Manuscript title

A Significance of MMP-1 in EGFR-TKI Resistant Lung Adenocarcinoma: A Potential of Therapeutic Target

Running title: A Role of MMP-1 in EGFR-TKI Resistant Lung Adenocarcinoma

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Abstract: Lung adenocarcinoma with EGFR-TKI (epidermal growth factor receptor-tyrosine kinase inhibitor) resistance was reported to harbor higher ability of invasion and migration than those sensitive to EGFR-TKI, but the function of MMPs (matrix metalloproteinases) has not been explored in EGFR-TKI resistant lung adenocarcinoma. In this study, the correlation between immunohistochemical status of MMP-1 and clinicopathological factors were analyzed in 89 lung adenocarcinoma. We performed microarray, migration assay and invasion assay using EGFR-TKI sensitive cell lines and EGFR-TKI resistant cell lines. To clarify the mechanism of MMP-1 induction, we treated lung adenocarcinoma cells with EGF and rapamycin, performed phosphorylation antibody array and analyzed the correlation between MMP-1 expression and EGFR or mTOR (mammalian target of rapamycin) pathway. As a result, we firstly demonstrated that MMP-1 played an important role in migration and invasion abilities of EGFR-TKI resistant lung adenocarcinoma, and that mTOR pathway could be associated with an induction of MMP-1. We demonstrated the significant positive correlation between MMP-1 status in lung adenocarcinoma cells and the history of smoking, and the subtype of invasive mucinous adenocarcinoma. In conclusion, This study provides insights into the development of a possible alternative therapy manipulating MMP-1 and mTOR signaling pathway in EGFR-TKI resistant lung adenocarcinoma.

Keywords: MMP-1, EGFR-TKI resistance, lung adenocarcinoma, mTOR

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Introduction

One of the most imminent clinical problems of lung cancer therapy is to improve the therapeutic strategy of the patients with lung adenocarcinoma developing EGFR-TKI (epidermal growth factor receptor-tyrosine kinase inhibitor) resistance. Recently, in addition to the first generation EGFR-TKI including gefitinib and erlotinib, second and third generation EGFR-TKI were developed and another targeted therapies focusing on anaplastic lymphoma kinase gene mutation and immune checkpoint such as programmed cell death 1 ligand 1 / programmed cell-death protein 1 pathway were employed to these patients developing therapeutic resistance [1-3]. It is true that these new therapies are sometimes very effective, but it is still important to develop the new therapies based upon the mechanisms of evolution of EGFR-TKI resistance.

Previously, it was reported that lung adenocarcinoma cells resistant to EGFR-TKI demonstrated higher ability of invasion and migration than those sensitive to EGFR-TKI sensitivity [4, 5]. Results of these studies did reveal that epithelial-mesenchymal transition, EGF pathway activation, and MET amplification were all significantly associated with the increase of invasion and migration abilities in lung adenocarcinoma cells resistant to EGFR-TKI. However, to the best of our knowledge, there have been no reports studying the functions of MMPs (matrix metalloproteinases), one of the well characterized factors to promote the abilities of invasion and migration, in EGFR-TKI resistant lung adenocarcinoma cells. Therefore, in this study, we focused on the function of MMPs, especially MMP-1, in EGFR-TKI resistant lung adenocarcinoma.

