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Alternative Efficacy-Predicting Markers for Paclitaxel instead of CHFR in Non-Small Cell Lung Cancer

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Running title: PTX and its Predictive Markers in NSCLC

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Abstract

Experiments using cancer cell lines have revealed that *CHFR* methylation is correlated with sensitivity to microtubule inhibitors. However, the evidence may not benefit actual clinical cases because it is hard to detect *CHFR* methylation without surgically resected samples. Thus, a more easily available marker that is linked to sensitivity to microtubule inhibitors might be necessary in NSCLC, especially in advanced cases. In this study, we show that *EGFR* gene status and smoking are significantly efficacy-predicting markers, in addition to *CHFR* methylation in NSCLC. Chemosensitivity to paclitaxel and six other chemotherapeutic agents was evaluated using the succinate dehydrogenase inhibition (SDI) method in 69 NSCLC cases, consisting of 48 adenocarcinomas, 20 squamous cell carcinomas and 1 large cell carcinoma. Next we evaluated the relationships between *CHFR* or *EGFR* status and clinicopathologic data. Methylation-specific PCR (MSP) and direct DNA sequencing were performed to detect aberrant methylation of *CHFR* and *EGFR* mutations, respectively. *CHFR* gene promoter methylation and *EGFR* gene mutation were observed in 11 cases (15.9%) and 7 cases (10.1%), respectively. The SDI method revealed that *CHFR* gene methylation was significantly related to high sensitivity to paclitaxel ($p<0.001$), while no sensitivity was found to other agents. Interestingly, *EGFR* with the wild genotype and smoking habit

were also significantly correlated with sensitivity to paclitaxel (p=0.014, 0.029, respectively). Our results demonstrate that the *EGFR* wild genotype and smoking habit, in addition to aberrant methylation of the *CHFR* gene, might be a predictor of clinical response to paclitaxel in NSCLC.

Keywords: CHFR, EGFR, Smoking habit, NSCLC, Paclitaxel

Introduction

Lung cancer is the leading cause of death due to cancer in advanced countries, and more than 60% of patients with non-small cell lung cancer (NSCLC) are at an advanced stage of the disease at diagnosis. [1] Even though the prognosis is still poor, the clinical usefulness of first-line chemotherapy has been established in advanced NSCLC. [2, 3] Paclitaxel, a microtubule inhibitor, is widely used as a first-line agent in advanced NSCLC. [2, 3] However, the response rate to paclitaxel as a single agent has been only 15-20%, and it improves median survival by only 2 months. [4, 5] The data suggest the possibility that a good many NSCLC cases may be paclitaxel-resistant, resulting in useless treatments. Despite the earnest efforts of global gene expression profiling analyses to detect chemosensitive marker, there are few useful and reliable markers for predicting chemosensitivity to paclitaxel, although many candidate genes potentially involved in sensitivity to other anti-cancer agents, such as the *excision repair cross-complementation 1* (*ERCC1*) gene for cisplatin sensitivity and the *ribonucleotide reductase M1* (*RRM1*) gene for gemcitabine sensitivity, have been identified. [6, 7] Therefore, a more easily available marker of sensitivity to microtubule inhibitors in NSCLC might be necessary, especially in advanced cases, through a search of clinical data and genetic or epigenetic alteration to predict a better response to paclitaxel for

made-to-order medicine.

Microtubules, filaments formed by the polymerization of heterodimeric α/β tubulin subunits, play a fundamental role in mitotic cell division. Microtubule inhibitors that disrupt the processes of microtubule depolymerization cause cell cycle arrest, resulting in the induction of apoptosis. [8, 9] Recently, *CHFR* (checkpoint gene with forkhead-associated domain and ring finger) was identified. [10] CHFR functions as a mitotic checkpoint by detecting mitotic stress induced by microtubule inhibitors such as paclitaxel, and under such conditions CHFR induces cell cycle arrest in the G2 phase (G2 arrest). [10] Cells with normal CHFR expression are arrested in the G2 phase by microtubule inhibitors and consequently are resistant to microtubule inhibitors. However, cells with a *CHFR* gene inactivated by aberrant methylation cannot be arrested in the G2 phase and proceed to mitosis, with subsequent cell death due to mitotic checkpoint impairment. [10] Therefore, aberrant methylation of the *CHFR* gene might be useful as a molecular marker for predicting sensitivity to microtubule inhibitors. A correlation between aberrant methylation of the *CHFR* gene and sensitivity to microtubule inhibitors has been reported in various kinds of human cancer cells in *in vitro* experiments. [11, 12] However, in NSCLC, it is still hard technically to detect *CHFR* methylation without surgically resected frozen samples.

In this study, we investigated chemosensitivity to paclitaxel using the succinate dehydrogenase inhibition (SDI) method, an *ex vivo* experiment, in 69 NSCLC cases. Next we evaluated the correlation between chemosensitivity and *CHFR* methylation, *EGFR* mutation, which is prevalent in NSCLC, and clinicopathologic data. We found that *EGFR* gene status and smoking habit are significant efficacy-predicting markers, in addition to *CHFR* methylation in NSCLC.

