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Combined inhibition of MET and EGFR suppresses proliferation of malignant mesothelioma cells

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Malignant pleural mesothelioma (MPM) is an aggressive neoplasm associated with asbestos exposure. Although expression and activation of receptor tyrosine kinases (RTKs), including MET, have been reported in most MPM, specific RTK inhibitors showed less than the expected response in MPM cells. To determine whether the lack of response of MET inhibitors was due to cooperation with other RTKs, we determined activation status of MET and other RTKs, including epidermal growth factor receptor (EGFR) family of 20 MPM cell lines, and tested whether dual RTK inhibition is an effective therapeutic strategy. We detected MET upregulation and phosphorylation (thus indicating activation) in 14 (70%) and 13 (65%) cell lines, but treatment with MET-specific inhibitors showed weak or modest effect of suppression in most of the cell lines. Phospho-RTK array analysis revealed that MET was simultaneously activated with other RTKs, including EGFR, ErbB2, ErbB3 and platelet-derived growth factor receptor-β. Combination of MET and EGFR inhibitors triggered stronger inhibition on cell proliferation and invasion of MPM cells than that of each in vitro. These results indicated that coactivation of RTKs was essential in mesothelioma cell proliferation and/or survival, thus suggesting that simultaneous inhibition of RTKs may be a more effective strategy for the development of molecular target therapy for MPM.

Introduction

Malignant pleural mesothelioma (MPM) is a highly aggressive neoplasm arising from mesothelial cells of the pleural cavity (1-4). There is often a long latent period between exposure to asbestos and clinical presentation. However, once the disease overtly presents, the clinical course of the patients is usually very rapid. Since MPM is resistant to conventional multimodal therapies, the prognosis of the patients with advanced stages is poor, with a median survival of 11-12 months after diagnosis (5,6). Hence, new approaches of the treatment of MPM are urgently needed.

Dysregulated activation of receptor tyrosine kinases (RTKs) plays a key role in tumorigenesis and progression in a variety of human

Abbreviations: EGFR, epidermal growth factor receptor; ERK, extracellular signal-regulated kinase; FCS, fetal calf serum; HGF, hepatocyte growth factor; MM, malignant mesothelioma; MPM, malignant pleural mesothelioma; mRNA, messenger RNA; PBS, phosphate-buffered saline; PCR, polymerase chain reaction; PDGFR, platelet-derived growth factor receptor; PI3-K, phosphoinositide 3-kinase; RTK, receptor tyrosine kinase; sh, short hairpin; TKI, tyrosine kinase inhibitor.

malignancies (7,8). A specific RTK that is activated in tumor cells is a potential therapeutic target, and EGFR tyrosine kinase inhibitors (TKIs), gefitinib and erlotinib, are one of the most successful TKIs that show a significant response in patients with non-small cell lung cancer, especially with activating mutation (9-11). However, a phase II clinical study of erlotinib treatment did not show an effect on MPM, although 96% of the specimens showed positive pEGFR (12). This may be due to the lack of EGFR mutation in MPM, as non-small cell lung cancer without EGFR mutation often shows resistance to EGFR TKIs (13).

MET is another RTK which mediates the activation of several signaling pathways, including phosphoinositide 3-kinase (PI3-K)/ AKT and Ras/mitogen-activated protein kinase cascades (14). Previous studies demonstrated that MET was expressed and activated in the majority of MPM cell lines and clinical specimens (15-18). However, MET inhibition caused growth inhibition in only a small subset of MPM cell lines regardless of frequent MET activation.

To develop molecular target therapies for expected RTKs that are shown to be activated in cancer cells, an understanding of the molecular mechanisms underlying acquired as well as intrinsic resistance of specific RTK inhibitors is extremely valuable. In the case of acquired resistance of EGFR TKI in lung cancer cells, a secondary mutation of T790M was shown to be an important mechanism (19). Engelman et al. (20) also demonstrated that MET gene amplification was an alternative mechanism. This indicated that PI3-K/AKT pathway in cancer cells can be activated by another RTK when the initial RTK with activating mutation is blocked. It also suggested that the reasons for the failure of specific RTK inhibitors against MPM cells may be multiple RTK activation as well as possible alterations of downstream molecules such as activating PI3-K mutation or loss of PTEN.

In the present study, to determine whether less than the expected response to MET inhibitors in MPM cells is due to cooperation with other RTKs, we first analyzed MET and other RTK activation status in MPM cells and then simultaneously blocked them with MET and other RTK inhibitors. Treatment with a combination of MET and EGFR TKIs showed stronger inhibition on cell proliferation and invasion of MPM cells than treatment by either one. Our results indicated that simultaneous inhibition of the activated RTKs was required for the efficient suppression of MPM cell proliferation and/or survival.

Materials and methods

Cell lines and primary specimens of malignant mesothelioma

Fourteen Japanese MPM cell lines, including ACC-MESO-1, -4, Y-MESO-8D, -9, -12, -14, -21, -22, -25, -26B, -27, -28, -29 and -30, were established in our laboratory as reported previously and described elsewhere, and the cells at 10-15 passages were used for assays (21). Four MPM cell lines, including NCI-H28, NCI-H2052, NCI-H2373, and MSTO-211H and one immortalized mesothelial cell line, MeT-5A, were purchased from the American Type Culture Collection (Rockville, MD) and cells at 3-5 passages after receiving from American Type Culture Collection were used. NCI-H290 and NCI-H2452 were the kind gift of Dr Adi F.Gazdar. All MPM cell lines were cultured in RPMI1640 medium supplemented with 10% fetal calf serum (FCS) and 1× antibiotic–antimycotic (Invitrogen, Carlsbad, CA) at 37°C in a humidified incubator with 5% CO_2. MeT-5A was cultured according to the instructions of the American Type Culture Collection. Two primary cultures of normal mesothelial cells at 3-5 passages derived from ascites of patients with gastric cancer, GAS-M1, and ovarian cancer, OV-M1, were also used as controls. Thirty-five primary specimens of malignant mesotheliomas (MMs) used were 23 pleural, 6 peritoneal, 2 lung, 1 heart, 1 mesentery, 1 bone and 1 kidney origin. Histological classification was 22 epithelial, 6 mixed, 5 spindle, 1 desmoplastic and 1 lymphohistiocytoid. Among 35 MMs, 16 were MPM tissue samples obtained from patients at Aichi Cancer Center Hospital, Nagoya University Hospital, Japanese Red Cross Nagoya First Hospital, Toyota Kosei Hospital and Kasugai City Hospital according to the institutional review board-approved protocol of each and the written informed consent from each patient. The other 19 MM samples were from the human mesothelioma tissue array from US Biomax (Rockville, MD).

Antibodies and reagents

Anti-MET antibody (MAB358) and anti-phosphorylation site (amino acid 1234/1235)-specific MET antibody (AF2480) were purchased from R&D Systems (Minneapolis, MN). Anti-AKT (#9272), anti-phospho-AKT (S473) (#9271), anti-extracellular signal-regulated kinase (ERK) 1/2 (#9102) and anti-phospho-ERK1/2 (#9107) antibodies were purchased from Cell Signaling Technology (Beverly, MA). Anti-β actin antibody (clone AC74) was purchased from Sigma (St Louis, MO).

AG1295, LY294002, PD153035, PD98059, SU5402 and SU11274 were purchased from EMD Chemicals (San Diego, CA), PHA-665752 from Tocris Bioscience (Ellisville, MO), and dissolved in dimethyl sulfoxide as 10 mmol/l stocks.

Preparation of DNA and RNA

Genomic DNA was extracted using a standard phenol–chloroform method. Total RNA was prepared using RNeasy Plus RNA extraction kit (Qiagen K.K., Tokyo, Japan), according to the manufacturer's protocol. Random-primed, first-strand complementary DNA was synthesized from 3 μg total RNA using Superscript II according to the manufacturer's instructions (Invitrogen).

Mutation analysis

Mutation analysis of all coding exons of the *MET* gene and exon 18–21 of the *EGFR* gene was carried out by direct sequencing after polymerase chain reaction (PCR) amplification of genomic DNA. Sequence of amplification primers was available on request.

Quantitative real-time reverse transcriptase–PCR

Quantitative real-time reverse transcriptase–PCR was performed using first-strand complementary DNA with TaqMan probes and TaqMan universal PCR Master Mix (Applied Biosystems, Foster City, CA). TaqMan probes for *MET* and *HGF* were purchased from Applied Biosystems, and the amplification was carried out with an ABI 7500 Real-Time PCR System (Applied Biosystems) according to the manufacturer's instructions. Glyceraldehyde-3-phosphate dehydrogenase (*GAPDH*) served as an endogenous control; the expression levels of *MET* and *HGF* in each of the samples were normalized on the basis of the corresponding *GAPDH* content and recorded as relative expression levels.

Western blot analysis

Preparation of total cell lysates and western blotting were carried out as described previously (21). In brief, cells growing confluently were rinsed twice with phosphate-buffered saline (PBS), lysed in sodium dodecyl sulfate sample buffer (62.5 mM Tris, pH 6.8, 2% sodium dodecyl sulfate, 2% 2-mercaptoethanol and 10% glycerol) and homogenized. Total cell lysate (30 μg) was subjected to sodium dodecyl sulfate–polyacrylamide gel electrophoresis and transferred to Immobilon-P polyvinylidene difluoride membranes (Millipore, Bedford, MA). Following blocking with 5% non-fat dry milk, the filters were incubated with the primary antibody, washed with PBS, reacted with the secondary antibody and then detected with ECL (Amersham Biosciences, Buckinghamshire, UK).

RNA interference vectors

Complementary short hairpin (sh) sequences were cloned into pLentiLox3.7 under control of a U6 promoter and transfected into HEK293FT cells along with the vectors of VSVG, RSV-REV and pMDLg/pRRE, to generate lentiviruses that transcribe shRNA. Sh-MET-RNA interference lentivirus vector (Sh-MET) contained a target sequence of the hairpin loop of MET (5'-GCCAGATTCTGCCG AACCA-3'). A control shRNA vector for luciferase (Sh-Luc) with the target sequence for Luciferase (5'-CGTACGCGGAATACTTCGA-3') was also constructed. The efficacy of each virus was tested by immunoblotting of whole-cell lysates 96 h after transducing NCI-H290 cells at the multiplicity of infection of 10.

Immunohistochemical analysis

Immunohistochemical analysis was carried out on formalin-fixed, paraffin-embedded tissue sections of mesothelioma specimens. Sections (5 μm thick) were deparaffinized in xylene and rehydrated in decreasing concentrations of ethanol. The antigens were retrieved by 45 min of heating at 98°C in 0.5% Immunosaver (Nisshin EM, Tokyo, Japan) in a waterbath. After fading the endogenous peroxidase activity with 3% aqueous H_2O_2 solution for 15 min, the sections were reacted with a primary antibody [anti-MET antibody, 3 μg/ml and anti-phospho-MET (Tyr1234/1235) antibody, 2 μg/ml] for 1 h at room temperature, washed with PBS, treated with a biotinylated secondary antibody for 15 min at room temperature and allowed to react for 15 min with the streptoavidin–peroxidase reagent using an Ultra-tech Kit (Beckman Coulter, Marseille, France). The 3,3'-diaminobenzidine tetrahydrochloride Liquid System (DakoCytomation, Glostrup, Denmark) was used to detect immunostaining. Normal rabbit immunoglobulin G, mouse immunoglobulin G or omission of primary antibodies served as negative controls. Immunoreactivity was evaluated independently by two investigators (K.K. and H.M.). The

intensity of staining was scored as strong (3+), moderate (2+), weak (1+) or negative (0), respectively.

Phospho-RTK array analysis

The Human Phospho-RTK Array Kit (R&D Systems) was used to determine the relative levels of tyrosine phosphorylation of RTKs. The membranes contained spotted antibodies corresponding to 42 distinct RTKs and both positive and negative controls. MPM cell lines were cultured on 10 cm plates in RPMI1640 media with 10% FCS confluently, changed into the media without FCS for 24 h and lysed in NP-40 lysis buffer (1% NP-40, 20 mM Tris–HCl, pH 8.0, 137 mM NaCl, 10% glycerol, 2 mM ethylenediaminetetraacetic acid, 1 mM sodium orthovanadate, 10 μg/ml aprotinin and 10 μg/ml leupeptin). The arrays were blocked in the provided blocking buffer and incubated with 500 μg of cell lysate overnight at 4°C. The arrays were washed and incubated with a horseradish peroxidase-conjugated phospho-tyrosine detection antibody, treated with ECL and exposed to film. Intensity of each spot detected with a phospho-RTK array was measured by Image analyzer, LAS-4000 (Fuji Film, Tokyo, Japan) and calculated for the relative intensity of the average signal of the pair of duplicated spots to the negative control spots. Background intensity was also determined and subtracted from each average signal. Phospho-RTKs with ratios over three times those of the highest negative control were listed (Table I).

