Original Study

Article title:

High expression of dihydropyrimidine dehydrogenase in lung adenocarcinoma is associated with mutations in epidermal growth factor receptor: implications for the treatment of NSCLC using 5-fluorouracil

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Microabstract

The regulation of dihydropyrimidine dehydrogenase (DPD)   in cancers with mutated epidermal growth factor receptor (*EGFR*) is unknown. In this study, the clinicopathological analysis of 96 NSCLC patients and cell based studies reveal high DPD expression in *EGFR* mutated tumors. This finding should be taken into account when designing a therapeutic strategy for the treatment of NSCLC.

Abstract

Background: It has been shown that 5-fluorouracil (5-FU) sensitivity in non-small cell lung cancer (NSCLC) is associated with epidermal growth factor receptor (*EGFR*) mutation status. However, the relationship between dihydropyrimidine dehydrogenase (DPD), a 5-FU degrading enzyme, and *EGFR* mutation status is unknown. Here, we focus on clinicopathological factors and *in vitro* correlations between DPD expression and *EGFR* mutation status.

Patients and Methods: *EGFR* mutations and mRNA levels of DPD and thymidylate synthase (TS) were analyzed in 47 resected NSCLCs by laser capture microdissection. In addition, relationships between *EGFR* mutation status and the immunohistochemical expression of DPD and TS in 49 patients with primary NSCLC, treated with a 5-FU derivative of S-1 postoperatively, were examined. Correlations between clinicopathological factors were evaluated. The effect of EGF on DPD expression was also investigated *in vitro* in various cell lines.

Results: Adenocarcinoma *in situ* (AIS) showed significantly higher DPD mRNA levels and more *EGFR* mutation frequency than other histological types (*P<*.05). DPD immuno-positive cases were more frequently observed in adenocarcinoma, in females and in non-smokers. DPD immune-positive cases were correlated with *EGFR* mutation status (*P<*.003). The prognoses of *EGFR* wild-type and mutated populations were similarly favorable with postoperative S-1 treatment, which

overcomes the problem of 5-FU degradation in mutated *EGFR*. *In vitro*, *EGFR* mutated cell lines showed high DPD mRNA and protein expression. **Conclusions:** High DPD expression was shown to be correlated with *EGFR* mutation in adenocarcinoma cells and tissues. This finding should be taken into consideration when using 5-FU to treat NSCLC patients.

Word count for Abstract: 250

Key words: adenocarcinoma *in situ*; 5-fluorouracil; dihydropyrimidine dehydrogenase; S-1; epidermal growth factor receptor mutation; epidermal growth factor receptor tyrosine kinase inhibitor

Introduction

The current standard regimen of chemotherapy for the treatment of wild types of epidermal growth factor receptor (*EGFR*) and echinoderm microtubule-associated protein-like 4 anaplastic lymphoma kinase (*EML4ALK*) non-small cell lung cancer (NSCLC) is intravenous administration of a platinum doublet.[1-2] At the same time, the use of anti-metabolite drugs, such as the fluoropyrimidine anti-cancer agent 5-fluorouracil (5-FU), is an additional option for NSCLC therapy. Previously the use of 5-FU was thought to be inappropriate for lung cancer therapy because the lung contains high levels of dihydropyrimidine dehydrogenase (DPD), which is known to degrade 5-FU.[3] However, there are now two types of 5-FU derivatives available which avoid the problem of degradation by DPD and can be administrated orally. Uracil-tegafur, which combines tegafur (a 5-FU prodrug) and uracil in a 1:4 molar ratio, has been approved for the treatment of patients with resected NSCLC in Asian countries such as Japan, Korea, Singapore and Thailand. Seminal randomized studies showed that adjuvant chemotherapy with uracil-tegafur improved the prognosis of patients with stage I adenocarcinoma 2 cm or greater in size, but not squamous cell carcinoma (SQCC).[4, 5] Another 5-FU derivative, termed S-1, consists of tegafur and two modulators gimeracil and oteracil. Gimeracil is a DPD inhibitor that prevents degradation of 5-FU in the body. Oteracil is an orotate phosphoribosyltransferase (OPRT) inhibitor, which

decreases the activity of 5-FU in normal gastrointestinal mucosa. The phase II and phase III trials of S-1 plus platinum documented that S-1 is effective against both adenocarcinoma (ADC) and squamous cell carcinoma (SQCC), suggesting that S-1 is a valid treatment option.[6,7]

EGFR mutation status is well known as a predictor of response to treatment by *EGFR* tyrosine kinase inhibitor (EGFR-TKI) and as the target of EGFR-TKIs.[8-10] In 2007, Suehisa and colleagues reported that adjuvant chemotherapy with uracil-tegafur significantly prolonged survival rates among patients with *EGFR* wild-type adenocarcinoma but not among patients with *EGFR* mutant tumors.[11] *In vitro* studies also demonstrated that *EGFR*-wild type cells are more sensitive to 5-FU than mutant cells. In the same period, Okabe and colleagues demonstrated that combined administration of 5-FU and EGFR-TKI had a synergistic antiproliferative effect *in vitro* on all NSCLC cell lines by the down-regulation of thymidylate synthase (TS). [12]These two studies indicate that a molecular relationship might exist between *EGFR* mutation status and 5-FU related enzymes. However, the results from these two studies seem to be paradoxical in that the target populations of EGFR-TKI and 5-FU differ in *EGFR* mutation status but the effects are the same and are synergistic in all NSCLC populations regardless of *EGFR* mutation status. In addition, down regulation of TS alone by EGFR-TKI does not seem to explain the phenomenon. In order to explain the discrepancy, it is

necessary to determine the relationship between DPD expression and *EGFR* mutation status.

In the present study, we examined the correlation between *EGFR* mutation status and DPD and TS expression in lung cancer cells and tissues. To verify this relationship, we analyzed 47 resected NSCLCs by laser capture microdissection and 49 patients with primary NSCLC who were treated with S-1 adjuvant chemotherapy. We then evaluated the relation between *EGFR* mutation status, and each of the 5-FU related enzymes and various clinicopathological factors. We also investigated the effect of epidermal growth factor (EGF) on DPD expression in both *EGFR* mutated and non-mutated cell lines.

Patients and Methods

For the laser microdissection study, tumor specimens were obtained from 47 patients with primary NSCLC who underwent surgery at Nagasaki University Hospital from June 1996 to April 2005. The follow up period was 7.3 to 66.4 months (median 60.9 months for overall and 60.8 months for relapse free). For the postoperative S-1 treatment study, a subset of patients was selected from a multi-center feasibility study that used S-1 as postoperative adjuvant chemotherapy in patients with curatively resected pathological stage IB-IIIA NSCLC.[13] The follow up period was 6.3 to 80.5 months (median 62.7 months for overall and 57.0 months

for relapse free). Tumor specimens were obtained from 49 patients from June 2005

to March 2007. The clinicopathological characteristics of the patients are

summarized in Table 1. Informed consent for use of the tumor specimens was

obtained from all patients, and the Ethics Review Board on Clinical Research of

Nagasaki University Hospital (protocol # 05062433) and each hospital approved

the study protocol.