Results

Aberrant hypermethylation of the *CHFR* gene and *EGFR* gene mutation in NSCLC and the association of each with clinicopathologic data

To elucidate the promoter aberrant methylation of the *CHFR* gene, methylation-specific PCR was carried out in the frozen sections of 69 lung cancers using genomic DNA obtained from gross dissection. Aberrant methylation of the *CHFR* promoter region was found in 11 of the 69 (15.9 %) cases (Figure 1a). Among them, 9 cases were squamous cell carcinomas and the remaining 2 were adenocarcinomas. Statistically, aberrant methylation of the *CHFR* promoter region was associated with smoking habit ($p=0.015$; Table IA) and tumor histology ($p<0.01$; Table IA). Univariate analysis of smoking habit, age, gender, pathological stage and tumor histology for the significance of *CHFR* methylation was performed. Smoking habit and tumor histology were significant factors ($p=0.021$, $p<0.01$, respectively), but there was no significance in age ($p=0.24$), gender ($p=0.40$) or pathological stage ($p=0.93$) (data not shown). We applied smoking habit, age and tumor histology, which showed *p*-values of 0.3 or less, to logistic multivariate analysis. The results showed smoking habit and tumor histology to be significant risk factors for *CHFR* methylation, with relative risks of 4.36 and 5.90, respectively (Table IB).

Mutational analysis for *EGFR* gene exons 18-21 was also performed. *EGFR* mutations were found in 7 of the 69 cases (10.1%). Six cases showed a T-to-G transversion at the second nucleotide of codon 858 in exon 21, resulting in substitution of leucine with arginine residue (Figure 1b). One deletion mutation occurred around codons 747–752 in exon 19 (Figure 1c). All of these mutations have previously been described, [13, 14] and all patients were adenocarcinoma with a pack-year index of less than 20. Statistically, *EGFR* gene mutation was conversely associated with smoking habit (*p=0.003*; Table IA).

Comparison of the sensitivity of seven anti-cancer agents using SDI method in NSCLC

The chemosensitivity of 69 NSCLC cases was examined for cisplatin (CDDP), carboplatin (CBDCA), paclitaxel (PTX), gemcitabine (GEM), vinorelbine (VNR), irinotecan (CPT-11), and etoposide (VP-16) using the SDI method. The averages of chemosensitivity rates among all cases were as follows: 61.57%± 16.85% for CDDP, 61.33%±12.49% for CBDCA, 48.28%±19.30% for PTX, 29.92%±11.27% for GEM, 36.58%±13.89% for VNR, 27.89%±21.22% for CPT-11 and 21.63%±16.61% for VP-16 (Figure 2, Table II). The mean chemosensitivity rate for platinum agents, CDDP

and CBDCA, were significantly higher than those for other agents. The mean chemosensitivity rate for VP-16, which has been used in small cell lung cancer (SCLC) but not in NSCLC, was lowest among the seven anti-cancer agents. The Scheffe test divided the seven anti-cancer agents into four groups and showed statistical significance among them (Figure 2, Table II).

Correlations between chemosensitivity and methylation of *CHFR* gene promoter, *EGFR* gene mutation and clinicopathologic data

We examined the correlations with *CHFR* gene methylation and chemosensitivity for CDDP, CBDCA, PTX, GEM, VNR, CPT-11 and VP-16. The sensitivity rate for PTX was significantly higher in the 11 *CHFR*-methylated cases than in the 58 *CHFR*-unmethylated cases ($p<0.001$; Figure 3c). A noteworthy finding was that the mean PTX sensitivity was higher than mean CDDP sensitivity in *CHFR*-methylated cases (Figure 3c). There were no significant differences in chemosensitivity rates between *CHFR*-methylated and ummethylated cases for CDDP, CBDCA, GEM, VNR, CPT-11 or VP-16 (Figure 3a, b and d-g).

Next, we compared chemosensitivity and *EGFR* gene mutation. The sensitivity rate for PTX was significantly higher in the 62 cases with *EGFR* wild-type than in the 7

cases with *EGFR* mutant (*p=0.014*; Figure 3h). There was no statistically significant difference in chemosensitivity rates regarding *EGFR* gene status for CDDP, CBDCA, GEM, VNR, CPT-11 or VP-16 (Table III).

Furthemore, we examined the association between clinicopathologic data and chemosensitivity for CDDP, CBDCA, PTX, GEM, VNR, CPT-11 and VP-16 in the 69 NSCLC cases. The sensitivity rate for PTX was significantly higher in smokers than in never-smokers (*p=0.029*; Figure 3i). There were no statistically significant differences in chemosensitivity rates regarding smoking status for CDDP, CBDCA, GEM, VNR, CPT-11 or VP-16 (Table III). There were no significant differences among chemosensitivity rates for seven anti-cancer agents and other clinicopathologic factors such as age, gender, tumor histology and pathologic stage (data not shown).

Finally, we examined PTX sensitivity between smokers with *CHFR* methylation and *EGFR*-wild type, and never-smokers with unmethylated *CHFR* and *EGFR* mutant. The sensitivity rate for PTX was significantly higher in smokers with *CHFR* methylation and *EGFR*-wild type than in never-smokers with unmethylated *CHFR* and *EGFR* mutant (*p<0.0001*; Figure 3j). By analyzing the 3 best variables, *CHFR*, *EGFR* and smoking status, simultaneously, the strength of our predictive model increased.

The strongest factor for PTX sensitivity

As shown in Figure 3, the sensitivity rate for PTX was significantly associated with *CHFR* methylation, *EGFR* mutation and smoking habit ($p<0.001$, $p=0.014$, 0.029, respectively). Hence, we evaluated the strongest factor in PTX sensitivity using receiver operator characteristic (ROC) curves (Figure 4a-c). *CHFR* methylation, *EGFR* mutation and smoking habit produced significant ROC curves with area under the ROC curves (AUC) of 0.5 or more, showing the sensitivity and specificity of chemosensitivity rate for PTX. The AUCs of *CHFR* methylation, *EGFR* mutation and smoking habit were 0.848, 0.747 and 0.667, respectively. *CHFR* methylation had the highest AUC among the three factors, indicating that the strongest factor for PTX sensitivity, followed by *EGFR* mutation and smoking habit.