Cell proliferation assay

For RTK inhibitors, 3000 MPM cells were seeded onto flat-bottomed 96-well plates and grown in RPMI1640 with 10% FCS. After 24 h, the cell medium was changed to 50 μl RPMI1640 medium with 5% FCS containing RTK inhibitor, and the cells were incubated for an additional 72 h. The final concentration of dimethyl sulfoxide was adjusted as 1% for both single- and dual-inhibitor treatment and for all various concentrations of RTK inhibitors. For RNA interference lentiviral transduction, 1.5×10^4 MPM cells were seeded onto flat-bottomed 24-well plates and grown in RPMI1640 with 10% FCS. After 24 h, cells were transduced with lentiviral vectors at the multiplicity of infection of 10, incubated for an additional 6 h and then changed with 500 μl RPMI1640 medium with 5% FCS. Cells were incubated for an additional 90 h. Each drug concentration for RTK inhibitors and viral transduction was applied to triplicate wells for cells.

Calorimetric assays were performed with adding of 10 μl (for 96-well plate) or 50 μl (for 24-well plate) of TetraColor One (Seikagaku, Tokyo, Japan) containing 2-(2-methoxy-4-nitrophenyl)-3-(4-nitrophenyl)-5-(2,4-disulfophenyl)-2H-tetrazolium, monosodium salt and 1-methoxy-5-methylphenazinium methylsulfate as electron carrier in each well and incubation at 37°C for 1 h. Absorbance was read at 450 nm using a multiplate reader. Growth inhibition was expressed as a mean ratio of absorbance reading from treated cells versus untreated cells.

Cell cycle analysis

In total, 1×10^6 cells were seeded into 15 mm plates, and indicated drugs were added 24 h later. After 48 h, the cells were trypsinized and centrifuged. After washing with PBS, cells were resuspended in 500 μl solution containing 50 μg/ml propidium iodine (Sigma), 0.1% Triton X-100 and 0.1% sodium citrate and incubated at room temperature for 10 min. The cell suspension was then added with 2 μl of 0.1 mg/ml RNaseA (Roche Applied Science, Basel, Switzerland) and incubated at room temperature for 30 min. Cell cycle profiles were analyzed by flow cytometry using FACSCalibur (Becton-Dickinson, Franklin Lakes, NJ) using Modfit LV software (Verity Software House, Topsham, ME).

Cell migration and invasion assays

Cell migration and invasion potential were measured by *in vitro* Boyden chamber assays (BD Biosciences Discovery Labware, Bedford, MA) according to the manufacturer's protocol. Briefly, MPM cells (5×10^4 cells for transwell migration assay and 1×10^5 for Matrigel invasion assay) in 0.5 ml of serum-free RPMI1640 medium with different inhibitors were added to the upper wells of uncoated and Matrigel-coated Boyden chambers with 8 μm pore membrane. The bottom chambers were filled with 5% FCS-containing medium as a chemo-attractant. After 22 h incubation, non-invasive cells were removed by scrubbing with a cotton swab. The cells that have migrated through the membrane and stuck to the lower surface of the membrane were fixed stained using Diff-Quik stain (Sysmex, Kobe, Japan). Values for migration and invasion were obtained by counting five predetermined fields per membrane at ×100 magnifications and representing the average of three independent experiments.

Results

Frequent expression and activation of MET in MPMs

To select representative MPM cell lines for cell proliferation assay with MET inhibitors and subsequent combination assay with other RTK inhibitors, we first examined the expression and phosphorylation

Table I. Summary of activated RTKs in 15 MPM and MeT-5A cell lines

RTKs	H290	H2373	MSTO-211H	ACC-MESO-1	ACC-MESO-4	Y-MESO-8D	Y-MESO-9	Y-MESO-12	Y-MESO-14	Y-MESO-22	Y-MESO-25	Y-MESO-27	Y-MESO-28	Y-MESO-29	Y-MESO-30	MeT-5A
HGFR	MET	MET	MET		MSPR	MET	MSPR	MET	MET		MET	MET		MET	MET	
EGFR	EGFR, ErbB2, ErbB3	EGFR, ErbB2, ErbB3	EGFR, ErbB2	EGFR	EGFR	EGFR	EGFR, ErbB3	MSPR, EGFR, ErbB2, ErbB3	MSPR, EGFR, ErbB2	MSPR, EGFR, ErbB2, ErbB3	EGFR	EGFR, ErbB3	MSPR, EGFR, ErbB3, ErbB4	EGFR, ErbB2, ErbB3	MSPR, EGFR, ErbB3	EGFR
PDGFR	PDGFRβ	PDGFRβ		PDGFRβ					PDGFRβ	PDGFRβ		PDGFRβ	PDGFRβ, M-CSFR			
VEGFR					VEGFR2		VEGFR2				VEGFR2		VEGFR2			
Others	DTK	AXL	DTK, FGFR3		DTK, Tie-2, EphA4	DTK, IGF1-R, EphA7		AXL, FGFR3, EphA4	DTK, EphA4	AXL, FGFR3, IGF1-R, EphA4, EphA7, EphB2		AXL	DTK, AXL, FGFR3, TrkB, EphA4, EphB2	DTK, FGFR3, RET	DTK, AXL, FGFR3, EphA4, EphA7, EphB2	DTK
Total no. of active RTKs	5	6	5	2	6	5	4	8	7	11	3	5	13	7	10	2

DTK, developmental tyrosine kinase; FGFR3, fibroblast growth factor receptor 3; HGFR, HGF receptor; IGF1-R, type I insulin-like growth factor receptor; M-CSFR, macrophage-colony stimulating factor receptor; MSPR, macrophage-stimulating protein receptor; VEGFR, vascular endothelial growth factor receptor.

(activation) status of MET in 20 MPM cell lines. Comparing with the expression level of MET messenger RNA (mRNA) in an immortalized mesothelial cell line, MeT-5A, which was arbitrarily set as 1.0, MET mRNA expression >2.0 was observed in 15 (75%) of 20 cell lines (Figure 1A), whereas the average level of two short-termed primary cultures of non-malignant mesothelial cells derived from ascites with cancer patients was 0.43 (supplementary Figure 1A is available at Carcinogenesis Online). Western blot analysis using total cell lysates from cell lines that were cultured for 24 h under serum-starved conditions also confirmed elevated MET protein in 14 cell lines compared with the level of MeT-5A, and we found that most cell lines with MET mRNA elevation also consistently showed an elevated level of MET protein. Using anti-phospho-MET antibody, we detected phospho-MET in 13 cell lines under serum-starved condition, including four strong, five moderate and four weak cell lines of phosphorylation (Figure 1B).

To verify that MET expression detected in MPM cell lines reflects primary MPMs, we investigated MET expression in 35 MM specimens, including 23 pleural and 6 peritoneal mesotheliomas. Immunohistochemical analysis detected 28 (80%) of 35 specimens positive for MET (Figure 1C and D), whereas normal mesothelial cells showed very faint staining (supplementary Figure 2 is available at Carcinogenesis Online). Nineteen of 28 MET-positive specimens also stained positive for phospho-MET. Comparison of the levels of MET and phospho-MET reactivity showed a positive correlation (Figure 1C and D and supplementary Figure 2 is available at Carcinogenesis Online). Among them, three specimens were the origins of three cell lines (Figure 1B and C). KD1050/Y-MESO-12 and KD1048/Y-MESO-9 primary/cell line pairs showed strong/high and negative/low expression, respectively, suggesting a similar tendency of MET expression between the primary tumor and cell line. However, KD1053/Y-MESO-14 showed inconsistency, i.e. moderate/low expression. One of the possible reasons was cell selection during establishment of cell culture.

Comparison of MET activation and AKT and ERK activation

To determine whether MET activation was a major determinant for activation of downstream signaling cascades in MPM cells, we analyzed the two significant MET downstream signaling cascades, AKT and ERK1/2, under serum-starved conditions. Regarding AKT, 12 MPM cell lines showed a relatively robust level of phospho-AKT, whereas eight MPM, MeT-5A and two non-malignant mesothelial cell lines showed an undetectable or very low level of phospho-AKT (Figure 1B and supplementary Figure 1B is available at Carcinogenesis Online). Between MET phosphorylation and AKT phosphorylation status, both positive signals were observed in nine cell lines and both were negative in four, suggesting a weak correlation. Furthermore, the levels of positivity of pMET and pAKT were not always consistent between cell lines, including strong pMET but weak pAKT, such as in NCI-H28 and Y-MESO-27 (Figure 1B, lane 1 and 17), and negative pMET but strong pAKT in Y-MESO-22 (Figure 1B, lane 14). These data suggested that MET activation was not a sole major determinant for AKT activation for most MPM cells.

Regarding activation of ERK1/2, the majority of cell lines and MeT-5A also exhibited a similar level of phospho-ERK1/2, indicating that phosphorylation of MET was not correlated with that of ERK1/2 (Figure 1B).

MET activation in MPM cell lines was not caused by its mutation

Since MET mutation has been reported in a subset of MPMs (17) and activating mutation of RTKs can be a strong indicator of the effectiveness of a specific TKI, we investigated whether or not the MET activation in the MPM cell lines was due to mutation. We sequenced all 21 exons that cover the entire coding region of MET in 20 cell lines including five cell lines that had been reported to have no mutation. However, we detected only single-nucleotide polymorphisms but not any mutation to change amino acid sequence in 15 cell lines as well as the five cell lines negative for mutation (supplementary Table I is available at Carcinogenesis Online).
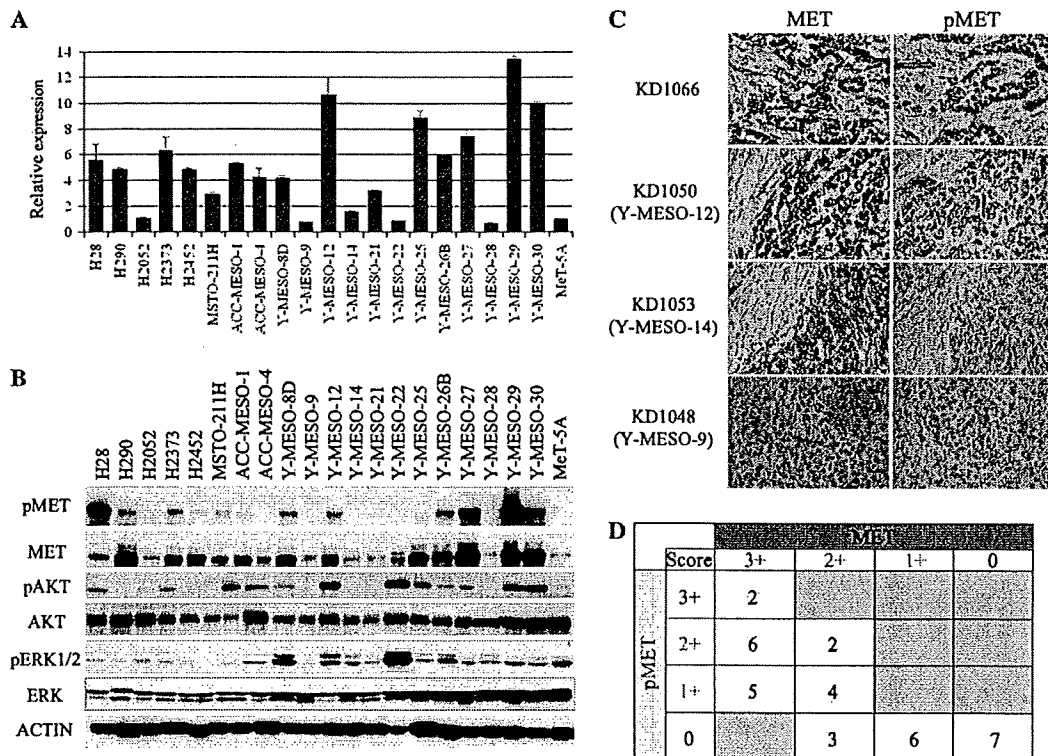
Fig. 1. Expression and activation of MET in MM cells. (A) Quantitative reverse transcriptase–PCR analysis of MET in 20 MPM cell lines and one immortalized mesothelial cell line, MeT-5A, with a primer set covering exon 7 to 8. RNAs were extracted from cell lines cultured in the media supplemented with 10% FCS. Relative expression of MeT-5A was arbitrarily set as 1.0. Data represent mean ± SD. (B) Western blot analysis of MET, AKT and ERK and their phosphorylated forms in MPM cell lines. Expression of β-actin was used as the control. Total cell lysates were extracted from cell lines cultured for 24 h under serum-starved conditions. Four cell lines (NCI-H28, Y-MESO-27, Y-MESO-29 and Y-MESO-30) expressed strong, five cell lines (NCI-H290, NCI-H2373, Y-MESO-8D, Y-MESO-12 and Y-MESO-26B) moderate and four cell lines (NCI-H2452, MSTO-211H, ACC-MESO-1 and Y-MESO-25) weak phospho-MET. All 13 cell lines and ACC-MESO-4 expressed MET at a clearly higher level than MeT-5A. (C) Immunohistochemical analysis of MET (left) and phospho-MET (right) in four primary MPM specimens. MET was strongly positive in KD1066 and KD1050, moderately in KD1053 and negative in KD1048. Phospho-MET was strongly positive in KD1066, moderately in KD1050, weakly in KD1053 and negative in KD1048. The names of cell lines established from each primary specimen were indicated in parentheses. (D) Correlation of MET and phospho-MET expression in 35 MM specimens, including 23 pleural and 6 peritoneal mesotheliomas. Intensity of staining was scored as strong (3+), moderate (2+), weak (1+) or negative (0), respectively.