MMPs are calcium-dependent zinc-containing endopeptidases and about 30 subtypes have been identified as a member of MMPs. MMPs are also known to degrade numerous kinds of extracellular matrix proteins. MMPs play an important role in reproduction and embryonic development in normal physiological conditions, and in tissue remodeling, invasion, migration, and so on in pathological conditions [6]. MMP-1 degrades the interstitial collagens types I, II, and III [7]. It expresses in normal stromal fibroblasts, macrophages, endothelial and epithelial cells, and trophoblastic cells [6]. However, MMP-1expression has been reported in various kinds of malignant cells and well known to promote metastasis and invasion, and to be related with poor prognosis in breast cancer, prostate cancer, gastric cancer, malignant melanoma, lung cancer, and other malignant neoplasms [8] [9] [10] [11] [12] [13]. While Min et al reported that high levels of MMP-1 protein were significantly associated with poor prognosis in NSCLC (non-small cell lung carcinoma) [12], the correlation between MMP-1 status and detail clinicopathological factors had not been examined. In previous *in vitro* study using A549 cells, with the absence of EGFR mutation, MMP-1 promoted the invasion ability [13]. However, the correlation between MMP-1 and EGFR-TKI resistant lung adenocarcinoma has not been studied. Itoh et al reported that signal transducers and activators of transcription 3 activity in response to EGF played pivotal roles in the process of MMP-1 induction in urinary bladder cancer [14]. In our present study, we therefore hypothesized that various activities of EGFR pathway associated with EGFR mutation and EGFR-TKI resistance could possibly change the expression levels of MMP-1 in EGFR-TKI resistant lung adenocarcinoma and contribute to the development of EGFR-TKI resistance.

In this present study, we investigated immunohistochemically the association between MMP-1 expression and clinicopathological factors in detail. Furthermore, the present study is the first to examine the effects of MMP-1 on EGFR-TKI resistant lung adenocarcinoma.

Results

mRNA expression of MMPs and related genes

We evaluated the mRNA expression levels of genes encoding MMPs in EGFR-TKI resistant (PC9/GR and PC9/ER) and EGFR-TKI sensitive lung adenocarcinoma cells (PC9/6m) using microarray analysis. Results were summarized in Table 1. Among these MMPs and their inhibitors, TIMPs (tissue inihibitor of metalloproteinases), the mRNA levels of *MMP-1*, *23* and *24* both in PC9/GR and PC9/ER were changed with >10-fold changes versus compared with those in PC9/6m. We then focused on MMP-1 expression.

Status of MMP-1 immunoreactivity in the patients with lung adenocarcinoma

Representative findings of immunohistochemsitry were summarized in Fig. 1a, 1b, 1c, 1d. MMP-1 immunoreactivity was detected in the cytoplasm. 55 cases were categorized as low expression group, and 34 cases as high expression group.

The correlation of MMP-1 immunoreactivity with the clinicopathological parameters of the patients examined was summarized in Table 2A. Significant positive association was detected between the status of MMP-1 immunoreactivity and smoking status ($p = 0.016$), Brinkmann index ($p = 0.038$), pT ($p = 0.029$), tumor size ($p = 0.007$), and subtype of invasive mucinous adenocarcinoma ($p < 0.001$). There were also significant positive correlations between subtype of invasive mucinous adenocarcinoma and smoking status or Brinkmann index (respectively, $p = 0.012$, $p = 0.038$). 5-year overall survival curve was illustrated in Fig. 1e. Significant positive correlation was detected between high MMP-1 group and adverse clinical outcome of all the patients examined following univariate analysis ($p = 0.021$). Multivariate analysis revealed that high MMP-1 status was a significant independent poor prognostic factor significantly ($p = 0.020$, relative risk (95%CI (confidence interval)) = 2.722 (1.170-6.330)) (Table 2B).

The mRNA expression of *MMP-1* gene in EGFR-TKI resistant lung adenocarcinoma cells was higher than that in EGFR-TKI sensitive ones.

The mRNA levels of *MMP-1* gene in two EGFR-TKI resistant lung adenocarcinoma cells (PC9/GR and PC9/ER) were significantly higher than those in EGFR-TKI sensitive ones (PC9/6m) (PC9/GR versus PC9/6m; $p = 0.0349$, PC9/ER versus PC9/6m; $p < 0.0001$, PC9/ER versus PC9/GR; $p < 0.0001$) (Fig. 2a). After seeding PC9 cells onto 6-well dishes (5×10^4 cells/ml) and treating those cells with gefitinib 5 µM, erlotinib 5 µM or DMSO (dimethyl sulfoxide) for control for 72 hours, we evaluated the mRNA expression levels of *MMP-1* gene. However, there was no significant change (gefitinib; $p = 0.4057$, erlotinib; $p = 0.6079$) (Fig. 2b).