In addition, we applied the *CHFR*, *EGFR* and smoking status to logistic analysis. The results showed *CHFR* and smoking status to be significant risk factors for PTX sensitivity with relative risks of 1.168 and 1.032 (Table IV).

Discussion

The majority of patients with NSCLC are diagnosed at an advanced stage (IIIB/IV) and subsequently succumb to the disease, usually within 2 years. [1] Treatment with the combination of platinum and paclitaxel (PTX) has been widely performed as the first-line chemotherapy in advanced NSCLC, but the median survival time and 1-year survival rate are still unsatisfactory, ranging from 12 to 15 months and 35% to 45%, respectively. [2, 3] However, it has been reported that this treatment strikingly reduced tumor size. [4, 5] The data suggest the possibility that some NSCLCs are sensitive to PTX while others are resistant. Regarding PTX, the correlation between aberrant methylation of the *CHFR* gene and sensitivity to PTX has been recently reported in various kinds of human cancer cells *in vitro*, but it is hard to detect *CHFR* methylation without surgically resected frozen samples. Therefore, the identification of the more easily available predictive markers of a response to PTX would help to determine a suitable chemotherapy for each individual. In this study, we showed that the *EGFR* wild genotype and smoking habit are significant efficacy-predicting markers, in addition to *CHFR* methylation in NSCLC. The evaluation of *EGFR* gene status is non-invasive more than *CHFR* gene analysis because formalin fixed tissue samples biopsied previously and bronchial washing samples besides surgically resected frozen samples

are available for *EGFR* gene mutation analysis

Evidence is accumulating that the aberrant methylation of genes related to cell cycle checkpoints is often associated with sensitivity to chemotherapeutic agents. [15, 16] Aberrant methylation of the *p73* gene promoter, which is a member of the *p53* family and is involved in cell cycle checkpoint function, is significantly correlated with sensitivity to alkylating agents in colon cancer cell lines. [15] In addition, aberrant methylation of the *14-3-3*α gene promoter, the G2-M phase checkpoint gene, is correlated with sensitivity to adriamycin in gastric cancer cell lines. [17] Using gastric and endometrial cancer cell lines, recent studies have indicated that cells with aberrant methylation of the *CHFR* gene are more sensitive to microtubule inhibitors than those without methylation. [11, 12] In the present study, we demonstrated that aberrant methylation of the *CHFR* gene promoter of clinical NSCLC samples was significantly related to sensitivity to PTX. Another investigator has reported that *CHFR* methylation status was associated with clinical response to PTX and docetaxel (DOC) in patients with advanced gastric cancer. [18] The data suggest that *CHFR* methylation status can be used to identify patients who should receive microtubule inhibitors. A previous study has also reported that sensitivity to CDDP in endometrial cancer cells with *CHFR* methylation was unaltered after demethylation, whereas sensitivity to PTX decreased

after demethylation, [19] while another study found no correlation between *CHFR* methylation and sensitivity to CDDP or VP-16 through experiments with cancer cell lines. [20] We also showed that *CHFR* methylation status did not affect sensitivity to other anti-cancer agents such as CDDP, CBDCA, GEM, VNR, CPT-11, or VP-16 (Figure 3). Our results are consistent with those of other studies that *CHFR* methylation status is important regarding sensitivity to microtubule inhibitors, but not to other anti-cancer agents.

Tsao et al. analyzed 1370 patients from the United States with stage III or IV NSCLC, and found that responses to first-line chemotherapy were not associated with patient age, gender, or histological subtype of tumors; [21] these results are all in accord with the present results. Regarding smoking status, those authors found that never-smokers had higher response rates than former or current smokers. [21] In opposition to their report, we showed that only the sensitivity rate to PTX was significantly higher in smokers than in never-smokers (Figure 3i). The precise mechanism underlying this difference remains to be determined, but interestingly, in our data, a logistic regression multivariable analysis revealed that smoking habit was a significant risk factor for *CHFR* gene methylation, which was also significantly related to sensitivity to PTX. The data might suggest that cigarette smoking is relevant to

aberrant methylation of the *CHFR* gene. In addition, we showed that the sensitivity rate for PTX was significantly higher in smokers with *CHFR* methylation and *EGFR*-wild type than in never-smokers with unmethylated *CHFR* and *EGFR* mutant (Figure 3j). The result may indicate that the sensitivity rate for PTX is significantly higher especially in smokers with *CHFR* methylation and *EGFR*-wild type among all smokers. To our knowledge, this is the first report to show a correlation between smoking habit and PTX sensitivity.

Recently, it was reported that a subgroup of patients with NSCLC had specific mutations in the *EGFR* gene that correlate with clinical responsiveness to the tyrosine kinase inhibitor gefitinib. [13, 14] *EGFR* gene mutations occur in 25-40% of NSCLC cases, [14] and are associated preferentially with NSCLC in non-smokers and adenocarcinoma with BAC features. In contrast, we demonstrated that reduced CHFR protein expression is associated with squamous cell carcinoma of NSCLC, smoking habit, and tumor differentiation. [22] In the present study, we showed that *EGFR* gene mutation of clinical NSCLC samples was significantly related to resistance to PTX in opposition to *CHFR* methylation. In opposition to our results, a recent clinical study showed that the response rate of paclitaxel plus carboplatin treatment in the *EGFR* mutation-positive subgroup is higher than that in the *EGFR* wild-type subgroup. [23] In this content, because

PTX inhibit not only microtubule depolymerization but also angiogenesis, there may be differences between clinical study *in vivo* and chemosensitivity test *ex vivo*. In addition, the difference may be attributable to the small sample size of the *EGFR* mutation-positive group in our results. However, recent studies have shown that activation of Akt, a downstream molecule of EGFR signals that promotes cell survival, migration, proliferation, and angiogenesis, increased PTX resistance by preventing apoptosis in human ovarian and bladder cell lines. Although further investigation is necessary, the present observation, together with these data, leads us to propose a hypothesis that activated Akt signaling induced by *EGFR* gene mutation may show resistance to PTX in NSCLC. [24, 25] In addition, recent study has reported that CHFR regulates the expression of Aurora-A by degradation of the protein, and that Aurora-A overexpression induces the activation of Akt. [26] Therefore, our results suggest that activated Akt signaling induced by Aurora-A overexpression due to *CHFR* methylation also may show resistance to PTX in NSCLC.