In addition, to test whether a ligand of MET, hepatocyte growth factor (HGF), was responsible for MET activation in MPM cell lines, we examined expression of HGF with real-time reverse transcriptase–PCR. One cell line, Y-MESO-8D, highly expressed HGF and three others expressed HGF similar to the level of MeT-5A, suggesting that autocrine activation of MET was not a major factor in most MPM cell lines *in vitro* (supplementary Figure 3 is available at *Carcinogenesis* Online).

Downregulation of MET induces modest inhibition of MPM cell proliferation

Next, we performed a knockdown experiment using shRNA expression vectors. Three MPM cell lines, NCI-H290, NCI-H2373 and Y-MESO-29, with elevated MET expression were analyzed. Ninety-six hours after transduction, Sh-MET-RNA interference vector induced efficient knockdown of MET protein in all three cell lines, with subsequent downregulation of phospho-MET (Figure 2A). Western blot analysis showed that shRNA-mediated MET knockdown reduced the pAKT levels to 65, 88 and 37% of control in NCI-H290, NCI-H2373, and Y-MESO-29, respectively (Figure 2A). In contrast, no reduction of phosphorylation status of ERK1/2 was observed in all three cell lines (Figure 2A). Inhibition of cell proliferation of these cell lines was determined with calorimetric assay. We found that downregulation of MET induced a weak and moderate inhibition for NCI-H290 and Y-MESO-29 cells, respectively, but not for NCI-H2373 cells,

indicating that MET inhibition was not very effective for suppression of MPM cell proliferation (Figure 2B).

We also studied the suppression of cell proliferation of 13 MPM cell lines using two TKIs specific to MET, SU11274 and PHA665752. After confirming that SU11274 treatment suppressed MET activation dose dependently without affecting MET expression levels (supplementary Figure 4 is available at *Carcinogenesis* Online), cell proliferation assay was performed 72 h after treatment. We found that two cell lines, NCI-H290 and Y-MESO-29, showed growth suppression of 30% compared with the control cells at 5 μM (Figure 2C). Meanwhile, the other 11 MPM cell lines and MeT-5A were relatively resistant to treatment by SU11274 (Figure 2C). Using the same panel of cell lines, we obtained similar results with PHA665752 treatment (data not shown).

Frequent coactivation of multiple RTKs in MPM cells

The results shown above indicated that, while MET downregulation induced suppression of the proliferation of several MPM cell lines, the effects seemed to be limited in a subset of MPMs and also relatively modest. This supported a hypothesis that other RTKs that were simultaneously coactivated in mesothelioma cells compensated for the inhibitory effect of MET downregulation. To test this, we examined the phosphorylation status of 42 RTKs simultaneously using a phospho-RTK array in 15 MPM cell lines under serum-starved conditions (Figure 3 and Table I). Ten of 15 cell lines showed MET phosphorylation, which was well
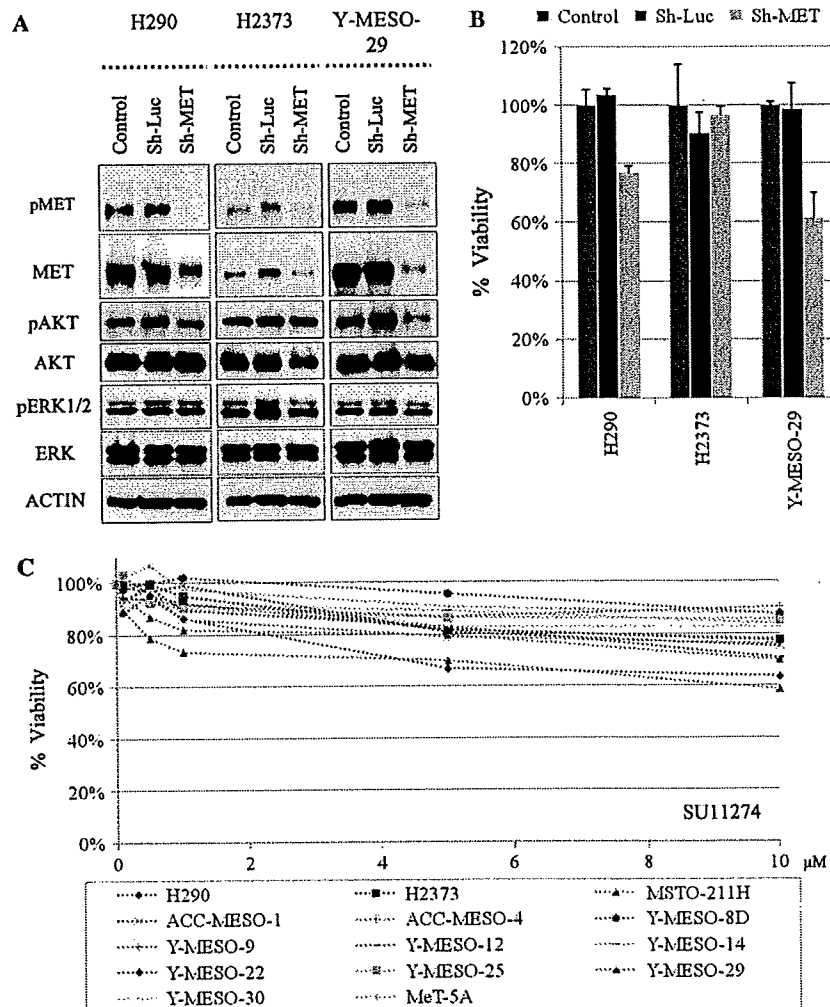
Fig. 2. Inhibition of MET with shRNA-mediated knockdown or a small inhibitory molecule in MPM cell lines. (A) Western blot analysis of NCI-H290, NCI-H2373 and Y-MESO-29 after shRNA-lentivirus transduction. Whole-cell lysates were extracted from each cell line without transduction or after transduction with control virus targeting Luciferase (Sh-Luc) or virus targeting MET (Sh-MET). Cell lines were finally cultured in the media supplemented with 5% FCS and antibodies used were the same as in Figure 1B. Note that signals of pAKT and pERK1/2 in H290 and H2373 stronger than the ones in Figure 1B were due to longer exposure of the films. The signals were also enhanced by the addition of FCS in the media. (B) Cell proliferation assay of three MPM cell lines after shRNA-lentivirus transduction. Cells were transduced with Sh-Luc or Sh-MET viruses or uninfected. Cell proliferation assay was performed 96 h later, and absorbance values were normalized to the non-infected control, which was arbitrarily set as 100%. (C) Cell proliferation assay of 13 MPM cell lines and MeT-5A cell line with MET-specific inhibitor, SU11274. Cells were grown in the presence of increasing concentrations of SU11274. After 72 h treatment, cell viability was determined by cell proliferation assay and expressed as a ratio of cell proliferation in comparison with each control cell treated with 1% dimethyl sulfoxide.

consistent with the western blot analysis data with anti-phospho-MET antibody. We detected that several RTKs (EGFR, ErbB2 and ErbB3) were frequently coactivated with MET (Figure 3). Noticeably, EGFR, with or without other family members (ErbB2, ErbB3 or ErbB4), was phosphorylated in all MPM cell lines tested (Table I). Since frequent mutation of EGFR has been detected in lung adenocarcinomas from Asian non-smokers, we sequenced exons 18–21 of EGFR in 20 MPM cell lines, but did not find any mutation (data not shown).

Several cell lines also exhibited phosphorylation of other RTKs, such as platelet-derived growth factor receptor (PDGFR)-β or vascular endothelial growth factor receptor. Totally, the average number of phosphorylated RTKs in each MPM cell line was 6.5 among 42 (Table I). Interestingly, the patterns of most activated RTKs between the serum-starved condition and the usual cell culture condition supplemented with 10% FCS were similar, suggesting that activation of RTKs expressed on MPM cells was serum-independent (supplementary Figure 5 is available at Carcinogenesis Online). In contrast, the

normal mesothelial-derived cell line, MeT-5A, exhibited only developmental tyrosine kinase and weak EGFR activation (Figure 3).

Simultaneous treatment with MET and EGFR inhibitors induced more inhibitory effect on MPM cell proliferation and invasion in vitro

As described above, we detected frequent phosphorylation of EGFR family members, especially EGFR, as well as MET. To determine whether a sole EGFR inhibitor also induces growth inhibition of MPM cells, we tested PD153035, an EGFR inhibitor, using 11 MPM and MeT-5A cell lines. Three cell lines (ACC-MESO-4, Y-MESO-12 and Y-MESO-22) showed moderate growth suppression by PD153035, whereas the eight other cell lines and MeT-5A exhibited weak suppression (supplementary Figure 6 is available at Carcinogenesis Online).

