Functional Analyses of Mutations in Receptor Tyrosine Kinase Genes in Non–Small Cell Lung Cancer: Double-Edged Sword of DDR2

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Abstract

Purpose: This study investigated whether mutations of receptor tyrosine kinase (RTK) genes detected using next-generation sequencing (NGS) are suitable therapeutic targets.

Experimental design: Fifty surgically resected non–small cell lung cancer (NSCLC) samples were target resequenced using NGS. We then investigated the functions of the identified RTK gene mutations, including their oncogenic potential, in vitro.

Results: Mutations in RTK genes were found in 20 samples (EGFR, 15; ERBB4, 1; ALK, 1; DDR2, 2; FGFR1, 1), mutations in MAPK pathway genes were found in nine samples (KRAS, 7; NRAS, 1; BRAF, 2), and mutations in PI3K pathway genes were found in three samples (PIK3CA, 1; PTEN, 3). Among the mutations in RTKs, the functions of four mutations were unclear (ERBB4 D245G; DDR2 H246R and E655K; FGFR1 A263V). These mutations did not exhibit any transformational activities. Neither the phosphorylation nor the protein expressions of RTKs were changed by the DDR2 H246R, ERBB4 D245G, and FGFR1 A263V mutations, although the expression level of the DDR2 protein harboring the E655K mutation was particularly low. Collagen stimulation decreased cellular proliferation through p38 activation in the DDR2 wild-type–overexpressed cell lines, whereas the growth-suppressive effect was weakened in DDR2 E655K–overexpressed cell lines. Furthermore, the DDR2 E655K protein strongly bound to ubiquitin ligase E3 (Cbl-b), and the mutant protein expression was increased after treatment with a proteasome inhibitor.

Conclusions: Our experimental findings suggest that RTK mutations are not always suitable as therapeutic targets. The DDR2 E655K mutation can play a role in cancer progression by reducing the growth-inhibitory effect of collagen. Clin Cancer Res; 1–9. ©2016 AACR.

Introduction

Lung cancer is the leading cause of cancer-related death worldwide, with approximately 1.6 million deaths each year (1). More than 80% of lung cancers are non–small cell lung cancer (NSCLC), which includes adenocarcinoma, squamous cell carcinoma, and large cell carcinoma. Among patients with advanced NSCLC, cytotoxic chemotherapy has a response rate of 20% to 35% and a median survival period of 10 to 12 months (2). Meanwhile, several oncogenic driver mutations have been recognized in NSCLC, including genes encoding EGFR and ALK. Small-molecule–based targeted therapies directed against these events have been successfully developed for improving patient prognosis (3–8). In addition, targeted therapeutic agents can improve the prognosis of approximately 50% of patients with lung adenocarcinomas carrying other rare gene alterations such as AKT1, BRAF, HER2, KRAS, MAP2K1, MET, RET, and ROS1 (9). In a subset of NSCLC, however, driver mutations capable of serving as therapeutic targets have not yet been identified. Therefore, the identification of new oncogenic driver mutations is required. Especially, receptor tyrosine kinases (RTK) have been shown not only to act as key regulators of normal cellular processes, but also to play a critical role in the development and progression of many cancers. Indeed, many drugs that target RTKs, such as EGFR, HER2, ALK, ABL, KIT, and PDGFR, have been approved for many cancers (10). Thus, the identification of alterations in RTK genes could provide a ticket for novel molecular-targeted therapy.

The next-generation sequencing (NGS) approach has drastically decreased the time and cost associated with comprehensive genome analysis and has been used as a research tool for understanding tumor molecular mechanisms, discovering novel drug targets, and screening candidate patients for clinical trials. NGS technologies have successfully identified novel mutations in a variety of cancers, including lung cancer (11, 12). Meanwhile, Alexandrov and colleagues reported the detection of approximately 300 somatic mutations in NSCLC (13), and the oncogenic potential of these mutations should be investigated using a molecular and cellular biologic approach.