In this study, *ex vivo* chemosensitivity was evaluated by the SDI test, which gives significant information about drug sensitivity. This chemosensitivity test has been reported to be a reliable, rapid, and convenient method for clinical use and to be associated with clinical response. [27] In this study, this method has been considered to be

a reliable tool because there was no significant difference between platinum agents, and there were significant differences between VP-16, which has been used in small cell lung cancer, and other all agents (Figure 2 and Table 2). A previous study has shown that the combined expressions of Rad 51 and ERCC1 was associated with resistance to platinum agents by the SDI test. [28] Therefore, if the simultaneous examination of clinical samples is possible, this chemosensitivity test can be used as a good surrogate method to investigate therapeutic markers.

In conclusion, we have revealed that the *EGFR* wild genotype and smoking habit, in addition to the aberrant methylation of the *CHFR* gene promoter, are associated with sensitivity to microtubule inhibitors in NSCLC. Our results suggest it may be possible to identify patients who benefit from treatment with microtubule inhibitors based on *EGFR* gene status and smoking habit, regardless of *CHFR* methylation. In the next step, we will explore the predicitive value of chemosensitivity data in the clinical tiral using paclitaxel based chemotherapy.

Materials and Methods

Lung cancer specimens

All clinical samples were obtained as part of the protocol approved by the Ethics Committee of the Kyushu University Hospital. Tumor sections were acquired from 69 patients with NSCLC who had undergone surgery at Kyushu University Hospital between 2002 and 2006. Informed consent was obtained from all patients. Tumor tissues for the SDI method were preserved in saline solution and for gene analysis in liquid nitrogen. The age of the patients ranged from 43 to 81 years (mean, 68 years), and included 50 males (72%) and 19 females (28%), 43 Stage I, 13 Stage II and 13 Stage III. Twenty-one patients (30%) were never-smokers and the remaining 48 were smokers. Histological diagnosis was determined by hematoxylin and eosin staining essentially based on the WHO criteria with minor modifications, [29] and was based on consensus among three pathologists (M.T., T.K., K.S.). The results revealed 48 adenocarcinomas, 20 squamous cell carcinomas, and 1 large cell carcinoma. Pathologic staging was classified according to the tumor-node-metastasis (TNM) classification system revised in 1997. [30]

Methylation analysis

Tumor cells were enriched under light-microscope-assisted gross dissection followed by DNA isolation using standard phenol and chloroform extraction. Sodium bisulfite conversion of genomic DNA was performed using the EZ DNA Methylation Kit (Zymo Research, Orange, CA, USA), as described previously. [22, 31] Methylation-specific PCR (MSP) was carried out with the following oligonucleotide primers, which were designed to be specific to either the methylated or unmethylated DNA sequence of the *CHFR* gene after sodium bisulfite conversion as described above. The methylated DNA-specific primers were MF (5'-ATATAATATGGCGTCGATC-3') and MR (5'-TCAACTAATCCGCGAAACG-3'). The unmethylated DNA-specific primers were UF (5'-ATATAATATGGTGTTGATT-3') and UR (5'-TCAACTAATCCACAAAACA-3'). PCR amplification consisted of 35 cycles of 94 ℃ for 1 min, 60 ℃ for 1 min, and 72 ℃ for 1 min (MF1 and MR1); and of 94 ℃ for 1 min, 48 ℃ for 1 min, and 72 ℃ for 1 min (UF1 and UR1), which generated a 206-bp PCR product. The resultant PCR products were separated on 2% agarose gels. Each MSP was repeated at least two times.

Sequence analysis of *EGFR* gene

We performed direct DNA sequencing to identify mutations in *EGFR* exons 18–21.

Tumor cells were enriched under light-microscope-assisted gross dissection. The isolated cells were used for RNA extraction by acid guanidinium thiocyanate-phenol-chloroform extraction methods. [32] First-strand cDNA was synthesized with 0.3 μg total RNA using a first-strand cDNA Synthesis Kit (Invitrogen, Carlsbad, CA, USA). The cDNAs were amplified using the following primers: 5'-TCCAAACTGCACCTACGGATGC-3' (forward) and 5'-CATCAACTCCCAAACGGTCACC-3' (reverse). PCR amplification consisted of 35 cycles (95°C for 45 seconds, 55°C for 30 seconds, and 72°C for 60 seconds). The sequences of the PCR products were determined using ABI Prism 310 (Applied Biosystems, Foster City, CA, USA). Amplification and sequencing were performed in duplicate for each tumor cell. The sequences were compared with the GenBank-archived human sequence of *EGFR* (accession no. NM 005228.3).

Chemosensitivity test

The succinate dehydrogenase inhibition (SDI) test was performed by the methods described previously. [27, 28] In brief, after removal of necrotic portions, tumor tissues were cut with scissors, and the fragments obtained were put into a sterile flask containing a mixture of enzymes, pronase (protease type XXV, Sigma Chemical, St.

