

Table 4. Number (%) of patients reporting adverse events leading to dose reduction, safety population

Parameter, n (%)	Inotuzumab ozogamicin treatment		
	1.3 mg/m ² (n = 3)	1.8 mg/m ² (n = 10)	Total (n = 13)
No. of patients with dose reductions			
No dose reductions	3 (100)	8 (80)	11 (85)
One or more dose reductions	0	2 (20)	2 (15)
No. of dose reductions per patient*			
One	0	2 (100)	2 (15)
Any adverse event leading to dose reduction†	0	2 (20)	2 (15)
Thrombocytopenia	0	1 (10)	1 (8)
Pleural effusion	0	1 (10)	1 (8)

*Percentages are based on number of patients with ≥ 1 dose reduction in each treatment group. †Totals at a higher level are not necessarily the sum of those at the lower levels since a patient was able to report two or more different adverse events within the higher level category.

for patients who received 1.8 mg/m² are shown in Figures 1 and 2, respectively. The peak concentration of inotuzumab ozogamicin was generally observed at or shortly after the termination of infusion with moderate intersubject variability. The peak total calicheamicin concentrations were observed typically within 4 h after the start of inotuzumab ozogamicin infusion with small intersubject variability.

The mean pharmacokinetic parameters for inotuzumab ozogamicin and total calicheamicin are shown in Tables 5 and 6, respectively. The AUC of inotuzumab ozogamicin tended to increase with increased dose and period. The $t_{1/2}$ was prolonged with repeated treatment cycles. These were reflected by substantial decreases in clearances.

The mean total calicheamicin C_{max} appeared to increase with dose. The AUC of total calicheamicin increased with increased dose and period. No antibodies to inotuzumab ozogamicin were detectable in patients' serum during the course of the study. The pharmacokinetics data indicate that the disposition of inotuzumab ozogamicin and total calicheamicin following IV treatment was nonlinear with dose or number of doses.

Efficacy. The best tumor response is presented in Table 7. Antitumor activity was observed at both dose levels. In the 1.3 mg/m² cohort, two out of three patients had CR, and one patient had CRu for an ORR of 100% (95% CI, 29–100%). In the 1.8 mg/m² cohort, one out of 10 patients had CR, three patients had CRu, and four patients had PR for an ORR of 80% (95% CI, 44–98%).

Table 5. Serum pharmacokinetic parameters of inotuzumab ozogamicin

Dose (Once/4 weeks)	Treatment Day (n)	Number of cycles	C_{max} (ng/mL) (%)	$t_{1/2}$ (h) (%)	AUC (ng h/mL) (%)	CL (L/h) (%)	V_{ss} (L) (%)
1.3 mg/m ²	1 (3)	1	463 (8)	NC	NC	NC	NC
	29 (3)	2	610 (17)	29.7 (30)	24166 (29)	0.08 (32)	3.27 (11)
	57 (3)	3	524 (18)	43.6 (18)	31642 (21)	0.06 (22)	3.79 (12)
1.8 mg/m ²	1 (10)	1	657 (41)	13.0 (30)	14266 (32)	0.24 (40)	4.06 (21)
	29 (8)	2	727 (27)	35.8 (43)	34518 (46)	0.11 (54)	4.40 (20)
	57 (5)	3	763 (20)	44.0 (32)	39677 (41)	0.09 (56)	4.89 (19)

Data are expressed as mean, and percent coefficient of variance is expressed in parentheses. AUC, total area under the concentration-time curve; CL, clearance; C_{max} , peak concentration; NC, not calculated; $t_{1/2}$, terminal-phase elimination half-life ($0.693/\lambda_z$); V_{ss} , steady-state volume of distribution.

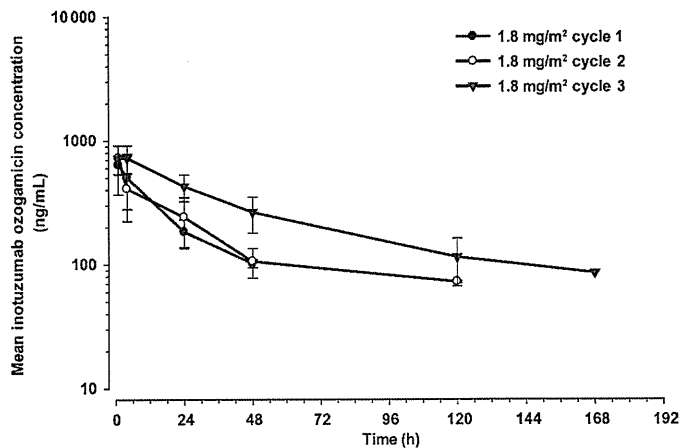


Fig. 1. Mean (SD) serum concentrations of inotuzumab ozogamicin after 1.8 mg/m² infusion of inotuzumab ozogamicin once every 4 weeks. Closed circle, cycle 1 (day 1, n = 10); open circle, cycle 2 (day 29, n = 8); closed triangle, cycle 3 (day 57, n = 5).

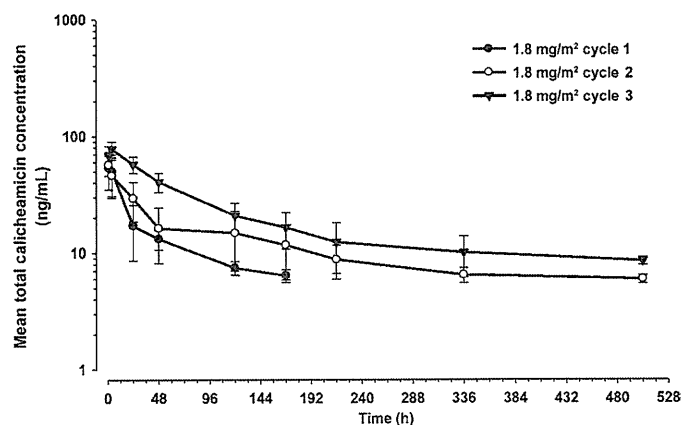


Fig. 2. Mean (SD) serum concentrations of total calicheamicin after 1.8 mg/m² infusion of inotuzumab ozogamicin once every 4 weeks. Closed circle, cycle 1 (day 1, n = 10); open circle, cycle 2 (day 29, n = 8); closed triangle, cycle 3 (day 57, n = 5).

Discussion

To improve the clinical outcome of patients with B-NHL who were pretreated with rituximab or rituximab-containing regimens, a number of new agents including antibodies, small mole-

Table 6. Serum pharmacokinetic parameters of total calicheamicin

Dose (Once/4 weeks)	Treatment Day (n)	Number of cycles	C _{max} (ng/mL) (%)	t _{1/2} (h) (%)	AUC (ng h/mL) (%)	CL (L/h) (%)	V _{ss} (L) (%)
1.3 mg/m ²	1 (3)	1	44.6 (17)	17.0 (39)	987 (44)	2.35 (58)	49.44 (13)
	29 (3)	2	52.4 (22)	150.6 (45)	5754 (40)	0.38 (48)	62.86 (30)
	57 (3)	3	56.6 (26)	216.3 (55)	8060 (37)	0.27 (43)	60.17 (25)
1.8 mg/m ²	1 (10)	1	59.0 (31)	49.6 (77)	2329 (51)	1.61 (54)	72.3 (28)
	29 (8)	2	59.4 (15)	162.4 (34)	7100 (48)	0.54 (62)	89.18 (41)
	57 (5)	3	78.2 (15)	172.7 (48)	9225 (32)	0.37 (44)	68.37 (26)

Data are expressed as mean, and percent coefficient of variance is expressed in parentheses. AUC, total area under the concentration-time curve; CL, clearance; C_{max}, peak concentration; NC, not calculated; t_{1/2}, terminal-phase elimination half-life (0.693/λ_z); V_{ss}, steady-state volume of distribution.

Table 7. The best tumor response during treatment: number (%) of patients in efficacy population

Best tumor response	Inotuzumab ozogamicin treatment		
	1.3 mg/m ² (n = 3)	1.8 mg/m ² (n = 10)	Total (n = 13)
CR, CRu	3 (100)	4 (40)	7 (54)
PR	0	4 (40)	4 (31)
OR	3 (100)	8 (80)	11 (85)
SD	0	2 (20)	2 (15)

CR, complete response; CRu, unconfirmed complete response; OR, overall response (CR + CRu + PR); PR, partial response; SD, stable disease.

cule, targeted agents, and chemotherapeutic drugs have been developed. However, new treatment modalities with improved toxicity profiles and better responses are needed. Inotuzumab ozogamicin (CMC-544), an antibody-targeted chemotherapy agent, has demonstrated an acceptable toxicity profile and high activity against relapsed or refractory patients with FL who were pretreated with rituximab or rituximab-containing treatment.

In a recent phase I, multicenter, open-label, dose escalation study of inotuzumab ozogamicin administered IV as a single agent in the USA and the European Union, inotuzumab ozogamicin was found to be reasonably well-tolerated with the MTD of 1.8 mg/m² administered every 4 weeks and with the major toxicity of grade 3 or greater thrombocytopenia, which was manageable with careful monitoring and platelet transfusion. Response rates of 69% in patients with FL and 33% in patients with DLBCL in the expanded cohort of this trial were observed.⁽¹²⁾

In the present phase I dose escalation study in Japanese patients with relapsed or refractory FL, who were pretreated with rituximab, the MTD of inotuzumab ozogamicin was determined to be 1.8 mg/m² administered once every 28 days, a value that was the same as that observed for non-Japanese patients.

Most common inotuzumab ozogamicin related adverse events were thrombocytopenia, leukopenia, lymphopenia, neutropenia, elevated AST, anorexia, and nausea, a finding that was very similar to the non-Japanese study. Adverse events (AEs) leading to dose delays were neutropenia and thrombocytopenia.

The pharmacokinetic profiles of inotuzumab ozogamicin and total calicheamicin indicated that disposition was non-linear and was associated with increases in drug exposure with increasing dose or number of doses. The pharmacokinetic profiles of inotuzumab ozogamicin and total calicheamicin in Japanese patients were similar to the values for non-Japanese patients. The study population was very limited, thus no definite conclusion can be made for Japanese patients. However, nonlinearities in drug disposition are known for antibodies⁽¹⁶⁾ and had been

observed previously for gemtuzumab ozogamicin.⁽¹⁷⁾ Saturable binding with target antigen is thought to influence antibody disposition, potentially leading to nonlinear distribution and elimination.

Potent antitumor activity for inotuzumab ozogamicin was observed at both the 1.3 and 1.8 mg/m² dose levels. In the 1.3 mg/m² cohort, all three patients had CR or CRu for an ORR of 100%. In the 1.8 mg/m² cohort, one out of 10 patients had CR, three patients had CRu, and four patients had PR for an ORR of 80%. Although the number of patients was limited, our preliminary ORR was greater in comparison to other reported antibody-based agents in the treatment of patients with FL and prior exposure to rituximab-containing regimens. For example, in a recent phase I/II study, veltuzumab, a humanized second-generation anti-CD20 monoclonal antibody, was reported to have an ORR of 44%.⁽¹⁸⁾ In another phase I/II, single-agent, dose escalation study, galiximab, an anti-CD80 antibody, demonstrated an ORR of only 11%.⁽¹⁹⁾ Fludarabine phosphate, one of the most effective drugs in the treatment of indolent B-NHL, had an ORR of 65%, when administered as a single agent.⁽²⁰⁾

The FLIPI scores in this study were good predictors of favorable outcome. Of the five patients who had low scores (low risk) two demonstrated CR, two had CRu, and one had PR. Of the six patients who had intermediate scores, one had CR, two had CRu, one had PR, and two had SD. The two patients with high FLIPI scores demonstrated only PR.