We recently reported that an ALK gene mutation, R1192G, was found in an NSCLC sample (14). This mutation behaves as
Translational Relevance

Activating mutations of receptor tyrosine kinases (RTK) have been regarded as therapeutic targets against non–small cell lung cancer (NSCLC). A next-generation sequencing (NGS) has the power to detect numerous mutations, and whether these mutations have oncogenic potential and can become therapeutic targets should be investigated. In this study, 50 NSCLC samples were target resequenced using NGS. Among the detected RTK mutations, the functions of 4 mutations were unclear. None of these mutations exhibited transformational activity or led to an elevation in phosphorylation levels in vitro. In contrast, the expression level of the DDR2 protein harboring the E655K mutation was particularly low and the growth-inhibitory effect of collagen through DDR2 was weakened, suggesting that the DDR2 E655K mutation plays a role in tumor progression. These experimental findings suggest that RTK mutations are not always suitable as therapeutic targets and that DDR2 is a double-edged sword in terms of cancer progression.

Materials and Methods

Patients
Fifty patients with NSCLC who underwent surgical resection at Kinki University Hospital between January 2012 and May 2012 were enrolled in this study. This study was retrospectively performed and was approved by the institutional review board of the Kinki University Faculty of Medicine (Osaka, Japan).

Isolation of genomic DNA
Genomic DNA was extracted from formalin-fixed, paraffin-embedded (FFPE) tumor samples using the QIAamp DNA FFPE Kit (Qiagen), and the concentration was determined using the aQuant-iT PicoGreen dsDNA Assay Kit (Life Technologies). Genomic DNA was extracted from formalin-fixed, paraffin-embedded (FFPE) tumor samples using the QIAamp DNA FFPE Kit (Qiagen), and the concentration was determined using the aQuant-iT PicoGreen dsDNA Assay Kit (Life Technologies). Genomic DNA was extracted from formalin-fixed, paraffin-embedded (FFPE) tumor samples using the QIAamp DNA FFPE Kit (Qiagen), and the concentration was determined using the aQuant-iT PicoGreen dsDNA Assay Kit (Life Technologies). Genomic DNA was extracted from formalin-fixed, paraffin-embedded (FFPE) tumor samples using the QIAamp DNA FFPE Kit (Qiagen), and the concentration was determined using the aQuant-iT PicoGreen dsDNA Assay Kit (Life Technologies).

Next-generation sequencing
The Ion AmpliSeq Colon and Lung Cancer Research Panel (Life Technologies), which targets 22 cancer-associated genes (Supplementary Table S1), was used to generate target amplicon libraries. The methods used in library construction and sequencing have been described previously (15). Variant calling was performed using the Ion Torrent Variant Caller (Life Technologies) with hg19 as a reference. Germline mutations were excluded using the Human Genetic Variation Database (http://www.genome.med.kyoto-u.ac.jp/SnpDB; ref. 16).

Cell culture
The HEK293 (human embryonic kidney), NH3T3 (mouse fibroblast), and A549 (human lung cancer) cell lines were main-tained in DMEM (Sigma-Aldrich) supplemented with 10% FBS (Gibco BRL). The H1299, PC-9, and H3122 (human lung cancer) cell line was maintained in RPMI medium (Sigma-Aldrich) supplemented with 10% FBS. The cells were incubated in a humidified atmosphere of 5% CO₂ and passed every 3 to 4 days. The HEK293, NH3T3, A549, and H1299 cell lines were obtained from ATCC in 2006, the PC-9 cell line was kindly provided by Dr. Y. Hayata (Tokyo Medical University, Tokyo, Japan) in 1993, and the H3122 cell line was kindly provided by Dr. P. A. Jänne (Dana-Farber Cancer Institute, Boston, MA) in 2010. The HEK293, A549, H1299, and PC-9 cell lines were authenticated using a short tandem repeat DNA (STR) method. The database did not include the STR pattern of the H3122 cell line, but the pattern did not match any of the other cell lines.