Louis, MO, USA), 0.1% collagenase (type I, Sigma), and DNase I (type I, Sigma) in McCoy's 5A solution with antibiotics. The enzymatic disaggregation was carried out for 20 min at 37 °C with gentle stirring, and was terminated by adding sufficient amounts of minimal essential medium (MEM). The cells were then passed through a nylon mesh, washed twice with MEM, pelleted, and resuspended in MEM. Aliquots (100 μl) of this single cell suspension were dispensed into 96-well microtiter plates (Corning 25860; Corning Glass, Corning, NY, USA) and then incubated at 37 °C in a humidified 5% CO_2 atmosphere for 3 days in the presence of anticancer drugs. Each anticancer drug was tested at 10 x the peak of plasma concentrations because SD activity optimally reflects cell viability at this concentration: [27, 28] cisplatin (CDDP), 20 μg/ml; carboplatin (CBDCA), 100 μg/ml; paclitaxel (PTX), 6 μg/ml; vinorelbine (VNR), 0.5 μg/ ml; gemcitabine (GEM), 200 μg/ml; irinotecan (CPT-11), 0.33 μg/ml; etoposide (VP-16), 2 μg/ml. Except for VP-16, all of these have been used as first-line chemotherapeutic agents in NSCLC. After incubation, the calorimetric reaction was initiated by adding 3-(4,5-dimethyl-2-thiazolyl)-2,5-diphenyl [2H] tetrazolium bromide (MTT) at a concentration of 0.4 mg/ml and sodium succinate in the range of 0–30 mM. The formazan formed from MTT was extracted with dimethyl sulfoxide, and the absorbance of the formazan was measured at 540 nm using a spectrophotometer (Labsystems

Multiskan JX; Thermo Bioanalysis, Helsinki, Finland). The SD activity was presented as the optical density per milligram of protein, and evaluated as the mean of 6-well microtiter plates for each anticancer drug.

Chemosensitivity rates were calculated as follows: chemosensitivity rate (%) = 100-(T-B)/(U-B) x 100% (T, treated cell: absorbance determined when tumor cells are exposed to drugs; U, untreated cell: absorbance of untreated cells; B, blank: absorbance when neither drug nor MTT was added). When a chemosensitivity rate was a negative value, it was assumed to be zero. [28]

Statistical analysis

Continuous and dichotomous variables were compared with the *t* test and Fisher's exact test, respectively. We evaluated the relative risk of exposures by calculating odds ratios using multivariate logistic regression. The correlations among clinicopathologic findings, methylation of *CHFR* gene promoter and *EGFR* gene mutation, and chemosensitivity were evaluated using the *t* test. Differences for chemosensitivity between groups were determined by using Scheffe's post-hoc multiple comparisons. Receiver operator characteristic (ROC) curve analysis was done by comparing the strongest factor to PTX sensitivity to estimate the area under the curve (AUC), which is

defined as an AUC of 0.5 or more (a straight line from the bottom left to top right corners, implying a decision rule no better than pure chance) as a significant value. A P value of less than 0.05 was regarded as statistically significant. Statistical analysis was performed using SPSS software version 9.0 (SPSS, Chicago, IL, USA).

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Legends to figures

Figure 1. (a) Methylation-specific PCR of the *CHFR* promoter region. U and M denote unmethylated DNA-specific amplification and methylated DNA-specific amplification, respectively. Aberrant methylation of the *CHFR* gene promoter is found in cases 3, 4, 7, 8, 12, 15, 22, 36 37, 62 and 66 of 69 primary NSCLCs. (b, c) Mutations in the *EGFR* gene exons 19 and 21 in non-small cell lung cancers. L858R mutations in *EGFR* exon 21 are found in cases 1, 18, 23, 29, 39 and 65 (b). L747-S752 deletion in *EGFR* exon 19 is found in case 50 (c).

Figure 2. Comparison of chemosensitivity rate for seven anti-cancer agents. Box plot indicates the 25th and 75th centiles (box), mean (horizontal bar), and the 5th and 95th centiles (vertical topped bars). Scheffe's test divides the agents into four groups: group 1 (GEM, CPT-11 and VP-16), group 2 (VNR, GEM and CPT-11), group 3 (PTX) and group 4 (CDDP and CBDCA). The mean chemosensitivity rate for platinum agents such as CDDP and CBDCA is significantly higher than that for other agents. The differences among the four groups are statistically significant ($p<0.05$) by Scheffe's test.

Figure 3. Correlation between the chemosensitivity of seven anti-cancer agents and

aberrant methylation of the *CHFR* gene promoter, *EGFR* mutation and smoking status. (a-g) *CHFR* gene promoter methylation is significantly related to high sensitivity to PTX (c), while no sensitivity is found to the other six anti-cancer agents (a, b and d-g). (h) *EGFR* wild genotype is significantly related to high sensitivity to PTX. (i) Smoking is significantly related to high sensitivity to PTX. (j) Smokers with *CHFR* methylation and *EGFR*-wild type are significantly related to high sensitivity to PTX. Bars indicate mean of the chemosensitivity rate.

Figure 4. Comparison of related factors with chemosensitivity for PTX. Receiver operator characteristic (ROC) curves for chemosensitivity rate of PTX for *CHFR* methylation-positive versus -negative (a); *EGFR* gene mutation versus *EGFR* wild genotype (b); and smokers versus never-smokers (c). *CHFR* methylation status (a), *EGFR* mutation status (b) and smoking status (c) produce significant ROC curves. *CHFR* methylation status shows the highest AUC value in ROC curve analysis.