MMP-1 induced the abilities of migration and invasion in the EGFR-TKI resistant lung adenocarcinoma cells with high expression of MMP-1

We compared the migration ability between PC9/ER, which is EGFR-TKI resistant lung adenocarcinoma cells with high expression of MMP-1, and PC9/6m using wound healing assay and

migration assay for 24 hours. As a result, PC9/ER demonstrated significantly higher migration ability than PC9/6m (wound healing assay; $p = 0.0084$, migration assay; $p = 0.0314$) (Fig. 3a, 3b). In addition, in PC9/ER, both migration and invasion abilities for 24 hours were significantly inhibited by knockdown of *MMP-1* gene using siMMP-1 treatment for totally 72 hours (wound healing assay; $p = 0.0018$, migration assay; $p = 0.0004$, invasion assay; $p = 0.0009$) (Fig. 3c, 3d, 3e). In this study, the inhibitory effect of siMMP-1 was confirmed by the decrease of protein and mRNA expression of MMP-1 (mRNA; $p = 0.0442$) (Fig. 3f).

mTOR pathway in the induction of MMP-1 expressoin in lung adenocarcinoma

After seeding PC9 cells, EGFR mutated, and A549 cells, not EGFR mutated, onto 6-well dishes (5×10^4 cells/ml) and treating those cells with EGF (25 ng/ml and 50 ng/ml) or acetic acid for control for 24 hours, we evaluated the mRNA expression levels of *MMP-1* gene. In both cells, EGF significantly increased the mRNA expression of *MMP-1* gene in a dose-dependent manner (PC9: 25ng/ml versus control; $p = 0.0006$, 50ng/ml versus control; $p < 0.0001$, 50ng/ml versus 25ng/ml; $p = 0.0046$, A549: 25ng/ml versus control; $p = 0.0296$, 50ng/ml versus control; $p = 0.0030$, 50ng/ml versus 25ng/ml; $p = 0.0447$) (Fig. 4a-b). In addition, PC9/ER showed increased levels of phosphorylation of EGFR than PC9/6m (Fig. 4c).

In order to further study which pathway under EGFR pathway could be associated with the induction of MMP-1 expression, we performed phosphorylation antibody array using PC9 treated with EGF 50 ng/ml or acetic acid for control for 24 hours. EGF treatment promoted the phosphorylation of Akt and S6 (Fig. 4d), both of which played important roles in mTOR pathway. Following the treatment of PC9 and PC9/ER with rapamycin 10 nM to inhibit mTOR pathway or DMSO as a control for 72 hours, we evaluated the mRNA levels of *MMP-1* gene expression. mRNA levels of *MMP-1* gene expression were significantly decreased by inhibition of mTOR pathway using rapamycin in both cells (PC9; $p = 0.0068$, PC9/ER; $p = 0.0112$) (Fig. 4e-f). These inhibitory effects of rapamycin on mTOR pathway were confirmed by the decrease of phosphorylation of mTOR. (Fig. 4g).

Discussion

To the best of our knowledge, this is the first study to demonstrate that MMP-1 plays an important role in migration and invasion abilities of EGFR-TKI resistant lung adenocarcinoma based on global analyses about MMPs, and that mTOR pathway is associated with induction of MMP-1 expression in lung adenocarcinoma including EGFR-TKI resistant cells. In addition, we firstly revealed the significant positive correlation between MMP-1 status in lung adenocarcinoma cells and the history of smoking, and the subtype of invasive mucinous adenocarcinoma in this study.