Plasmid construction and viral transfection
The methods used in this section have been described previously (14). The full-length wild-type (WT) ERBB4, FGFR1, and DDR2 cDNAs were obtained from Addgene. The point mutations were amplified using the PrimeSTAR Mutagenesis Basal Kit (TaKaRa). Full-length coding sequences (CDS) were introduced into a pQCLIN retroviral vector (Clontech) together with enhanced GFP (EGFP) following the internal ribosome entry site sequence (IRES) to monitor the expression of the inserts indirectly. A vector harboring EGFP alone was used to establish the control transfectants. Using these constructions, DDR2, ERBB4, or FGFR1-overexpressed HEK293 and NIH3T3 cell lines and DDR2-overexpressed A549, H1299, PC-9, and H3122 cell lines were created. The DDR2-overexpressed cell lines were designated as HEK293-DDR2 WT, HEK293-DDR2 H246R, HEK293-DDR2 E655K, HEK293-DDR2 S768R, HEK293-EGFP, NIH3T3-DDR2 WT, NIH3T3-DDR2 H246R, NIH3T3-DDR2 E655K, NIH3T3-DDR2 S768R, NIH3T3-EGFP, A549-DDR2 WT, A549-DDR2 E655K, A549-DDR2 S768R, A549-EGFP, H1299-DDR2 WT, H1299-DDR2 E655K, H1299-DDR2 S768R, H1299-EGFP, PC-9-DDR2 WT, PC-9-DDR2 E655K, PC-9-EGFP, H3122-DDR2 WT, H3122-DDR2 E655K, and H3122-EGFP, respectively.

Antibodies and reagents
Rabbit antibodies specific for DDR2, ERBB4, phospho-ERBB4, FGFR1, phospho-FGFR, ERK1/2, phospho-ERK1/2, JNK, phospho-JNK, clun, phospho-clun, p38, p53, phospho-p53, p21, p27, and β-actin, and mouse antibodies specific for phospho-p38 and phospho-tyrosine were obtained from Cell Signaling Technology. Rabbit antibodies specific for p16 and HBPl were obtained from Abcam. Collagen I (from calf skin), SB202190 (a p38 inhibitor), and MG132 (a proteasome inhibitor) were obtained from Sigma-Aldrich and dissolved in 0.1 mol/L acetic acid (Collagen I) or dimethyl sulfoxide (SB202190 and MG132).

Real-time reverse-transcription PCR
One microgram of total RNA was converted to complementary DNA (cDNA) using a GeneAmp RNA-PCR Kit (Applied Biosystems). The cDNAs were then used for quantitative PCR analysis with SYBR Premix Ex Taq (TaKaRa). The PCR evaluation was performed using Thermal Cycler Dice (TaKaRa), as described previously (17). GAPDH was used as an internal control to normalize and compare each sample. The following primers specific for DDR2 and GAPD were used: DDR2-F, 5'-AATCCAGC-TATATGCCGC-3'; DDR2-R, 5'-GTGTGCAAGTCACTGTGCAG-3'; GAPDH-F, 5'-TCAAGGATTTGCGCTGCT-3'; GAPDH-R, 5'-CCGGTTGTGTCTCGTTC-3';
GAPD-F, 5'-GCACCGCTCAAGGCTGAGA-3'; and GAPD-R, 5'-ATGGTGTTGAAGCCCGATG-3'.

Amplification of full-length DDR2 CDS

The full-length DDR2 CDS was amplified from the cDNAs using the specific primers, and the amplicons were visualized using agarose gel electrophoresis.

Cell proliferation assay

Cell growth was assessed using a 3-(4, 5-dimethyl-thiazolyl-2-yl)-2,5-diphenyltetrazolium bromide (MTT) assay that detects the dehydrogenase activity of viable cells. The methods used in this section have been described previously (17). The experiment was performed in triplicate.