In conclusion, the results from this phase I study suggest that inotuzumab ozogamicin is safe, well tolerated, and shows promising efficacy in Japanese patients with relapsed or refractory FL pretreated with rituximab-containing therapy. In addition, pharmacokinetics and efficacy in this study are comparable with those in preceding studies in non-Japanese patients. These results therefore warrant further investigation of inotuzumab ozogamicin in relapsed or refractory B-NHL.

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Disclosure Statement

This study was funded by Wyeth which was acquired by Pfizer, Inc., in October 2009. Dr. Junko Ohata was an employee of Wyeth K.K. at the time of the study. Dr. Chiho Ono is an employee of Wyeth K.K. No other potential conflict of interest relevant to the article is reported.

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Sorafenib-induced erythema multiforme for metastatic renal cell carcinoma

Sorafenib is an active agent for cytokine-refractory renal cell carcinoma (RCC) patients [1]. Skin toxicity such as hand-foot syndrome (HFS) is one of the frequent adverse events of sorafenib. In the phase II study conducted in Japan, grade 3 skin toxicity occurred in 13.7% of 131 patients with RCC, but all of those skin toxic effects were HFS or rash/desquamation [2]. We here report three cases of erythema multiforme (EM) associated with sorafenib therapy. EM, Stevens–Johnson syndrome, and toxic epidermal necrolysis are mucocutaneous diseases associated with significant morbidity and mortality. The term ‘Stevens–Johnson syndrome’ has been widely accepted as a synonym for EM major [3].

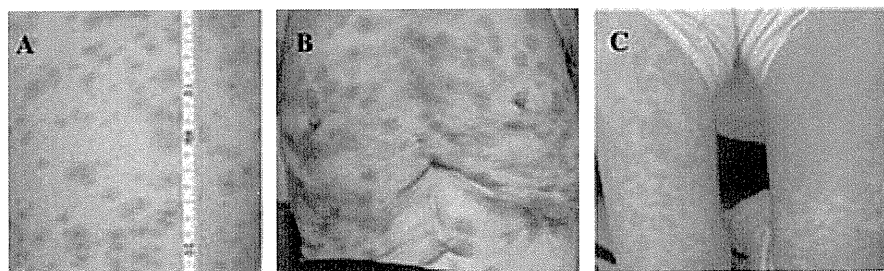


Figure 1. Erythema multiforme of three cases. Target-like erythematous skin rash of left femoral lesion of case 1 (A), case 2 (B), and case 3 (C).

case 1

A 25-year-old female with pulmonary metastases of papillary RCC received sorafenib 800 mg/day. At day 8, erythema appeared on lower legs and spread over 50% of body surface area in 2 days (Figure 1A) with HFS. She also suffered intermittent fever up to 39.5°C from day 8. Serum examination excluded the viral infection, including measles, herpes simplex, or herpes zoster. Skin biopsy of femoral region at day 12 revealed superficial and perivascular lymphocyte infiltration and necrotic keratinocytes, compatible with EM. Skin rash disappeared within days after discontinuation of sorafenib without steroid treatment or antimicrobial treatment.

case 2

An 80-year-old man with multiple pulmonary metastases of papillary RCC was treated with sorafenib 800 mg/day. From the 8th to 12th day of sorafenib, erythema spread over his whole body (Figure 1B) with grade 2 HFS, mild stomatitis, and grade 3 fatigue. EM and HFS disappeared within 2 weeks after discontinuation of sorafenib and oral prednisolone 10 mg/day. When sorafenib (400 mg/day) was rechallenged, EM with high fever reappeared within 24 h and the sorafenib was discontinued at once.

case 3

A 70-year-old female with metastatic clear cell RCC developed erythema spread to whole body 15 days after starting sorafenib 800 mg/day (Figure 1C). Eruption disappeared within 2 weeks after discontinuation of sorafenib and topical treatment without steroid. She was treated with sunitinib 50 mg/day and EM has not appeared.

Rash, desquamation, and HFS are most frequent skin symptoms with sorafenib. They are dose dependent and disappear with discontinuation of sorafenib. In most cases, restart with the same dose is possible and the symptoms may resolve without dose modification. MacGregor et al. [4] reported sorafenib-induced EM in malignant melanoma patient. They carried out skin biopsy and showed the same findings with that of our case. In that report, the patient was rechallenged with the reduced dose of sorafenib (100 mg) but developed the same eruption within 24 h, and they discontinued sorafenib. There have been only two other reports about sorafenib-induced EM [5, 6], but we experienced three EM patients, one of which was confirmed by biopsy, of 16 RCC patients we have treated with sorafenib in a year.

Furthermore, postmarketing surveillance of sorafenib-treated patients in Japan reported that 108 cases of so-called EM occurred in 2889 cases from February 2008 to October 2009, so occurrence rate of EM might be different between Japanese and Caucasians. A recent study suggests that polymorphisms in specific genes encoding for metabolizing enzymes, efflux transporters, and drug targets are associated with toxic effects of sunitinib, another angiogenesis inhibitor [7]. In addition, the population-related pharmacogenomics might contribute to differences in adverse events and responses of antitumor agents between patients in Japan and those in the United States [8]. Further study is necessary to elucidate this discrepancy of EM occurrence rate between Japanese and Caucasians.

In clinical practice, it is very difficult to diagnose whether the drug-induced skin rash is caused by allergic or toxic mechanisms. To use sorafenib safely, restarting of sorafenib needs careful monitoring because the possibility of an allergic mechanism cannot be ruled out. Patients with skin lesions due to allergic mechanisms may not have benefits from sorafenib treatment because of early treatment failure. At present, we cannot recommend the rechallenge of sorafenib for these patients as two of three patients including our case recurred EM.

Sorafenib is now one of few standard agents for metastatic RCC. Molecular mechanism of this type of toxicity remains unknown. Further investigation is necessary to disclose the mechanism and establish the effective therapy for sorafenib-induced EM, which might not be a rare adverse event in Japanese patients.

M. Kodaira¹, S. Takahashi^{1*}, K. Takeuchi², T. Yuasa¹
T. Saotome¹, J. Yonese³, I. Fukui³ & K. Hatake¹

¹Department of Medical Oncology, Cancer Institute Hospital of Japanese Foundation for Cancer Research, ²Department of Pathology and ³Department of Urology, Cancer Institute of Japanese Foundation for Cancer Research, Tokyo, Japan
(*E-mail: stakahas@jfc.or.jp)

disclosure

None of the authors declare conflicts of interest.

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Role of Insulin-Like Growth Factor Binding Protein 2 in Lung Adenocarcinoma

IGF-Independent Antiapoptotic Effect Via Caspase-3

Toshiro Migita,* Tadahito Narita,*[†] Reimi Asaka,* Erika Miyagi,* Hiroko Nagano,* Kimie Nomura,* Masaaki Matsuura,^{‡§} Yukitoshi Satoh,[¶] Sakae Okumura,[¶] Ken Nakagawa,[¶] Hiroyuki Seimiya,^{||} and Yuichi Ishikawa*

From the Divisions of Pathology,* and Cancer Genomics,[‡] The Cancer Institute, Tokyo; Zenyaku Kogyo Co., Ltd.,[†] Tokyo; the Bioinformatics Group,[§] Genome Center, Tokyo; the Department of Thoracic Surgical Oncology,[¶] and the Division of Molecular Biotherapy,^{||} Cancer Chemotherapy Center, The Cancer Institute Hospital, Japanese Foundation for Cancer Research, Tokyo, Japan

Insulin-like growth factor (IGF) signaling plays a pivotal role in cell proliferation and mitogenesis. Secreted IGF-binding proteins (IGFBPs) are important modulators of IGF bioavailability; however, their intracellular functions remain elusive. We sought to assess the antiapoptotic properties of intracellular IGFBP-2 in lung adenocarcinomas. IGFBP-2 overexpression resulted in a decrease in procaspase-3 expression; however, it did not influence the phosphorylation status of either IGF receptor or its downstream targets, including Akt and extracellular signal-regulated kinase. Apoptosis induced by camptothecin was significantly inhibited by IGFBP-2 overexpression in NCI-H522 cells. Conversely, selective knockdown of IGFBP-2 using small-interfering RNA resulted in an increase in procaspase-3 expression and sensitization to camptothecin-induced apoptosis in NCI-H522 cells. LY294002, an inhibitor of phosphatidylinositol 3-kinase, caused a decrease in IGFBP-2 levels and enhanced apoptosis in combination with camptothecin. Immunohistochemistry demonstrated that intracellular IGFBP-2 was highly expressed in lung adenocarcinomas compared with normal epithelium. Intracellular IGFBP-2 and procaspase-3 were expressed in a mutually exclusive manner. These findings suggest that intracellular IGFBP-2 regulates caspase-3 expression and contributes to the inhibitory effect on apoptosis independent of IGF. IGFBP-2, therefore, may offer a novel therapeutic target and serve as an antiapoptotic

biomarker for lung adenocarcinoma. (Am J Pathol 2010, 176:1756–1766; DOI: 10.2353/ajpath.2010.090500)

Insulin-like growth factor-I and -II (IGF-I and -II) are important regulators of cellular metabolism, growth, and survival. When IGFs bind to their receptors, the type I and type II IGF receptors (IGF-IR or IGF-IIR), they activate the downstream signaling cascades via the phosphorylation of tyrosine kinase. Activated IGF-1R transmits signals to the major distinct pathways mitogen-activated protein kinase and phosphatidylinositol 3-kinase (PI3K), signaling pathways that are highly implicated in the development and progression of neoplasia. IGF's bioavailability is regulated by six high affinity IGF binding proteins (IGFBPs). Secreted IGFBPs by cancer cells interfere primarily with IGF-I or -II through the formation of IGF-IGFBPs complex, which in turn exert an inhibitory effect on IGF-mediated biological functions.

IGF-independent functions of extracellular IGFBPs have long been discussed. Secreted and membrane-associated IGFBP-2 directly binds to proteoglycans and integrins,^{1–5} demonstrating IGFBP-2 as a negative or positive regulator of cell adhesion, migration, and invasion in an IGF-independent manner. In the same way, IGFBP-2 positively or negatively regulates cell growth

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Current address of T.M.: Division of Molecular Biotherapy, Cancer Chemotherapy Center, Japanese Foundation for Cancer Research, Tokyo, Japan.

