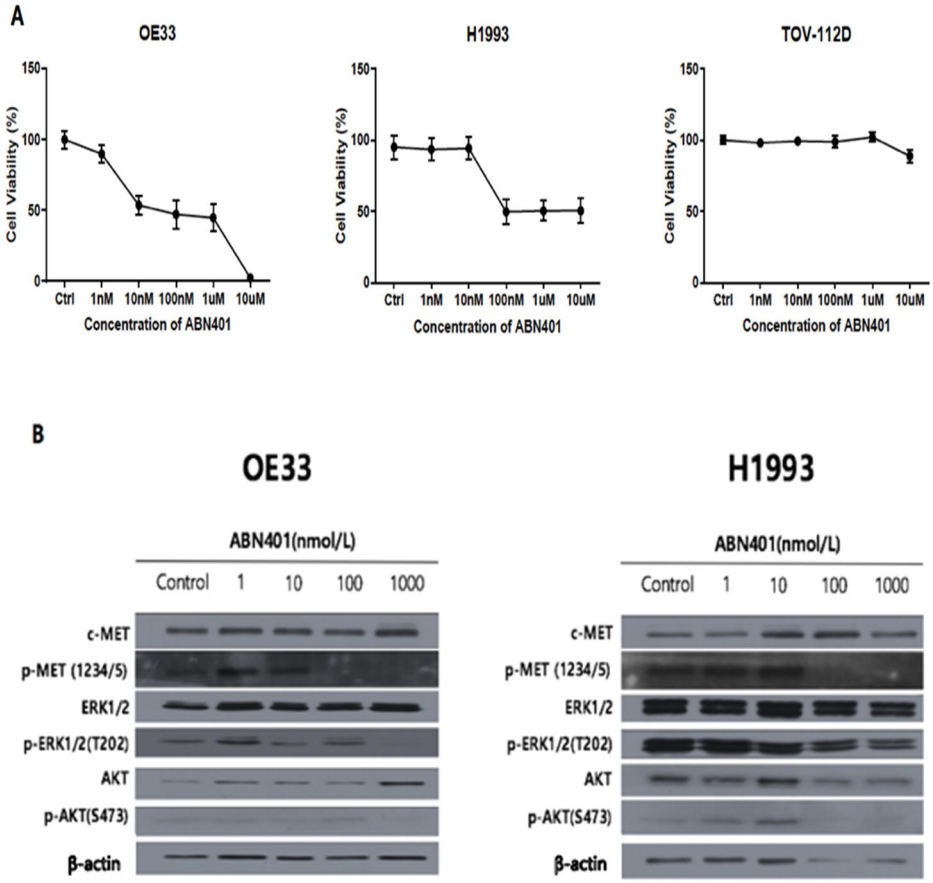

Figure 3. ABN401's inhibitory effect and MET selectivity on cell lines. Cells were treated with ABN401 for 10-fold increases from 1nM to 1uM. (A) $5x10^3$/well were seeded on 96 well for 12 hours then treated with ABN401. After 72 hours of incubation, WST reagent was used. (B) 72 hour after ABN401 treatment, cells were harvested then protein level was analyzed by western blotting. β-actin was used as the control.