Focus formation assay

A focus formation assay was performed as described previously (14). The transfectant NIH3T3 cell lines were cultured for 2 to 3 weeks in DMEM supplemented with 5% FBS. The focus formations were then photographed using a light microscope (IX71; Olympus).

Western blotting

A Western blotting analysis was performed as described previously (17). Cells were washed twice with PBS and lysed by incubating in Lysis A buffer containing 1% Triton X-100, 20 mmol/L Tris-HCl (pH 7.0), 5 mmol/L EDTA, 50 mmol/L sodium chloride, 10 mmol/L sodium pyrophosphate, 50 mmol/L sodium fluoride, 1 mmol/L sodium orthovanadate, a protease inhibitor cocktail (Complete Mini; Roche Diagnostics), and phosphatase inhibitor cocktail (Sigma-Aldrich). Proteins were resolved using SDS-PAGE and were transferred to a polyvinylidene difluoride membrane (Immobilon; Millipore). After blocking with Tris-buffered saline (TBS) containing 0.02% Tween 20 and 5% nonfat milk, the strips of membrane were exposed to the primary antibody overnight. The strips were then incubated with HRP-conjugated secondary antibody, and the proteins were visualized using an ECL Western Blotting Detection System (GE Healthcare).

Immunoprecipitation

An immunoprecipitation analysis was performed as described previously (18). Briefly, cells were isolated and lysed in Lysis buffer A. The protein extract was incubated with anti-DDR2 antibody overnight at 4°C. Protein G-agarose beads (Santa Cruz Biotechnology) were then added and allowed to bind to the complex for 90 minutes at 4°C. After the beads were washed with Lysis buffer A five times, the immunoprecipitated proteins were resolved using SDS-PAGE and subjected to an immunoblotting analysis, as described above.

Statistical analysis

Continuous variables were compared using the Student t test. A two-tailed P value less than 0.05 was considered statistically significant. All the analyses were performed using Microsoft Excel (Microsoft).

Results

Gene mutation profile in NSCLC samples

The patient characteristics are summarized in Table 1. The median age of the patients was 71 years (range, 50–86 years).

There were 30 male patients (60.0%) and 20 female patients (40.0%). Thirty-nine of the patients had stage I disease (78.0%), eight had stage II (16.0%), and three had stage III (6.0%). Adenocarcinoma was the major histologic type (39/50, 78.0%); 13 patients were never-smokers (26.0%), and 29 were current smokers or former smokers (58.0%).

Two of the 50 samples could not be sequenced because of the quality of the DNA. These mutations were mutually exclusive with each other.

To investigate the biologic function of these four RTK genes in vitro, HEK293 and NIH3T3 cell lines overexpressing DDR2, ERBB4, or FGFR1 were retrovirally created. The HEK293-DDR2 E655K cell line had a particularly low DDR2 protein expression level, compared with that of the HEK293-DDR2 WT...
pressed cell lines (ylation of each RTK was not elevated in the mutation-overexpression (Fig. 2A and Supplementary Fig. S1). The phosphor-

more, the DDR2 E655K protein exhibited a fragmented ladder (Fig. 2A), whereas the other mutations did not affect protein

mRNA. Further-

results (Supplementary Fig. S2), suggesting that the effect of DDR2 E655K mutation can be observed in various background NSCLC. The phenotype of DDR2 S768R, which has been reported to be an activating mutation and to be associated with the response to dasatinib, was compared with that of DDR2 E655K. In contrast to DDR2 E655K, DDR2 S768R elevated the phosphorylation of DDR2 without collagen I and exhibited a transformational ability (Fig. 2B and C). Without collagen I, the cellular proliferation of DDR2 S768R-overexpressed cell lines was significantly enhanced, compared with that in the controls. In contrast, under exposure to collagen I, the cellular proliferation was particularly reduced, similar to that in the DDR2 WT-overexpressed cell line. In addition, the proliferation of DDR2 E655K-overexpressed cell lines was significantly enhanced, compared with that of DDR2 S768R-overexpressed cell lines, under exposure to collagen I (Fig. 2D and Supplementary Fig. S3A). These results suggest that the DDR2 E655K mutation may play a role in tumor progression by reducing the growth-inhibitory effect of collagen and that the DDR2 E655K mutation is phenotypically different from the activating DDR2 S768R mutation.