Table IA. Correlation between *CHFR* methylation status and *EGFR* mutation, and clinicopathologic parameters

	CHFR methylation			*EGFR* mutation		
	Negative(n=58)	Positive (n=11)	*p*-value	Negative(n=62)	Positive(n=7)	*p*-value
Age (y)	68±10.04	71.2±5.7	*0.16*	68.5±9.71	64.3±5.9	*0.27*
Gender						
Male	40	9		45	4	
Female	18	2	*0.49*	17	3	*0.41*
Smoking status						
Smoker	37	11		47	1	
Never smoker	21	0	*0.015*	15	6	*0.003*
Pathologic stage						
I	37	6		37	6	
II	9	4		13	0	
III	12	1	*0.36*	12	1	*0.62*
Tumor histology						
Adenocarcinoma	46	2		41	7	
Squamous cell carcinoma	11	9		20	0	
Large cell carcinoma	1	0	*<0.01*	1	0	*0.14*

Table IB. Factor analysis for methylation status of *CHFR* gene

Parameter	Regression coefficient	SE	Relative Risk	95% CI	*p*-value
Age	0.0650	0.049	1.07	0.97-1.18	*0.187*
Smoking status	1.5231	0.715	4.36	1.26-18.34	*0.0232*
Tumor histology	1.7756	0.742	5.90	1.38-25.27	*0.0167*

Logistic regression analysis

Table II. Post-hoc Scheffe Test – mean differences between groups

		Subset for $\alpha = 0.05$			
Anti-cancer drugs	n	1	2	3	4
VP16	69	22.635			
CPT11	69	27.886	27.886		
GEM	69	29.926	29.926		
VNR	69		36.575		
PTX	69			48.281	
CBDCA	69				61.339
CDDP	69				61.570
Significance		0.332	0.136	1.000	1.000

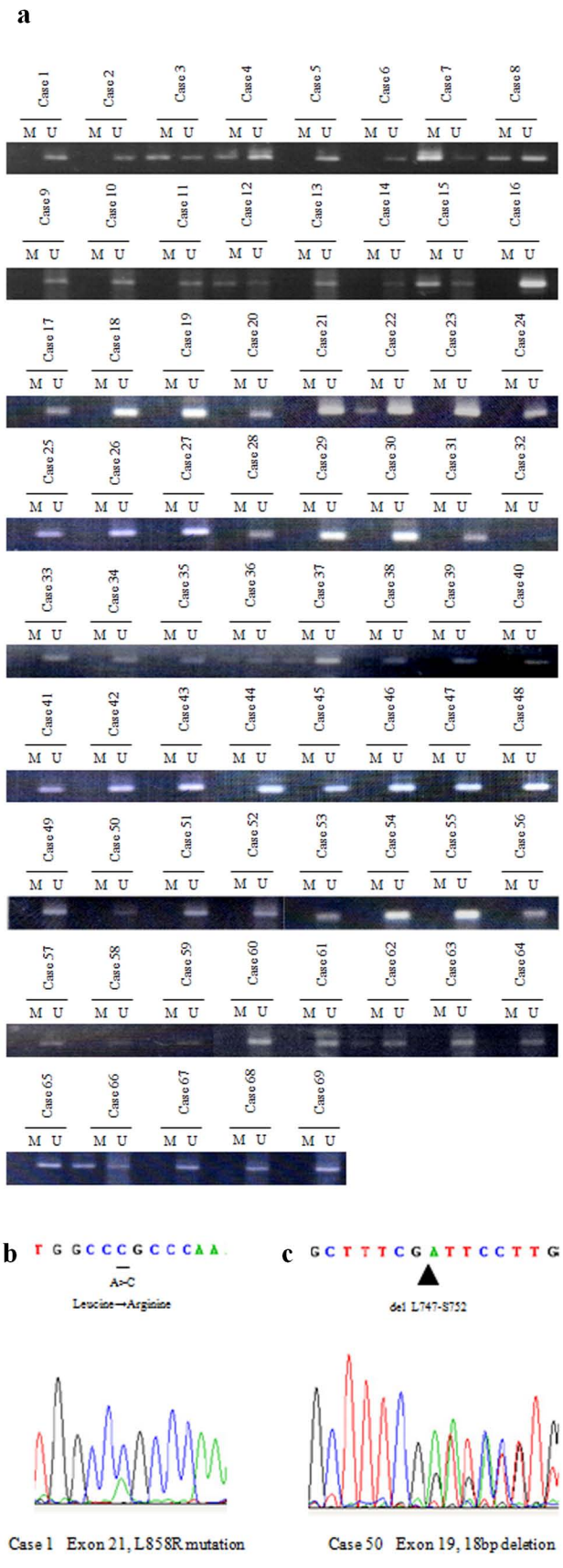
Table III. The correlation with *CHFR* methylation status, *EGFR* gene mutation, smoking status, and chemosensitivity

Parameters	CDDP	CBDCA	PTX	GEM	VNR	CPT11	VP16
CHFR methylation							
Negative (n=58)	62.1±17.4	61.8±13.2	45.7±19.7	29.8±11.9	36.5±14.6	27.8±21.1	23.1±16.9
Positive (n=11)	58.8±14.0	59.1±8.18	62.1±8.49	30.5±7.95	37.1±9.51	28.5±23.1	20.2±15.5
p-value	*0.50*	*0.39*	***<0.001***	*0.82*	*0.87*	*0.92*	*0.58*
EGFR mutation							
Negative (n=62)	61.3±17.3	61.0±13.0	49.8±19.5	30.0±11.6	36.7±14.2	27.9±21.6	23.1±16.3
Positive (n=7)	64.0±13.5	64.0±7.13	34.7±11.7	28.9±8.60	35.2±12.2	28.0±19.5	18.6±20.2
p-value	*0.64*	*0.38*	***0.014***	*0.76*	*0.77*	*0.99*	*0.59*
Smoking status							
Never smoker	64.3±13.2	64.5±12.1	40.6±16.9	29.6±12.3	39.1±13.2	29.3±22.4	24.8±16.9
Smoker	60.4±18.2	59.9±12.5	51.6±19.4	30.1±10.9	35.5±14.2	25.0±20.8	21.7±16.6
p-value	*0.37*	*0.16*	***0.029***	*0.89*	*0.33*	*0.51*	*0.47*