Tissue preparation and histopathological evaluation

The surgically resected tumors were fixed in 10% buffered formalin and

embedded in paraffin. Representative hematoxylin-eosin-stained sections were

reviewed. Clinical and pathological diagnoses were categorized and classified

according to the latest 7th TNM staging system guideline (Union of International

Cancer Control: UICC. Ver. 7).[14,15] Vascular invasion was determined by the

identification of intravascular tumor clots in the lumen of lymphatic or blood

vessels, as described previously.[16]

Mutational analysis for EGFR

DNA was extracted from 2 to 3 serial, 10-μm-thick sections using the QIAamp

DNA Mini kit (Qiagen, Tokyo, Japan) according to the protocol described in the

manufacturer's instructions. The hot-spot mutations of codon 12 in *KRAS* and

EGFR, along with the 9- to 18-bp deletions in exon 19 and the L858R missense

mutation in exon 21, were analyzed using the mutant-enriched polymerase chain

reaction (PCR)-restriction fragment length polymorphism (RFLP) method

described previously.[17] Briefly, in order to examine the mutation in exon 19, we

used ex19 S-1 and ex19 AS-1 for the 1st PCR. PCR amplification was performed

with 50 to 100 ng of genomic DNA with Go Taq® Green Master Mix (Promega,

Madison, WI, USA). We also used ex21 f-s and ex21 r-s to examine the mutation

in exon 21. DNA was amplified for 35 cycles at 94°C - 30 sec, 60°C - 30 sec, 72°C

- 30 sec followed by 5 minutes of extension at 72°C in the 1st PCR. For the 1st

RFLP, the restriction enzyme MseI was used to digest the TTAA sequence in the

wild-type genes, as this is frequently absent in exon 19 deletion mutants. In

addition, the restriction enzyme MscI was used to digest the TGGCCA sequence in

the wild-type genes, since the mutant type L858R in exon 21 is not digested. The

1st PCR product from these restriction enzymes was incubated at 37°C for 12

hours. For the 2nd PCR, we used ex19 HR-F and ex19 HR-R to examine the

mutation in exon 19. To examine the mutation in exon 21, we used ex21 HR-F and

ex21 r-s. The 1st RFLP product was amplified for 20 cycles at 94°C - 30 sec, 60°C

- 30 sec, 72°C - 30 sec followed by 5 minutes of extension at 72°C in the 2nd PCR.

For the 2nd RFLP, the restriction enzyme Sau96I was used to digest the GGNCC

sequence. Since the wild-type DNA is not digested, even partial digestion of the

PCR product indicates the presence of a mutation in exon 21. The 2nd PCR

product from this restriction enzyme was incubated at 37°C for 12 hours. This step

was not required for the detection of a mutation in exon 19. After the 2nd PCR or

2nd RFLP, the product was then analyzed by 6% or 8% Polyacrylamide Gel

Electrophoresis. Primer pairs used for PCR in this study are shown in

Supplemental Table 1.

In order to confirm the accuracy of *EGFR* mutation status, mutant samples were

detected with mutant-enriched PCR-RFLP, by nested PCR assay based on the

direct sequence using a previously described procedure.[8] Briefly, ex19seqS-1 and

ex19seqAS-1 primer pairs were used for the 1st PCR, ex19seqS-2 and ex19seqAS-

2 primer pairs were used for the 2nd PCR to examine a mutation in exon 19. We

also used ex21seqS-1 and ex21seqAS-1 for the 1st PCR, ex21seqS-2 and

ex21seqAS-2 for the 2nd PCR to examine a mutation in exon 21. Direct

sequencing was performed with the 3100 Genetic Analyzer (Applied Biosystems,

Tokyo, Japan), and the results were analyzed using Sequencing Analysis 5.1.1

software (Applied Biosystems) to compare variations. The sequences were

compared with the GenBank sequence for human *EGFR* (accession number

NC000007). The entire procedure is summarized in supplemental methods and

supplemental Figure 1.

Mutational analysis for KRAS

The hot-spot mutations in codon 12 in *KRAS* were also detected by the mutant-enriched PCR- direct DNA sequencing method. The 1st PCR was done using the primer pairs K-F-2 and K-R-2. DNA was amplified for 35 cycles at 95°C - 30 sec, 56°C - 30 sec, 72°C - 30 sec followed by 5 minutes of extension at 72°C in the 1st PCR. For RFLP, the restriction enzyme MvaI was used to digest the CCA/TGG sequence in the wild-type genes, which is not digested in the mutant. The 1st PCR product from this restriction enzyme was incubated at 37°C for 12 hours. The 2nd PCR was performed using a primer pair K-F-2 and K3-R-2. The RFLP product was amplified for 20 cycles at 95°C - 30 sec, 62°C - 30 sec, 72°C - 30 sec followed by 5 minutes of extension at 72°C in the 2nd PCR. After the 2nd PCR, the product was then analyzed by 2% agarose gel electrophoresis. Direct sequencing was performed with the 3100 Genetic Analyzer (Applied Biosystems, Tokyo, Japan), and the results were analyzed using Sequencing Analysis 5.1.1 software (Applied Biosystems) to compare variations. The sequences were compared with the GenBank sequence for human KRAS (accession number NC000012). The procedure is summarized in supplemental methods and supplemental Figure 1.

Laser capture microdissection study

Using a previously described procedure,[18] 10-μm-thick sections were stained with neutral fast red to enable laser capture microdissection (P.A.L.M. Microlaser Technologies AG, Munich, Germany) of tumor cells only. RNA was isolated from formalin-fixed, paraffin-embedded specimens using a novel, proprietary procedure (Response Genetics, Los Angeles, CA: United States Patent Number 6,248,535). After RNA isolation, cDNA was derived from each sample using a previously described procedure.[19] For the quantitative RT-PCR, the specifics of the procedure and primers used were described in our previous report.[18,19]

Immunohistochemical staining for DPD, and TS

5-μm-thick sections were prepared for immunohistochemical staining. Details of the entire procedure are as described previously.[18] The primary polyclonal antibodies against DPD and TS were both used at a concentration of 1:1000. These antibodies were generously donated by Taiho Pharmaceutical Co, Japan. The secondary antibody for DPD was biotinylated anti-rabbit IgG and peroxidase-labeled streptavidin (Histofine Simple Stain MAX-PO, Nichirei, Tokyo, Japan). For TS, the VECTASTAIN Elite ABC kit reagents (Vector Laboratories, Burlingame, CA) were used as the secondary antibody. Antibody binding was visualized using DAB (DAKO, Santa Barbara, CA).

Two pathologists, without knowledge of the clinicopathological data, independently evaluated all immunohistochemical staining (Supplemental Figure 2).

Cell culture study

The human lung adenocarcinoma cell line, A549 and PC9 were obtained from DS Pharma Biomedical (Osaka, Japan) and Immuno-Biological Laboratories (Gunma, Japan). NCI-H1975, NCI-H1299 and NCI-H1437 were obtained from the American Type Culture Collection (Manassas, VA, USA). In all cell lines, mutation screening for *EGFR* and *KRAS* was performed as described above. The results confirmed that A549 has a G12S point mutation in *KRAS,* PC9 has a 746-750 deletion in *EGFR* exon 19, and H1975 has a L858R point mutation in *EGFR* exon 21. H1299 and H1437 are wild type for both *EGFR* and *KRAS*.

All cells were maintained in RPMI1640 (Invitrogen Japan, Tokyo, Japan). The media contained 10% fetal bovine serum, 1% penicillin-streptomycin (Nacalai Tesque, Kyoto, Japan), and 2 mM L-glutamine (Invitrogen Japan, Tokyo, Japan). Cells were incubated in a humid atmosphere containing 95% air and 5% CO_2.

RNA from cultured cells was extracted using the RNeasy Mini Kit (QIAGEN, Tokyo, Japan) and cDNA was produced by the PrimeScript RT Master Mix (Takara, Siga, Japan), according to the protocol described in the manufacturer's

instructions. Genomic DNA in the cultured cells was extracted using the

QuickGene DNA tissue kit S (Fujifilm, Tokyo, Japan), according to the protocol

described in the manufacturer's instructions.

PCR reactions were performed using 20 ng of cDNA with the LightCycler 480

SYBR Green I Master (Roche Molecular Biochemicals, Indianapolis, IN, USA)

according to the protocol described in the manufacturer's instructions. Quantitative

RT-PCR was performed on a Roche LightCycler 480 system (Roche Molecular

Biochemicals). The primer pairs used for this RT-PCR are shown in Table S1.