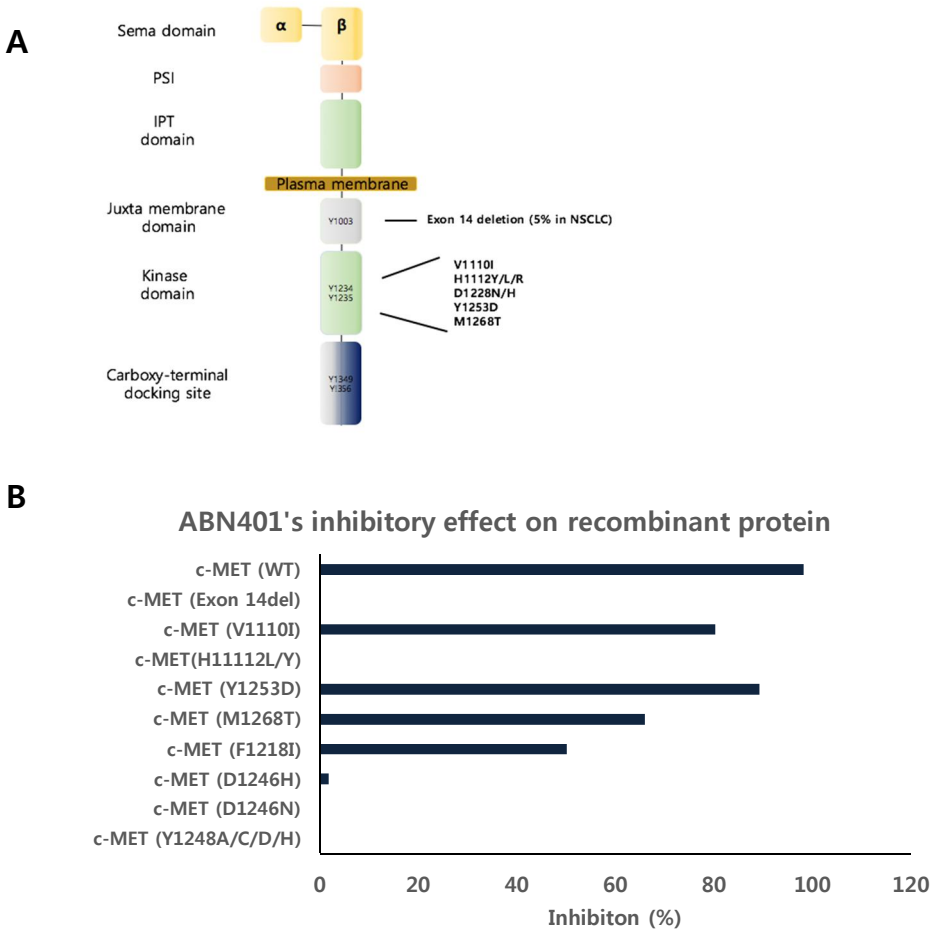

Figure 4. Inhibitory effect of ABN401 in recombinant protein.

(A) Schematic representation of location of *MET* mutations. (B) Recombinant protein assay data and mutations selected for cell based assay. For recombinant protein assay, WT, V1110I, Y1253D, M1268T, and F1218I, D1246N/H, and Y1248A/C/D/H mutations were conducted. For cell based assay, WT, exon 14 deletion, V1110I, H1112L/Y, D1246N/H Y1253D, and M1268T mutations were conducted.

Table 2. Characteristics of Mutations on *MET*

Mutation	Characteristics	reference
Exon14 deletion	• Reported in about 3% in lung adenocarcinoma patients • Increase cellular growth • Deleted site includes CBL E3 ubiquitination binding site	[21] [22]
V1110I	• Transforming ability • Constitutively activating mutation • Have contact with activation loop proteins	[15] [17] [18]
H1112Y	• Germline mutation • Intermediately activates MET • Near ATP binding site	[13] [16] [20]
H1112L	• Weakly activating MET	[19]
D1246N/H	• Increase of MET endocytosis/recycling level • Weakly activates MET • Known resistance mechanism for Type I inhibitor	[24] [20]
Y1253D	• Activating mutation • Associated with patient survival	[6]
M1268T	• Increase of MET endocytosis/recycling level • Strongly activates MET • Have contact with activation loop proteins	[14] [18] [20]

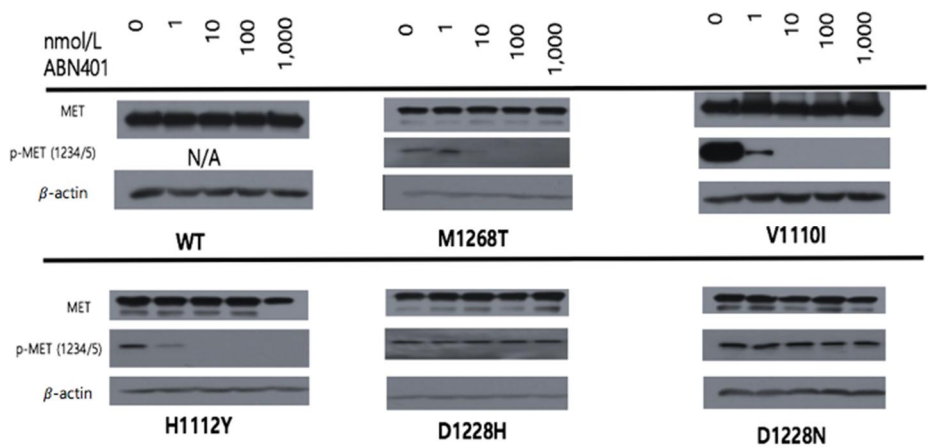

Figure 5. Inhibitory effect of ABN401 in *MET* gene transfected Ba/F3 cell line. Ba/F3 cell lines were treated with ABN401 for 10-fold increases from 1nM to 1uM for 3.5hours then harvested for western blotting. β-actin was used as the control.