Address reprint requests to Toshiro Migita, M.D., Ph.D., Division of Molecular Biotherapy, Cancer Chemotherapy Center, Japanese Foundation for Cancer Research, 3-8-31, Ariake, Koto-ku, Tokyo 135-8550, Japan. E-mail: toshiro.migita@jfccr.or.jp.

and survival in certain types of cancers *in vitro*.^{2,6-11} In *in vivo* studies, the growth of mice colorectal adenomas induced by chemical carcinogen was inhibited when they were crossed with IGFBP-2 transgenic mice¹²; however, in contrast, IGFBP-2 exerts oncogenic effects in brain-specific transgenic mice.¹³ Thus, increased IGFBP-2 confers advantage or disadvantage for tumor growth, depending on cell type and physiological conditions.^{2,14}

Despite these two opposite effects of IGFBP-2 on biological behaviors of cancers, biochemistry and molecular pathology have demonstrated that IGFBP-2 is overexpressed in a wide variety of human malignancies, including glioma,¹⁵ prostate cancer,¹⁶ lung cancer,¹⁷⁻¹⁹ colorectal cancer,²⁰ ovarian cancer,²¹ adrenocortical tumor,²² breast cancer,²³ and leukemia.²⁴ Importantly, IGFBP-2 is frequently overexpressed in advanced cancers and is suggested to be involved in the metastatic process.²⁵ Several potential mechanisms of cancer progression mediated by secreted IGFBP-2 are discussed,¹⁴ but little study has been conducted to the analysis of intracellular-IGFBP-2 functions.

Our aim for this study is to examine the effect of intracellular IGFBP-2 on apoptosis in lung cancer cells and elucidate its molecular mechanism. We also examine the significance of intracellular IGFBP-2 and procaspase-3 in clinical samples and explore the therapeutic implications.

Materials and Methods

Cell Culture and Clinical Samples

The human lung adenocarcinoma cell lines A549, NCI-H460, NCI-H23, NCI-H522, HOP62, COR-L105, and PC14 were obtained from the American Type Culture Collection (Manassas, VA) and grown in RPMI 1640 media supplemented with 10% fetal bovine serum (both medium and serum were from Gibco-BRL, Tokyo, Japan) and 1% penicillin/streptomycin in an atmosphere of 5% CO₂ at 37°C, as previously described.²⁶

We also analyzed the mRNA and protein expression in 24 pairs of primary lung adenocarcinomas and corresponding normal lung tissues. All experiments were performed by using a protocol approved by the Institutional Review Board of the Japanese Foundation for Cancer Research (number 2007-1058).

Transient and Stable Transfections

IGFBP-2 cDNA expression construct in pcDNA3.1/Neo (Invitrogen, Carlsbad, CA) was a generous gift from Dr. Hiroaki Kataoka (Section of Oncopathology and Regenerative Biology, Department of Pathology, University of Miyazaki, Japan).²⁷ Cells were plated at 7×10^5 per well in 60-mm dishes and transfected in triplicate by using the FuGENE 6 Transfection Reagent according to the manufacturer's protocol (Roche Diagnostics, Inc., Indianapolis, IN). We established stable cell lines COR-L105, NCI-H522, and HOP62 overexpressing IGFBP-2 after 4 weeks of selection in 400 μ g/ml of neomycin.

RNA Preparation and Real-Time RT-PCR

The cells and frozen tissue were collected for RNA extraction by using an RNeasy Kit (Qiagen, Valencia, CA), and total RNA was applied for first-strand cDNA synthesis with a high capacity cDNA Reverse Transcriptase kit (Applied Biosystems, Foster City, CA). Gene-specific probes and primer were obtained from Universal ProbeLibrary (number 25, Roche Applied Science, Tokyo, Japan), and primer sequences were as follows: 5'-TTGCA-GACAATGGCGATGACC-3' (IGFBP-2 forward); 5'-GGG-ATGTGCAGGGAGTAGAGG-3' (IGFBP-2 reverse). PCR was performed in 96-well plates by using the LightCycler 480 System (Roche Applied Science). All reactions were performed at least in triplicate. The relative amounts of all mRNAs were calculated by using the comparative threshold cycle (CT) method after normalization to human β 2 microglobulin.

Cell Lysis and Immunoblotting

To obtain total protein lysates, frozen tissue and cells were homogenized and dissolved in radioimmunoprecipitation assay buffer (150 mmol/L of NaCl, 1.0% Nonidet P40, 0.5% sodium deoxycholate, 0.1% SDS, 50 mmol/L of Tris, pH 7.6) containing proteinase inhibitors and phosphatase inhibitors (Nacalai Tesque, Kyoto, Japan). The protein concentration of each lysate was determined by using a protein assay reagent kit (BioRad, Hercules, CA). The total cell lysate was applied on 4% to 12% SDS-polyacrylamide gel electrophoresis. After electrophoresis, the proteins were transferred electrophoretically from the gel to polyvinylidene difluoride membranes (Millipore, Bedford, MA). The membranes were then blocked for 1 hour in blocking buffer (5% low-fat dried milk in Tris-buffered saline) and probed with the primary antibodies overnight. After being washed, the protein content was made visible with horseradish-peroxidase-conjugated secondary antibodies followed by enhanced chemiluminescence (Amersham, Piscataway, NJ). Signal densities were quantitatively determined by ImageJ 1.36 b software (NIH, Bethesda, MD). The primary antibodies used were raised against IGFBP-2 (C-18, Santa Cruz Biotechnology, Santa Cruz, CA), caspase-3, phosphorylated (Tyr1135/1136) and total IGF-1R β , phosphorylated (Ser 473) and total Akt, phosphorylated (Thr 202/Tyr 204) and total Erk1/2, cleaved poly ADP-ribose polymerase (PARP; all obtained from Cell Signaling Technology, Danvers, MA), and β -actin (Sigma, St. Louis, MO). LY294002 was purchased from Sigma.

Caspase Activity Assay

Caspase activities were measured by using the Caspase-Glo 3/7 assay kit according to the manufacturer's instruction (Promega, Madison, WI). Cells (5×10^3 cells/well) were placed in a 96-well culture plate, followed by treatment with dimethyl sulfoxide (DMSO) vehicle or 200 nmol/L of camptothecin for 24 hours. One hundred microliters of Caspase-Glo 3/7 reagent was added to each well and incubated for 1 hour at room temperature.

The culture media with the reagent served as blank, and blank control value was subtracted from each sample value. Luminescence of all samples was measured by using a Tecan Spectrafluor Plus (Wako, Osaka, Japan).

Enzyme-Linked Immunosorbent Assay

IGFBP-2 concentrations in media of cell culture were determined with IGFBP-2 Duoset enzyme-linked immunosorbent assay (ELISA) Development system (R and D Systems, Minneapolis, MN) according to the manufacturer's protocol. Briefly, capture antibody was plated in a 96-well microplate and incubated overnight at room temperature. One hundred microliters of supernatant of culture media or IGFBP-2 standard were added into plate and incubate for 2 hours at room temperature, followed by the immunoreaction with IGFBP-2 detection antibody. IGFBP-2 concentration was calculated from the standard curve. All experiments were performed in duplicate or triplicate.

RNA Interference

Small-interfering RNA (siRNA) oligonucleotides for IGFBP-2 (Santa Cruz Biotechnology) and a negative control (Invitrogen) were transfected into the cells. Transfection was performed by using Lipofectamine RNAiMAX (Invitrogen) according to the manufacturer's protocol. Briefly, 60 pmol of siRNA and 10 μ l of Lipofectamine RNAiMAX were mixed in 1 ml of Opti-MEM medium (10 nmol/L of final siRNA concentration). After 20 minutes of incubation, the mixture was added to the suspended cells and these were plated on dishes. Cells were harvested at 24-hour intervals until 72 hours after transfection.

Cell Proliferation and Apoptosis

Cell proliferation was measured as the number of viable cells, as evaluated at 450 nm optical density by using Cell Count reagent SF (Nacalai Tesque). Apoptotic cells were determined by Hoechst 33342 staining, and the apoptosis rate (percent of total population) was evaluated by counting apoptotic and nonapoptotic cells in at least three randomly selected fields.

Immunohistochemistry

Tissue microarrays were constructed from 169 paraffin-embedded lung adenocarcinomas. Briefly, H&E-stained sections containing representative tumor regions were selected. Tissues were punched from cancer areas of each donor block by using tissue cylinders with a diameter of 2 mm and then brought into a recipient paraffin block. Three tumor cores were taken per patient.

Immunohistochemistry was performed on 5- μ m thick, formalin-fixed, paraffin-embedded sections by using primary antibodies for IGFBP-2 (C-18, Santa Cruz Biotechnology) and procaspase-3 (Cell Signaling Technology). Antigen retrieval was performed for 30 minutes in citrate buffer for each antibody. The slides were developed by using the labeled streptavidin biotinylated peroxidase method

(Nichirei, Tokyo, Japan) according to the manufacturer's instructions. 3,3'-Diaminobenzidine tetrahydro-chloride was used as the chromogen, and hematoxylin was used as the counterstain. A549 xenografts in nude mice were previously established²⁶ and were used as a positive control. The primary antibody was omitted for negative controls. All immunohistochemical staining was accomplished with a Dako Autostainer (DakoCytomation, Carpinteria, CA) under the same conditions. The staining intensity of IGFBP-2 and procaspase-3 was scored semiquantitatively: positive in less than 25% of cancer cells (weak), positive in 25% to 50% of cancer cells (moderate), and positive in more than 50% of cancer cells (strong). Representative score of each patient was defined as the highest score across three cores.

Statistical Analysis

For *in vitro* experiments, statistical analysis was performed by using Welch's *t*-tests. Comparisons of IGFBP-2 mRNA levels in clinical samples were made by using paired *t*-test analysis. Dose/time dependency of drugs was determined by the confidence interval (CI) based test of slope of the linear regression. Concentrations of drugs that suppressed cell proliferation to 50% of levels exhibited by control cells (IC50) were derived from the dose-response curve. Correlation between IGFBP-2 and caspase-3 expression in immunohistochemistry was evaluated by performing the Fisher's exact test. For all analyses, $P \leq 0.05$ was considered statistically significant. Statistical analyses were performed by using the statistical programming language of R (<http://www.R-project.org>; accessed February 1, 2010) and Statistika (Statsoft, Inc., Tulsa, OK).

Results

IGFBP-2 Is Expressed and Secreted in Lung Adenocarcinoma Cell Lines

At first, intracellular IGFBP-2 expression levels were examined in various lung cancer cell lines by the use of Western blot. IGFBP-2 was highly expressed in A549, NCI-H460 cells, but expressed at very low levels in HOP62 and COR-L105 cells (Figure 1A).

The levels of secreted IGFBP-2 in media were measured by ELISA. Secreted IGFBP-2 levels correlated with intracellular protein levels obtained by Western blot (Figure 1B).