Cycle conditions consisted of denaturation (95°C - 10 min), 50 cycles of

amplification (95°C - 10 sec, 60°C - 10 sec, 72°C - 6 sec), melting (95°C - 5 sec,

65°C - 1 min, 97°C - 0 sec), and cooling (40°C - 30 sec). Quantification data were

analyzed with the LightCycler analysis software (Roche Molecular Biochemicals).

EGF administration in cell culture study

Human epidermal growth factor (EGF) was obtained from Cell Signaling

Technology (Danvers, MA, USA). To examine the relationship between DPD

expression and *EGFR* mutation status, we administered various concentrations of

EGF (0 to 100 ng/ml) to the cell lines. Cells in 2000 μl of culture medium per well

were seeded into 6-well plates and cultured at 37°C for 24 h. The culture medium

was then replaced with serum-free medium (RPMI 1640 containing 2mM L-

glutamine), and 100 μl of PBS containing various amounts of EGF. After

additional incubation at 37°C for 48 h, RNA was extracted from cell lysates as

described above.

Western blots

Cells were washed twice with PBS and lysed in Passive Lysis Buffer (Promega,

Madison, WI, USA). The lysates were centrifuged at 14,000 rpm for 1 min at 4°C,

and the protein concentration of the supernatants was determined using the Pierce

BCA Protein Assay Kit (Thermo scientific, Rockford, IL, USA). For

immunoblotting, supernatant proteins (10 μg) from cell lysates were separated by

sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE). The

proteins were blotted onto a polyvinylidene difluoride membrane and incubated

with anti-DPYD rabbit monoclonal IgG (Cell Signaling Technology, Danvers, MA,

USA) or anti-actin rabbit polyclonal IgG (Abcam, Cambridge, MA, USA) as the

primary antibody, followed by horseradish peroxidase-conjugated goat anti-rabbit

IgG (Amersham, Buckinghamshire, UK). Immunolabeled proteins were visualized

using the Luminescent Image Analyzer LAS-1000plus (Fujifilm, Tokyo, Japan)

after incubation with Chemi-Lumi One Super (Nacalai Tesque, Kyoto, Japan).

Statistical analysis

The chi-square test was used to analyze associations between *EGFR* and *KRAS* mutation, DPD and TS immunohistochemical expression, and clinicopathological factors associated with S-1 adjuvant chemotherapy. The Kaplan-Meier method was used to estimate overall survival (OS) and relapse-free survival (RFS). The log-rank test was used to test for differences between estimated time-to-event curves. Student's t-test was used to analyze associations between expression of DPD and TS mRNA and clinicopathological factors. A *P*-value of 0.05 was regarded as statistically significant and all comparisons were 2-sided. JMP 10.0 statistical software (SAS Campus Drive Cary, NC) was used to perform all analysis.

RESULTS

Correlation among pathology, DPD and TS mRNA levels, and EGFR mutation status in NSCLC

With regard to histopathological types, ADC had significantly higher DPD mRNA levels than SQCC (*P* = .006) (Figure 1). When we subdivided ADC into AIS and invasive ADC (including minimally invasive ADC), the difference in DPD mRNA levels was more remarkable between AIS and SQCC (*P* < .001) than between ADC and SQCC (*P* = .247). Although the TS mRNA level found in SQCC was slightly higher than that in ADC, we did not detect a significant trend.

The Chi-square analysis revealed that AIS had an obviously higher *EGFR* mutation rate than ADC and SQCC ($P < .0001$) (Table 2).

Correlation between clinicopathological factors and EGFR mutation status and immunohistochemical staining for DPD and TS in the specimens obtained from the phase II study

Table 3 shows the relationships between *EGFR* mutation status and various clinicopathological parameters including the immunohistochemical evaluation for DPD and TS in NSCLC tissue obtained from patients in the phase II study. *EGFR* mutations were only detected in ADC and were significantly correlated with female ($P = .017$) and non-smoker ($P = .001$) groups. In the immunohistochemical examination, high DPD expression was significantly correlated with female sex ($P = .003$), non-smoking status ($P = .019$), and *EGFR* mutation status ($P = .003$). For TS immunostaining, there were no correlations among any of the clinicopathological factors including *EGFR* mutation status.

EGFR mutation status and prognosis

In the microdissection study, the prognosis of the *EGFR* mutation positive group was significantly better than for the negative group ($P = .033$) (Figure 2A). The 5-year relapse-free survival rate also indicated that the *EGFR* mutation

positive group tended to have a better prognosis than the negative group ($P = .084$)

(Figure 2B). Conversely, in the postoperative S-1 administration study, the

prognoses of the *EGFR* mutation positive group and negative group were similar

($P = .921$) (Figure 2C). In addition, relapse-free survival rates of the *EGFR*

mutation positive and negative group were 63.6% and 71.2% ($P = .584$) (Figure

2D).

Basal DPD mRNA and protein expression in human lung adenocarcinoma cell lines

The expression level of DPD mRNA in the lung ADC cell lines was quantified

by real-time RT-PCR (Figure 3A). The basal DPD mRNA expression levels in the

EGFR mutant cell lines PC9 and H1975 were higher than that of *EGFR* wild-type

cells ($P < .003$). From western blot analysis, the expression of DPD protein was

higher in *EGFR* mutant cells than in *EGFR* wild-type cells (Figure 3B).

Effects of EGF administration on DPD mRNA expression

Figure 4 shows the DPD mRNA expression of each cell line after the

administration of EGF. Of the five cell lines examined, only *EGFR* mutant cell

lines had a statistically significant increase in DPD mRNA expression after the

administration of EGF. In the PC9 cell line, which harbors a deletion of exon 19

(E746-A750) with *EGFR* amplification,[20] DPD mRNA expression significantly

increased at 0.1 to 1.0 ng/ml added EGF and remained high with increasing

concentrations of EGF (P = .0006). In H1975 cell lines, which harbor point

mutations in exon 21 of *EGFR* (L858R and T790M) but does not have *EGFR*

amplification,[20] DPD mRNA expression was significantly increased at 0.1 ng/ml

EGF (P = 0.0083) and decreased at higher concentrations of added EGF thereafter.

In the *EGFR* wild-type cell lines, there was no effect on DPD mRNA expression

with added EGF.

Discussion

The present studies reveal a clinical correlation between *EGFR* mutation status

and DPD expression. Our results demonstrate that higher DPD mRNA expression

occurs in adenocarcinomas (ADC), especially adenocarcinoma *in situ* (AIS)

(Figure 1). These results are consistent with the report of Kaira and colleagues that

higher DPD expressions were found in adenocarcinomas as compared with non-

adenocarcinomas.[21] DPD is a rate-limiting enzyme for degrading 5-FU in the liver

and other tissues.[22] A high level of DPD in tumor cells accelerates inactivation of

5-FU thus reducing the effectiveness of 5-FU therapy. Therefore, AIS and invasive

ADC might have differing sensitivities to 5-FU, indicating that these carcinomas

have different biological properties. This study also shows that a higher frequency

of *EGFR* mutation occurs in AIS (Table 2). This result is accordant with several studies that found *EGFR* gene mutation clusters in bronchioloalveolar carcinoma (BAC) (now classified as AIS) and that this characteristic of BAC is a predictor of response to EGFR-TKI.[23-25]

The clinicopathological analysis revealed that higher DPD protein levels were found in females than in males and in non-smokers than in smokers. In our previous study, significantly higher DPD mRNA expression and DPD protein levels were also correlated with females and non-smokers.[18] These two populations overlap in the target population of EGFR-TKI therapy.[9] Accordingly, a statistically significant correlation was observed between *EGFR* mutation status and DPD protein expression (Table 3). These data support the results of Suehisa and colleagues that showed sensitivity to 5-FU is lower in *EGFR* mutant cell lines than in *EGFR* wild-type human lung cancer cell lines.[11] Because DPD is a rate-limiting enzyme for degradation of 5-FU, patients with *EGFR*-mutated tumors might not respond to uracil-tegafur treatment because of their high DPD activity.