These data suggested that because most MPM cells harbor activation of multiple RTKs simultaneously, inhibition of single RTK may be insufficient to suppress MPM cell proliferation, strongly indicating the need for dual or multiple RTK inhibition. Since MET and EGFR
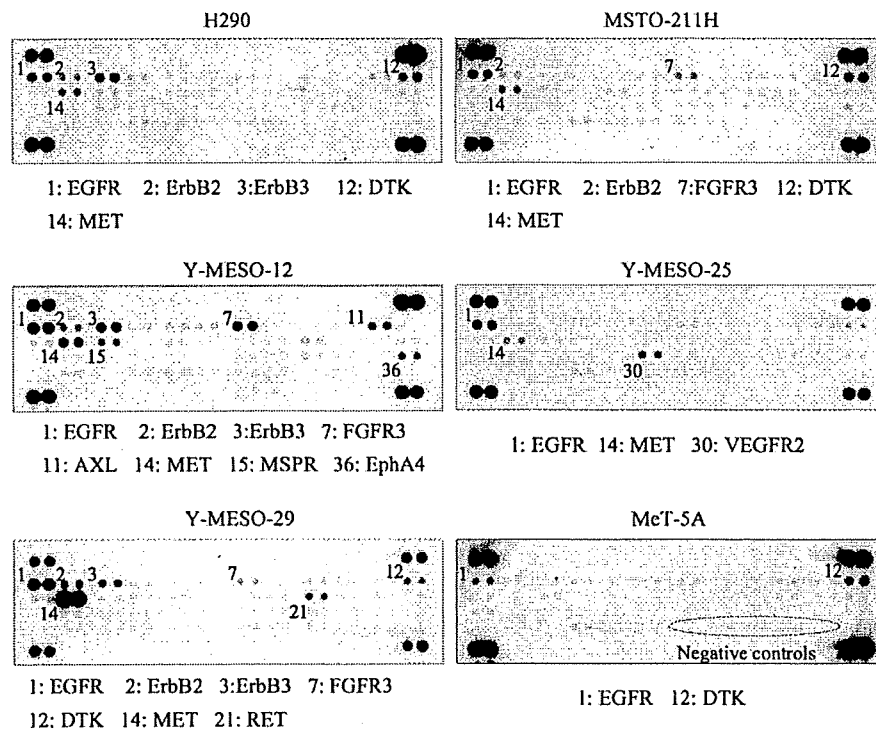
Fig. 3. Coactivation of multiple RTKs with MET in mesothelioma cell lines. Total cell lysate from each mesothelioma cell line that was cultured under serum-starved conditions for 24 h was subjected to phospho-RTK array. Positive duplicated spots for individual RTK were numbered and indicated below the array. Two dots at each corner indicate positive controls and eight dots at the lower right indicate negative controls (see oval dotted line in MeT-5A).

were the most commonly phosphorylated in MPM cells, we tested whether simultaneous treatment by MET and EGFR inhibitors induces a greater inhibitory effect on MPM cell proliferation. We tested two MPM cell lines, NCI-H2373 and NCI-H290, both of which showed phosphorylation of MET and EGFR (Figures 3 and 4B). In combination with these inhibitors, NCI-H2373 cells showed more significant growth suppression compared with each inhibitor alone (Figure 4A). We also tested PDGFR and fibroblast growth factor receptor inhibitors (AG1295 and SU5402, respectively) alone or in combination. Cell proliferation of NCI-H2373 was suppressed additively in the combination of PDGFR and EGFR inhibitors, but not with MET and PDGFR inhibitors (Figure 4A). Phospho-RTK analysis revealed that combination of EGFR and MET inhibitors also reduced the tyrosine phosphorylation of ErbB2 and ErbB3 in addition to MET and EGFR, but not AXL or PDGFR-β in the NCI-H2373 cells (Figure 4B). Western blot analysis showed that AKT phosphorylation was also significantly reduced by simultaneous treatment with EGFR and MET inhibitors (Figure 4C).

NCI-H290 exhibited phosphorylation of six RTKs, including EGFR and MET (Figure 3 and Table I). Single inhibition of EGFR or MET partially reduced the cell growth and AKT activation (Figure 5A and B). In contrast, the combination of the two inhibitors completely abrogated phosphorylation of AKT and induced significant inhibition of cell proliferation (Figure 5A and B). Fluorescence-activated cell sorting analysis also showed treatment-induced cell apoptosis and G₁–S arrest of the cell cycle (Figure 5C). We also tested five other cell lines (MSTO-211H, Y-MESO-8D, Y-MESO-12, Y-MESO-25 and Y-MESO-30) with combined treatment by EGFR and MET inhibitors and by each treatment alone. Combined treatment with both inhibitors more effectively suppressed growth of all cell lines than with either one (data not shown).

We also studied whether simultaneous inhibition of RTKs affected cell migration and invasion in vitro. Using Boyden chamber assays, we detected that combined treatment by EGFR and MET inhibitors

suppressed cell migration and invasion of three MPM cells (supplementary Figure 7 is available at Carcinogenesis Online).

MPM cell proliferation dependent on PI3-K/AKT pathway

Finally, we performed cell proliferation assay using mitogen-activated protein kinase/ERK kinase inhibitor, PD98059, and PI3-K inhibitor, LY294002, to determine on which downstream signaling cascades of RTKs MPM cells were dependent for cell proliferation. LY294002 treatment induced significant inhibition of cell proliferation in all five MPM cell lines tested (supplementary Figure 8A is available at Carcinogenesis Online). However, PD98059 treatment showed moderate in NCI-H290 and H2373, weak in Y-MESO-30 and no suppression in the other cell lines regardless of effective inhibition of phospho-ERK (supplementary Figure 8A and B is available at Carcinogenesis Online). These results suggested that MPM cell proliferation and/or survival was more dependent on the PI3-K/AKT-signaling cascade than the mitogen-activated protein kinase/ERK kinase-signaling cascade.

Discussion

In the present study, we demonstrated that multiple RTKs, especially the MET and EGFR family, were simultaneously activated in most MPMs, and dual inhibition of MET and EGFR was more effective for suppression of MPM cell proliferation and/or survival than single inhibition, with cell migration and invasion potentials also more suppressed in vitro.

Molecular target therapy has been developed to specifically inhibit consistently activated oncogene products, including RTKs. One of the most successful cases is treatment with EGFR TKI for non-small cell lung cancer with an EGFR mutation (9–11). In MPM, high EGFR expression was reported in several studies, and EGFR inhibitors, gefitinib and erlotinib, were applied in phase II studies for patients with MPM (12,22). However, these studies showed no significant tumor response. The lack of EGFR mutation in MPM may be one of the reasons for the unresponsiveness (23).
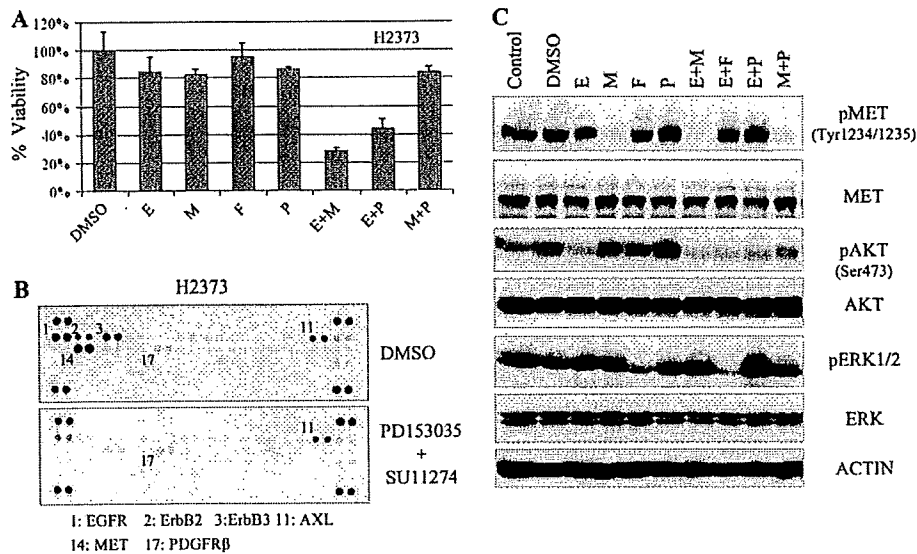
Fig. 4. Reduction of cell viability and AKT phosphorylation in NCI-H2373 cells with treatment by multiple RTK inhibitors. (A) Cell proliferation assay. NCI-H2373 cells with a single or combination treatment by RTK inhibitors or 1% dimethyl sulfoxide (DMSO) as negative control. Abbreviations of E, M, F and P stand for PD153035 (EGFR inhibitor), SU11274 (MET inhibitor), SU5402 (fibroblast growth factor receptor inhibitor) and AG1295 (PDGFR inhibitor), respectively. Final concentration of each inhibitor in the medium with 5% FCS was 5 μM. Cell viability with 1% dimethyl sulfoxide was arbitrarily set as 100%. (B) Phospho-RTK array analysis. Strong phosphorylation of EGFR, ErbB2, ErbB3, AXL and MET and weak phosphorylation of PDGFR-β in NCI-H2373 cells were detected with 1% dimethyl sulfoxide (DMSO) treatment. Phosphorylation of EGFR, ErbB2, ErbB3 and MET (duplicated spots 1, 2, 3 and 14) was markedly reduced in the presence of PD153035 and SU11274. Phosphorylation of AXL and PDGFRβ (duplicated spots 11 and 17) did not show any significant change after treatment. (C) Western blot analysis of NCI-H2373 cells after treatment with RTK inhibitors.
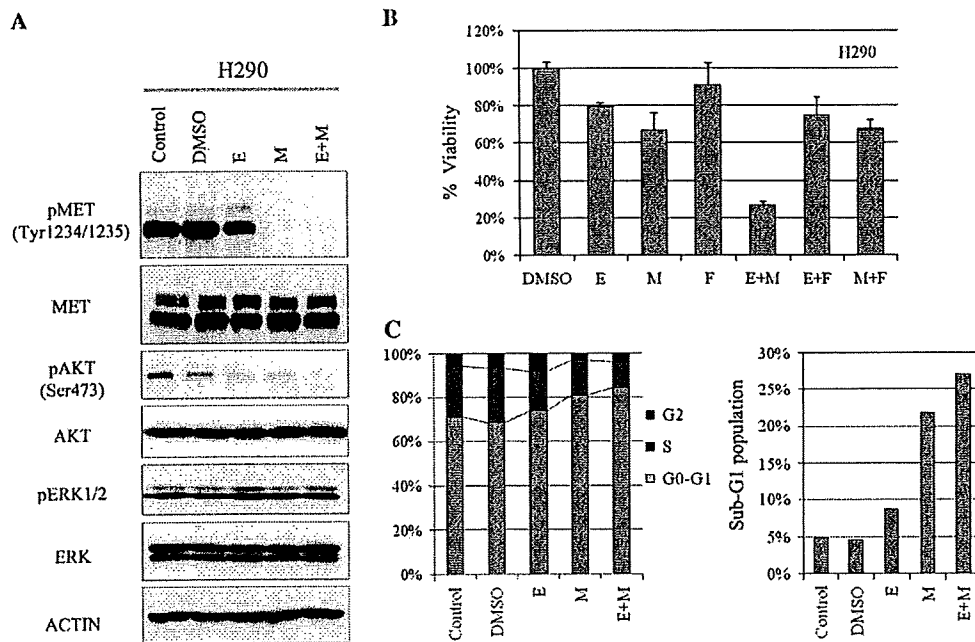

Fig. 5. Inhibition of cell viability of NCI-H290 cell line with multiple RTK inhibitors. (A) Western blot analysis. RTK inhibitor treatment was performed for 24 h with PD153035 (final concentration 5 μM), SU11274 (final concentration 5 μM) or combination of both in 5% serum-containing medium and then whole-cell lysates of NCI-H290 were analyzed. (B) Cell proliferation assay. Cell viabilities of NCI-H290 cells were determined 72 h after single or combination treatment by RTK inhibitors. The cell viability with 1% dimethyl sulfoxide was arbitrarily set as 100%. (C) Cell cycle analysis after treatment with RTK inhibitors. NCI-H290 cells at the G_0/G_1 phase of the cell cycle (left) and at the sub-G_1 fraction (right) were significantly increased by treatment with RTK inhibitors in combination comparing with dimethyl sulfoxide control or single RTK inhibitor. Abbreviations of E, M and F for RTK inhibitors are the same in the Figure 4 legend.

To develop new therapeutic strategies, it is necessary to understand detailed mechanisms of intrinsic resistance against TKIs that would be expected to be effective based on frequent activation of their corresponding RTKs. There are several possible mechanisms for resistance, which include TKI-resistant mutation of RTK, compensation by the activation of other RTKs and alterations of downstream molecules, especially a mutation of the Ras-family gene or the PI3-K catalytic subunit gene, *PIK3CA*, and loss of PTEN (19,24). One of the reasons we first focused on analyzing the *MET* gene was that a subset of MPMs was reported to harbor a point mutation, which suggested that MET would be a good candidate RTK for target therapy of MPM (17). Among 43 MPMs and 7 cell lines, Jagadeeswaran *et al.* (17) found point mutations of *MET* in four MPM specimens and two cell lines (NCI-H513 and NCI-H2596). Meanwhile, Mukohara *et al.* (18) found none in 10 cell lines. Among 20 cell lines we examined in the present study, five cell lines (NCI-H28, NCI-H2052, NICI-H2373, NCI-H2452 and MSTO-211H) were reported to be negative for mutation. Thus, 15 cell lines were newly investigated for *MET* mutation in the present study, 14 Japanese MM cell lines and NCI-H290. Taken together, possible rationales for our failure to detect *MET* mutation in the present study may be that *MET* mutation, if any, is a relatively infrequent event in MPM or that there is an ethnic difference in mutation rates of the *MET* gene. In addition, gene amplification of *MET* was reported in several cancers, including lung and colorectal cancers (25,26). Although we performed array-based comparative genomic hybridization analyses with 2304 bacterial artificial chromosome and P-1 derived artificial chromosome clones (27) and with 244K oligo probes (data not shown), clear gene amplification of the *MET* locus was not detected in 14 MPM cell lines that we established, suggesting that elevation of *MET* mRNA did not result from gene amplification.