IGFBP-2 Expression Is Regulated Transcriptionally and Posttranslationally

IGFBP-2 expression is physiologically up-regulated by the energy restriction or insulin-dependent diabetes mellitus.^{28,29} To determine whether the supplement of nutrients can alter IGFBP-2 expression in lung cancer cells, we examined the effects of glucose or serum depletion on IGFBP-2 expression in A549 cells. Glucose depletion significantly reduced IGFBP-2 levels at both protein and mRNA levels ($P = 0.0017$), whereas serum depletion did not ($P = 0.311$; Figure 2A). IGFBP-2 protein and mRNA levels were dependent on glucose concentration (Figure 2B). These findings suggest that IGFBP-2 expression in

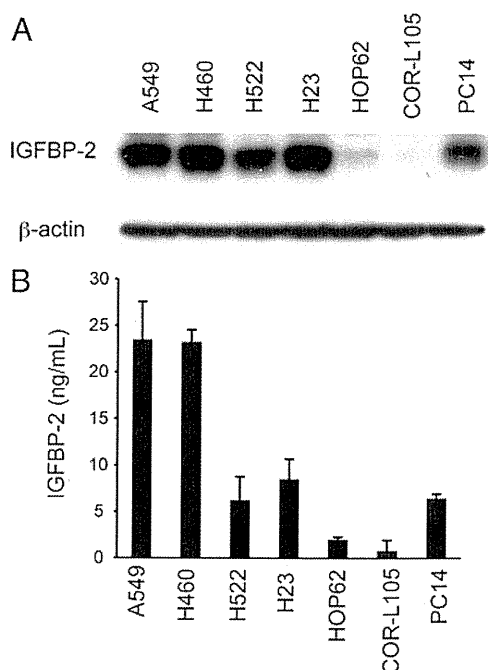


Figure 1. A: Basal levels of intracellular IGFBP-2 protein in seven lung adenocarcinoma cell lines. Cells (5×10^5) were plated in a 60-mm dish and cultured for 48 hours. The protein extracts from each cell line were resolved by SDS-polyacrylamide gel electrophoresis and blotted with an antibody against IGFBP-2. β -actin served as internal control. A representative data from two independent experiments is shown. **B:** Conditioned media containing a different amount of IGFBP-2 in lung adenocarcinoma cell lines. Secreted IGFBP-2 was measured, under the same conditions as above, by ELISA. Values represent means \pm SD.

cancer cells is glucose-dependent and is regulated by a mechanism that is distinct from normal cells.

It has been reported that IGFBP-2 expression is regulated by the PI3K-PEN (phosphatase and tensin homolog deleted on chromosome 10) pathway in prostate and glioblastoma cells.³⁰ Thus, extracellular and intracellular IGFBP-2 levels were evaluated in lung cancer cells treated with LY294002, a PI3K inhibitor. PEN protein was detected in all cell lines, except PC14, as described previously.²⁶ Secretion of IGFBP-2 protein was suppressed in all cell lines by the treatment of LY294002 to varying degrees (Figure 2C). The effect of LY294002 on IGFBP-2 expression showed a significant dose dependence ($P = 0.0048$) and time course dependence (95% CI: 0.134 to 0.18, control; 0.029 to 0.043, LY294002) in A549 cells (Figure 2, D and E). Intracellular IGFBP-2 levels were also decreased with LY294002 (Figure 2F). Interestingly, a fraction of IGFBP-2 protein was degraded into approximately 20 kDa after treatment with LY294002 (Figure 2F). Conversely, IGFBP-2 mRNA was significantly increased with LY294002 ($P < 0.005$; Figure 2G), suggesting the existence of a compensatory feedback mechanism.

IGFBP-2 Overexpression Suppresses Pro-caspase-3 Expression and Confers Resistance for Drug-induced Apoptosis

To address whether IGFBP-2 is involved in apoptotic event, IGFBP-2 was enforced in cells with low endoge-

nous IGFBP-2 levels, and then caspase expression was examined. IGFBP-2 overexpression resulted in a remarkable increase in intracellular IGFBP-2 levels in COR-L105, NCI-H522, and HOP62 cells compared with vector control (Figure 3A). Secreted IGFBP-2 levels of these cells were also increased corresponding to the levels of intracellular IGFBP-2 (Figure 3B). Intriguingly, IGFBP-2 overexpression resulted in a substantial decrease in pro-caspase-3 expression (Figure 3A). However, caspase-9 was not decreased (Figure 3A), suggesting IGFBP-2 specifically inhibits caspase-3 expression. Despite a higher amount of IGFBP-2 secretion into media, no significant changes were found in the IGF signaling pathway including phosphorylation statuses of IGF-1R, Akt, or Erk1/2 (Figure 3A). These findings suggest that IGFBP-2-mediated caspase-3 inhibition occurs in an IGF-independent manner.

Next, to examine whether IGFBP-2 involves in apoptotic event, we compared the sensitivity of IGFBP-2 overexpressing cells and vector control cells to an apoptosis inducer, camptothecin. IGFBP-2 overexpressing and vector control H522 cells were exposed to 20 to 1000 nmol/L of camptothecin for 24 hours, and the cell proliferation and caspase-3 activity were analyzed. The results indicated that IGFBP-2 overexpressing H522 cells were significantly resistant to camptothecin (EV, IC₅₀ = 686 nmol/L; BP-2, IC₅₀ > 1000 nmol/L; Figure 3C). As expected, caspase-3 activity was significantly decreased in IGFBP-2 overexpressing cells compared with vector control cells on treatment with camptothecin ($P < 0.02$; Figure 3D). Apoptosis was evaluated by Hoechst 33342 staining and PARP cleavage. Enforced IGFBP-2 significantly inhibited PARP cleavage, as determined by Western blot (Figure 3E), and reduced camptothecin-induced apoptotic cells in H522 cells ($P = 0.003$; Figure 3F). Similar results were obtained with the treatment of cisplatin or etoposide (data not shown).

IGFBP-2 Inhibition Up-Regulates Pro-caspase-3 Expression and Promotes Drug-Induced Apoptosis

To further elucidate the effects of IGFBP-2 on caspase-3, gene silencing for IGFBP-2 was performed in A549 and H522 cells. IGFBP-2 knockdown induced an increase in pro-caspase-3 expression until 72 hours after siRNA treatment in both cell lines (Figure 4A). No significant active form of cleaved caspase-3 was identified (data not shown). As is the results with IGFBP-2 overexpression, no substantial change was found in caspase-9. In addition, IGFBP-2 siRNA also decreased the phosphorylation status of IGF-1R. This effect might be because of a rapid decrease in both intracellular and extracellular IGFBP-2. Although IGFBP-2 knockdown resulted in morphological changes such as shrinkage in A549 cells, no substantial increase in apoptosis was identified by Hoechst 33342 staining or PARP cleavage (data not shown).

We now asked whether IGFBP-2 inhibition sensitizes cells for drug-induced apoptosis. Figure 4B shows the cell proliferation of IGFBP-2 knockdown and negative control cells with a treatment of camptothecin. IGFBP-2

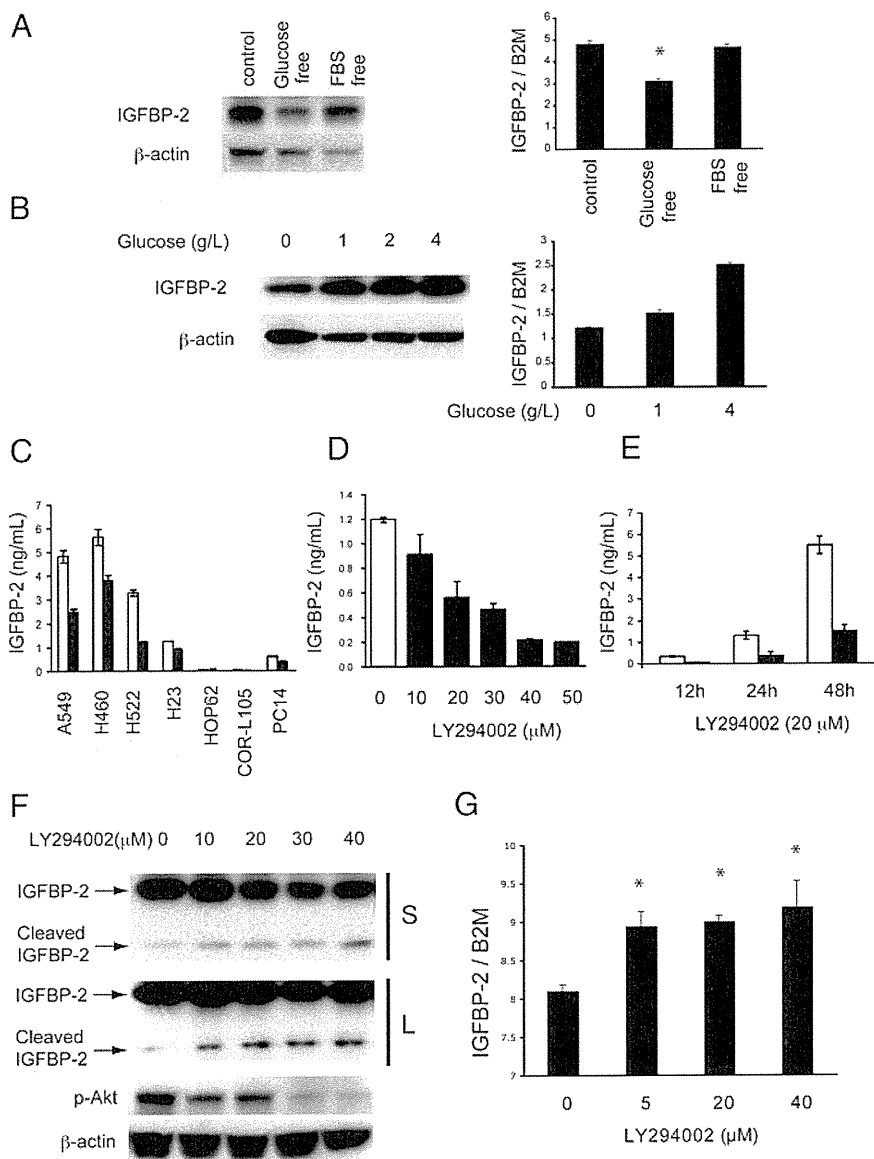


Figure 2. A: The effect of glucose and fetal bovine serum on intracellular IGFBP-2 levels. A549 cells (5×10^5) were incubated for 2 hours in fetal bovine serum (FBS) free media, followed by a 24-hour incubation in glucose free, FBS free, or regular media. The cells were then harvested and subjected to both immunoblotting and quantitative RT-PCR for IGFBP-2. IGFBP-2 mRNA was normalized to human $\beta 2$ microglobulin (B2M). Values represent means \pm SD. Statistical analysis was performed by Welch's *t*-test. **P* < 0.01. **B:** A549 cells were cultured in media with the indicated concentrations of glucose for 24 hours. IGFBP-2 expression levels were measured by both immunoblotting and quantitative RT-PCR. Values represent means \pm SD. Significant slope of regression line between IGFBP-2 mRNA and glucose concentration was obtained (*P* = 0.012). **C:** Effect of LY294002, a PI3K inhibitor, on extracellular IGFBP-2 levels. Seven lung adenocarcinoma cell lines were treated either with a vehicle control DMSO (white bar) or 20 μ mol/L of LY294002 (black bar) for 24 hours. Secreted IGFBP-2 was measured by ELISA as described before. **D:** A549 cells were treated with the indicated concentration of LY294002 for 24 hours. A significant slope of regression line between secreted IGFBP-2 and LY294002 concentration was obtained (*P* = 0.0048). **E:** Time course of IGFBP-2 secretion in A549 cells treated with 20 μ mol/L of LY294002. The 95% CI based test of slope regression was significant (*P* < 0.05): 0.134 to 0.18 vs. 0.029 to 0.043, in control DMSO and LY294002, respectively. **F:** The effect of LY294002 on intracellular levels of IGFBP-2. A549 cells were treated with the indicated concentration of LY294002, followed by immunoblotting for IGFBP-2, phosphorylated Akt (Ser 473), and β -actin. S, short exposure; L, long exposure. **G:** A549 cells were treated with the indicated concentration of LY294002, and IGFBP-2 mRNA levels were evaluated by a real-time RT-PCR. Values represent means \pm SD. Statistical analysis was performed by Welch's *t*-test. **P* < 0.01.