Results of our present *in vitro* studies including microarray analyses did firstly demonstrated that MMP-1 expression was significantly increased in EGFR-TKI resistant adenocarcinoma cells than EGFR-TKI sensitive ones. There was a significant difference of MMP-1 expression between PC9/GR cells and PC9/ER cells. These results indicated that PC9/ER cells harbored high expression of MMP-1 and that this difference was not due to the differences of EGFR-TKI, because gefitinib and erlotinib were both well-known to have almost the same mechanisms of action as well as similar efficacy, toxicity, and resistance mechanism [15].

In some previous reports, they said that MMP-1 promoted the invasion ability of cancer cells including A549, which is lung adenocarcinoma cells without EGFR mutation [13]. Consistently, the results of this present study also showed that MMP-1 was associated with the increase of migration and invasion abilities in EGFR-TKI resistant cell clone with high expression of MMP-1.

We subsequently studied the mechanism of MMP-1 induction in lung adenocarcinoma cells. Results of previously published studies demonstrated that the mechanisms of MMP-1 induction could possibly depend on each tumor [14, 16-18] , but those in lung carcinoma have virtually unknown. Results of global analyses using phosphorylation antibody array in our present study did reveal that EGFR pathway, especially mTOR related EGFR pathway was significantly associated with the induction of MMP-1 expression in PC9 cells and PC9/ER cells. These results were consistent with the previous report that EGFR pathway is important for the induction of MMP-1 expression in urinary bladder cancer [14], endometrial cancer [18], and others. The reason why EGFR-TKI resistant cells demonstrated significantly high expression of MMP-1 could be reasonably postulated as follows; in EGFR-TKI resistant lung adenocarcinoma cells, it is difficult to inhibit the increased EGFR pathway activity due to EGFR mutation. Therefore, EGFR pathway could be more constantly activated than EGFR-TKI sensitive lung adenocarcinoma cells. This hypothesis means that the EGFR pathway activity increases and subsequently induces MMP-1 expression after obtaining of EGFR-TKI resistant. In addition, results of our present study did reveal that treatment of PC9 cells with EGFR-TKI for only short-time did not change the MMP-1 expression, which is also consistent with the hypothesis above.

MMP-1 was also reported as a poor prognostic factor in NSCLC [12], which is consistent with results of our present study. In addition, the status of MMP-1 immunoreactivity was significantly and positively correlated with pT in our present study, although it did not with pN. These results indicated that MMP-1 might act on migration more strongly than invasion, because MMP-1 can degrade collagens types I, II, III while MMP-1 cannot degrade collagen types IV [7], which is an important factor consisting basement membrane of lymphatic and blood vessels [19]. Therefore, this could be the reason why

carcinoma cells had less incidence of lymphatic or vascular invasion despite high expression of MMP-1, but it awaits further investigations for clarification. We also firstly revealed the significant positive correlation between MMP-1 expression in lung adenocarcinoma cells and the history of smoking, and the subtype of invasive mucinous adenocarcinoma. Smoking was reported to activate mTOR pathway [20], and that there was significant correlation between mucinous type of lung adenocarcinoma and *k-ras* mutation, and between smoking and *k-ras* mutation [21] [22]. According to the result of our present study and these reports, the association of MMP-1 expression with smoking and invasive mucinous adenocarcinoma may be due to increased activity of mTOR pathway through smoking, but further studies are also required to clarify this interesting hypothesis, because we used only 89 cases and did not investigate the involvement of k-ras mutations to MMP-1 induction. The tendency of negative correlation between MMP-1 expression in lung adenocarcinoma cells and lepidic subtype could be because lung adenocarcinoma with lepidic subtype had low potential of invasion. On the other hands, the tendency of positive correlation between MMP-1 expression in lung adenocarcinoma cells and pleomorphic subtype could be because lung adenocarcinoma with pleomorphic subtype had high ability of invasion.

In conclusion, in this study, we firstly revealed that MMP-1 could promote migration and invasion through an activation of mTOR pathway especially in EGFR-TKI resistant lung adenocarcinoma cells harboring high MMP-1 expression. Results of this study could also provide possibility of mTOR pathway suppression in EGFR TKI resistant lung adenocarcinoma patients.