Among the 20 MPM cell lines studied, 15 (75%) cell lines showed elevated MET at the mRNA level and 14 (70%) at the protein level. Four MM cell lines (NCI-H28, H2052, H2452 and MSTO-211H) have also been reported to show elevated MET protein in a previous study (17). Comparing our data with theirs, we found that the levels of MET expression in all but the NCI-H28 cell line were almost within 2-fold variation, which seemed to be similar results in-between. In addition, the MET expression level of two short-termed primary cultures of non-malignant mesothelial cells was about half that of MeT-5A, which also supported the idea that the 2-fold elevation of MET (which was considered to be high in this study) might be biologically significant for MPM cell proliferation *in vitro*.

Phospho-RTK array analysis showed that the EGFR family, EGFR, ErbB2 or ErbB3, was frequently coactivated with MET, which suggested that MET and EGFR family activation may compensate each other for the persistent downstream signaling activation (28). It was also interesting that several cell lines harbored coactivation of PDGFR-β, vascular endothelial growth factor receptor, MSP-R and fibroblast growth factor receptor 3 with EGFR or MET. In fact, overexpression of PDGFR-β or MSP-R was also detected in these cell lines with western blot analysis (data not shown). Overexpression of PDGFR-β was also previously reported in MPM (29–31), and imatinib mesylate (Gleevec), which inhibits both c-Kit and PDGFR-α/β, was applied for a phase II trial for MPM patients (32,33). However, no obvious tumor response was observed, which also suggested that a single RTK inhibitor was not sufficient to inhibit MPM cell proliferation.

Regarding the downstream molecules of RTKs, Altomare *et al.* (24) reported activation of AKT in 65% of MM specimens and loss of PTEN in one of nine human mesothelioma cell lines. We also observed two cell lines, ACC-MESO-1 and Y-MESO-25, which exhibited homozygous deletion of *PTEN*, whereas no mutation of the *PIK3CA* gene was detected in the other 19 MPM cell lines (34). Taken together, genetic alterations of the downstream molecule gene also seemed to be infrequent for PI3-K/AKT activation in MPM cells.

In conclusion, our results indicated that activation of multiple RTKs is critical for cell proliferation and/or survival of MPM cells. Among RTKs, MET and EGFR were thought to be the most significantly involved in MPM proliferation and/or survival via PI3-K/AKT-signaling cascade activation. Our results thus suggested that inhibition of multiple RTKs may serve to develop a more effective target therapy for patients with MPM in the future.

Supplementary material

Supplementary Figures 1–8 and Table I can be found at http://carcin.oxfordjournals.org/

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Combined Survival Analysis of Prospective Clinical Trials of Gefitinib for Non–Small Cell Lung Cancer with *EGFR* Mutations

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Abstract

Purpose: Somatic mutations of the epidermal growth factor receptor (*EGFR*) gene are associated with an increased response to gefitinib in patients with non–small cell lung cancer. We have examined the impact of gefitinib on progression-free survival and overall survival in patients with *EGFR* mutation–positive non–small cell lung cancer.

Experimental Design: We searched for all clinical trials that prospectively evaluated the efficacy of gefitinib for advanced non–small cell lung cancer with *EGFR* mutations in Japan. We did a combined analysis based on individual patient data from the identified trials.

Results: Seven eligible trials were identified for a total of 148 non–small cell lung cancer patients with *EGFR* mutations. The overall response rate to gefitinib was 76.4% [95% confidence interval (95% CI), 69.5-83.2]. The median progression-free survival and overall survival were 9.7 months (95% CI, 8.2-11.1) and 24.3 months (95% CI, 19.8-28.2), respectively. Good performance status and chemotherapy-naïve status were significantly associated with a longer progression-free survival or overall survival. Of the 148 patients, 87 received gefitinib as a first-line therapy, whereas 61 received systemic chemotherapy before gefitinib treatment. The median progression-free survival after the start of first-line therapy was significantly longer in the gefitinib-first group than in the chemotherapy-first group (10.7 versus 6.0 months; *P* < 0.001), whereas no significant difference in median overall survival was apparent between the two groups (27.7 versus 25.7 months; *P* = 0.782).

Conclusions: Gefitinib monotherapy confers substantial clinical benefit in terms of progression-free survival and overall survival in non–small cell lung cancer patients with *EGFR* mutations. Randomized trials comparing chemotherapy with gefitinib as a first-line treatment are warranted in such patients.

Non–small cell lung cancer is the leading cause of death related to cancer worldwide (1). Cytotoxic chemotherapy remains the mainstay of treatment for patients with metastatic non–small cell lung cancer on the basis of the associated moderate improvement in survival and quality of life (2-4). The poor outlook even for patients with advanced non–small cell lung cancer who receive such chemotherapy has prompted a search for new therapeutic approaches.

The epidermal growth factor receptor (EGFR) is frequently overexpressed in non–small cell lung cancer and has been implicated in the pathogenesis of this disease (5, 6). Given the biological importance of EGFR signaling in non–small cell lung cancer, EGFR-specific tyrosine kinase inhibitors, including gefitinib and erlotinib, have been extensively studied in patients with this condition (7-10). We and others have shown that a clinical response to these agents is more common in women than in men, in Japanese than in individuals from Europe or the United States, in patients with adenocarcinoma than in those with other histologic subtypes of cancer, and in individuals who have never

Translational Relevance

Somatic mutations of the epidermal growth factor receptor (EGFR) are associated with response of advanced non-small cell lung cancer to EGFR-specific tyrosine kinase inhibitors such as gefitinib and erlotinib. Prospective phase II trials of gefitinib monotherapy for advanced non-small cell lung cancer with *EGFR* mutations have found higher response rates than those observed with conventional chemotherapy. However, overall survival data have not been available because of the short follow-up period in these trials. We have now analyzed updated individual patient data from seven Japanese prospective phase II trials of gefitinib monotherapy, including a total of 148 *EGFR* mutation-positive individuals. We found that gefitinib confers a highly favorable progression-free survival (9.7 months) and overall survival (24.3 months) in such patients. Furthermore, an exploratory comparison between gefitinib and systemic chemotherapy in the first-line setting suggests that gefitinib monotherapy is an effective first-line treatment for *EGFR* mutation-positive non-small cell lung cancer. These results have potentially important implications for the treatment of non-small cell lung cancer associated with *EGFR* mutations.

smoked than in those with a history of smoking (11). Somatic activating mutations in the EGFR gene (*EGFR*) have also been identified as a major determinant of the clinical response to treatment with gefitinib or erlotinib (12-14). *EGFR* mutations are present more frequently in never-smokers, females, individuals with adenocarcinoma, and patients of East Asian ethnicity (15-18), the same groups identified clinically as most likely to respond to treatment with EGFR tyrosine kinase inhibitors.

Several prospective clinical trials of gefitinib or erlotinib for treatment of non-small cell lung cancer patients with *EGFR* mutations have been done to date (18-26). These trials have shown radiographic response rates ranging from 55% to 82% and a median progression-free survival of 8.9 to 13.3 months. These values are three to four times those historically observed with platinum-based chemotherapy as a first-line treatment for advanced non-small cell lung cancer. As the data accumulate, it seems clear that non-small cell lung cancer patients with *EGFR* mutations exhibit a distinct clinical response to treatment with EGFR tyrosine kinase inhibitors. An improvement in overall survival conferred by treatment with these drugs is also expected in patients harboring *EGFR* mutations. However, it was not possible to evaluate overall survival in most of the clinical trials at the time of publication because the number of patients was not sufficiently large and the follow-up period was not long enough to obtain precise estimates of survival outcome.

We have now done a combined analysis based on individual patient data from prospective phase II trials of gefitinib monotherapy in non-small cell lung cancer patients with *EGFR* mutations. The present study was designated I-CAMP for Iressa Combined Analysis of Mutation Positives. Our main aim was to update the effects of gefitinib treatment on survival end points in the selected population of patients. We further explored the efficacy of gefitinib administration as a first-line treatment for *EGFR* mutation-positive patients in comparison with conventional cytotoxic chemotherapy.

Materials and Methods

Study selection. We searched for all clinical trials conducted in Japan that prospectively evaluated the efficacy of gefitinib monotherapy for advanced non-small cell lung cancer associated with *EGFR* mutations. The search was done with PubMed and the Proceedings of the American Society of Clinical Oncology covering the period from 2004 to 2008. Seven phase II trials were identified, all of which were published (19-25). All identified trials, including genomic analysis of stored or collected tumor tissue, were approved by institutional review boards, and *EGFR* mutations were determined either by direct sequencing, by common fragment analysis of PCR-mediated amplification products for exon 19 deletions and cycleave real-time PCR for the L858R point mutation (26), or by the peptide nucleic acid-locked nucleic acid PCR clamp method (27). All trials had the same treatment schedule, consisting of the oral administration of 250 mg of gefitinib once a day. In some trials, gefitinib was the first-line treatment, whereas in others, it was administered after cytotoxic chemotherapy. The primary end point of these trials was tumor response rate, which was assessed according to the Response Evaluation Criteria in Solid Tumors (28).

Collection of individual patient data. The study was done in accordance with the Declaration of Helsinki (1964, amended in 2000) and the Ethical Guidelines for Epidemiologic Study (Ministry of Health, Labor, and Welfare of Japan, 2002). The primary objective of the study was to determine the impact of gefitinib treatment for *EGFR* mutation-positive non-small cell lung cancer on overall survival on the basis of examination of individual data from 148 patients enrolled in the seven selected trials. Secondary objectives included evaluation of response, progression-free survival, and safety for gefitinib, and to compare progression-free survival and overall survival for first-line gefitinib treatment with those for first-line chemotherapy administered before gefitinib. The medical records of patients in the seven identified studies were reviewed for patient characteristics, drug side effects, tumor response, progression-free survival, and overall survival. Patient characteristics noted included sex, age, Eastern Cooperative Oncology Group (ECOG) performance status, tumor histology, tumor-node-metastasis staging, postoperative disease recurrence, smoking history, previous chemotherapy, and type of *EGFR* mutation. All adverse events with a grade of ≥3 according

Table 1. Patient characteristics

Characteristic	No. of patients (*n* = 148)
Histology (adeno/nonadeno)	143/5
Median age (range), y	65 (33-89)
Sex (female/male)	102/46
Smoking status (never-smoker/smoker)	105/43
Tumor stage (IIIB/IV)	19/129
ECOG PS (0/1/2/3/4)	58/69/14/3/4
No. of previous chemotherapy regimens (0/1/2/3)	85/48/14/1
EGFR mutation (ex 19 del/L858R/other*)	88/56/4

Abbreviations: adeno, adenocarcinoma; nonadeno, nonadenocarcinoma; PS, performance status; ex 19 del, exon 19 deletion.
*Exon 19 deletion + L747P, L858R + L858K, exon 19 deletion + 26-bp deletion + AT insertion, or exon 19 deletion + L858R.
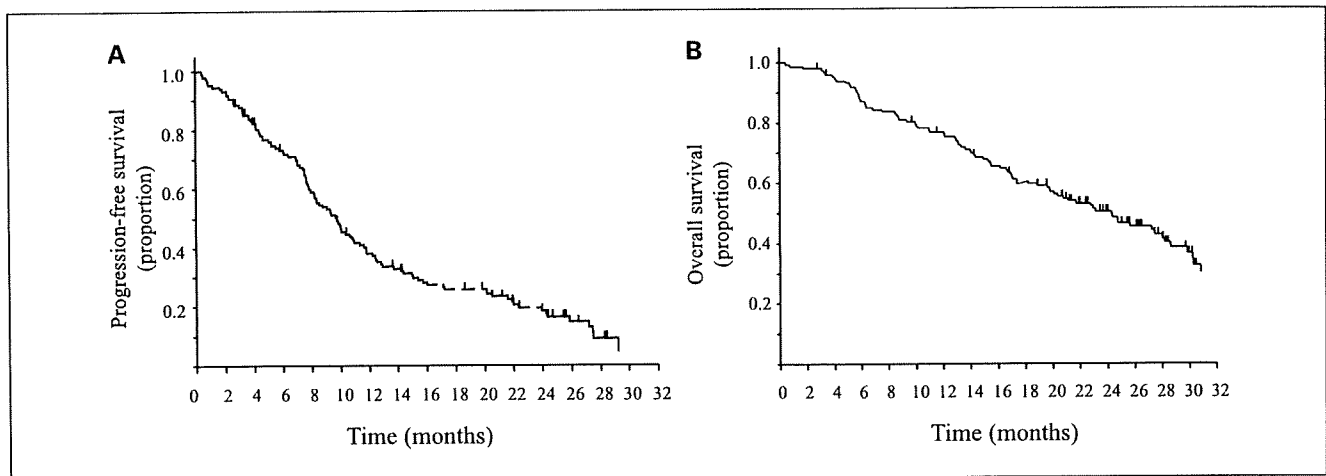
Fig. 1. Kaplan-Meier analysis of progression-free survival (*A*) and overall survival (*B*) for all 148 patients after initiation of gefitinib treatment.

to the National Cancer Institute–Common Toxicity Criteria (version 3.0) were recorded, as was interstitial lung disease of any grade. The clinical data for first-line chemotherapy administered before gefitinib were obtained retrospectively. In accordance with Response Evaluation Criteria in Solid Tumors, objective tumor responses were classified as complete response, partial response, stable disease, or progressive disease. For analysis of progression-free survival and overall survival, the day of initiation of gefitinib treatment, the day tumor progression was detected, and the last day that survival was evaluated or the day of death were noted. In addition, clinically important prognostic factors were examined.