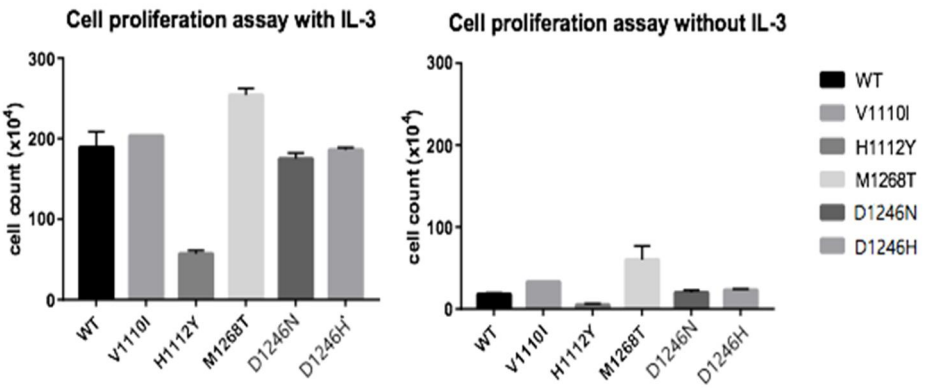

Figure 6. Cell proliferation assay for *MET* mutant transfected Ba/F3 cell lines. $5x10^5$ cells were seeded on 6well plate and cell proliferation assay was performed on Ba/F3 cell line in presence or absence of IL-3. Number of live cells was counted 72 hours after seeding.

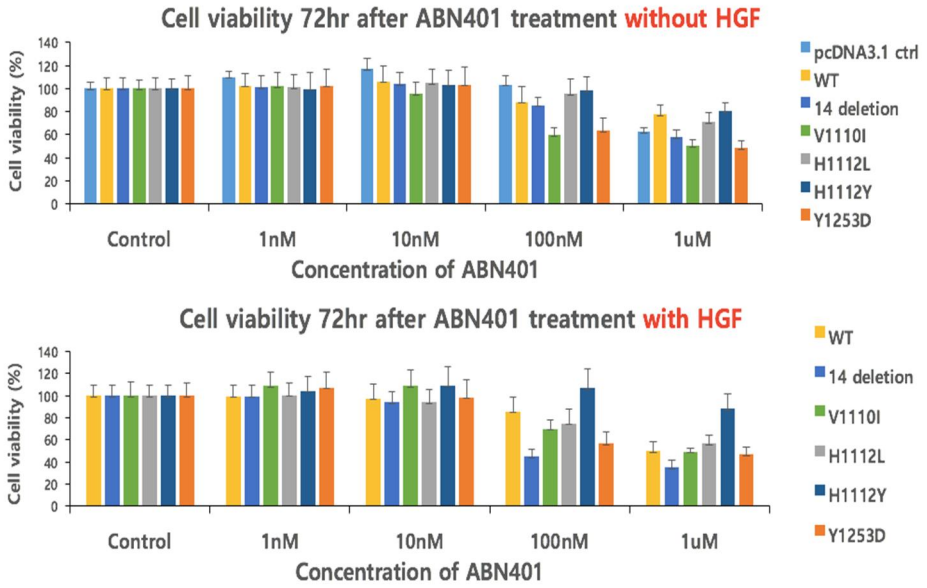

Figure 7. Cell viability assay in presence or absence of HGF in NIH-3T3 cell line. Cells were treated with ABN401 for 72 hours 10-fold increase from 1nM to 1uM. Cell viability with HGF treatment was pre-incubated with HGF (100ng/ml) for 48 hours then treated with ABN401 with HGF (100ng/ml) for 72 hours.