Table IV. Logistic regression analysis for PTX sensitivity

	R	*p*-value	Odds ratio
CHFR status	0.29	0.0083	1.168
EGFR status	-0.18	0.0627	0.959
Smoking status	0.19	0.0237	1.032

Figure 1


a
Case 1
Case 2
Case 3
Case 4
Case 5
Case 6
Case 7
Case 8
Case 9
Case 10
Case 11
Case 12
Case 13
Case 14
Case 15
Case 16
Case 17
Case 18
Case 19
Case 20
Case 21
Case 22
Case 23
Case 24
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Case 50
Case 51
Case 52
Case 53
Case 54
Case 55
Case 56
Case 57
Case 58
Case 59
Case 60
Case 61
Case 62
Case 63
Case 64
Case 65
Case 66
Case 67
Case 68
Case 69
M U
b
T G G C C C G C C C A A
A>C
Leucine→Arginine
c
G C T T T C G A T T C C T T G
del L747-S752
Case 1 Exon 21, L858R mutation
Case 50 Exon 19, 18bp deletion

Figure 2

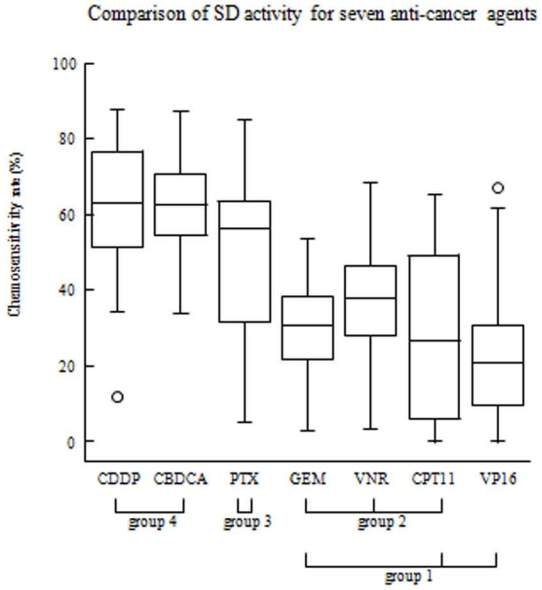

Comparison of SD activity for seven anti-cancer agents
Chemosensitivity rate (%)
100
80
60
40
20
0
CDDP
CBDCA
PTX
GEM
VNR
CPT11
VP16
group 4
group 3
group 2
group 1