Statistical analysis. Overall survival was defined as the time from the initiation of gefitinib monotherapy until death from any cause. Progression-free survival was defined as the time from the initiation of gefitinib monotherapy to the first observation of disease progression or death from any cause. Progression-free survival and overall survival were analyzed by the Kaplan-Meier method and were compared between groups by the log-rank test. Prognostic factors for progression-free survival and overall survival were examined by the Cox regression model, with adjustment for covariates, including sex (female versus male), smoking history (never-smoker versus smoker), tumor-node-metastasis stage (IIIB versus advanced), ECOG performance status (0 or 1 versus 2 to 4), the number of previous chemotherapy regimens (0 versus 1 to 3), and type of *EGFR* mutation (L858R versus exon 19 deletion). Differences in characteristics between patient groups according to first-line therapy were evaluated by the χ^2 test. A *P* of <0.05 was considered statistically significant.

Results

Patient characteristics. The characteristics of the 148 *EGFR* mutation–positive non–small cell lung cancer patients are summarized in Table 1. The median age was 65 years, with a range of 33 to 89 years. A total of 102 patients (69%) were women, and 105 patients (71%) were never-smokers. The most common tumor histology was adenocarcinoma, which was present in 143 patients (97%). Whereas 88 patients had a single *EGFR* mutation consisting of an exon 19 deletion, 56 patients had a single mutation consisting of L858R in exon 21 and the remaining 4 patients had double or triple mutations involving an exon 19 deletion or L858R.

Drug safety and toxicity. Most treatment-related toxicity was mild, being of National Cancer Institute–Common Toxicity Criteria grade 1 or 2. Adverse events of grade 3 or 4 included skin rash (2.7%), diarrhea (1.4%), interstitial lung disease (2.7%), and abnormal liver function, including elevated aspartate aminotransferase or alanine aminotransferase (8.1%). There were no treatment-related deaths.

Response and survival. Eleven patients (7%) showed a complete response and 102 individuals (69%) achieved a partial response to gefitinib monotherapy, yielding an objective response rate of 76.4% [95% confidence interval (95% CI), 69.5-83.2] and substantiating the individual observations of

Table 2. Cox regression analysis of progression-free survival and overall survival after gefitinib treatment (*n* = 148)

Variable	PFS			OS		
	HR	95% CI	*P*	HR	95% CI	*P*
Sex (female/male)	0.63	0.37-1.09	0.098	0.65	0.35-1.22	0.182
Never-smoker/smoker	0.93	0.51-1.66	0.794	0.82	0.42-1.61	0.570
Tumor stage (IIIB/IV)	1.42	0.81-2.48	0.219	1.83	0.96-3.48	0.067
ECOG PS (0-1/2-4)	0.58	0.33-1.01	0.056	0.27	0.15-0.48	<0.0001
Previous chemotherapies (0/1-3)	0.57	0.38-0.86	0.007	0.60	0.37-0.95	0.031
EGFR mutation (L858R/ex 19 del)	0.93	0.62-1.40	0.730	0.83	0.52-1.33	0.438

Abbreviations: HR, hazard ratio; PFS, progression-free survival; OS, overall survival.

Table 3. Patient characteristics at the onset of first-line treatment with gefitinib or chemotherapy

Characteristic	Gefinitib (n = 87)	Chemotherapy (n = 61)	P
Histology (adeno/nonadeno)	85/2	58/3	0.403
Median age (range), y	66 (33-89)	61 (33-79)	0.032
Sex (female/male)	64/23	38/23	0.145
Smoking status (never-smoker/smoker)	68/19	37/24	0.021
Tumor stage (IIIB/IV)	8/79	11/50	0.111
ECOG PS (0/1/2/3/4)	33/37/11/3/3	25/32/3/0/1	0.026
EGFR mutation (ex 19 del/L858R/other)	50/36/1	38/20/3	0.256

NOTE: Ps for differences between the two groups of patients were determined by the χ^2 test, with that for ECOG performance status being determined for comparison of the proportion of patients with a status of 2 to 4.

each of the relatively small phase II trials. Twenty-three patients (16%) had stable disease, and nine (6%) had progressive disease.

At the time of analysis, the median follow-up time was 20.7 months. The median progression-free survival was 9.7 months (95% CI, 8.2-11.1), and the 1-year progression-free survival rate was 37.7% (95% CI, 29.7-45.7; Fig. 1A). The median overall survival was 24.3 months (95% CI, 19.8-28.2), and the 1-year overall survival rate was 76.7% (95% CI, 69.8-83.6; Fig. 1B). Cox regression analysis revealed that an ECOG performance status of 0 or 1 and chemotherapy-naïve status were significantly associated with a longer progression-free survival or overall survival (Table 2).

Comparison between gefitinib and cytotoxic chemotherapy as first-line treatment. Of the 148 EGFR mutation-positive non-small cell lung cancer patients, 87 received gefitinib as first-line therapy whereas 61 received systemic chemotherapy as first-line treatment, followed by gefitinib. Clinical information was assembled retrospectively for the patients who received first-line chemotherapy before gefitinib treatment. The clinicopathologic data for these two groups of patients are shown in Table 3. The proportion of patients with a poor ECOG performance status ($\geq$2) was higher in the first-line gefitinib group (20%) than in the first-line chemotherapy group (7%; P = 0.026). The response rate was significantly higher for the first-line gefitinib group than for the first-line chemotherapy group (79.3% versus 24.6%; P < 0.001; Table 4). Kaplan-Meier analysis of progression-free survival and overall survival after the start of first-line antitumor therapy is shown in Fig. 2. The log-rank test revealed that progression-free survival was significantly longer in the first-line gefitinib group than in the first-line chemotherapy group (median of 10.7 versus 6.0 months; Fig. 2A), whereas there was no significant difference in overall survival between the two groups of patients (median of 27.7 versus 25.7 months, respectively; Fig. 2B). Cox regression analysis yielded similar results for progression-free survival and overall survival.

Discussion

EGFR mutations were first associated with non-small cell lung cancer in 2004, and several prospective phase II trials of gefitinib or erlotinib for treatment of non-small cell lung cancer patients with activating EGFR mutations have subsequently been reported (12-14). The primary end point of these prospective trials was objective response rate, with the result

that overall survival data were not complete because of the short follow-up periods. We have now analyzed the updated individual data for 148 EGFR mutation-positive non-small cell lung cancer patients enrolled in seven prospective phase II trials of gefitinib monotherapy in Japan. The median progression-free survival and overall survival were 9.7 months (95% CI, 8.2-11.1) and 24.3 months (95% CI, 19.8-28.2), respectively. These findings reveal a markedly improved outcome with gefitinib therapy compared with that typically observed with systemic chemotherapy in patients with advanced non-small cell lung cancer.

The number of EGFR mutation-positive patients examined in the present study is sufficient to allow comparison of progression-free survival and overall survival among non-small cell lung cancer patients with different clinicopathologic characteristics. Previous studies have shown that EGFR mutations are more frequent in females, individuals with no history of smoking, and patients with adenocarcinoma, and that these characteristics are also associated with a higher response rate and longer survival after gefitinib treatment (16-18). We have now found that sex and smoking status were not significantly associated with progression-free survival or overall survival among patients with EGFR mutations, indicating that such mutations, regardless of sex and smoking status, are the most appropriate determinant for gefitinib treatment. These findings suggest that analysis of EGFR mutation status is warranted for

Table 4. Tumor response to first-line treatment with gefitinib or chemotherapy

Tumor response	Gefitinib	Chemotherapy*
Complete response	7	0
Partial response	62	15
Stable disease	11	31
Progressive disease	6	10
Unknown	1	5
Total	87	61
Response rate (95% CI), %	79.3 (70.8-87.8)	24.6 (13.8-35.4)

NOTE: P < 0.001 for difference in response rate between the two groups (χ^2 test).
*The chemotherapy regimens included platinum doublet (n = 39), nonplatinum doublet (n = 8), single agent (n = 9), and unknown (n = 5).
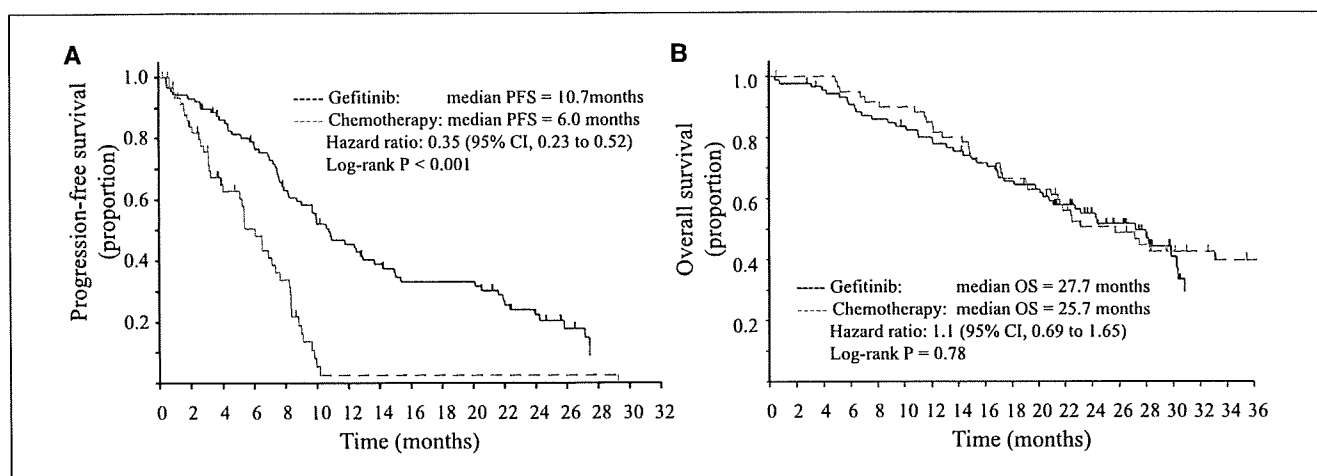
Fig. 2. Comparison of progression-free survival (*A*) or overall survival (*B*) after the initiation of first-line treatment with gefitinib or chemotherapy.

treatment selection even in male smokers with adenocarcinoma. Studies on North American patients have indicated that those with deletions of exon 19 of *EGFR* have a better response rate, progression-free survival, and overall survival after EGFR tyrosine kinase inhibitor treatment than do those with the L858R mutation in exon 21 (33, 34); however, the present study did not detect a significant difference in progression-free survival or overall survival between the gefitinib-treated patients with exon 19 deletions and those with L858R. Our finding is consistent with previous retrospective analysis of East Asian cohorts showing similar survival benefit of EGFR tyrosine kinase inhibitor treatment in patients with either type of mutation (35, 36). This apparent difference between North American and East Asian populations might be due to the type of EGFR tyrosine kinase inhibitor (gefitinib or erlotinib) studied, given that all patients in the East Asian cohorts and our present study were treated with gefitinib whereas the North American patients included those treated with erlotinib or gefitinib. Although the biological basis for a possible ethnic difference in EGFR tyrosine kinase inhibitor efficacy according to the type of *EGFR* mutation remains unknown, it seems that East Asian patients with exon 19 deletions or with L858R benefit equally from gefitinib treatment.