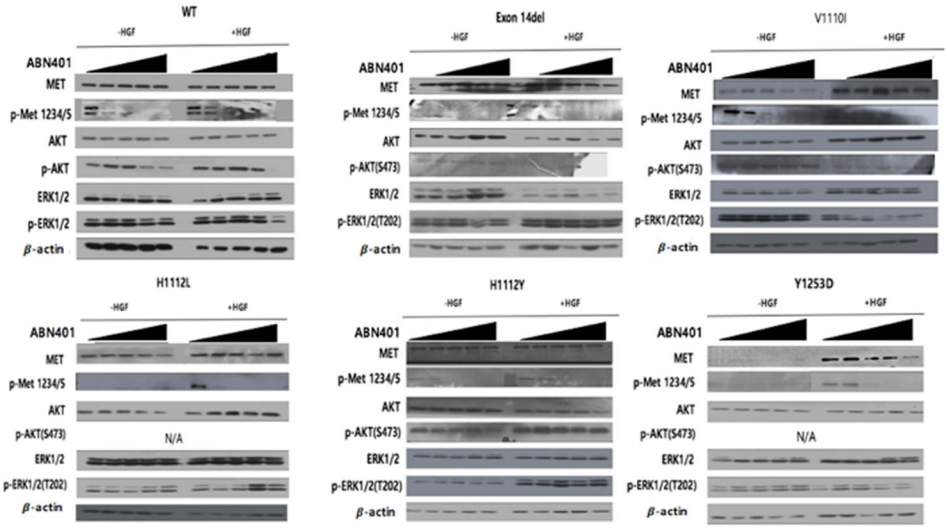

Figure 8. MET downstream effectors in presence or absence of HGF in NIH-3T3 cell line. 24 hours after ABN401 treatment 10-fold increase from 1nM to 1uM in presence or absence of HGF, cells were harvested and analyzed by western blot. β-actin was used as the control.

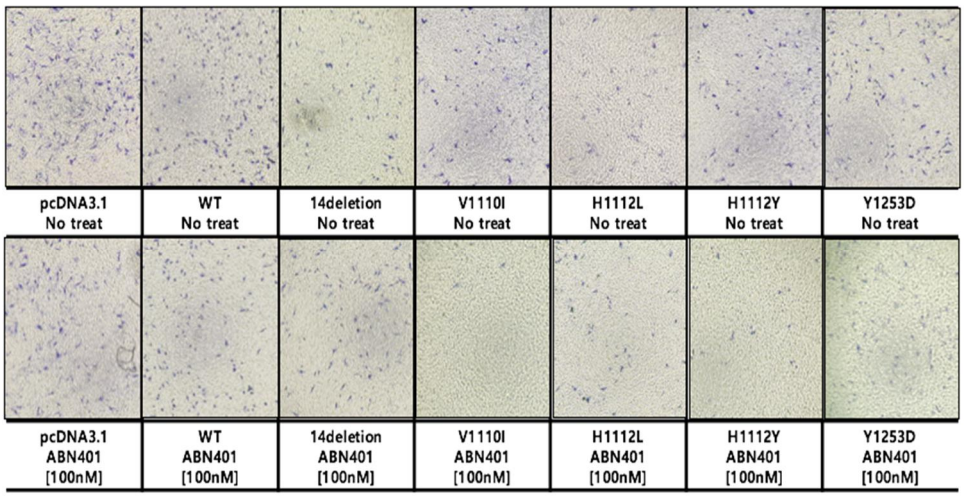

Figure 9. ABN401 inhibits cell migration for *MET* mutant transfected NIH-3T3 cell line. First row is a picture of 6 hours after no ABN401 treated NIH-3T3 cell line. Second row is a picture of 6 hours after 100nM of ABN401 treated NIH-3T3 cell lines.

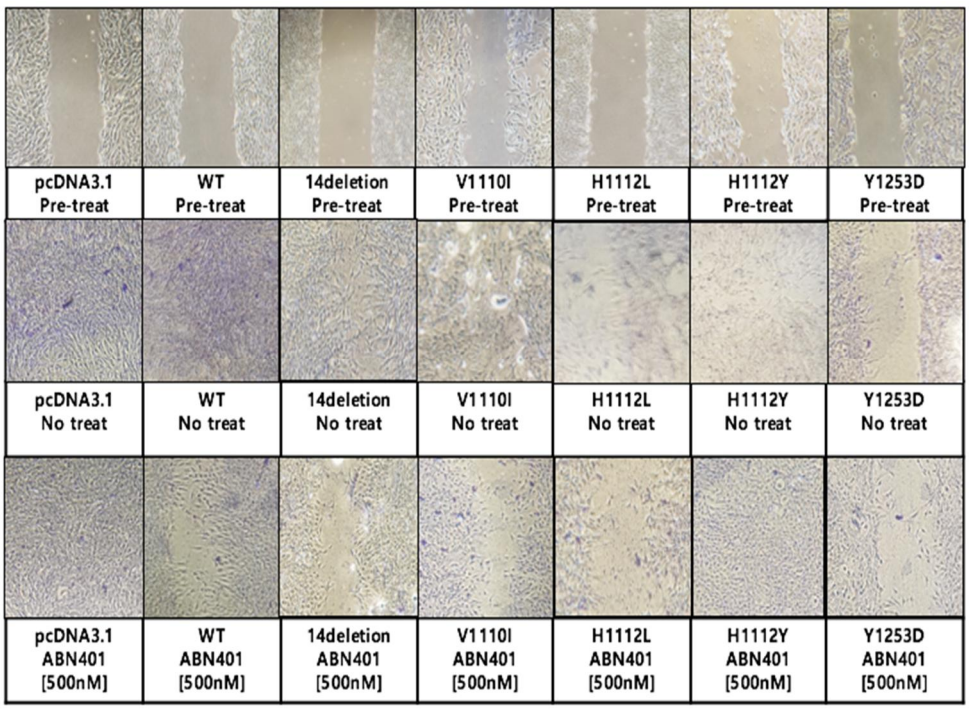

Figure 10. ABN401 inhibits cell migration and cell-cell interaction in *MET* mutant transfected NIH-3T3 cell line. First row is a picture taken right after scratch. Second row is a picture of 48 hours after no drug-treated cell lines. Third row is a picture of 48 hours after 500nM of ABN401 treated cell lines.