Materials and methods

Reagents and angibodies

The following materials were commercially obtained: Gefitinib 5 µM (Biaffin, Kassel, Germany); Erlotinib 5 µM (kindly provided from Roche Diagnostics, Mannheim, Germany); EGF 25 ng/ml, 50 ng/ml (Sigma-Aldrich, Saint Louis, MO, USA); Rapamycin 10 nM (Cayman Chemical, Ann Arbor, MI, USA). Antibodies were obtained from the following sources: MMP-1 (Abcam, Cambridge, UK); EGFR, pEGFR, mTOR (mammalian target of rapamycin), pmTOR (Cell Signaling Technology, Beverly, MA, USA); β-actin (Sigma-Aldrich).

Microarray

Total RNA was extracted with care from PC9/6m, PC9/GR and PC9/ER as described below. Next, 44 K Whole Human genome arrays (G4112F; Agilent Technologies, Inc., Santa Clara, CA) were prepared and were hybridized with linearly amplified and labeled total RNA, according to the manufacturer's protocol. cRNAs probes were labeled using Low Input Linear Amplification and Labeling kit (Agilent Technologies). Fluorescently labeled probes were purified using RNeasy Mini kit (Qiagen), according to the manufacturer's instructions. Results were extracted using Feature Extraction software (Agilent Technologies) and were analyzed using Genespring GX11 software (Agilent Technologies) to obtain gene expression ratios.

Patients and tissue preparation

Surgical pathology specimens of 89 lung adenocarcinoma were retrieved from the patients operated at department of Thoracic Surgery, Tohoku University Hospital (Sendai, Japan). The patients did not receive chemotherapy or radiation prior to surgery. These specimens had been all fixed in 10 % formalin and embedded in paraffin wax. Relevant clinical data were retrieved from the review of the patients' charts. The Ethics Committee of the Tohoku University School of Medicine approved the research protocol (2013-581).

Immunohistochemistry

We used rabbit monoclonal antibody against MMP-1. This antibody was reported to specially recognize human MMP-1 by both immunoblotting and immunohistochemistry [23]. Histofine Kit (Nichirei Co. Ltd., Tokyo, Japan) based on streptavidin–biotin amplification method was used in this study. Antigen retrieval for MMP-1 was performed using autoclave treatment with ethylenediaminetetraacetic acid (pH 9.0). The primary antibody was diluted by 1:500. Antigen–antibody complexes were visualized using 3,30-diaminobenzidine solution (1 mM DAB, 50 mM Tris–HCL buffer (pH 7.6), and 0.006% H_2O_2) and by counterstaining with hematoxylin. Human placenta tissue was used for positive control [24].

Adenocarcinoma cells presenting higher immunointensity than background were designated as MMP-1 positive. A modified H-score was used for scoring of MMP-1 expression. Briefly, the modified H-score was obtained by adding the percentage of strongly stained cells ($2\times$) with that of weakly stained cells ($1\times$), which provided a possible range of 0–200. MMP-1 expression was defined as follows; the modified H-score >90 is high expression, and $\leqq$ 90 is low expression.

Cell culture

Human lung adenocarcinoma cell lines used in this present study were as follows: PC9 (Riken Cell Bank, Tsukuba, Japan), A549 (American Type Cell Culture Collection (ATCC), Manassas, VA, USA). PC9/6m, PC9/ER, PC9/GR were established in our laboratory previously [25]. Particularly, PC9 (Immuno-biological Laboratories (IBL), Gunma, Japan) were exposed to increasing concentrations of gefitinib and erlotinib to generate the gefitinib or erlotinib -resistant cell lines (PC9/GR and PC9/ER, respectively). These doses were gradually increased to 10 nM (2 months), 1 μM (2 months) and 5 μM (2 months). PC9 cells were also cultured for 6 months (PC9/6m) in regular medium in order to eliminate the effects of long-term cell culture. These cells have EGFR mutation as follows; PC9, PC9/6m and PC9/GR: Exon 19 deletion, PC9/ER: Exon 19 deletion, L858R mutation, and T790M mutation.