Our survival data of the microdissection studies indicate that the *EGFR* mutation positive group has a better prognosis than the negative group after operation (Figure 2A and 2B). The finding is consistent with previous reports indicating that *EGFR* mutation status is a postoperative predictive marker of a good prognosis.[26, 27] However, in the postoperative S-1-treated population, the

survival curve of the *EGFR* mutation positive group overlapped with that of the negative group. The explanation is that S-1 might be more effective in patients that are *EGFR* mutation negative, referred to as wild-type, with improved survival rates comparable to the level of the *EGFR* mutation-positive population, thus resulting in the same prognosis for both populations. However, given that the 70.7% of 5-year overall survival rate of the *EGFR* mutation positive group is favorable in stage IB to IIIA patients, there is a possibility that S-1 might be able to suppress 5-FU degradation in *EGFR* mutated tumors with high DPD because S-1 has 180 times higher DPD suppressive effect than uracil-tegafur. As a result, S-1 postoperative treatment might show some prognostic improvement even in *EGFR* mutant populations. Survival differences classified by TS expression or DPD expression might be of interest. However, there was no significant survival difference between the DPD positive and negative groups and the TS-positive and negative groups in both cohorts of the microdissection study and the postoperative S-1-treated study (data not shown).

Okabe and colleagues concluded that the suppression of TS by EGFR-TKI induced the synergistic antitumor effect with 5-FU and EGFR-TKI.[12] Using a nude mouse NSCLC model, they demonstrated that both single S-1 and S-1 plus EGFR-TKI exerted antitumor effects on lung cancer cell lines regardless of *EGFR* mutation status.[12] However, they did not mention the strong DPD inhibitory effect

of S-1 which might affect the antitumor efficacy of 5-FU in lung cancer cell lines. Our results indicate that high DPD activity is another key factor determining the effectiveness of 5-FU derivatives in *EGFR* mutant populations and cells. The real synergetic effect could be established by doing clinical studies, as well as treated samples, using the combination of both S-1 and EGFR-TKI.

Phosphorylation of EGFR is responsible for activation of JAK-STAT, MAP kinase, and the Phosphatidylinositol-3 kinase/AKT signal cascade, which regulates a variety of proteins. Because AIS tends to have high *EGFR* mutation rates with higher DPD mRNA levels, it is likely that an unknown mechanism for molecular regulation exists between the EGFR cascade and DPD expression, similar to the correlation between EGFR signal transduction and TS expression.[12] In this study, no trend was observed when comparing TS mRNA levels in EGFR mutated or wild type cell lines, or in the EGF administration exam (data not shown). Interestingly, *EGFR* mutated cell lines showed high DPD mRNA and protein expression in base line (Figure 3). Further, EGF administration caused a fluctuation in DPD mRNA expression in *EGFR* mutant cell lines but not in wild-type cell lines (Figure 4). This result is consistent with the report of Sordella and colleagues that EGF phosphorylates different parts of the EGFR C-terminal tyrosine residues between *EGFR* wild-type and mutant, and thus, it activates selective downstream signaling pathways.[28] It is curious that administration of an EGF dose below or

equal to 1 ng/ml resulted in an increase in DPD expression, but EGF

concentrations above 1 ng/ml caused a decrease in DPD expression. This same

phenomenon was observed by Kashima et al. using uterine cervical carcinoma

SKG-IIIB cells [29]. It is likely, as the authors in that report speculate, that the

anabolic pathway became dominant in cells with stimulated growth and that DPD,

an enzyme of the catabolic pathway, was inhibited at a higher EGF concentration.
[29]

There are some limitations in this study. First, our sample size may not be large

enough to discuss the prognostic analysis. Larger scale analysis might be necessary

to elucidate the prognostic impact of *EGFR* mutation status for S-1 treated

populations. Second, although we did not test for other 5-FU related genes in this

study, such additional information is unlikely to be sufficient to explain the lack of

5-FU efficacy as this depends on many genes in the 5-FU pathway. Detailed

studies of signal cascades in DPD transcriptional regulation should be undertaken.

Published analysis of the DPD promoter identified several potential *cis*- acting

regulatory elements including binding sites for transcription factors Sp-1, AP-2,

NF-kB, and Egr families.[30,31] Further studies are necessary to clarify the crosstalk

between EGFR signaling and DPD transcription.

Conclusion

High DPD expression was shown to be correlated with *EGFR* mutation in adenocarcinoma. In particular, AIS had both high DPD mRNA expression and high *EGFR* mutation frequency. Given that *EGFR* mutant cells had higher DPD mRNA and protein levels, and EGF administration increased DPD mRNA in such cell lines, DPD activity might be another key factor determining the effectiveness of 5-FU derivatives in *EGFR* mutant populations and cells. Further analysis about transcriptional regulation of the EGFR cascade and DPD expression should be investigated.

Clinical practice points

- Previously the use of 5-FU was thought to be inappropriate for lung cancer therapy. However, in Asian countries, there are now two types of 5-FU derivatives, which avoid the problem of degradation by dihydropyrimidine dehydrogenase (DPD), are now approved and available as an option for NSCLC therapy. It has been shown that *EGFR* mutation status affects the efficacy of the 5-FU derivatives for NSCLC treatment. However, the effects of *EGFR* mutation status on DPD expression have not been elucidated.

- The major findings of the present study were the positive correlation between DPD expression and *EGFR* mutations. Histologically, higher DPD mRNA expression occurs in adenocarcinomas, especially adenocarcinoma *in situ* (AIS). In addition, AIS showed a significantly higher *EGFR* mutation frequency than other histological types. Clinically, DPD immuno-positive cases were more frequently observed in females and in non-smokers, which are overlapping *EGFR* mutated populations. Accordingly, DPD immune-positive cases were correlated with *EGFR* mutation status. The cell culture based study also revealed high DPD mRNA and protein expression in *EGFR* mutated cell lines and that added EGF increases DPD mRNA only in such cell lines. This finding indicates that DPD regulation might be different depending on *EGFR* mutation status.

- The clinical impact of these results is to help select the appropriate treatment for NSCLC considering the effect of *EGFR* mutation status on 5-FU treatment. The present study indicates that 5-FU derivatives might have more benefit for *EGFR* mutation negative populations and less benefit for *EGFR* mutation positive populations because of DPD activity in NSCLC.

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Disclosure

All authors have no conflicts of interest.

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Figure legends

Fig. 1.   Box-and-whisker plots of mRNA levels according to histopathological type. Lines within the boxes represent median values; the upper and lower lines of the boxes represent the 25th and 75th percentiles, respectively, and the upper and lower bars outside the boxes represent the 90th and 10th percentiles, respectively. The dots represent outlier values. *p* -values were evaluated by Student t-test using log (data) ($p < 0.05$).

Fig. 2.   Kaplan-Meier overall and relapse free survival curves for all patients according to *EGFR* mutation status in the laser microdissection study (n = 47; the follow up period was 7.3 to 66.4 months) (A and B) and postoperative S-1 treated study (n = 49; the follow up period was 6.3 to 80.5 months) (C and D).