Platinum-based doublet chemotherapy is the standard of care for most patients with advanced non–small cell lung cancer (2, 3). The substantial clinical benefits of gefitinib treatment in EGFR mutation–positive non–small cell lung cancer patients raise the question about whether first-line gefitinib treatment is more beneficial than systemic chemotherapy in this genotype-defined population. Given that the impact of first-line systemic chemotherapy on EGFR mutation–positive non–small cell lung cancer patients has not been fully evaluated, we compared progression-free survival and overall survival between mutation-positive patients who received first-line gefitinib treatment and those treated initially with systemic chemotherapy. We found that first-line gefitinib treatment yielded a significantly longer progression-free survival than did systemic chemotherapy in EGFR mutation–positive non–small cell lung cancer patients, supporting the use of gefitinib as an initial therapy in this patient population. This finding is consistent with a subset analysis of a recently

completed randomized phase III study known as Iressa Pan-Asia Study, which showed that first-line gefitinib treatment significantly improved the progression-free survival of EGFR mutation–positive patients with advanced non–small cell lung cancer compared with treatment with carboplatin and paclitaxel (37). We further showed that the significant difference in progression-free survival of EGFR mutation–positive patients according to first-line therapy was not associated with a difference in overall survival likely because all patients treated with systemic chemotherapy as a first-line treatment received gefitinib as a subsequent treatment. This finding suggests that the survival benefit of gefitinib treatment for patients with EGFR mutations is substantial, even when the drug is administered as a second-line therapy, and it raises the question of whether gefitinib is more effective in such patients as a first-line therapy or is equally effective when administered after systemic chemotherapy. Cox regression analysis in the present study revealed that progression-free survival after gefitinib treatment was significantly longer in the chemotherapy-naive patients than in those who had received previous chemotherapy. Although the impact of systemic chemotherapy on the subsequent efficacy of gefitinib in EGFR mutation–positive patients remains ill defined, our data raise the possibility that systemic chemotherapy may induce biological effects that lead to gefitinib resistance. Elucidation of such effects will be difficult given the challenges associated with repeated tumor biopsy in non–small cell lung cancer patients after the initiation of chemotherapy. Recent randomized phase III studies found that the tolerability profile of gefitinib was better than that of systemic chemotherapy, resulting in improvement in quality of life (9, 10). Taken together, these data provide support for the treatment of chemotherapy-naive, EGFR mutation–positive non–small cell lung cancer patients with gefitinib, although well-designed randomized trials that compare EGFR tyrosine kinase inhibitors with standard chemotherapy and monitor quality of life in such patients are warranted.

In conclusion, our combined analysis of updated individual patient data from seven Japanese phase II trials confirmed that gefitinib monotherapy yields substantial clinical benefits in terms of a high response rate and prolonged progression-free

survival and overall survival in advanced non–small cell lung cancer patients with *EGFR* mutations. Our results have important potential implications for clinical practice. The median survival time of ~2 years achieved in patients with *EGFR* mutation–positive non–small cell lung cancer by treatment with gefitinib supports the notion that this group of patients constitutes a clinically distinct population. Furthermore, our exploratory comparison between gefitinib and systemic chemotherapy as a first-line treatment suggests that gefitinib monotherapy is a potentially important first-line treatment option for *EGFR* mutation–positive non–small cell

lung cancer. We are currently doing phase III randomized studies comparing platinum-based chemotherapy with gefitinib in chemotherapy-naïve non–small cell lung cancer patients with *EGFR* mutations. Such ongoing phase III clinical trials will help determine whether gefitinib monotherapy becomes the standard of care for *EGFR* mutation–positive non–small cell lung cancer.

Disclosure of Potential Conflicts of Interest

No potential conflicts of interest were disclosed.

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ORIGINAL ARTICLE

hOGG1 Ser326Cys polymorphism and risk of lung cancer by histological type

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Human 8-oxoguanine DNA glycosylase 1 (*hOGG1*) has a major role in the repair of 8-hydroxyguanine, a major promutagenic DNA lesion. The genetic polymorphism rs1052133, which leads to substitution of the amino acid at codon 326 from Ser to Cys, shows functional differences, namely a decrease in enzyme activity in *hOGG1*-Cys326. Although several studies have investigated the association between rs1052133 and lung cancer susceptibility, the effect of this locus on lung cancer according to histology remains unclear. We therefore conducted a case–control study with 515 incident lung cancer cases and 1030 age- and sex-matched controls without cancer, and further conducted a meta-analysis. In overall analysis, the homozygous Cys/Cys genotype showed a significant association with lung cancer compared to Ser allele carrier status (odds ratio (OR)=1.31, 95% confidence interval (CI)=1.02–1.69). By histology-based analysis, the Cys/Cys genotype showed a significantly positive association with small-cell carcinoma (OR=2.40, 95% CI=1.32–4.49) and marginally significant association with adenocarcinoma (OR=1.32, 95% CI=0.98–1.77). A meta-analysis of previous and our present study revealed that this polymorphism is positively associated with adenocarcinoma, although suggestive associations were also found for squamous- and small-cell lung cancers. These results indicate that rs1052133 contributes to the risk of adenocarcinoma of lung.
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Keywords: *hOGG1*; lung cancer; polymorphism

INTRODUCTION

Cancer is linked to environmental exposure to various carcinogens, of which tobacco smoke is a well-known example. Exposure leads to various types of DNA damage, such as oxidative damage. Genetic variations in DNA repair genes are associated with DNA repair capacity, suggesting a consequent association with cancer risk.[1]

8-Hydroxyguanine, produced by reactive oxygen species in tobacco smoke, is a major form of DNA damage.[2] This alteration to the DNA structure causes G:C to T:A transversions, and may thus be responsible for mutations that lead to carcinogenesis.[3] Human 8-oxoguanine DNA glycosylase 1 (*hOGG1*) has been extensively studied as the main enzyme involved in the repair of 8-oxoG DNA adducts. Although it has a major role in the repair of 8-hydroxyguanine, however, its role in carcinogenesis has not been well elucidated.[4] Genetic polymorphisms of *hOGG1* have been documented, and the polymorphism Ser326Cys (rs1052133) is associated with complementation activity for *Escherichia coli* mutants that are defective in the repair of 8-hydroxyguanine. Activity in the repair of 8-hydroxyguanine

is greater with the *hOGG1*-Ser326 protein than the *hOGG1*-Cys326 protein,[5] and the possible contribution of this locus to the risk of a variety of human cancers has been reported.[6]

A number of studies[7–14] and systematic approaches[15–17] have examined the role of the Ser326Cys polymorphism in lung cancer susceptibility. One meta-analysis showed that the overall odds ratio (OR) of homozygotes for the *hOGG1*-326Cys allele against those for the *hOGG1*-326Ser allele was 1.24 (95% confidence interval (CI)=1.01–1.53), suggesting that the locus is involved in susceptibility to overall lung cancer.[17] In contrast, another meta-analysis reported no significant association.[15] A recent pooled analysis from the International Lung Cancer Consortium involving a substantial number of cases and controls showed a suggestive association for this polymorphism in Caucasians.[16] One question that remains unanswered is whether the impact of rs1052133 differs according to histological subtype of lung cancer.

Here, we evaluated the role of the *hOGG1* Ser326Cys polymorphism in lung cancer susceptibility among a Japanese population in

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consideration of histology. We also conducted a meta-analysis of the literature to evaluate the impact of this polymorphism by histology.

MATERIALS AND METHODS

Subjects

The case subjects were 515 patients who were newly and histologically diagnosed with lung cancer and who had no history of cancer. Controls were randomly selected from among the 2395 cancer-free individuals and matched by age (± 3 years) and sex to cases in a 1:2 case/control ratio. All subjects were recruited within the framework of the Hospital-based Epidemiologic Research Program at Aichi Cancer Center (HERPACC), as described elsewhere,[18,19] and were exactly the same cohort we reported on in a previous paper.[20] In brief, information on lifestyle factors was collected using a self-administered questionnaire from all first-visit outpatients at Aichi Cancer Center Central Hospital aged 18–79 who were enrolled in the HERPACC between January 2001 and November 2005. Response was checked by a trained interviewer. Outpatients were also asked to provide blood samples. Each patient was asked about their lifestyle when healthy or before the current symptoms developed. Approximately 95% of eligible subjects completed the questionnaire and 60% provide blood samples. The data were loaded into the HERPACC database and routinely linked with the hospital-based cancer registry system to update the data on cancer incidence. All participants gave written informed consent and the study was approved by institutional ethical committee of Aichi Cancer Center.

Genotyping of *hOGG1*

DNA from each sample was extracted from the buffy coat fraction using a BioRobot EZ1 with an EZ1 DNA Blood 350 μl kit or QIAamp DNA Blood mini kit (Qiagen KK, Tokyo, Japan). Polymorphisms of *hOGG1* Ser326Cys were examined based on TaqMan assays by Applied Biosystems (Foster City, CA, USA). The principle of the TaqMan real-time polymerase chain reaction (PCR) assay system using fluorogenic probes and 5′ nuclease has been described by Livak.[21] All of the assays were carried out in 96-well PCR plates using a 7500 Fast Real-Time PCR System (Applied Biosystems) coupled with the 7500 Fast System SDS software. Amplification reactions (5 μl) were carried out in duplicate with 30 ng of template DNA, 2× TaqMan Universal Master Mix buffer (Applied Biosystems) and 20× primer and probe mix (Applied Biosystems). Thermal cycling was initiated with a first denaturation step of 20 s at 95 °C, and then by 40 cycles of 3 s at 95 °C and 30 s at 62 °C. Genotyping quality was statistically assessed using the Hardy–Weinberg test in our laboratory; when allelic distributions for controls departed from the Hardy–Weinberg frequency, genotyping was assessed using another method.

Consumption of tobacco, alcohol, fruits and vegetables

Cumulative smoking dose was evaluated as pack-years (PY), the product of the number of packs consumed per day and the number of years of smoking. Smoking habit was entered in the four categories of never, former, and current smokers of <40 and ≧40 PY. Former smokers were defined as those who quit smoking at least 1 year before the survey. Drinking habit was categorized in the three categories of never, former and current drinkers. Former drinkers were defined as those who quit drinking at least 1 year before the survey. Consumption of fruits and vegetables was determined using a semiquantitative food frequency questionnaire (SQFFQ), described in detail elsewhere.[22] Briefly, the SQFFQ consisted of 47 single food items with frequencies in eight frequency categories. We estimated average daily intake by multiplying the frequency of intake by the serving size of food (in grams). Energy-adjusted intake of fruits and vegetables was calculated by the residual method.[23] The SQFFQ was validated using a 3-day weighed dietary record as standard, which showed that reproducibility and validity were acceptable.[24]

Statistical analysis

To assess the strength of associations between *hOGG1* polymorphism and risk of lung cancer, we estimated ORs with 95% CIs, using conditional logistic models adjusted for potential confounders. For stratified analyses exploring interactions, we applied unconditional logistic regression models because matching was not retained after stratification by smoking and drinking habit and carotene intake in conditional models. Fruit and vegetable intake was

categorized into three levels by applying thresholds of tertiles among controls. Potential confounders considered in the multivariate analyses were age, sex, smoking habit (never smokers, former smokers, current smokers of less than 40 or 40 or more PY), drinking habit (never, former and current drinkers), total energy intake (as a continuous variable), and dietary fruit and vegetable intake (g per day, tertiles). Missing values for each covariate were treated as dummy variables and were included in the model. Trend for genotype was assessed by application of a score test value for each genotype (0, homozygous for reference allele or combined reference genotypes; 1, heterozygote or one reference genotype and 2, homozygous nonreference allele or nonreference genotype). Differences in categorized demographic variables between cases and controls were tested by the χ^2-test. Mean values for age and total energy intake were compared for cases and controls by Wilcoxon's signed-rank test. Accordance with the Hardy–Weinberg equilibrium was checked for controls using the χ^2-test and the exact *P*-value was used to assess any discrepancies between