These cells were maintained in RPMI 1640 (Sigma-Aldrich, Saint Louis, MO, USA) supplemented with 10% fetal bovine serum (FBS; Nichirei Co. Ltd.) and 1% penicillin/streptomycin at 37°C in a humidified incubator containing 5% CO2.

Real-time RT-PCR

Total RNA was extracted carefully from cultured cells using TRI reagent (Molecular Research Center Inc., Cincinnati, Ohio, USA) and was reverse transcribed to

cDNA using a reverse transcription kit (QuantiTect Reverse Transcription Kit; Qiagen, Hilden, Germany). Levels of mRNA expression were semi-quantified by performing real-time RT-PCR in LightCycler System (Roche Diagnositics GmbH). The PCR mixture (20 μl) included 0.5 μM of *MMP-1* primer or 1 μM of ribosomal protein L13a (*RPL13A*) primer and 2× QuantiTect SYBR Green PCR Master Mix (Qiagen). PCR protocol is as follows: initial denaturation at 95°C for 5 min, followed by 40 amplification cycles of 95°C for 10 sec and annealing at 60°C for 30 sec. The primers used for PCR are as follows: *MMP-1* forward, 5′-CAGATTCTACATGCGCACAAATCCCTTC-3′; *MMP-1* reverse, 5′-TGTCGGCAAATTCGTAAGCAGCTTCA-3′; *RPL13A* forward, 5′-CCTGGAGGAGAAGAGGAAAG-3′; and *RPL13A* reverse, 5′-TTGAGGACCTCTGTGTATTT-3′. mRNA levels of *MMP-1* are expressed as the ratio of *RPL13A* mRNA levels.

Western blotting

Total protein was extracted using PhosphoSafe Extraction Reagent (Biosciences Inc., Darmstadt, Germany) from cultured cells. Following the measurement of protein concentration (Protein Assay Rapid Kit Wako; Wako), the total protein was individually subjected to SDS-PAGE (SuperSep Ace; Wako). These proteins were transferred onto Hybond P polyvinylidene difluoride membrane (GE Healthcare, Buckinghamshire, UK). Then, the proteins on the membrane was blocked in 5% non-fat dry skim milk powder (Wako) for over 1h at room temperature, and were incubated with primary antibodies for 24 hours at 4°C using ImmunoShot (Cosmo Bio Co., Ltd., Tokyo, Japan). The dilution of primary antibodies used in this study were as follows: MMP-1, 1:2000; EGFR, 1:1000; pEGFR, 1:500; mTOR, 1:1000; pmTOR, 1:500; β-actin, 1/1000. These antibody-protein complexes on the blot were detected using ECL-plus Western blotting detection reagents (GE Healthcare) following incubation with anti-rabbit or anti-mouse IgG horseradish peroxidase (GE Healthcare) at room temperature.

RNA interference

siRNA (Small interference RNA) targeting MMP-1 used in this study were purchased from Sigma-Aldrich as follows: siMMP-1 sense, 5′-GCAUAUCGAUGCUGCUCUUTT-3′; and siMMP-1 anti-sense, 5′-AAGAGCAGCAUCGAUAUGCTT-3′. Silencer Select Negative Control 1 siRNA (Ambion, Austin, TX, USA) served as a negative control (siControl). Each siRNA 50 nM was transfected into cells $(1 \times 10^5$ cells/ml) using Lipofectamine RNAi MAX reagent (Invitrogen) for 72 hours. Knock-down efficiency was assessed by RT–PCR or immunoblotting.