Fig. 3.   Relative DPD mRNA expression ratio compared to β-actin (A) and Western blot analysis (B) in the *KRAS* mutation-positive cell line (A549), *EGFR* mutation-positive cell lines (PC9 and H1975), and wild-type cell lines (H1299 and H1437) (*n* = 5).
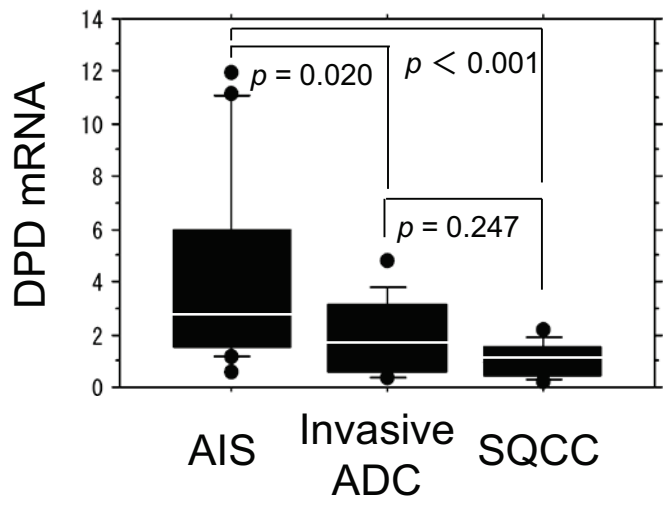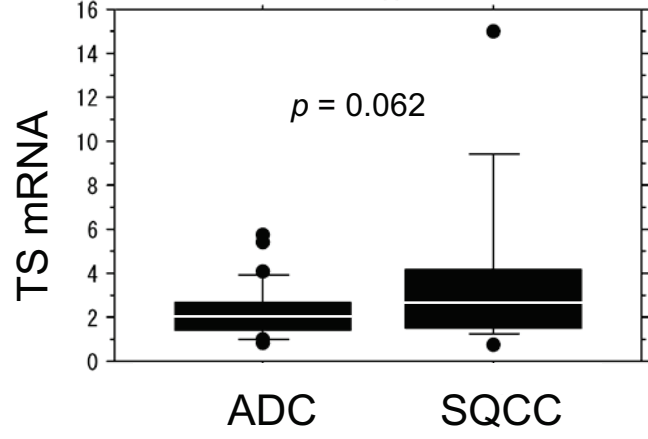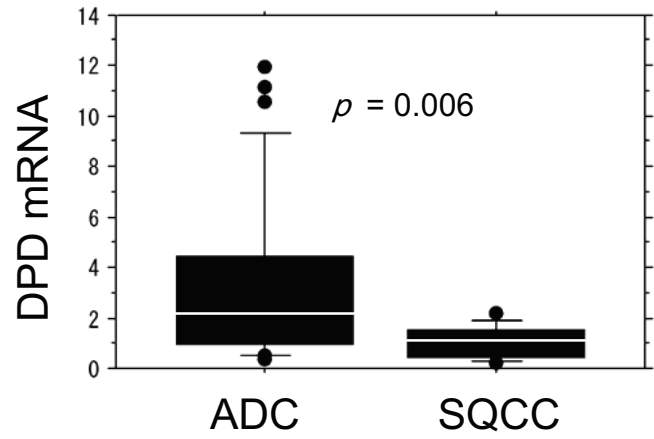
Fig. 4.   Effects of EGF on DPD mRNA expression in each cell line (*n* = 4). Low concentration of EGF increased DPD mRNA level in *EGFR*-mutated cell lines

(PC9 and H1975). The dots represent outlier values ($p < 0.05$). EGF had no effect on DPD mRNA level in *KRAS* mutation-positive cell line (A549) and in *EGFR* mutation wild-type cell lines (H1299 and H1437).

Supplemental Fig. 1.   Flow chart of the mutation analysis. Details of the entire procedure are provided in the supplemental methods.

Supplemental Fig. 2.   Immunohistochemical staining. The intensity of staining was classified as 0 (no staining), +1 (weak staining), +2 (distinct staining), or +3 (very strong staining). Grades 0 and 1 were further categorized as negative, and grades 2 and 3 as positive.

Figure 1

Figure 2

A

Overall survival rate

p = 0.033

EGFR mutation
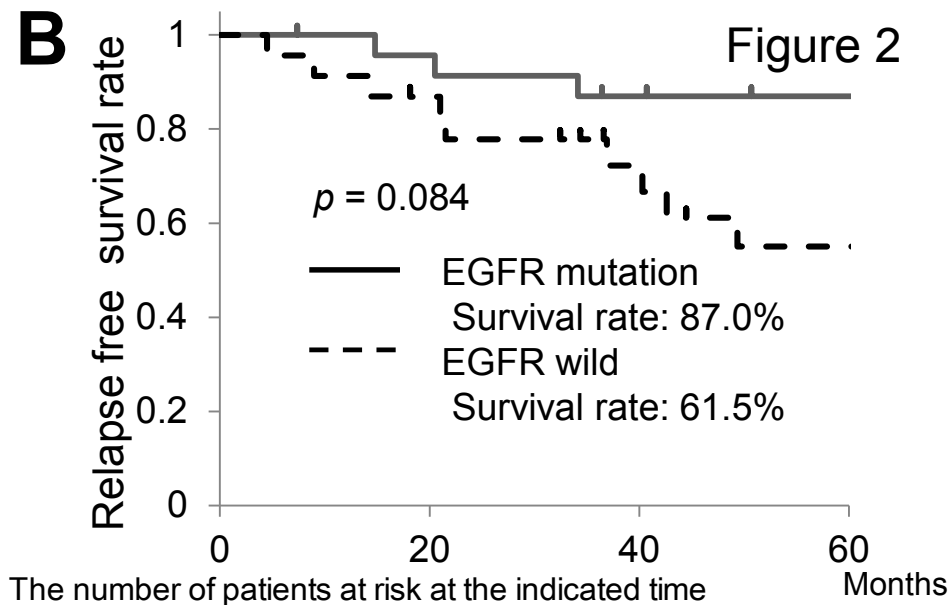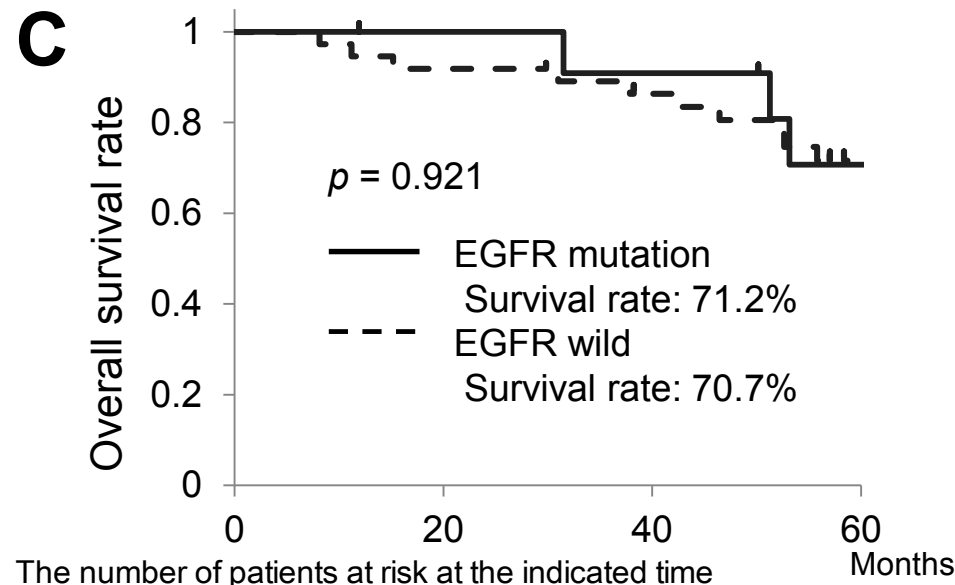Survival rate: 87.0%
EGFR wild
Survival rate: 55.2%