The p38–p53–p21 pathway was activated by collagen stimulation in the DDR2 WT-overexpressed cell line

To elucidate the mechanism of the cellular inhibitory effect induced by collagen, we assessed the activity of a signal pathway in response to collagen I stimulation in DDR2-overexpressed A549 cell lines using a Western blotting analysis. Previous studies have revealed that DDR2 activates a number of important signal pathways, including the MAPK pathway (21), whereas no differences in the phosphorylation of ERK1/2, cJun, or JNK were observed among the A549-EGFP, A549-DDR2 WT, and A549-DDR2 E655K cell lines. In contrast, the phosphorylation of p38 was detected only in the A549-DDR2 WT cell line and was enhanced 6 or 24 hours after collagen I stimulation (Fig. 4A). In addition, the phosphorylation of p53 and a subsequent increased in p21 expression were observed in the A549-DDR2 WT cell line.

Growth-inhibitory effect of collagen was reduced in the DDR2 E655K-overexpressed cell lines

We next investigated the effect of the DDR2 mutations on cellular proliferation in the HEK293 cell lines. Under exposure to its ligand, collagen I, the cellular proliferations of the HEK293-DDR2 WT and HEK293-DDR2 H246R cell lines were significantly reduced, compared with that of the HEK293-EGFP cell line, but a similar reduction was not observed in the HEK293-DDR2 E655K cell line (Fig. 2D). Because the sample harboring the DDR2 E655K mutation also carried the BRAF K601E mutation (Supplementary Table S2), we mainly used the NSCLC cell lines with an activated mutation and to be associated with the response to dasatinib.
Functions of identified DDR2 mutations. A, protein expression of DDR2 in transfectant HEK293 cell lines. The DDR2 mRNA expression level was evaluated using real-time RT-PCR, and GAPDH was used as an internal control. The full-length DDR2 CDS was visualized using agarose gel electrophoresis. DDR2 protein expression was evaluated using Western blotting. The HEK293-DDR2 E655K cell line had a particularly low level of DDR2 protein expression, compared with that in the HEK293-DDR2 WT cell line, despite an equal expression of DDR2 mRNA. The DDR2 protein produced a fragmented ladder in the HEK293-DDR2 E655K cell line. DDR2 S768R was used as a positive control, and β-actin was used as an internal control. Columns, mean of independent triplicate experiments; Bars, SD. B, phosphorylation of DDR2 in transfectant HEK293 cell lines. The phosphorylation of DDR2 was detected using a phospho-tyrosine antibody with or without stimulation with collagen I (10 μg/mL). In both DDR2 H246R and E655K-overexpressed cell lines, the phosphorylation level of DDR2 was not elevated, compared with that in the HEK293-DDR2 WT cell lines, even after exposure to collagen I. The phosphorylation of DDR2 could not be detected in the HEK293-DDR2 E655K cell line because of the low level of DDR2 protein expression. DDR2 S768R was used as a positive control and elevated the phosphorylation of DDR2. β-Actin was used as an internal control. C, transformational ability in transfectant NIH3T3 cell lines. The DDR2-overexpressed NIH3T3 cell lines were created and cultured until they reached confluence. None of the mutations exhibited a transformational ability except for DDR2 S768R. The KRAS-overexpressed NIH3T3 cell line was used as a positive control. Scale bars, 100 μm. D, cellular proliferation of transfectant HEK293 cell lines. The proliferation of the HEK293-DDR2 S768R cell line was significantly reduced, compared with that of HEK293-EGFP, whereas a reduction was not observed in the HEK293-DDR2 E655K cell line. In the HEK293-DDR2 S768R cell lines, under exposure to collagen I, the cellular proliferation was particularly reduced, similar to that in the HEK293-DDR2 WT cell line. Furthermore, the proliferation of the HEK293-DDR2 E655K cell line was significantly enhanced, compared with that of the HEK293-DDR2 S768R cell line, under exposure to collagen I. Columns, mean of independent triplicate experiments; bars, SD; *P < 0.05; n.s., not significant.