Figure 3

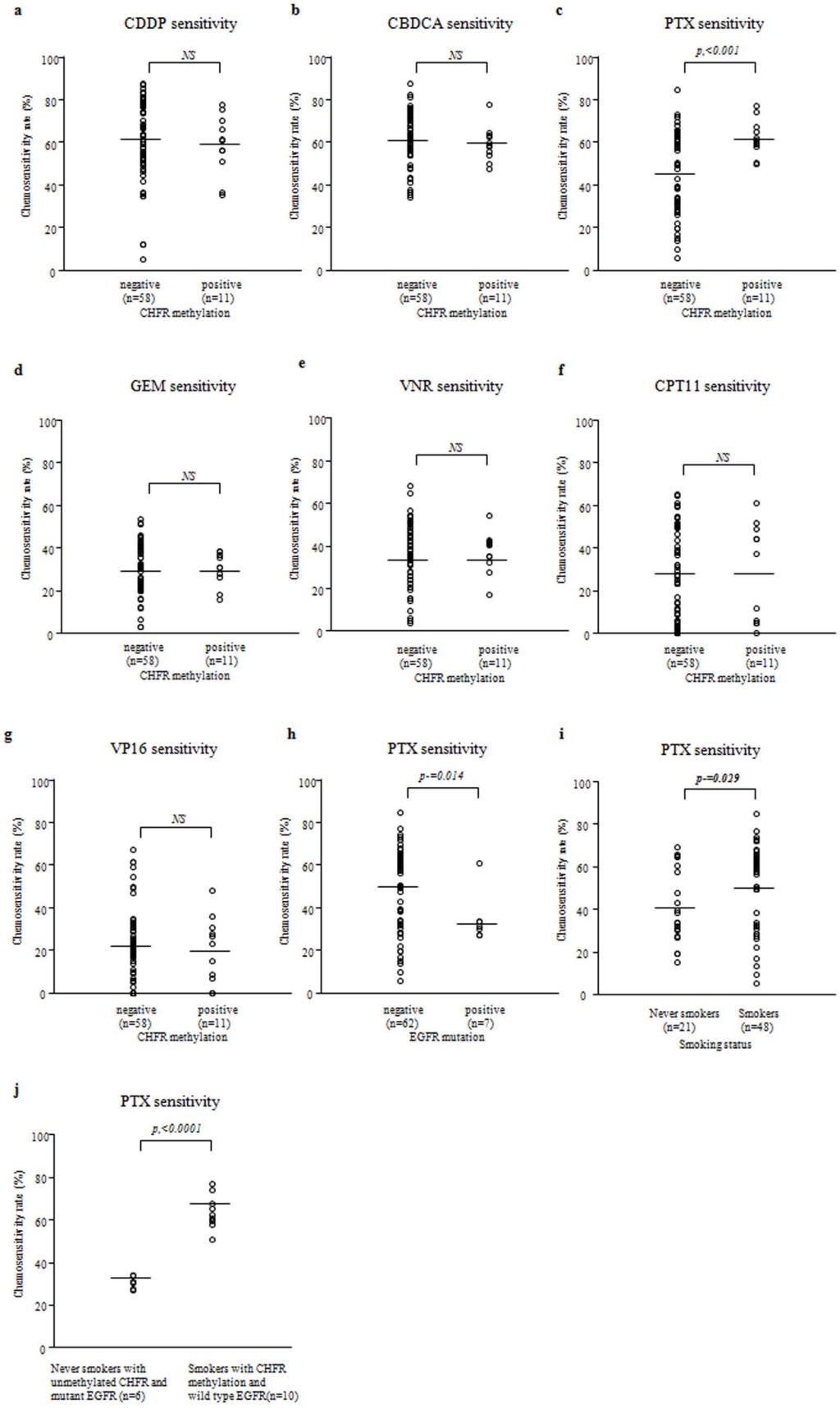
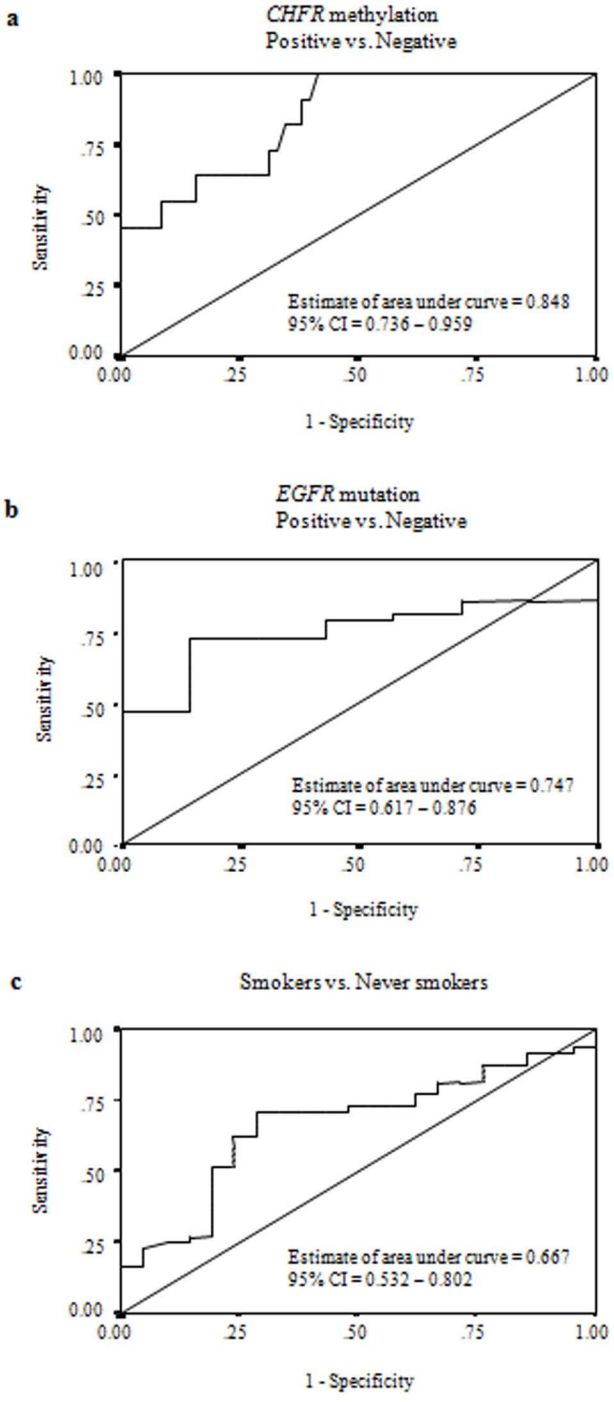

a
CDDP sensitivity
NS
Chemosensitivity rate (%)
negative (n=58)
positive (n=11)
CHFR methylation
b
CBDCA sensitivity
NS
Chemosensitivity rate (%)
negative (n=58)
positive (n=11)
CHFR methylation
c
PTX sensitivity
p,<0.001
Chemosensitivity rate (%)
negative (n=58)
positive (n=11)
CHFR methylation
d
GEM sensitivity
NS
Chemosensitivity rate (%)
negative (n=58)
positive (n=11)
CHFR methylation
e
VNR sensitivity
NS
Chemosensitivity rate (%)
negative (n=58)
positive (n=11)
CHFR methylation
f
CPT11 sensitivity
NS
Chemosensitivity rate (%)
negative (n=58)
positive (n=11)
CHFR methylation
g
VP16 sensitivity
NS
Chemosensitivity rate (%)
negative (n=58)
positive (n=11)
CHFR methylation
h
PTX sensitivity
p-=0.014
Chemosensitivity rate (%)
negative (n=62)
positive (n=7)
EGFR mutation
i
PTX sensitivity
p-=0.029
Chemosensitivity rate (%)
Never smokers (n=21)
Smokers (n=48)
Smoking status
j
PTX sensitivity
p,<0.0001
Chemosensitivity rate (%)
Never smokers with unmethylated CHFR and mutant EGFR (n=6)
Smokers with CHFR methylation and wild type EGFR(n=10)

Figure 4


a
CHFR methylation
Positive vs. Negative
Sensitivity
1 - Specificity
Estimate of area under curve = 0.848
95% CI = 0.736 – 0.959
b
EGFR mutation
Positive vs. Negative
Sensitivity
1 - Specificity
Estimate of area under curve = 0.747
95% CI = 0.617 – 0.876
c
Smokers vs. Never smokers
Sensitivity
1 - Specificity
Estimate of area under curve = 0.667
95% CI = 0.532 – 0.802