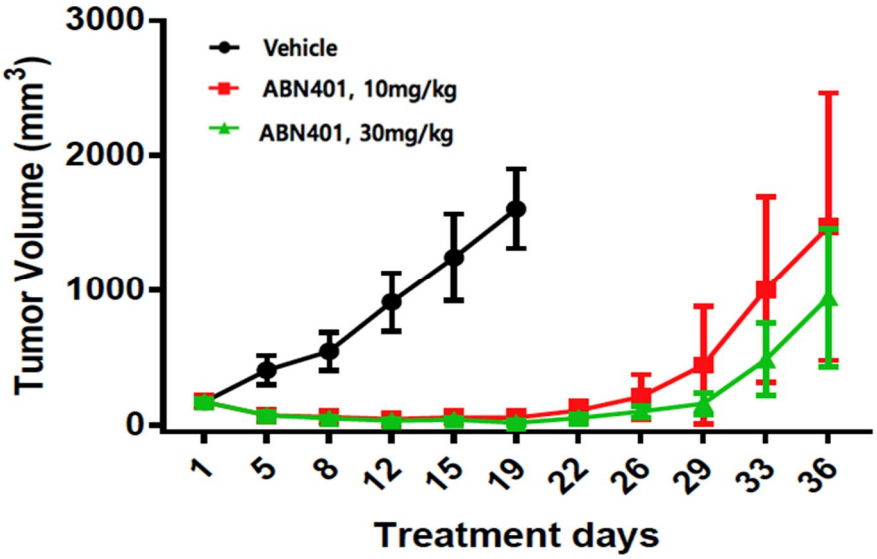

Figure 11. Effect of ABN401 on PDX models with *MET* exon 14 deletion mutation. *MET* amplified and Exon 14 deleted lung cancer PDX model was treated with ABN401. Each cycle was composed of five-day treatment and two days of resting. Three cycles of treatment were performed. Tumor volume was measured twice per week and data represents mean ±SD for each group.

DISCUSSION

Globally, many clinical trials related to MET is ongoing. Even though there is great effort put into developing MET targeting drug, there is no FDA approved MET targeting drug yet. Because of MET's special characteristics, which are either it can be a driver or bypass mechanism for cancer, MET has been considered as promising target for new drug. To fulfill this medical unmet need, highly specific MET inhibitor, ABN401 was designed. MET targeting drugs can be classified as two kinds which are antibody that binds to extracellular domain of MET and chemical drug that can bind to intracellular tyrosine kinase domain of MET. Antibody that targets extracellular domain of MET has limitation that it cannot inhibit cancer driven by *MET* mutation. Unlike antibody, tyrosine kinase inhibitor, ABN401 can potentially inhibit mutation derived cancer by binding to ATP pocket of MET. Binding of ABN401 to MET blocks downstream effectors. As a result, it decreased viability of cancer cell lines. MET tyrosine kinase inhibitors can be classified as three types. Type I and II inhibitors are ATP competitive inhibitors while type III inhibitor inhibits MET by binding to allosteric binding site. ABN401 is type Ia inhibitor, which interacts with MET Y1248 site and solvent front G1181 site [31,32]. ABN401's binding stability is determined by bonding. However,

ABN401's ability to form hydrogen bond with G1181 might not be able to rescue solvent front mutations. Because of this limitation, identifying mutations that ABN401 cannot exhibit inhibitory effect is important for patients to prescribe proper drug.

ABN401's selectivity for MET was confirmed by cell viability assay and western blot assay. Two *MET* expressing cell lines, OE33 and H1993 cell lines' viability was decreased while no-*MET* expressing cell line's viability was not affected with treatment of ABN401.