Other relevant downstream targets of p38 (HBP1, p27, and p16) were not changed after collagen I stimulation, even in the A549-DDR2 WT cell line (Supplementary Fig. S4). When the A549-DDR2 WT cell line was treated with a p38 inhibitor (SB202190), the phosphorylation of p38 was decreased and the suppression of proliferation was cancelled (Fig. 4C). In the A549-DDR2 S768R cell line, the phosphorylation of p38 was enhanced after collagen I stimulation and the p38 inhibitor also cancelled the suppression of proliferation (Supplementary Fig. S3B and S3C). These results suggest that the tumor-suppressive function of DDR2 was caused by the activation of the p38–p53–p21 pathway. In the A549-DDR2 E655K cell line, which had a low level of DDR2 protein expression, the pathway was not activated and the proliferation potency was maintained even with collagen I stimulation. In addition, the DDR2 E655K mutation was phenotypically different from the activating DDR2 S768R mutation.

Double-Edged Sword of DDR2

On the basis of the presence of a fragmented ladder for the DDR2 E655K protein in the Western blotting analysis, we hypothesized that this mutant protein was in an unstable condition. As previously reported, Cbl-b, a family of E3 ubiquitin ligases, interacts with DDR2 and promotes its ubiquitination, resulting in its degradation (22). We next examined the interaction of DDR2 with Cbl-b using immunoprecipitation. As shown in Fig. 5A, a strong binding between the DDR2 E655K protein and Cbl-b, compared with that for the DDR2 WT protein, was observed. Moreover, when the HEK293-DDR2 E655K cell line was treated with a proteasome inhibitor (MG132), the expression of DDR2 protein increased with no change in the DDR2 mRNA expression levels (Fig. 5B). Furthermore, a growth-inhibitory effect of collagen I was observed in the HEK293-DDR2 E655K cell line, similar to the HEK293-DDR2 WT cell line, when the cells...
were stimulated with MG132 (Supplementary Fig. S5). These results indicate that the DDR2 E655K protein binds strongly to Cbl-b and that the protein is immediately broken down by the ubiquitin–proteasome pathway (Fig. 5C).

Discussion

In this article, we performed a gene mutation analysis of 50 NSCLC samples using NGS technology and four RTK gene mutations with unknown functions were identified (ERBB4 D245G; DDR2 H246R and E655K; FGFR1 A263V). In vitro, these mutations in RTK genes did not accelerate phosphorylation or suitable therapeutic targets, as other data have shown. However, our experimental findings have shown an interesting phenotype: the DDR2 E655K mutation plays a role in the progression of cancer by reducing the growth-inhibitory effect of collagen. In addition, our experimental findings suggest that the DDR2 E655K mutation is phenotypically different from the activating DDR2 S768R mutation. To the best of our knowledge, this is the first functional analysis of RTK gene mutations to show the role of DDR2 E655K mutation in cancer progression.