knockdown cells were more sensitive to camptothecin rather than vector control cells (95% CI: -2.7×10^{-4} to -1.6×10^{-4} vs. -4.5×10^{-4} to -3.0×10^{-4} , in negative control and IGFBP-2 siRNA, respectively; Figure 4B). In caspase-3 activity assay (Figure 4C), there were no significant changes in caspase-3 activity between negative control and IGFBP-2 siRNA with DMSO treatment (white bars). When cells were treated with camptothecin, IGFBP-2 siRNA significantly increased caspase-3 activity than negative control siRNA (black bars). The sensitivity to camptothecin was significantly potentiated by IGFBP-2 inhibition (*P* < 0.0001). Apoptosis was significantly increased in cells with IGFBP-2 siRNA compared with negative control siRNA (*P* = 0.0009; Figure 4D). Cleaved PARP was more substantial in IGFBP-2 siRNA treated cells compared with vector control H522 cells (Figure 4E). As a PI3K inhibitor induced IGFBP-2 degradation (Figure 2F), we examined whether a PI3K inhibitor has an additive effect on apoptosis with camptothecin. As

expected, combination therapy of LY294002 and camptothecin enhanced PARP cleavage in H522 cells when compared with camptothecin or LY294002 alone. IGFBP-2 levels were inversely correlated with the increase in the levels of cleaved PARP (Figure 4F, left panels). In contrast, there were no substantial effects of LY294002 on PARP cleavage in COR-L105 cells, which have low IGFBP-2 levels (Figure 4F, right panels).

These data strongly suggest that IGFBP-2 regulates apoptosis via caspase-3. Moreover, IGFBP-2 becomes a therapeutic target as well as a biomarker for the treatment of PI3K inhibitors.

Tissue IGFBP-2 Is Overexpressed in Lung Adenocarcinoma

Next, we examined tissue expression levels of IGFBP-2 in human lung adenocarcinoma and normal tissue by using

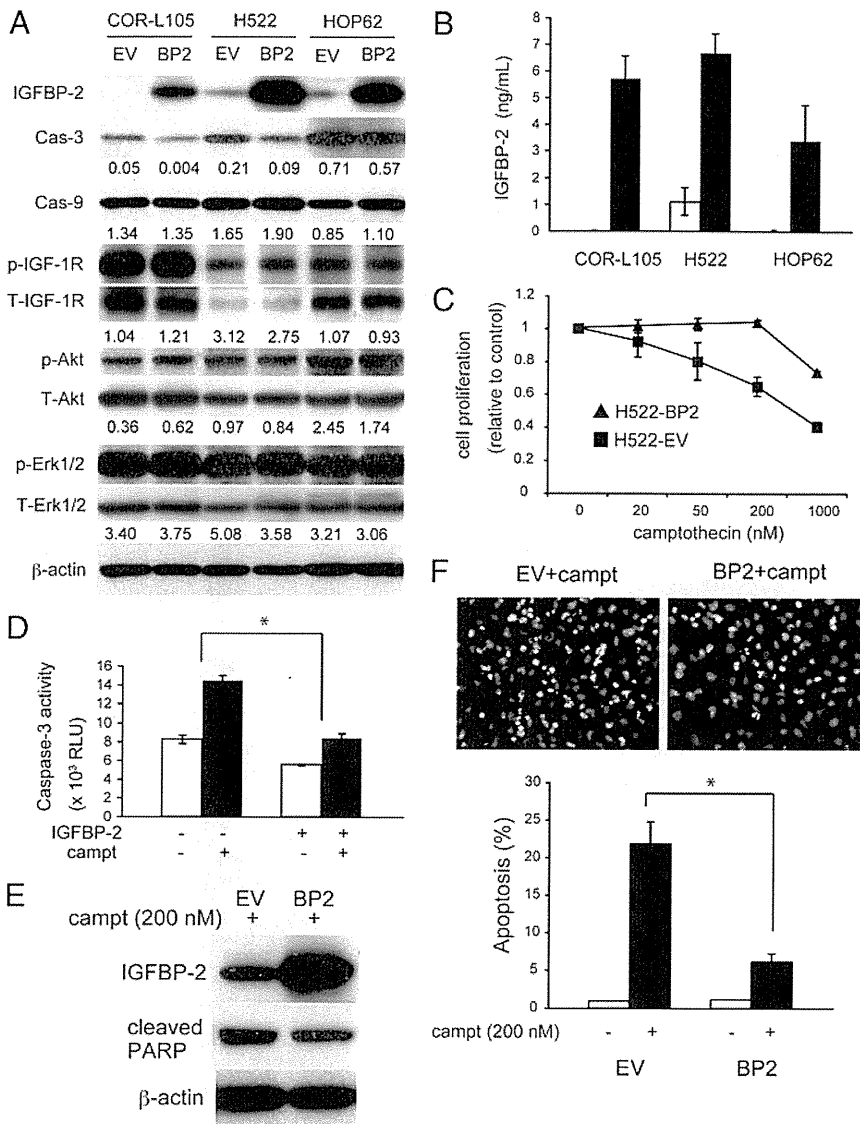


Figure 3. A: IGFBP-2 overexpression inhibits procaspase-3 expression independent of the IGF signaling pathway. Empty vector (EV) and IGFBP-2 (BP2) were transfected in COR-L105, NCI-H522, and HOP62 lung adenocarcinoma cell lines, and stably IGFBP-2 overexpressing cells were obtained. Whole cell lysates were subjected to SDS-polyacrylamide gel electrophoresis, followed by immunoblotting for IGFBP-2, procaspase-3, procaspase-9, phosphorylated and total IGF-1R, phosphorylated and total Akt, phosphorylated and total Erk1/2, and β-actin. Signal densities were quantified by ImageJ, and then procaspase-3/β-actin, procaspase-9/β-actin, p-IGF1R/T-IGF1R, p-Akt/T-Akt, and p-Erk1/2/T-Erk1/2 ratios were calculated. **B:** Secreted IGFBP-2 levels were measured by ELISA in three different stable vector- and IGFBP-2-transfected (white and black bars, respectively) cell lines. Data represent means ± SD. **C:** IGFBP-2 overexpressing and empty vector NCI-H522 cells were plated in 96 wells and treated with indicated concentration of camptothecin for 24 hours. Cell proliferation was determined by microplate reader using cell count reagent. Data represent means ± SD. The IC50 values were 686 nmol/L and more than 1000 nmol/L in empty vector and IGFBP-2 cells, respectively. **D:** Caspase-3 assay in IGFBP-2 overexpressing and empty vector NCI-H522 cells. Cells were plated in 96 wells and treated with 200 nmol/L of camptothecin for 24 hours. Caspase-3 activity was determined by a microplate reader. Data represent means ± SD. Statistical analysis of comparison between empty vector and IGFBP-2 overexpressing cells was performed by Welch's *t*-test. EV (camptothecin/DMSO) versus BP2 (camptothecin/DMSO); **P* < 0.02. **E:** Apoptosis was also evaluated by immunoblotting for PARP cleavage with whole cell lysate. **F:** Twenty-four hours after exposure of 200 nmol/L of camptothecin, cells were stained with Hoechst 33342. Apoptotic and nonapoptotic cells were counted by microscopy at least in three different areas, and the apoptotic rate was represented. Values represent means ± SD. Statistical analysis was performed by Welch's *t*-test. **P* < 0.01.

a real-time RT-PCR and Western blotting. IGFBP-2 mRNA was significantly higher in tumors than in paired normal tissue, as examined by a real-time RT-PCR (*P* = 0.021; Figure 5A). A higher amount of IGFBP-2 protein was also frequently observed in tumor tissue compared with in paired normal tissue (Figure 5B).

Inverse Relationship between IGFBP-2 and Caspase-3 Expression

Finally, immunohistochemical analysis was performed on tissue microarray including 169 cases of lung adenocarcinoma. IGFBP-2 expression was mostly confined to cancer cells, whereas normal lung epithelium revealed very low or undetectable IGFBP-2 levels (Figure 6A, arrowheads). In most cases, IGFBP-2 was localized in cytoplasm of lung adenocarcinoma cells, as shown in Figure 6A. Membraneous IGFBP-2 expression was found in only 3 of 169 cases (1.8%; Figure 6B). IGFBP-2 was expressed in early precursor lesions, and its expression

levels increased gradually as the lesions progress from benign (Figure 6C, arrows) to malignant cells (Figure 6C, arrowheads). In particular, a strong IGFBP-2 expression was found in cancer cells with high nuclear grade distinct from ones with low nuclear grade even within the same gland (Figure 6D). It should be noted that the mutually exclusive expression between IGFBP-2 (Figure 6E, left panel, arrowheads and Figure 6F, left panel, upper area) and procaspase-3 (Figure 6E, right panel, arrowheads and Figure 6F, right panel, lower area) was frequently observed in lung adenocarcinomas. To summarize the immunohistochemical data, a significant inverse correlation between the groups in the numbers of patients with IGFBP-2 and procaspase-3 expression was observed in lung adenocarcinomas (Table 1).

Discussion

The IGF signaling pathway plays a pivotal role in cellular proliferation, differentiation, survival, and metabolism.