In silico study suggests ABN401's possible key residues and type of interaction between ABN401 and MET. Compare to other MET inhibitors, such as volitinib, AMG337, Cabozantinib, and Capmatinib, ABN401 has big active site because of its size (Data not shown). Salt bridge formation with D1249, E1251, and D1182 can increase bonding sustainability. Formation of pi-stacking bond with Y1248 is essential for ABN401 to bind MET [32]. With this *in silico* result, recombinant protein assay was conducted to find out whether ABN401 can inhibit selected mutations. As expected, ABN401 could not inhibit mutations on Y1248 site. In addition to mutation on essential bonding site, well known type I resistance mutation, D1248N/H mutation was not rescued by ABN401 [32]. Even though recombinant protein assay can identify mutations that ABN401 can inhibit binding of ATP to MET mutant, this assay has limitation that it cannot represent each mutation's characteristics.

To find out each mutation's characteristics and ABN401's effect on those

mutations, NIH-3T3 cell line was used to find out ABN401's inhibitory effect on various mutations with several different assays. Cell viability assay was conducted for each mutation and western blot assay were performed in presence or absence of HGF. Mutations which are known as activating mutations show no difference in cell viability whether HGF was present or not. However, exon 14 deletion and H1112L mutations, which are known as non-activating mutation and weakly activating mutation respectively, needed HGF to decrease cell viability [6,17,18,19,20]. Interestingly, while H1112L mutation showed effect on ABN401, H1112Y mutation did not show inhibitory effect even though only single amino acid was changed from same site. This leads to the idea that confirmation change induced by single amino acid can block binding of ABN401 to MET. Phosphorylation of MET mutation was decreased when ABN401 was treated. NIH-3T3 and Ba/F3 western blot assay data suggest that ABN401 has inhibitory effect on exon 14 deletion, H1112L, H1112Y, V1110I, Y1253D, and M1268T while it does not show inhibitory effect on D1246N and D1246H mutations. Downstream effector for NIH-3T3 cell lines was not inhibited even in presence of ABN401. We assume that this phenomenon was occurred because *MET* has role in may pathways other than AKT and ERK1/2 pathway [34]. Even though downstream effectors did not change in presence of ABN401, PDX model study showed ABN401 can inhibit tumor growth of *MET* amplified and exon 14 deleted lung cancer model. This also supports that other pathway might have role in ABN401's effect.

Trans-well assay and wound healing assay were conducted to find out the

migration ability for each mutation. Other than finding out cell's migration ability, wound healing assay can evaluate cell-cell interaction. Interestingly, trans-well assay and wound healing assay result did not have correlation. Wound healing assay result showed that except for Y1253D mutation, other four mutations in NIH-3T3 cell line showed migration ability. Among those four mutations, ABN401 showed inhibitory effect on exon 14 deletion, V1110I, and H1112L mutation. Trans-well assay showed that ABN401 cannot inhibit migration ability of exon 14 deletion and Y1253D mutation. V1110I, H1112L, and H1112Y mutation's migration ability was inhibited by ABN401 in trans-well assay. Interestingly, Y1253D mutation did not show migration ability from wound healing assay while it showed on trans-well migration. Also, H1112Y mutation had been showing no inhibitory effect by ABN401 in cell viability assay and wound healing assay. However, in trans-well migration assay, H1112Y mutation's migration was inhibited in presence of ABN401.

To achieve the purpose of this study, which is to find the ABN401's efficacious effect on each *MET* mutation, the key was to establish *MET* gene addicted cell lines. This idea led to choose Ba/F3 cell line, which is a IL-3 dependent mouse pro-B cell line that can survive without IL-3 when cell is addicted to receptor [7]. However, the result of the experiment suggests that *MET* gene cannot induce transformation of *MET* mutant-transfected Ba/F3 cell lines. When cell viability assay was conducted, viability was not changed even though ABN401 was treated (Data not shown). Also, Ba/F3 could not proliferate when IL-3 was not added into

the culture medium. Because of this issue, NIH-3T3 cell line was alternatively used for this experiment. Cell viability of NIH-3T3 was affected by concentration of ABN401. However, even with treatment of ABN401, level of downstream effectors did not change. This leads to the idea that either NIH-3T3 was not successfully addicted to *MET* gene or other downstream effectors have a role in MET pathway.

While performing western blot assay, *MET* gene was expressed at 190kDa and 145kDa for Ba/F3 cell line. It is known that MET pre-cursor is appeared at 170kDa and glycosylation of MET forms 190kDa MET heterodimer. After the heterodimer formation, proteolytic cleavage by furin cleaves heterodimeric MET to alpha (50kDa) and beta subunit(145kDa) [30]. We hypothesized that Ba/F3 lacks post-translational modification pathway, which is caused by lack of Furin. Further studies are required to find out expression of furin in Ba/F3 cell line. If there is no furin expression in native Ba/F3 cell line, we suggest co-transfection of furin and *MET* gene to conduct *MET* addicted cell line.