Wound healing assay

PC9/6m, PC9/GR and PC9/ER were seeded onto 6-well dishes $(1 \times 10^5$ cells/ml) in regular medium. PC9/ER transfected with siRNA for 48 hours were seeded onto 6-well dishes $(1 \times 10^5$ cells/ml) in RPMI 1640 with siRNA and 10% FBS without penicillin/streptomycin. After several hours, scratch wound was created using a p200 micropipette tip into confluent cells. After that, PC9/6m, PC9/GR and PC9/ER were cultured in regular medium, and PC9/ER transfected with siRNA was cultured in RPMI 1640 with siRNA and 10% FBS without penicillin/streptomycin for 24 hours. Images were subsequently

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captured in four different fields per well using phase-contrast microscopy at 0 and 24 h after wounding, and wound areas were calculated.

Migration assay

Migration assays were performed using Falcon Cell Culture Inserts containing membranes with 8-μm pore size (Corning Co, NY, USA) and 24-well dishes. PC9/6m, PC9/GR and PC9/ER were seeded onto upper chambers (5×10^4 cells/ml). Both in upper and lower champers, regular medium was used. PC9/ER transfected with siRNA for 48 hours were seeded onto upper chambres (5×10^4 cells/ml). Both in upper and lower champers, RPMI 1640 with siRNA and 10% FBS without penicillin/streptomycin was used. After 24 hours, the cells on the upper surface of the membrane were mechanically removed with cotton swabs. The migrating cells on the under surface were fixed in 100 % methanol and stained with Toluidine Blue. The membranes were subsequently mounted on glass slides. We evaluated the average number of the cells from 5 random microscopic fields ($\times$ 400 magnification).

Invasion assay

Invasion assays were performed using BioCoat Matrigel Invasion Chamber (Becton Dickinson, Bedford, MA, USA), which consisted of a 24-well companion plate with cell culture inserts containing 8-μm pore size filters coated with the basement membrane Matrigel. PC9/ER transfected with siRNA for 48 hours were seeded onto upper chambres (5×10^4 cells/ml). Both in upper and lower champers, RPMI 1640 with siRNA and 10% FBS without penicillin/streptomycin was used. After 24 hours, the cells on the upper surface of the membrane were mechanically removed with cotton swabs. The invading cells on the under surface were fixed in 100 % methanol and stained with Toluidine Blue. The membranes were subsequently mounted on glass slides. We evaluated the average number of the cells from 5 random microscopic fields ($\times$ 400 magnification).

Phosphorylation antibody array

Total protein was extracted as described above from PC9 cells seeded onto 10cm dishes (5×10^4 cells/ml) and treated with EGF 50 ng/ml or acetic acid for control for 24 hours. PathScan RTK Signaling Antibody Array Kit (Cell Signaling) was used for phosphorylation antibody array according to the manufacturer's instructions.

Statistical analysis

Statistical analysis was performed using StatView 5.0 J software (SAS Institute, Cary, NC, USA) and IBM SPSS Statistics 23 (IBM Corporation, New York, USA). Statistical differences between the two groups of immunohistochemical analyses were evaluated by t-test or χ2 tests. Statistical analyses of *in vitro* study were evaluated by ANOVA or Bonferroni test. 5-year overall survival curves were generated according to the Kaplan–Meier method and the statistical significance was calculated using the logrank test. Both uni- and multivariate analyses were performed using a Cox's proportional hazard model. Statistical significance was defined as p < 0.05 in this study.

Author contributions

RS and YM conceived and designed the study; RS performed most of the experiments and drafted the manuscript; NI and SH contributed to immunohistochemistry and in vitro analyses; CI, MK, and YO collected and stored all the samples; HY-O and HS supervised all experiments; YM and HS edited the manuscript. All authors read and approved the final manuscript.

Conflict of interest statement

HY-O has ownership interest in CHUGAI pharmaceutical Co., Gotemba, Japan. HS received research funding from CHUGAI pharmaceutical Co.