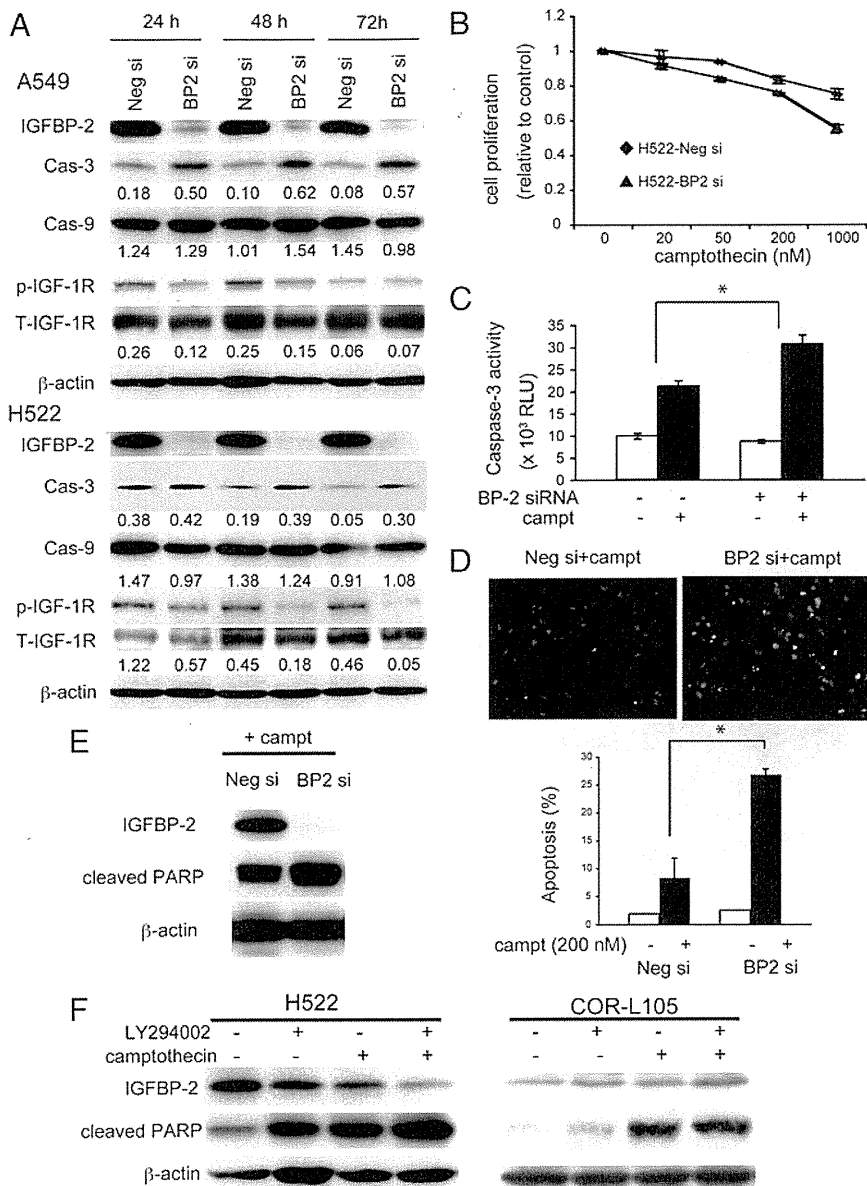


Figure 4. A: Specific IGFBP-2 inhibition resulted in the increase in procaspase-3. A549 or NCI-H522 cells were transfected with negative control or IGFBP-2 siRNA oligonucleotides, followed by immunoblot for IGFBP-2, procaspase-3, procaspase-9, phosphorylated and total IGF-1R, and β -actin at indicated times after transfection. Signal densities were quantified by ImageJ, and then procaspase-3/ β -actin, Procaspase-9/ β -actin, and p-IGF1R/T-IGF1R ratios were calculated. B: NCI-H522 cells were treated with negative control or IGFBP-2 siRNA for 24 hours and then exposed to different concentrations of camptothecin for 24 hours. Cell proliferation was determined as described before. Data represent means \pm SD. A 95% CI based test of slope regression was significant ($P < 0.05$): $-2.7E-04$ to $-1.6E-04$ in negative siRNA vs. $-4.5E-04$ to $-3.0E-04$ in IGFBP-2 siRNA. C: Caspase-3 assay in NCI-H522 cells treated with negative control or IGFBP-2 siRNA. NCI-H522 cells were treated with negative control or IGFBP-2 siRNA for 24 hours and then exposed to 200 nmol/L of camptothecin for 24 hours. Caspase-3 activity was determined by a microplate reader. Data represent means \pm SD. Statistical analysis of comparison between negative control and IGFBP-2 siRNA was performed by Welch's *t*-test. $*P < 0.0001$. D: Twenty-four hours after exposure of 200 nmol/L of camptothecin, siRNA-treated NCI-H522 cells were stained with Hoechst 33342. The apoptotic rate was measured as described previously. Values represent means \pm SD. Statistical analysis was performed by Welch's *t*-test. $*P < 0.001$. E: Apoptosis was also evaluated by immunoblot for PARP cleavage in NCI-H522 cells. F: NCI-H522 and COR-L105 cells were treated with 20 μ mol/L of LY294002 or 200 nmol/L of camptothecin or combination of LY294002 and camptothecin for 24 hours. Immunoblot was performed with IGFBP-2, cleaved PARP, and β -actin antibodies.

IGFBPs are circulating proteins and function as modulators of IGF signaling through sequestration of IGFs in serum and the extracellular fluid. Increased levels of serum IGFBP-2 are found in certain pathophysiological conditions including fasting, diabetes mellitus, growth hormone deficiency, hepatic or renal failure, and cancer.³¹ In cancer, IGFBP-2 exerts various biological functions by virtue of IGF-dependent or -independent mechanisms. Soluble IGFBP-2 binds to IGFs and consequently inhibits IGF signaling in various human cancers, including lung cancer.^{19,32-34} Membrane-associated IGFBP-2 stimulates or inhibits cell proliferation and migration through a direct binding to serum and extracellular matrix molecules, such as cell surface integrin receptors, proteoglycans, and heparin.^{2-5,35} Meanwhile, a number of studies demonstrate that intracellular IGFBP-2 promotes cancer cell growth in various cell types.^{9,11,36} Moreover, IGFBP-2 overexpression confers resistance to apoptosis induced

by chemotherapy in breast cancer cells⁶ and by androgen ablation in prostate cancer.⁹ Serum IGFBP-2 can be used for prediction of chemotherapy response and prognosis in ovarian cancer³⁷ and acute lymphoblastic leukemia.³⁸ Notably, IGFBP-2 is a marker for antiestrogen resistance, but not for cell growth in human breast cancer cells.³⁹ These observations invoke that intracellular IGFBP-2 mainly contributes to cancer cell survival independently of secreted IGFBP-2.

In the present study, we have shown that (1) intracellular IGFBP-2 regulates caspase-3 expression in an IGF independent manner; (2) IGFBP-2 overexpression prevents camptothecin-induced apoptosis, whereas IGFBP-2 inhibition promotes apoptosis; and (3) there is an inverse expression pattern between intracellular IGFBP-2 and caspase-3 in human lung adenocarcinomas.

We demonstrated a novel mechanism of antiapoptotic effect of IGFBP-2 via procaspase-3 inhibition in lung can-

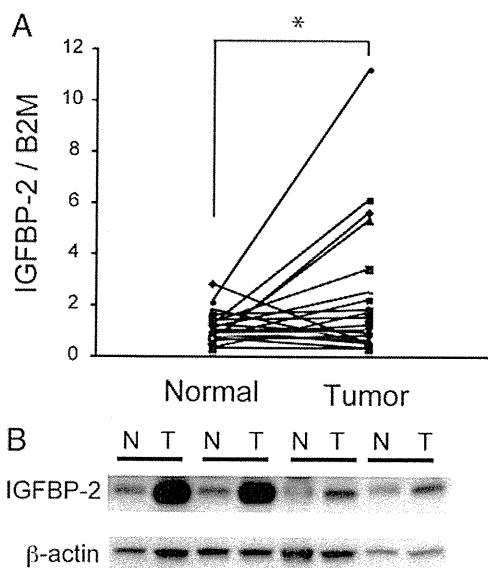


Figure 5. A: IGFBP-2 mRNA expression was measured by real-time RT-PCR in 24 pairs of human normal and corresponding tumor tissue. The mRNA levels of IGFBP-2 are presented as arbitrary units for the mRNA levels of human $\beta 2$ microglobulin (B2M). A paired *t*-test was used for statistical significance (**P* = 0.021). **B:** Representative picture of Western blots. IGFBP-2 protein levels were measured with four pairs of normal (N) and corresponding tumor (T) tissue from lung adenocarcinoma patients.

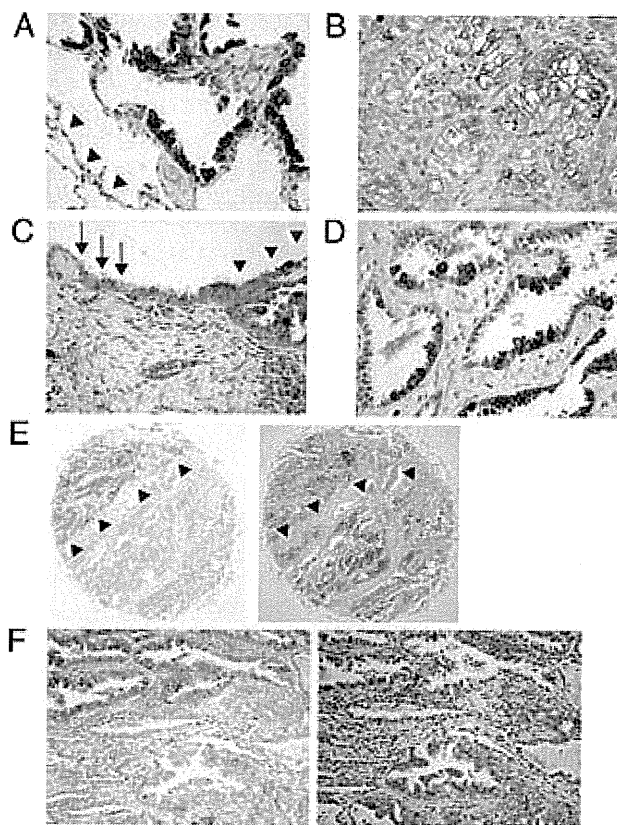


Figure 6. A: Representative pictures of immunohistochemistry for IGFBP-2 in lung adenocarcinomas. Note a strong immunoreactivity in cytoplasm of cancer cells, whereas almost negligible in normal epithelium (arrowheads). **B:** Typical membranous IGFBP-2 expression. **C:** IGFBP-2 expression is gradually increased from benign cells (arrows) to malignant cells (arrowheads). **D:** Strong IGFBP-2 expression is only localized in cancer cells with high nuclear grade. **E:** Representative mutually exclusive expression between IGFBP-2 (left, arrowheads) and procaspase-3 (right, arrowheads) in serial sections on tissue microarray. **F:** Another case also demonstrates an inverse expression pattern between IGFBP-2 (left) and procaspase-3 (right) in serial sections. Original magnification: $\times 400$ (A–D, and F); $\times 100$ (E).

cer. Caspases are cysteine proteases that play essential roles in mammalian apoptosis. Procaspase-3 cleavage and consequent activation is the final step of caspase cascades in response to various apoptotic stimuli. Several authors have proved that enforced procaspase-3 potentiates sensitivity to chemotherapy and promotes apoptosis.^{40–42} In lung cancer, decreased caspase-3 expression has been shown as a poorer prognostic factor in non-small-cell lung cancer.^{43–45}

Our results raise the important question regarding the regulatory mechanisms involved in caspase-3 inhibition via IGFBP-2. A recent report has shown that transcriptional factor Sp1 activates the caspase-3 promoter.⁴⁶ Mammalian IGFBP-2 also has the Sp1 binding regions upstream of the transcriptional start site.⁴⁷ One possible explanation for the regulation of caspase-3 via IGFBP-2 is that IGFBP-2 overexpression in cancer cells inhibits Sp1 through negative feedback mechanism, and thereby inhibits caspase-3 gene and protein expression. Another possibility is PTEN. IGFBP-2 has been identified as the most significant molecular signature for loss of PTEN in brain and prostate cancer.³⁰ It has been shown that PTEN is cleaved by caspase-3 in a PTEN phosphorylation-regulated manner.⁴⁸ IGFBP-2 overexpression may induce PTEN up-regulation and protein stabilization through feedback mechanisms, and thereby negatively regulating caspase-3 activation. Future studies will help to identify the precise regulatory mechanism of caspase-3 mediated by IGFBP-2. Recent studies demonstrate caspase-3 has apoptosis-independent physiological functions, including differentiation, maturation, proliferation, and immune response.^{49,50} Thus, caspase-3 may contribute to lung cancer development and progression by multiple functions including apoptosis.