Months
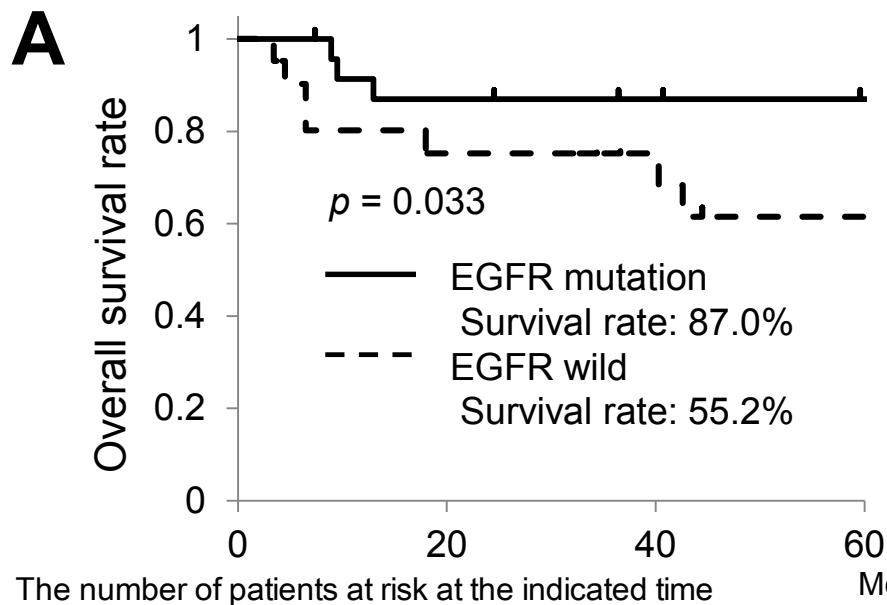
The number of patients at risk at the indicated time

EGFR mutation	24	23	20	18
EGFR wild	23	20	14	10

B

Relapse free survival rate

p = 0.084

EGFR mutation
Survival rate: 87.0%
EGFR wild
Survival rate: 61.5%

Months

The number of patients at risk at the indicated time

EGFR mutation	24	21	19	17
EGFR wild	23	15	12	9

C

Overall survival rate

p = 0.921

EGFR mutation
Survival rate: 71.2%
EGFR wild
Survival rate: 70.7%

Months

The number of patients at risk at the indicated time

EGFR mutation	12	12	11	8
EGFR wild	37	35	31	22

D

Relapse free survival rate

p = 0.584

EGFR mutation
Survival rate: 63.6%
EGFR wild
Survival rate: 71.2%

Months
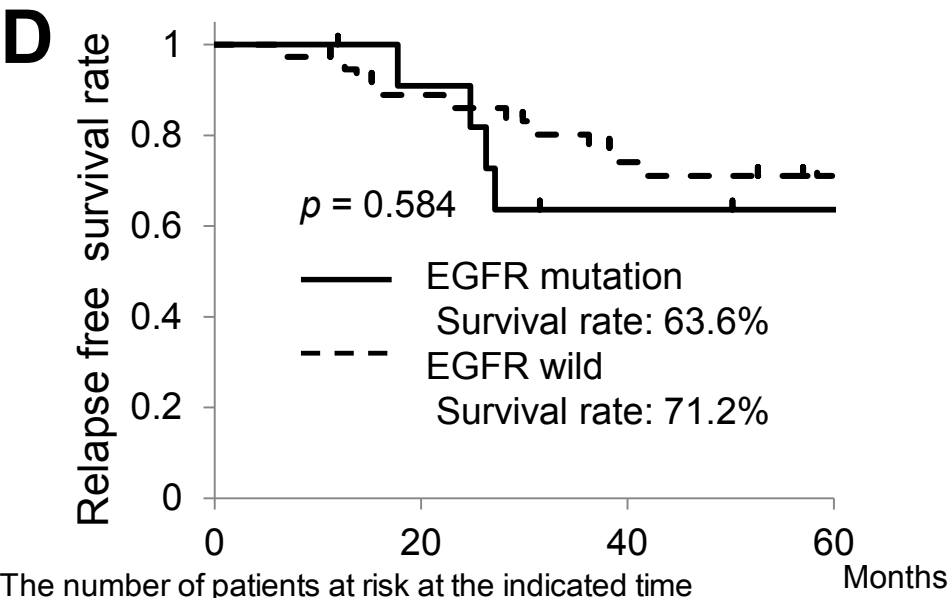
The number of patients at risk at the indicated time

EGFR mutation	12	11	7	6
EGFR wild	37	32	25	19
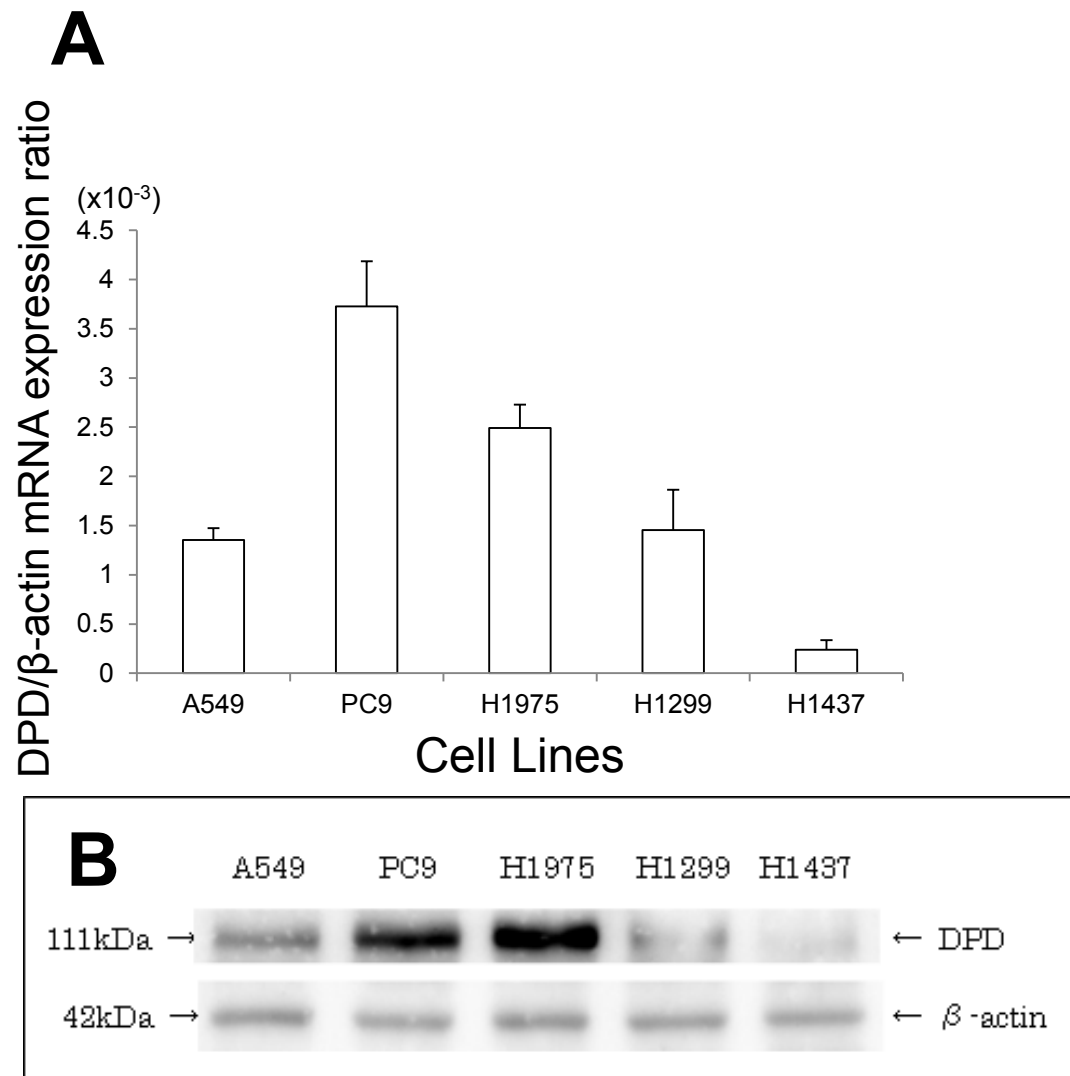
Figure 3