Genetic alterations were detected in 78.2% (38/48) of the patients. With the exception of TP53 mutations (37.5%, 18/48), EGFR mutations were the major gene alterations in this cohort (31.3%, 15/48), and all of these mutations were well-known activating mutations (exon 19 deletions or exon 21 L858R point mutation). The EGFR mutation was frequently found in women, patients with adenocarcinoma, and never smokers, similar to the results of previous studies (23, 24). Regarding the other oncogenic driver mutations, the KRAS and BRAF mutations were detected at frequencies of 14.6% and 4.2%, respectively. Similar to the EGFR mutations, all the KRAS and BRAF mutations were detected in adenocarcinomas. In contrast to the EGFR mutations, however, most KRAS mutations were observed in current or former smokers, and BRAF mutations were also detected only in patients with a smoking history. These frequencies and clinicopathologic correlations with alterations of oncogenic driver genes were almost concordant with those reported in previous studies (19, 20). An exclusionary relationship among driver gene mutations has been reported (20). In this study, the DDR2 E655K mutation coexisted with the BRAF K601E activating oncogenic mutation, but as mentioned above, the DDR2 E655K mutation was not an activating oncogenic mutation, which is compatible with the above-mentioned exclusiveness.

DDR2 is a RTK that is activated by collagen in the extracellular matrix. The activation of DDR2 has been associated with a number of cellular phenotypes, including proliferation, migration, transformation, and differentiation. There is some argument concerning the function of DDR2 in cancer progression, that is, DDR2 has a bilateral character, acting as either an oncogene or a...
cancer-suppressor gene. Hammerman and colleagues showed that a subset of DDR2 mutations, including L63V, I638F, and S768R, mediated an oncogenic effect (25). A recent study demonstrated that the inhibition of DDR2 suppressed the in vivo and in vitro growth of human hepatocellular carcinoma cell lines (26). In contrast, the activation of DDR2 by collagen has been shown to inhibit the proliferation of human melanoma and fibrosarcoma cell lines (27, 28). The loss of DDR2 has also been shown to predispose hepatic tissues to colon carcinoma cell growth and metastasis (29). Moreover, wild-type DDR2 expression in the HEK293 cell line greatly reduced colony formation in the presence of collagen (30). Collagen is the major structural component of the extracellular matrix and triggers many intercellular signal pathways regulating cellular growth, migration, and differentiation (31). In particular, collagen acts as a physical barrier for cell migration and interferes with the proliferative ability of both normal and cancer cells (32). Thus, the inhibition of cancer cell growth by collagen has been well documented (27, 33), and our results were consistent with these published data, indicating that the extracellular matrix and triggers many intercellular signal pathways regulating cellular growth, migration, and differentiation (31). In particular, collagen acts as a physical barrier for cell migration and interferes with the proliferative ability of both normal and cancer cells (32). Thus, the inhibition of cancer cell growth by collagen has been well documented (27, 33), and our results were consistent with these published data, indicating that the inhibition of DDR2 suppressed the in vivo and in vitro growth of human hepatocellular carcinoma cell lines (26). In contrast, the activation of DDR2 by collagen has been shown to inhibit the proliferation of human melanoma and fibrosarcoma cell lines (27, 28). The loss of DDR2 has also been shown to predispose hepatic tissues to colon carcinoma cell growth and metastasis (29).

Figure 4.
Growth-inhibitory effect of collagen via the activation of the p38 signal. A, Western blotting of the MAPK signal pathways. No differences in the phosphorylation of ERK1/2, c-Jun, or JNK were observed among the A549-EGFP, A549-DDR2 WT, and A549-DDR2 E655K cell lines, even after exposure to collagen. In contrast, the phosphorylation of p38 was detected only in the A549-DDR2 WT cell line and was enhanced 6 or 24 hours after stimulation with collagen I. β-Actin was used as an internal control. B, Western blotting of p53 and p21. Six or 24 hours after collagen I stimulation, the phosphorylation of p53 and a subsequent increase in p21 expression was observed in the A549-DDR2 WT cell line, but not in the others. β-Actin was used as an internal control. C, cellular proliferation and Western blotting of the A549-DDR2 WT and A549-DDR2 E655K cell lines with or without exposure to a p38 inhibitor (SB202190, 1 μM) in the presence of collagen I (10 μg/mL). The cellular growth was examined using an MTT assay. When the A549-DDR2 WT cell line was treated with SB202190, the suppression of proliferation by collagen stimulation was cancelled. In contrast, no significant change in the A549-DDR2 E655K cell line was observed. The phosphorylation level of p38, which was elevated by the collagen stimulation, was reduced by SB202190 in the A549-DDR2 WT cell line. In contrast, the phosphorylation level, which was not elevated by the stimulation, was not changed by SB202190 in the A549-DDR2 E655K cell line. β-Actin was used as an internal control. Columns, mean of independent triplicate experiments; bars, SD; *P < 0.05; n.s., not significant.