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국 문 초 록

Hepatocyte Growth Factor Receptor (HGFR)은 Mesenchymal-epithelial transition factor (MET)이라고도 알려져 있으며, HGF는 MET의 유일한 리간드로 알려져있다. MET은 상처 회복, 혈관신생, 간 재생에 영향을 끼친다. HGF가 MET에 결합하면, MET의 활성이 이루어지고, 이는 암세포의 침략과 전이를 일으키는 원인이 된다. MET의 종양을 형성하는 특성은 증폭, 과발현, 돌연변이에 의하여 일어나며, 이러한 변화들은 MET을 계속해서 활성시키는 원인이 되는 것으로 알려져있다. 이러한 변화들은 세포의 증식, 운동성, 생존에 영향을 주는 신호전달체계를 활성시키게 된다. 여러 암종에 걸쳐서 MET의 변화가 일어나는 것이 알려져 있으며, 현재 글로벌 제약회사들이 MET을 타깃하는 약물을 개발하고 있으나 현재 FDA의 승인을 받은 약물이 없는 상태이다.

ABN401은 MET 특이적으로 ATP와 경쟁하여 MET의 활성을 막는 약물이다. 위 연구는 ABN401의 MET 특이적 억제 효과와 임상에서 많이 발견되는 MET 돌연변이에서의 억제효과를 확인하는 실험을 수행하였다. MET 특이성을 확인하는 실험에서 MET이 증폭되어 있는 세포주에서 ABN401의 효능을 확인할 수 있었으며, MET이 발현이 많이

되어있지 않은 세포주에서 ABN401의 효능이 없는 것을 확인하였다. *In vitro*, *in silico*, 재조합 단백질실험에서 ABN401의 효능을 평가한 뒤, ABN401의 돌연변이 별 효능에 대하여 평가를 수행하였다. 그 결과 몇몇의 돌연변이에서 MET의 인산화가 약물의 농도에 따라 저해되는 것을 확인하였으며, 돌연변이에 의한 특징 중 하나인 운동성이 약물을 처리함에 따라 감소하는 것을 확인할 수 있었다. 위 연구 결과는 ABN401이 강력한 MET 저해제이며, MET 유전자에 변화가 있는 환자들에게 효능이 있음을 시사한다. 또한, ABN401의 돌연변이 별 효능 평가 결과는 임상에서 환자군 선별에 중요한 지표로 사용될 수 있음을 시사하는 바이다.

주요어: MET, 돌연변이, ABN401, Hepatocyte growth factor (HGF), 환자군 선별

학 번: 2017-28614

감사의 글

2 년 동안 석사 생활을 하면서 실험적인 부분이외에도 배운 것이 많습니다. 사람 사이의 관계에 대해서 배웠고, 사람을 대하는 방법에 대해 배운 것이 2년 동안의 가장 큰 성과라는 생각이 듭니다. 조그만 실험실에서 많은 루머와 소문들에 휩싸여도 보고, 이런저런 크고 작은 일을 겪으면서 제 나름의 사람을 대하는 규칙을 정하게 된 것 같습니다. 그 과정에서 지도 교수님이신 신영기 교수님께 감사하게 생각하고 있습니다. 제작년 9월에 제 표정을 보고 무슨 일이 있냐고 걱정해 주시며 힘들어 하던 저를 도와주셨던 그때 그 경험은 항상 소중히 간직하고 있습니다. 또한, 회사로 보내서 제게 뜻깊은 경험을 할 수 있게 해주신 것에 대하여 정말 감사하게 생각하고 있습니다. 회사에 파견되어 인턴을 함으로 인해 제가 하고 싶은 일에 대한 답을 찾을 수 있게 되었습니다.

많이 뵌적은 없지만 MT를 갔었을 때 같은 차를 타고 가면서 많은 이야기를 해주시고, 항상 유쾌하신 최준석 교수님 감사드립니다. 또한, 제 논문에 대해 아낌없는 조언을 해주신 이호영 교수님께 정말 감사드립니다.

많이 뵌적은 없지만, 제가 수업 조교를 할 때 "니가 민지니?" 라고 밝게 웃으시며 저를 맞이 해주신 이미옥 교수님께도 감사 말씀드립니다.