Figure 4
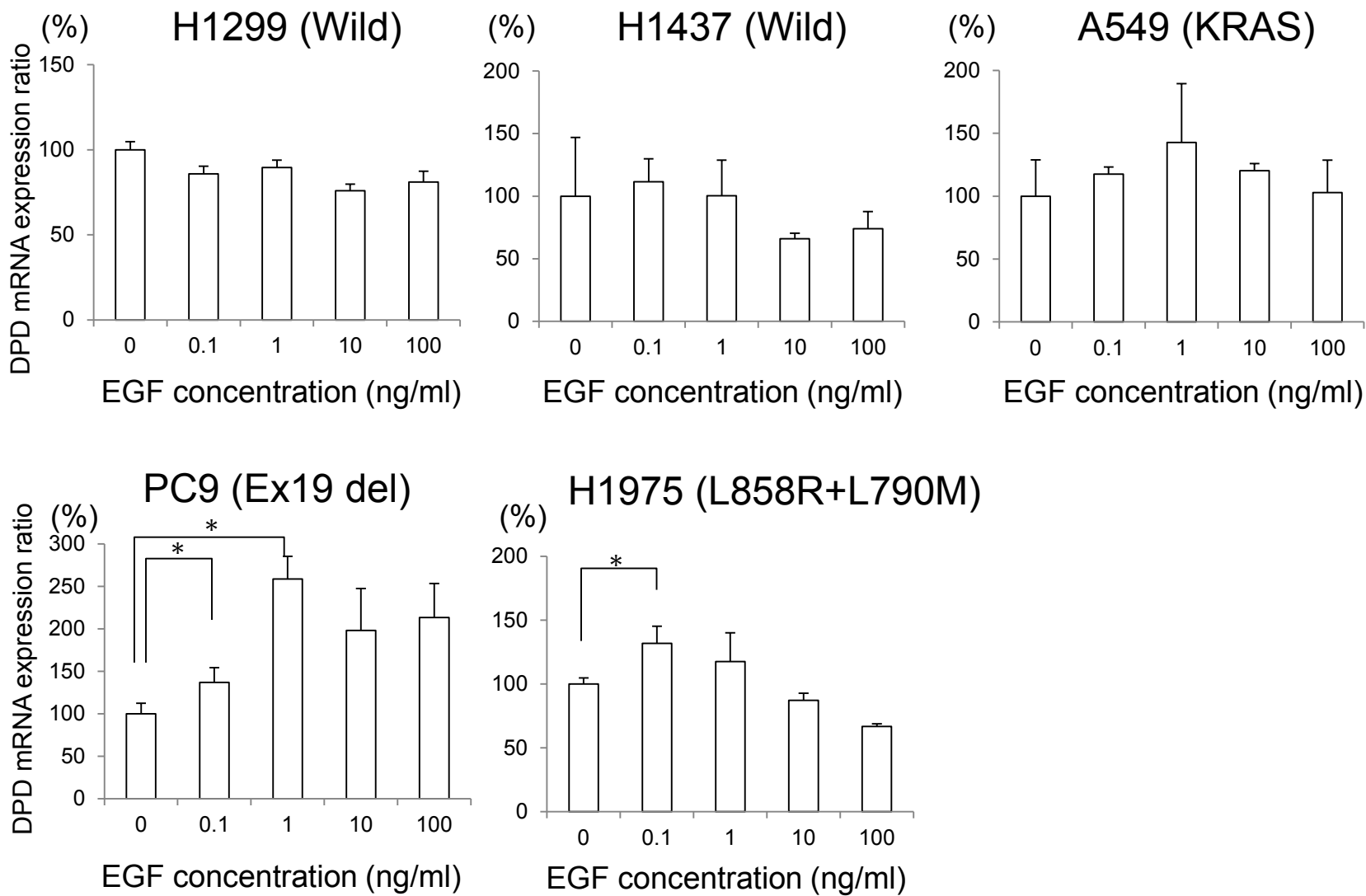
Method Flow Chart for Mutation Analysis

<EGFR Mutation>   <KRAS Mutation>

1. DNA extraction	1. DNA extraction
2. Mutant-enriched PCR-RFLP	2. Mutant-enriched PCR-RFLP
3. Polyacrylamide Gel Electrophoresis	3. Direct Sequence

Mutant Type	Wild Type		Mutant Type	Wild Type
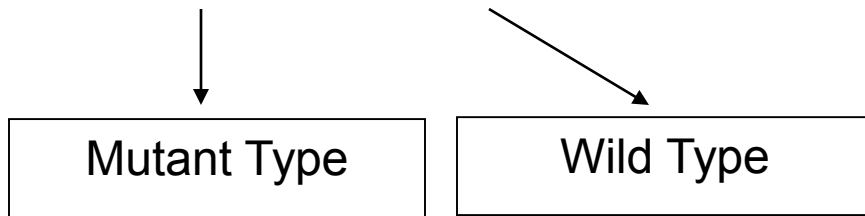
Additional confirmation

4. Nested PCR

5. Direct Sequence
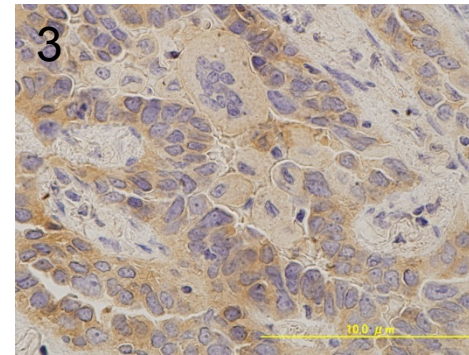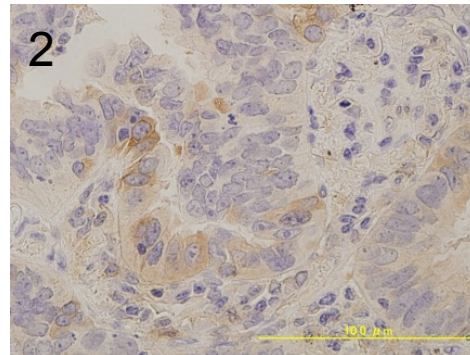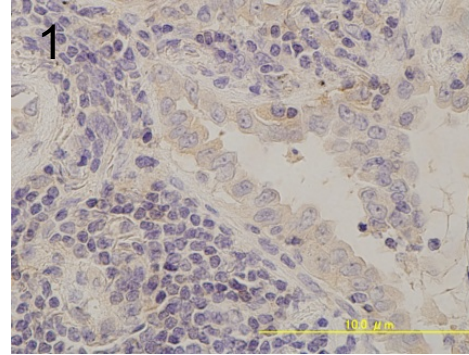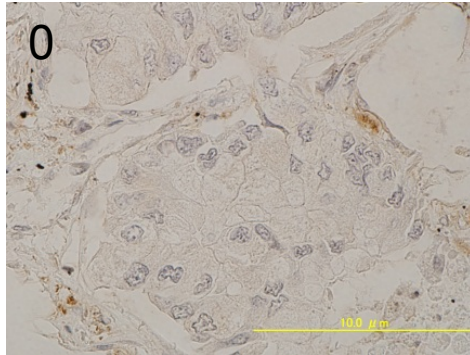
Table 1. Clinicopathological Data of Patients

Characteristic	Microdissection study (n = 47)	S-1 study (n = 49)
Gender		
Male	34	34
Female	13	15
Age (years)		
≥ 70	24	27
< 70	23	22
Smoking		
Current or former	34	38
Never	13	11
Histopathology		
ADC (AIS)	34 (20)	30 (0)
SQCC	13	16
AD-SQC	0	2
Carcinoid	0	1
Differentiation		
Well	23	10[a]
Moderate	18	36[a]
Poor	6	0[a]
Pathological Stage		
IA	28	0
IB	10	26
IIA	0	15
IIB	3	1
IIIA	4	7
IIIB	2	0

Abbreviations: ADC; adenocarcinoma, AIS; adenocarcinoma in situ, SQCC; squamous cell carcinoma, AD-SQC; adenosquamous cell carcinoma

[a]There was no information about differentiation in three cases (2 cases of adenosquamous carcinoma and one carcinoid).