Similar to previous studies, our experimental findings have demonstrated that the activation of p38 through DDR2 phosphorylates p53, leading to the induction of p21 and causing cell-cycle arrest (35).

Ubiquitination has been well recognized as a major mechanism for the negative regulation of the RTK signal, which is carried out by a cascade of enzymatic reactions involving E1 (ubiquitin-activating enzyme), E2 (ubiquitin-conjugating enzyme), and E3 (ubiquitin ligase; ref.36). The Cbl family, which consists of three homologues known as C-Cbl, Cbl-b, and Cbl-c in mammals, belongs to the E3 ubiquitin ligases (37). Cbl protein-mediated ubiquitination can target activated RTK for degradation, either by facilitating endocytic sorting into lysosomes or by promoting proteasomal degradation (38). In a previous study, Cbl-b was experimentally shown to function as a negative regulator in the DDR2 by promoting the ubiquitination and degradation of DDR2 (22). All three members of the Cbl family of proteins share a highly homologous N-terminal tyrosine kinase binding region, which serves as the structural platform for binding to phospho-tyrosine residues (39). DDR2 has three phospho-tyrosine residues (Tyr-736, Tyr-740, and Tyr-741) in the tyrosine kinase domain (563-849). The amino acid position at 655 is close to these residues. From our experimental findings, this position seems to be influential in binding to Cbl, and the E655K mutation has a great effect on the DDR2–Cbl-b interaction resulting in subsequent degradation (Fig. 5C). Although one lung squamous cell carcinoma sample with this DDR2 E655K...
mutation has been reported in The Cancer Genome Atlas dataset, this is the first functional analysis study to show the effect of the DDR2 amino acid at position 655 on the Cbl-b interaction and its role in cancer progression.

As much data, including the present results, have shown, mutations in cancer are not always activating mutations or suitable therapeutic targets. In addition, because unexpected functions, like that of DDR2 E655K in this study, are sometimes discovered, the functional analysis of mutations detected in clinical samples is extremely important for understanding tumor biology. NGS technology is capable of detecting a huge number of mutations, and approximately 300 somatic mutations have been detected in NSCLC (13). Thus, analyzing the functions of all known mutations one by one is difficult. Several computational methods have been developed to predict the effects of amino acid substitutions on protein function by evolutionary sequence comparison, structural constraints, or the physicochemical attributes of amino acids (40, 41). Several reports, however, have indicated performance disparities between these technologies because of a gap in prediction accuracy, sensitivity, and specificity, indicating that more robust and integrated systems are needed (40, 41).

In conclusion, we investigated the function of 4 RTK gene mutations that were found in 50 NSCLC samples sequenced using NGS. None of these mutations had any effect on phosphorylation or transforming activity, and they are unlikely to become novel therapeutic targets, indicating that RTK gene mutations do not always exhibit an oncogenic capability and are not always suitable therapeutic targets, as much data has shown. In contrast, the novel tumor-progressive function of the DDR2 E655K mutation and the nature of DDR2 as a double-edged sword in terms of cancer progression were revealed. Detail functional analyses based on clinical samples are important for understanding tumor molecular mechanisms, and further efficient systems for predicting the functions of mutations should be developed.

Disclosure of Potential Conflicts of Interest
No potential conflicts of interest were disclosed.

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References

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