처음 81동에 갔을 때 멤버였던 호빈오빠, 주석오빠, 준형오빠에게 부족한 저를 항상 도와주고 챙겨주셔서 감사했습니다. 항상 열심히 하고, 누구보다 성실한 호빈오빠, 많이 힘들어 보지이만 분명 노력한 만큼의 대가는 주어진다고 생각합니다!! 제 사수였던 주석오빠!! 처음 몇달 간은 오빠때문에 진짜 힘들었던 것 아시죠??? 하지만 그 이후에 많은 것을

배울 수 있었습니다. 오빠가 시키는 것들이 처음에는 너무 힘들고 하기 싫었었는데 결국에는 제게 도움이 되는 일이었다는 것을 많이 느끼면서 졸업하게 되는 것 같습니다. 오빠도 아시겠지만, 제가 오빠에게 의지를 많이 했고, 그만큼 오빠에게 고마움을 느끼고 있다는 것을 알고있었으면 합니다. 하지만 다른 부사수를 들인다면 항상 웃어주세요.. 오빠는 눈이 커서 안 웃으면 무섭게 생겼어요.... 준형오빠!!! 진짜 오빠 없었으면 너무너무 힘들었을 것 같아요. 오빠를 보면서 자기주장을 하는 방법을 배운 것 같아요. 너무 눈치만 보면서 살고 항상 남한테 치이고 오는 저에게 제 잘못이 아니라고, 제 편을 들어주셔서 항상 든든했어요. 제 석사 2년동안 실험실 생활에서 많이 의지를 한 사람 중 한 사람이 오빠라는 걸 꼭 알아줬으면 좋겠고, 오빠 덕분에 많이 웃으면서 지낼 수 있었어요.
중간에 81동에 온 방장님, 종완오빠, 태웅오빠, 상한오빠! 항상 열심히 하고 하고 싶은 일에 있어서 좋은 결과가 있었으면 좋겠습니다. 특히 종완오빠!! 처음에는 오빠에 대해 잘 몰랐는데 오빠가 81동와서 처음에 제가 느꼈던 것 보다 많이 달라진 것 같아서 너무 보기 좋아요. 저희랑 같이 들어와서 결국 한학기 더하지만 오빠가 한학기 더 한 만큼 더 좋은 결과를 가지고 졸업할 수 있을 것이라고 믿어요.
21동에 있었던 은혜언니, 혜빈이, 민훈이, 혜은언니, 네거씨, 범모오빠! 많이 본적이 없어서 서로 잘 모르는 것이 항상 안타까웠어요. 서로에 대해 더 잘 알았으면 좋았을 것이라는 생각을 항상 했는데 결국 졸업하게 되어서 안타깝습니다. 혜은언니!! 온지 얼마 안되서 너무 힘들 텐데 그래도 21동에 갈 때마다 항상 웃어주고 재밌게 이야기해줘서 너무 고마워요. 언니 덕분에 21동에 갈 때 언니가 있을까 하고 기대하게 가게 되더라고요. 앞으로 힘들겠지만 그래도 화이팅입니다! 그리고 한나언니! 가끔 언니랑 카톡하고 얘기하면서 너무 즐거웠어요. 앞으로도 연락 자주

해 주세요.

마지막으로 성수선배, 희정쌤, 영문쌤, 하연쌤, 태원언니, 소장님, 박박사님께도 감사드립니다. 처음에 81동 왔을 때 제게 관심을 주시고 항상 응원해 주셨던 성수선배! 선배덕분에 제가 제 주제 이외에 다른 것도 경험해 볼 수 있는 기회가 있었습니다. 희정쌤! 중간데 제 사수가 되어 주셔서 너무 감사드려요. 쌤 덕분에 저에 대한 자신감을 가질 수 있었습니다. 영문쌤! 고맙습니다. 회사에 있을 때 제 편들어주시고, 항상 저를 생각해주신거 너무 잘 알아요. 원래 제가 지나간 사람들에게는 연락을 잘 못하는데 성수선배, 희정쌤, 영문쌤은 오래 같이 있지 못했지만 계속 연락하고 싶은 분들이십니다. 하연쌤! 저를 도와주실 의무가 없는데도 불구하고, 제가 부탁해서 제게 실험을 가르쳐 주셔서 너무 감사했습니다. 항상 밝게 웃으시고, 행복해 보이셔서 덩달아 저도 행복해지는 것 같습니다. 소장님과 박박사님! 회사에서 두분 덕분에 많은 것을 배웠습니다. 회의에서 두 분이 논의하시는 것을 들으면서 박사는 이런 생각을 할 줄 알아야 하는 구나 라는 생각을 많이 하게 되었습니다.

그리고 가장 중요한 우리 가족에게 고맙고 사랑한다는 말을 하고 싶습니다. 항상 제 편이 되어주는 부모님과, 착하고 이쁜, 정말 최고의 동생 서희, 그리고 우리 막내 민석이!! 사랑합니다.