Table 2. Correlation Between EGFR Mutation Status and Pathological Histology in Patients with NSCLC (n = 93)

EGFR mutation status	AIS (n = 20)	Invasive ADC (n = 44)	SQCC (n = 29)	p-value
Positive	17	17	2	< 0.0001
Negative	3	27	27	

A chi-square test for independence. 47 cases in the microdissection study and 46 cases in the postoperative S-1 treated study were combined. Three patients with carcinoid (n = 1) or adenosquamous cell carcinoma (n = 2) were excluded. Abbreviations: AIS; adenocarcinoma *in situ*, ADC; adenocarcinoma (invasive ADC including 3 minimally invasive ADC), SQCC; squamous cell carcinoma, EGFR; epidermal growth factor receptor

Table 3. Correlation among Clinicopathological Characteristics, EGFR Mutation Status, and Immunohistochemical Expression of DPD or TS in Patients with NSCLC in S-1 Phase II Study (n = 49)

Characteristics	No. of patients	*EGFR* mutation Mutant 12	Wild 37	*p*	DPD Positive 10	Negative 39	*p*	TS Positive 16	Negative 33	*p*
Gender										
Male	34	5	29	0.017[a]	3	31	0.003[a]	12	22	0.553
Female	15	7	8		7	8		4	11	
Age, Years										
≥ 70	27	5	22	0.282	5	22	0.716	11	16	0.181
< 70	22	7	15		5	17		5	17	
Smoking										
Never	11	6	5	0.001[a]	5	6	0.019[a]	4	7	0.766
Current or former	38	6	32		2	23		12	26	
Histology										
ADC	30	12	18	0.002[a]	8	22	0.172	10	20	0.899
Non-ADC	19	0	19		2	17		6	13	
Tumor status										
pT1	12	3	9	0.962	3	9	0.650	3	9	0.515
pT2-3	37	9	28		7	30		13	24	
Nodal Status										
pN0	28	7	21	0.924	6	22	0.838	11	17	0.253

				P value			P value			P value
pN1-2	21	5	16		4	17		5	16	
Lymphatic invasion										
Negative	44	11	33	0.805	8	36	0.251	14	30	0.712
Positive	5	1	4		2	3		2	3	
Vessel invasion										
Negative	42	11	31	0.498	9	33	0.664	13	29	0.534
Positive	7	1	6		1	6		3	4	
Differentiation										
Well	10	5	5		2	8		4	6	
Moderately	24	6	18	0.070	5	19	0.981	6	18	0.581
Poorly	13	1	12		3	10		5	8	
EGFR										
Mutant	12	(-)	(-)		6	6	0.003[a]	2	10	0.174
Wild	37	(-)	(-)		4	33		14	23	
KRAS										
Mutant	14	2	12	0.294	3	11	0.911	3	11	0.289
Wild	35	10	25		7	28		13	22	

A chi-square test for independence (* P value < 0.05). Abbreviations: EGFR; epidermal growth factor receptor, KRAS; Kirsten rat sarcoma 2 viral oncogene homolog, DPD; dihydropyrimidine dehydrogenase, TS; thymidylate synthase, ADC; adenocarcinoma

Supplemental Table 1. Sequences of Primers Used for PCR in Each Method

Primer	Sequence
For mutant-enriched PCR-RFLP	
ex19 S-1	ATCCCAGAAGGTGAGAAAGATAAAATTC
ex19 AS-1	CCTGAGGTTCAGAGCCATGGA
ex19 HR-F	AAAATTCCCGTCGCTATCA
ex19 HR-R	AAGCAGAAACTCACATCG
ex21 f-s	CGCAGCATGTCAAGATCACAG
ex21 r-s	ACTTTGCCTCCTTCTGCATGG
ex21 HR-F	AGATCACAGATTTTGGGC
K-F-2	AACTTGTGGTAGTTGGACCT
K-R-2	TCAAAGAATGGTCCTGCACC
K3-R-2	GGTCCTGCACCAGTAATATG
For PCR-direct DNA sequencing	
ex19seqAS-1	CATAGAAAGTGAACATTTAGGATGTG
ex19seqS-2	CCTTAGGTGCGGCTCCACAGC
ex19seqAS-2	CATTTAGGATGTGGAGATGAGC
ex21seqS-1	CTAACGTTCGCCAGCCATAAGTCC
ex21seqAS-1	GCTGCGAGCTCACCCAGAATGTCTGG
ex21seqS-2	CAGCCATAAGTCCTCGACGTGG
ex21seqAS-2	CATCCTCCCCTGCATGTGTTAAAC
For RT-RCR	
DPD-S	GTTGTGGCTATGATTGATGA
DPD-AS	ATTCACAGATAAGGGTACGC
β-actin-S	GCAAAGACCTGTACGCCAAC
β-actin-AS	CTAGAAGCATTTGCGGTGGA

Abbreviations: RFLP; restriction fragment length polymorphism, RT; reverse

transcription, DPD; dihydropyrimidine dehydrogenase

Supplemental Table 2. Patient characteristicts and univariate analysis of 5 year survival in microdissection study cohort

Parameter	Number of patients	Oveall Survival		Relapse Free Survival	
		%Survival	P-value	%Survival	P-value
Age (years)					
<70	23	78.3 ± 0.1	0.611	81.3 ± 0.1	0.806
≧70	24	66.8 ± 0.1		76.4 ± 0.1	
Gender					
Male	34	65.1 ± 0.1	0.109	74.1 ± 0.1	0.262
Female	13	91.7 ± 0.1		91.7 ± 0.1	
Smoking					
Smoker	34	64.6 ± 0.1	0.092	73.3 ± 0.1	0.221
Never-smoker	13	92.3 ± 0.1		91.7 ± 0.1	
Histology					
Adenocarcinoma	34	80.8 ± 0.1	0.016	78.3 ± 0.1	0.867
Non-adenocarcinoma	13	46.4 ± 0.2		81.8 ± 0.1	
Tumor Status					
T1	30	86.0 ± 0.1	0.007	92.7 ± 0.0	0.010
T2-4	17	46.5 ± 0.1		58.3 ± 0.1	
Nodal Status					
N0	40	75.6 ± 0.1	0.190	80.7 ± 0.1	0.352
N1-2	7	51.4 ± 0.2		66.7 ± 0.2	
Lymphatic Invasion					
Negative	23	100.0 ± 0.0	<0.001	100.0 ± 0.0	0.001
Positive	24	47.0 ± 0.1		56.6 ± 0.1	

Vessel Invasion					
Negative	32	82.2 ± 0.1		83.4 ± 0.1	
			0.007		0.203
Positive	15	49.9 ± 0.1		67.1 ± 0.1	
Differentiation					
Well	23	95.2 ± 0.0		95.2 ± 0.0	
			0.002		0.009
Moderate or Poor	24	51.9 ± 0.1		61.8 ± 0.1	
EGFR Mutaion					
Mutated Type	24	87.0 ± 0.1		87.0 ± 0.1	
			0.033		0.084
Wild type	23	55.2 ± 0.1		61.5 ± 0.1	

Logrank test for independence, Data are mean ± S.D., EGFR: epidermal growth factor receptor