Because IGF signaling was not altered by the overexpression of intracellular IGFBP-2, our data suggest that intracellular and secreted IGFBP-2 are functionally independent. Interestingly, IGFBP-5 is another cancer-associated IGFBP, and it has been reported that intracellular IGFBP-5 induces growth inhibition and caspase-dependent apoptosis of breast cancer cells, whereas adding secreted-IGFBP-5 was not internalized and had no effects on growth and apoptosis.⁵¹ Further, endogenous and exogenous IGFBP-5 is suggested to exhibit opposing actions on cell survival in osteosarcoma cells.⁵² IGFBP-3, a most major IGFBP in serum, also induces

Table 1. Inverse Relationship between IGFBP-2 and Caspase-3 Expression in 169 Cases of Lung Adenocarcinomas

IGFBP-2	Caspase-3		
	Weak	Moderate	Strong
Weak	48	20	10
Moderate	40	9	2
Strong	40	0	0

Fisher's exact test was used for statistical significance (*P* = 0.0002). Data represent the number of patients.

growth inhibition and apoptosis in cancer cells, but it does not require the cell surface binding and nuclear translocation of IGFBP-3 in breast and prostate cancer.^{53,54} These lines of evidence prompt us to propose that intracellular IGFBP-2 elicits antiapoptosis effects on cancer cells via intracrine mechanism, independent of secreted IGFBP-2. Although not yet identified in IGFBP-2, the posttranslational modification (ie, glycosylation) of secreted IGFBP-3 or -5 can be involved in the functional difference between intracellular and secreted form.⁵¹

There are a number of lines of evidence that IGFBP-3 is able to induce apoptosis and potentiate the apoptotic effects of UV or chemotherapy.^{55,56} The inverse relationship between IGFBP-2 and IGFBP-3 expression at tissue and serum levels in a variety of cancers, including prostate,^{31,55,57} ovarian,⁵⁸ and testicular cancer, has been well recognized.⁵⁹ We also found a relatively inverse relationship between secreted levels of IGFBP-2 and IGFBP-3 in lung adenocarcinoma cell lines (unpublished data). Remarkably, IGFBP-2 is predominantly expressed in cytoplasm and nucleus of lung epithelium when exposed to hyperoxia, whereas IGFBP-3 is localized in the extracellular compartment.⁶⁰ These findings suggest that IGFBP-2 and -3 may be differentially regulated and also exert a distinct action for cell proliferation and apoptosis in different compartments.

Our immunohistochemical analysis demonstrated that most adenocarcinomas revealed a cytoplasmic IGFBP-2 expression pattern, and a significant inverse association between IGFBP-2 and procaspase-3 expression. These results support the evidence that intracellular IGFBP-2 regulates procaspase-3 expression *in vitro*, thereby inhibiting apoptosis. Interestingly, IGFBP-2 expression showed a marked heterogeneity within lung adenocarcinoma tissue. At cellular levels, a strong IGFBP-2 expression was found in cancer cells having high nuclear grade. This finding suggests IGFBP-2 overexpression in cancer cells is caused by adaptive mechanisms in tumor microenvironment and confers aggressive biological nature to survive under the toxic conditions.

IGFBP-2 protein is degraded by proteases such as matrix metalloprotease-1 and -7, calpain, as well as by basic fibroblast growth factor and an androgen blockade.⁶¹⁻⁶⁴ We found IGFBP-2 protein was degraded by a treatment of PI3K inhibitor in A549 cells. Because a various new PI3K inhibitors have been entered clinical trials,⁶⁵ IGFBP-2 would be a useful biomarker for the treatment with PI3K inhibitors in lung cancer as well as in glioma, prostate, and breast cancers.^{30,66} Further, our results suggested that IGFBP-2 is a therapeutic target in lung cancer, in line with the results in breast and ovarian cancers.^{6,10} In general, lung adenocarcinomas typically showed a resistance to multiple cancer chemotherapy. Because cytoplasmic IGFBP-2 may provide cancer cells with an antiapoptotic ability, IGFBP-2 is an attractive therapeutic target especially for chemotherapy resistant tumors. The combination of chemotherapy and the IGFBP-2 or PI3K inhibitors may also potentiate drug-sensitivity.

Lung cancer is the leading cause of cancer death worldwide. Despite the availability of some cytotoxins and molecular target therapy, the efficacy of these agents is limited.

It has thus become increasingly necessary to identify novel approaches to treat lung cancer. We propose that IGFBP-2 is not only a useful biomarker for predicting chemotherapy response, but also a novel therapeutic target in lung cancer.

Acknowledgments

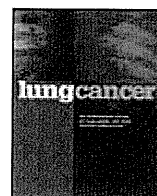
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Activation status of receptor tyrosine kinase downstream pathways in primary lung adenocarcinoma with reference of *KRAS* and *EGFR* mutations

Miyako Hiramatsu^{a,b}, Hironori Ninomiya^a, Kentaro Inamura^a, Kimie Nomura^a, Kengo Takeuchi^a, Yukitoshi Satoh^{a,d}, Sakae Okumura^d, Ken Nakagawa^d, Takao Yamori^c, Masaaki Matsuura^e, Toshiaki Morikawa^b, Yuichi Ishikawa^{a,*}

^a Division of Pathology, The Cancer Institute, Japanese Foundation for Cancer Research (JFCR), 3-8-31 Ariake, Koto-ku, Tokyo 135-8550, Japan

^b Department of Thoracic Surgery, Tokyo Jikei University of Medicine, Japan

^c Division of Molecular Pharmacology, The Cancer Chemotherapy Center, Japanese Foundation for Cancer Research (JFCR), Japan

^d Department of Thoracic Surgical Oncology, The Cancer Institute Hospital, Japanese Foundation for Cancer Research (JFCR), Japan

^e Division of Cancer Genomics, The Cancer Institute, Japanese Foundation for Cancer Research (JFCR), Japan

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ABSTRACT

The activation status of signal transduction pathways involving receptor tyrosine kinases and its association with *EGFR* or *KRAS* mutations have been widely studied using cancer cell lines, although it is still uncertain in primary tumors.

To study the activation status of main components of growth factor-induced pathways, phosphorylated Akt (pAkt), extracellular signal-regulated kinases 1 and 2 (pERK) and other downstream proteins were immunohistochemically examined using surgical samples of 193 primary lung adenocarcinomas. Also, thyroid transcription factor-1 (TTF-1) expression and mutation status of *EGFR* and *KRAS* were examined.

Advanced tumor stages ($p < 0.001$), negative TTF-1 expression ($p < 0.001$) and Akt activation ($p = 0.015$) were independent and significant poor prognostic markers. Akt activation related to advanced stage ($p = 0.021$), invasiveness ($p = 0.004$), and not to mutations. TTF-1 expression associated with never-smoker ($p = 0.013$), pre- or minimally invasiveness ($p < 0.001$) and *EGFR* mutations ($p = 0.017$) as well as with pERK ($p = 0.039$) expression. *EGFR* mutations did not correlated with pAkt and pERK expression, which was different from the results based on cultured cells, while *KRAS* mutations were solely and significantly linked to ERK activation ($p = 0.009$).

In lung adenocarcinoma, tumors with TTF-1 expression have distinct characteristics regarding mutations, signal protein activation and clinical issues. Moreover, this property was revealed to be important in outcome estimation at any tumor stage, whereas Akt activation is abnormally affected according to the tumor stage regardless of their cell origin. The signal proteins were differently related to mutation status from cultured cells.

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1. Introduction

Lung cancer is one of the leading causes of cancer-related deaths worldwide [1] and adenocarcinoma is recently becoming a frequent histologic type among non-small-cell lung cancers (NSCLCs) in many countries. Gefitinib, an inhibitor of epidermal growth factor receptor (*EGFR*) tyrosine kinase, has shown remarkable efficacy for control of a subset of lung adenocarcinomas, reflecting improved understanding of the underlying biology [2]. Especially the detection of somatic mutations in *EGFR* shed light on the mechanisms of acquisition of tumor growth advantage, featuring dysregulated signal transduction in tumor cells [3,4].

Akt, a serine/threonine kinase, and extracellular signal-regulated kinases 1 and 2 (ERK) are major target proteins, downstream of *EGFR* and various other oncoproteins such as Ras and Raf. They are known to be activated in a wide spectrum of human cancer together with various downstream substrates such as glycogen synthase kinase 3-beta (GSK3B), mammalian target of Rapamycin (mTOR), p70 ribosomal protein S6 kinase (S6K) and forkhead proteins FKHR/FKHL1 (FKHR) [5–8] and to play central roles in tumorigenesis or cell proliferation. The present study was performed to elucidate, by immunohistochemistry (IHC), whether there might be selective activation of downstream pathways of receptor tyrosine kinases (RTKs) in lung adenocarcinomas, depending on tumor-cell lineage or with/without *EGFR* and *KRAS* mutations. We further evaluated the clinicopathological and prognostic significance of such activation in various adenocarcinoma subtypes. Since we earlier revealed by expression profiling that

* Corresponding author. Tel.: +81 3 3570 0448; fax: +81 3 3570 0558.
E-mail address: ishikawa@jfcrc.or.jp (Y. Ishikawa).

adenocarcinoma cell lines might have different characteristics of gene expression from clinical adenocarcinomas [9], we here used tissue materials of surgically resected adenocarcinomas rather than cell lines.

2. Materials and methods

2.1. Patients and pathological review

A series of 193 Japanese cases with primary lung adenocarcinoma surgically resected between 1998 and 2001 at the Department of Thoracic Surgical Oncology, The Cancer Institute Hospital, Japanese Foundation for Cancer Research (JFCR), Tokyo, were selected for the present study. Informed consent was obtained from all the subjects at the time of surgery. This study was approved by the Institutional Review Board of the JFCR. All patients were staged pathologically according to the 5th edition of the UICC-TNM staging system [10]. For accuracy of survival analysis, only death of lung cancer was counted as cause-specific death. Smoking history was ascertained with all patients in detail.

Histological diagnosis was made according to the WHO classification [11], using sections through the largest cut surface of each tumor stained by hematoxylin–eosin and alcian-blue methods and PAS reaction. However, with its subdivision of lung adenocarcinomas, more than 80% tumors fell into the mixed subtype category. We therefore additionally used a noninvasive/invasive dichotomy as well as a predominance classification for invasive carcinomas, which is mostly based on the WHO classification except for the mixed subtype, such as bronchioloalveolar carcinoma (BAC) predominant, papillary predominant, acinar predominant, etc. The noninvasive carcinoma includes BAC. In the predominance classification of invasive carcinomas, we diagnosed by a component that makes up the predominant portion in the largest cut surface, or the cut surface containing a solid part shown by CT scans, of a tumor. Also, we employed a concept of “minimally invasive adenocarcinomas”, which were defined to be lesions where an invasive area of less than 5 mm in diameter or less.