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Figure legend

Figure 1:

Immunohistochemistry of MMP-1 in patients with lung adenocarcinoma. (a, b, c, d) Representative findings of MMP-1 immunohistochemistry in the patients with lung adenocarcinoma and human placenta for positive control (Bar: 100 µm). MMP-1 immunoreactivity (a: negative, b: weakly stained, c: strongly stained, d: positive control) was detected in the cytoplasm of carcinoma cells and trophoblast cells. (e) 5-year overall survival rate of 89 patients with lung adenocarcinoma. 55 patients presented low expression, and 34 patients presented high expression of MMP-1. Significant correlation was detected between high immunoreactivity of MMP-1 and poor clinical outcome of all the patients examined by univariate analysis ($p = 0.021$).

Figure 2:

The mRNA expression of *MMP-1* gene in EGFR-TKI resistant lung adenocarcinoma cells. (a) The mRNA levels of *MMP-1* gene in two EGFR-TKI resistant lung adenocarcinoma cells (PC9/GR and PC9/ER) were significantly higher than those in EGFR-TKI sensitive ones (PC9/6m) (PC9/GR versus PC9/6m; $p = 0.0349$, PC9/ER versus PC9/6m; $p < 0.0001$, PC9/ER versus PC9/GR; $p < 0.0001$). (b) There was no significant change between PC9 cells treated with gefitinib 5 µM or erlotinib 5 µM, and those with DMSO for control (gefitinib; $p = 0.4057$, erlotinib; $p = 0.6079$) for 72 hours. Bars, mean ± SD (n = 3); *$p < 0.05$.

Figure 3:

Correlation between MMP-1 expression and the abilities of migration and invasion in the EGFR-TKI resistant lung adenocarcinoma cells with high expression of MMP-1. (a, b) PC9/ER demonstrated significantly higher migration ability for 24 hours than PC9/6m. (a) Wound healing assay; $p = 0.0084$. (b) Migration assay; $p = 0.0314$. (c, d, e) Both migration and invasion abilities for 24 hours in PC9/ER were significantly inhibited by knockdown of *MMP-1* gene using siMMP-1 treatment for totally 72 hours. siControl was used for control. (c) Wound healing assay; $p = 0.0018$. (d) Migration assay; $p = 0.0004$. (e) Invasion assay; $p = 0.0009$. (f) The inhibitory effect of siMMP-1 was confirmed by the decrease of protein and mRNA expression of MMP-1 (mRNA; $p = 0.0442$). Bars, mean ± SD (n = 3); *$p < 0.05$.

Figure 4

The induction of MMP-1 in lung adenocarcinoma through EGFR pathway, especially mTOR pathway. (a, b) PC9 cells, which have EGFR mutation, and A549 cells, which do not have EGFR mutation, were treated with EGF (25 ng/ml and 50 ng/ml) or acetic acid for control for 24 hours. In both cells, EGF significantly increased the mRNA expression of *MMP-1* gene in a dose-dependent manner (PC9: 25ng/ml versus control; $p = 0.0006$, 50ng/ml versus control; $p < 0.0001$, 50ng/ml versus 25ng/ml; $p = 0.0046$, A549: 25ng/ml versus control; $p = 0.0296$, 50ng/ml versus control; $p = 0.0030$, 50ng/ml versus 25ng/ml; $p = 0.0447$). (c) PC9/ER showed high levels of phosphorylation of EGFR than PC9/6m. (d)

Phosphorylation antibody array using PC9 treated with EGF 50 ng/ml or acetic acid for control for 24 hours. EGF treatment promoted the phosphorylation of Akt and S6. Arrow: positive control spots. Arrow head: negative control spots. (e, f) PC9 and PC9/ER were treated with rapamycin 10 nM to inhibit mTOR pathway or DMSO for control for 72 hours. In both cells, the mRNA levels of *MMP-1* gene expression was significantly decreased by inhibition of mTOR pathway using rapamycin (PC9; $p = 0.0068$, PC9/ER; $p = 0.0112$). (g) Inhibitory effect of rapamycin on mTOR pathway was confirmed by the decrease of phosphorylation of mTOR. Bars, mean ± SD (n = 3); *$p < 0.05$.