Table 1 Characteristics of case and control subjects

	Cases (n=515) n (%)	Controls (n=1030) n (%)	P-value
Age			
<50	53 (10.3)	108 (10.5)	
50–59	142 (27.6)	283 (27.5)	
60–69	193 (37.5)	389 (37.8)	
70–79	127 (24.7)	250 (24.3)	1.00
Mean age (range)	61.9 (23–79)	61.8 (26–79)	0.87
Sex			
Male	381 (74.0)	762 (74.0)	
Female	134 (26.0)	268 (26.0)	1.00
Smoking (Pack-years)			
<5	136 (26.4)	424 (41.2)	
5–19.9	31 (6.0)	118 (11.5)	
20–39.9	88 (17.1)	208 (20.2)	
>40	258 (50.1)	275 (26.7)	<0.001
Unknown	2 (0.4)	5 (0.5)	
Drinking status			
Never	196 (38.1)	378 (36.7)	
Former[a]	15 (2.9)	56 (5.4)	
Current	304 (59.0)	596 (57.9)	0.08
Fruit/vegetable consumption (g per day)			
Tertile 1 (<118.4)	199 (38.8)	342 (33.2)	
Tertile 2 (118.4–211.3)	140 (27.3)	341 (33.1)	
Tertile 3 (>211.4)	166 (32.4)	341 (33.1)	0.03
Unknown	8 (1.6)	6 (0.6)	
Total energy intake (kcal, s.d.)[b]	1670 (371)	1676 (352)	1.00
Histology			
AD	316 (61.4)		
SQ	91 (17.7)		
SM	55 (10.7)		
LA	40 (7.8)		
Others	13 (2.5)		

Abbreviations: AD, adenocarcinoma; LA, large-cell carcinoma; SM, small-cell carcinoma; SQ, squamous-cell carcinoma.
[a]Former drinkers were defined as subjects who had quit drinking at least 1 year previously.
[b]Energy-adjusted.

genotypes and allele frequencies, with a P-value of less than 0.05 considered statistically significant. All analyses were performed using STATA version 10.1 (Stata, College Station, TX, USA).

Meta-analysis

We conducted a meta-analysis of relevant articles reporting associations between the hOGG1 polymorphism and lung cancer in consideration of the histological subtypes adenocarcinoma, squamous-cell carcinoma and small-cell carcinoma. Medline was searched for papers published between January 1995 and March 2009 and indexed with the terms (lung neoplasms AND (hOGG1 OR OGG1)). Inclusion criteria were (1) reporting of ORs or risk ratios calculated by comparing the Ser/Ser to the Cys/Cys or Cys allele carrier according to histological subtype; (2) a cohort, nested case–control, population-based case–control or hospital-based case–control study design and (3) use of cancer-free controls. All potentially relevant papers were independently reviewed by at least two investigators (TO and KM) and any disagreements were resolved by consensus. The reference lists of studies identified through the search process were also checked. Among the 65 papers identified through this process, 7 were considered eligible.[5,7–11,17] Two investigators (TO and KM) abstracted the data independently. We used OR from a random-effect model as a summary statistic for association.[25] Heterogeneity among the studies was examined based on the Q and I^2 statistics. The latter indicates the proportion of variation in summary estimates attributable to heterogeneity.[26] We determined which model to use to calculate summary OR and its 95% CI, a random- or fixed-effect model, based on significance in the Q statistics. The meta-analysis was conducted using the 'metan' command[27] in STATA version 10.1.

RESULTS

Characteristics of the 515 cases and 1030 controls are shown in Table 1. Age and sex were appropriately matched. Smoking habits differed remarkably between cases and controls, with the proportion of current smokers of 40 PY or more significantly higher in cases. Former drinkers tended to be more common among cases, albeit without statistical significance. Consumption of fruits and vegetables was significantly lower among cases. The distribution of histological type among cases was as follows: adenocarcinoma, 61.4% ($n=316$);

squamous-cell carcinoma, 17.7% ($n=91$); small-cell carcinoma, 10.7% ($n=55$); large cell carcinoma, 7.8% ($n=40$) and others, 2.5% ($n=13$).

Table 2 presents the frequency distribution of hOGG1 genotypes and ORs with 95% CI for lung cancer cases compared with controls. No significant dissociation from the Hardy–Weinberg equilibrium was observed among controls. In overall analysis, Cys/Cys showed a significantly positive association with lung cancer. The confounder-adjusted OR for Cys/Cys relative to Ser/Ser+Ser/Cys was 1.31 (1.02–1.69, $P=0.036$). In histology-based analysis, those with the Cys/Cys genotype were at significantly increased risk of small-cell carcinoma and marginally significantly increased risk of adenocarcinoma, compared to those with the Ser/Cys and Ser/Ser genotypes combined. No significant associations were observed for squamous-cell carcinoma.

Table 3 shows associations between hOGG1 Ser326Cys polymorphism combined with smoking and lung cancer risk. In overall analysis, the effect of cumulative smoking dose was stronger in those with Cys/Cys. In analyses by histology, a similar trend was observed for adenocarcinoma and small-cell carcinoma but not for squamous-cell carcinoma. This trend was more prominent for small-cell carcinoma. Adjusted ORs for heavy smoking (PY≧40) were 26.3 (5.34–129.6) for the Ser allele carrier and 72.3 (14.6–358.2) for those with the Cys/Cys.

To further examine the impact of hOGG1 Ser326Cys polymorphism according to histology, we conducted a meta-analysis. Table 4 shows a summary of studies that have investigated the association between hOGG1 Ser326Cys polymorphism and lung cancer risk, including the present study. As shown in Figure 1, hOGG1 Ser326Cys polymorphism summary ORs showed a significant association with adenocarcinoma (OR=1.44, 95% CI=1.18–1.77) with no significant heterogeneity. Although squamous-cell carcinoma showed a similarly increased risk (OR=1.81, 95% CI=1.06–3.07), the significant heterogeneity across studies ($I^2=58.5$) was a limitation. Although without significance and from a limited number of studies, the pooled estimate was 2.05 (0.91–4.63), suggesting an increased risk for small-cell carcinoma.

Table 2 hOGG1 genotype distribution and ORs for lung cancer

Genotype	Cases n=515	Controls n=1030	OR1 (95% CI)[a]	P-value	OR2 (95% CI)[b]	P-value
Overall						
Ser/Ser	117	250	1.00 (reference)		1.00 (reference)	
Ser/Cys	257	544	1.01 (0.77–1.32)		0.96 (0.72–1.26)	
Cys/Cys	141	236	1.28 (0.94–1.73)	0.054	1.27 (0.93–1.75)	0.047
Ser/Ser+Ser/Cys	374	794	1.00 (reference)		1.00 (reference)	
Cys/Cys	141	236	1.27 (1.00–1.62)	0.05	1.31 (1.02–1.69)	0.036
Adenocarcinoma						
Ser/Ser+Ser/Cys	227	794	1.00 (reference)		1.00 (reference)	
Cys/Cys	89	236	1.29 (0.97–1.72)	0.085	1.32 (0.98–1.77)	0.066
Squamous-cell carcinoma						
Ser/Ser+Ser/Cys	72	794	1.00 (reference)		1.00 (reference)	
Cys/Cys	19	236	0.99 (0.58–1.70)	0.98	1.10 (0.63–1.94)	0.73
Small-cell carcinoma						
Ser/Ser+Ser/Cys	34	794	1.00 (reference)		1.00 (reference)	
Cys/Cys	21	236	2.22 (1.26–3.92)	0.006	2.40 (1.22–4.12)	0.009

Abbreviations: CI, confidence interval; OR, odds ratio.
[a]Adjusted for age and sex.
[b]Adjusted for age, sex, smoking habit, drinking habit, total energy intake and energy-adjusted fruit/vegetable intake.

Table 3 Associations between *hOGG1* Ser326Cys polymorphisms and smoking by PY on lung cancer risk

Histology	Ser (+)		Cys/Cys	
	Case/Control	OR (95% CI)[b]	Case/Control	OR (95% CI)[b]
Overall[a]				
Smoking (pack-years)		Ser (+)		Cys/Cys
<5	95/317	1.0 (reference)	41/107	1.35 (0.88–2.09)
5–19.9	22/89	1.26 (0.72–2.21)	9/29	1.29 (0.55–3.03)
20–39.9	66/160	2.38 (1.53–3.68)	22/48	2.54 (1.39–4.63)
>40	191/223	5.26 (3.54–7.78)	67/52	7.44 (4.53–12.2)
Adenocarcinoma				
Smoking		Ser (+)		Cys/Cys
<5	89/317	1.0 (reference)	39/107	1.36 (0.87–2.13)
5–19.9	15/89	0.95 (0.50–1.79)	5/29	0.70 (0.23–2.13)
20–39.9	39/160	1.62 (0.98–2.66)	13/48	1.75 (0.86–3.54)
>40	84/223	2.75 (1.77–4.28)	30/52	3.99 (2.25–7.08)
Squamous-cell carcinoma				
Smoking		Ser (+)		Cys/Cys
5–19.9	3/406	1.0 (reference)	3/136	3.22 (0.64–16.3)
20–39.9	13/160	6.99 (1.93–25.4)	5/48	8.99 (2.04–39.5)
>40	56/223	19.5 (5.87–64.3)	11/52	16.6 (4.37–63.0)
Small-cell carcinoma				
Smoking		Ser (+)		Cys/Cys
5–19.9	2/406	1.0 (reference)	1/136	1.50 (0.13–16.7)
20–39.9	8/160	12.6 (2.39–66.2)	4/48	18.8 (3.09–114.3)
>40	24/223	26.3 (5.34–129.6)	16/52	72.3 (14.6–358.2)

Abbreviations: CI, confidence intervals; OR, odds ratios.
[a]Five controls and two cases are excluded from analysis because of smoking information unknown.
[b]ORs were adjusted for age, sex, smoking habit, drinking habit, total energy intake and energy-adjusted fruit/vegetable intake.

Table 4 Summary of published studies examining association between *OGG1* polymorphism and lung cancer risk according to histology

Author	Year	Subjects in each study						Odds ratio (95% CI) for Cys/Cys relative to Ser/Ser		
		Case								
		Total	Adeno	Squamous	Small	Control	Ethnicities	Adeno	Squamous	Small
Sugimura et al.[7]	1999	241	1974	78	118	197	Japanese	1.34 (0.53–3.39)	2.27 (0.92–5.60)	0.51 (0.09–2.87)
Wikman et al.[8]	2000	105	50	50	NA	105	Caucasian	1.84 (0.41–14.41)	1.76 (0.24–13.1)	NE
Ito et al.[9]	2002	138	138	0	0	241	Japanese	0.81 (0.44–1.52)	NE	NE
Le Marchand et al.[10]	2002	298	141	66	43	405	Caucasian, Japanese and Hawaiian	2.1[a] (1.1–3.9)	3.7[a] (1.7–8.3)	3.4[a] (1.1–10.4)
Park et al.[11]	2004	179	63	56	32	358	Caucasian	4.20 (1.10–15.8)	4.8 (1.1–21.0)	NE
Hung et al.[17]	2005	2188	499	902	0	2198	Caucasian	1.66 (1.04–2.66)	1.02 (0.63–1.64)	NE
Kohno et al.[12]	2006	1097	1097	0	0	394	Japanese	1.47 (1.02–2.13)	NE	NE
Our study	2009	515	316	91	55	1030	Japanese	1.32 (0.98–1.77)	1.10 (0.63–1.94)	2.40 (1.22–4.12)

Abbreviations: CI, confidence intervals; NE, not estimated; OR, odd ratios.
[a]ORs are calculated as that of the homozygous Cys/Cys genotype compared to those with the Ser/Ser and Ser/Cys genotype combined.

DISCUSSION

In this case–control study, we found that the *hOGG1* 326Cys/Cys genotype, which results in weaker activity, was associated with a significantly increased risk of lung cancer overall. By subtype we found a significant association of the Cys/Cys genotype with small-cell carcinoma and a marginally significant association with adeno-carcinoma. Moreover, in our subsequent meta-analysis of epidemiological studies based on histology, we observed that this genotype was associated with an increased risk of adenocarcinoma. Although results for squamous- and small-cell carcinoma were not conclusive,