2.2. Tissue microarrays

Tumor tissues were fixed in 15% neutral formalin and embedded in paraffin. Three histologically representative sites were selected per tumor, considering the well-known heterogeneity of lung adenocarcinomas (including the peripheral boundary and the central part of each tumor) and tissue microarrays were constructed as follows. Selected points of the donor paraffin blocks of the largest cut surface were punched with a 2-mm-diameter coring needle, and transferred to the array in the recipient block using a manual tissue arrayer (KIN-1, Azumaya, Inc, Japan). 48 human tissue rods (16 tumors) were embedded in one tissue array block. Based on our preliminary examinations using whole sections of tumor for several cases, we adopted 2 mm needles and three points to take tissues, rather than smaller needles and only one or two points. As controls, 3 mouse xenografts were selected from the panel of 39 cell lines (termed JFCR 39) [12] and embedded together with clinical samples in each array block as detailed below.

2.3. Protein expression analysis

Phosphorylated protein levels of Akt, ERK, GSK3B, mTOR, S6K and FKHR were immunohistochemically examined using antibodies for phosphorylated proteins designated by pAkt, etc. Also, thyroid transcription factor-1 (TTF-1) was examined for cell lineage analysis. The primary antibodies and citrate buffer used in this study are listed in Suppl. Table 1 and details of our immunohistochemical technique are also available on this. Antigen–antibody

complexes were detected by labeling with the Envision+/HRP system (DAKO, Carpinteria, CA, USA), using 3,3'-diaminobenzidine tetrahydrochloride as the chromogen and hematoxylin as counterstain. As well as using some normal cells as internal controls, mouse xenografts of the three cancer cell lines (PC-3, a prostate cancer line, showing high pAkt and low pERK expression, U251, a brain tumor line, showing moderate pAkt and low pERK expression and HTB26, a breast cancer line, showing low pAkt and high pERK expression) were included as external controls (Fig. 1), because we sought better quantification of immunoreactivity of each antibody by using well-known cell lines with well-documented reactivity. Immunoreactivity of each case was evaluated for all the tumor cells (or other cells of interest) appearing in all the three portions, applying the staining results for these xenografts. Essentially, for pAkt and pFKHR cytoplasmic staining (Fig. 1 and Suppl. Fig. 1), and for pS6K and pGSK3B whole cell staining were evaluated respectively, referring to the U251 levels. For pmTOR staining, comparison was with HTB26. All these were recorded as dichotomous parameters categorized as “negative” (weaker than or equal to xenograft staining) and “positive” (stronger than xenograft staining). For pERK immunoreactivity, the percentages of cells with positive staining were recorded and a score of 10% or less was categorized as “negative” and a score of more than 10% as “positive”.

2.4. Mutation analysis of EGFR and KRAS

The mutation status of four exons of the *EGFR* gene and three codons of the *KRAS* gene was evaluated in the subset ($n=93$) of the 193 cases. The primer sequences for exons 18 and 21 of *EGFR* were as follows (forward and reverse, respectively), exon 18 (5'-TCCAAATGAGCTGGCAAGTG-3' and 5'-TCCCAAATACTCAGTGAACAAA-3'), exon 21 (5'-GATGCAGAGCTTCTCCCAT-3' and 5'-ATACAGCTAGTGGGAAG GCA-3'). For *KRAS*, codon 12 and codon 13 (5'-CCTATGTGTGACATGTTCT-3' and 5'-CTATTGTTGGATCATATTCG-3'), codon 61 (5'-TTCC-TACAGGAAGCAAGTA-3' and 5'-GGCAAATACAAAAGAAAGC C-3'). All PCR assays were carried out in a 20 μ L volume that contained 0.2 μ L of Taq DNA polymerase (NEB Phusion TM High Fidelity DNA polymerase sets, Finnzymes Oy, Finland). DNA was amplified for 35 cycles at 98 °C for 10 s, 60 °C for 30 s, and 72 °C for 30 s, followed by a 7 min extension at 72 °C. All PCR products were incubated with exonuclease I and shrimp alkaline phosphatase (USB corporation, Exo SAP-IT, OH, USA) according to the manufacturer's instructions and then sequenced directly by a cycle sequencing method (Beckman Coulter Inc, DTL5-Quick Start Kit, CA, USA). All sequence variants were confirmed by sequencing the products of independent PCR amplifications in both directions. To detect deletion in exon 19 and insertion in exon 20 of *EGFR*, common fragment analysis was used. Sample DNA was amplified with a Cy5-labeled primer set as follows: exon 19 (5' Cy5-GTCTTCCTCTCTCTGTTCAT-3' and 5'-TGTGGAGA GTGAGCAGGGTCT-3'), exon 20 (5' Cy5-ACCATGCGAAGCCCACTGA-3' and 5'-TCCTTATCTCCCCTCCCCGTAT-3') and any deletion or insertion mutation was detected as a new peak of amplified products in the electrophoregram.

2.5. Statistical analysis

Statistical analyses were accomplished with STATA software, version 9 (Stata Corp. LP, College Station, TX, USA) and statistical programming language of R [13]. We studied the relationships between the survival and other clinicopathological factors and phospho-protein expression by univariate analyses of log-rank test. Then multivariate analyses using Cox regression model together with an AIC (Akaike Information Criteria) stepwise selection were applied to those factors to evaluate their relative risks. Then, correlation coefficients between each clinicopathological or

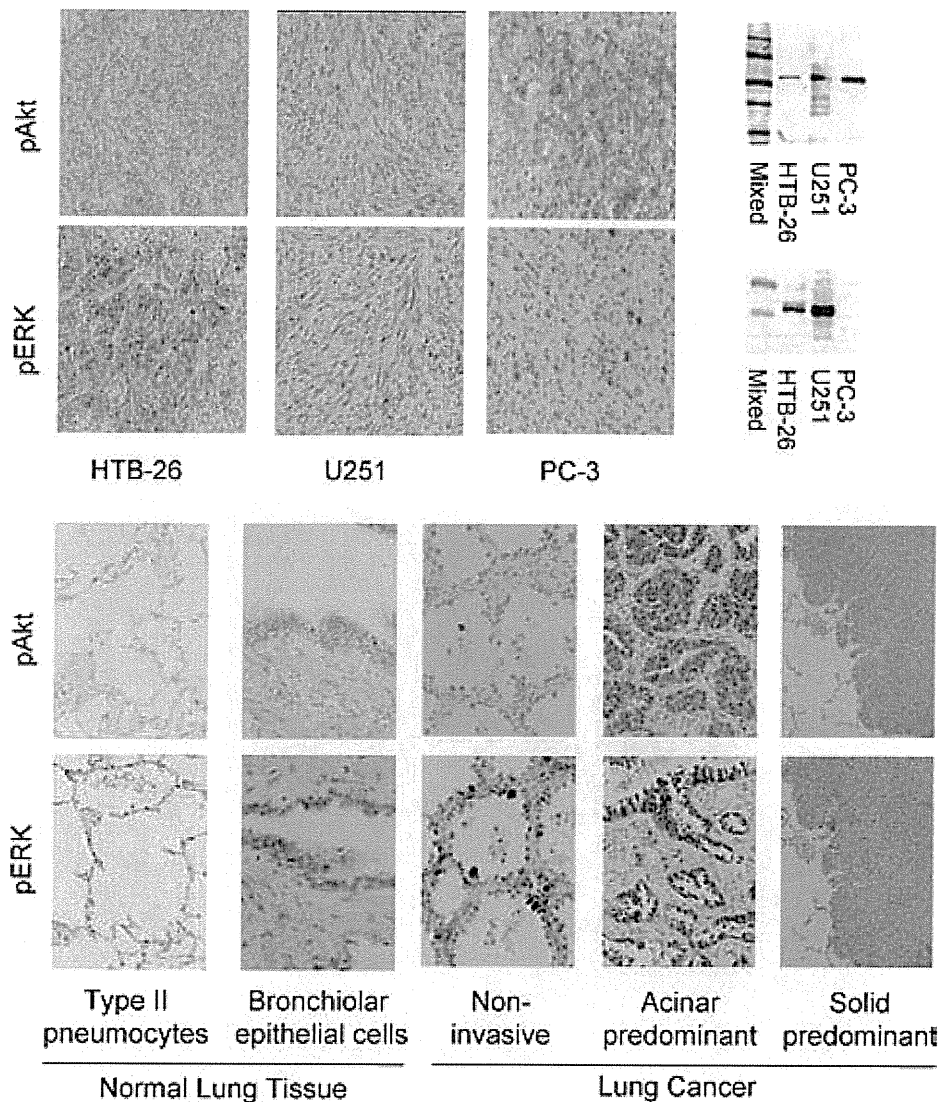


Fig. 1. Immunohistochemical staining of xenografts with specific antibody against pAkt (the upper row) and pERK1/2 (the lower row). (a) HTB-26; xenograft of breast cancer cell negative for pAkt and positive for pERK1/2; (b) U251; xenograft of glioma cell moderately positive for both pAkt and pERK1/2; (c) PC-3; xenograft of prostate cancer cell positive for pAkt and negative for pERK1/2. Almost all the cells with mitotic figure were scattered and were positive for pERK1/2. Immunohistochemical staining of resected lung specimen with specific antibody against pAkt (the upper row) and pERK1/2 (the lower row). Normal lung tissues (left side) and lung adenocarcinoma of noninvasive, acinar predominant and solid predominant histology (the right side). See that pAkt is almost homogeneously stained compared to that of pERK1/2.

immunohistochemical parameter were calculated and *p*-values for the statistical significance were given by a two-tails test checking a null hypothesis about zero Pearson's correlation coefficient between two variables.

Two-sided *p*-value below 0.05 was designated statistically significant.

3. Results

Patient characteristics of the 193 cases and differences in survival according to each clinicopathological factor or protein expression were shown in Table 1.

3.1. Patients and pathological review

The gender distribution was equal, the median age was 63 years and the median follow-up period was 2066 days (5.66 years) (ranged 133–3292 days). Pathological review revealed that more than 80% of the cases were classified as the adenocarcinoma with mixed subtype according to the current WHO classification. There-

fore results based on the predominance classification together with invasive/noninvasive dichotomy were presented here. 22.8% ($n=44$) of the cases were classified as pre- and minimally invasive adenocarcinomas. The rest (77.2%; $n=149$) were invasive adenocarcinoma in which papillary (including micropapillary), acinar, solid patterns or patterns of other variants are predominantly recognized. The rates were 61.1% ($n=118$), 9.9% ($n=19$) and 6.2% ($n=12$), respectively.

3.2. Immunohistochemical study and the EGFR/KRAS mutation status

We observed rather homogeneous and tumor-specific staining patterns for pAkt although the intensity was low, 37.8% (73/193) of surgically excised lung adenocarcinomas being positive. The positive rates for pGSK3B, pmTOR, pS6K, pFKHR and TTF-1 were 30.6% ($n=59$), 34.7% ($n=68$), 52.3% ($n=101$), 40.4% ($n=78$) and 79.8% ($n=154$), respectively. The pERK staining pattern was characteristically heterogeneous. The rate of positive tumor cells ranging from 5% to 100% and the staining was not tumor-cell specific but rather