

Clinical Significance of Insulin-like Growth Factor-binding Protein-3 Expression in Stage I Non-Small Cell Lung Cancer¹

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ABSTRACT

The activities of insulin-like growth factors (IGFs), including mitogenic and antiapoptotic properties, are modulated by a family of high-affinity insulin-like growth factor-binding proteins (IGFBPs), of which IGFBP-3 is the major serum carrier protein. Even though it is well known that IGFBP-3 plays an important role in cell proliferation, the expression of IGFBP-3 and its significance in primary non-small cell lung cancer (NSCLC) samples are unknown. This study explored IGFBP-3 expression in tumor samples from 74 patients with a diagnosis of pathological stage I NSCLC to determine if the expression status of IGFBP-3 influences the prognosis of patients with NSCLC. Two-sided statistical analyses were performed to correlate the clinical parameters and the prognostic effect with the IGFBP-3 expression level in this cohort. Reduced IGFBP-3 expression was found in 42 (56.8%) of 74 samples, and it was more frequent in large cell carcinoma than in squamous cell carcinoma and adenocarcinoma, although this difference was not statistically significant. This phenomenon was not associated with the other clinicopathological parameters tested, such as age, sex, histological grade, and smoking history. Significant statistical correlation between IGFBP-3 expression and disease-specific survival was noted ($P = 0.019$ by log-rank test). Although statistically nonsignificant, patients with decreased IGFBP-3 expression had shorter overall, disease-free, and event-free survival rates than did patients with normal IGFBP-3 expression. In a multivariate analysis

using IGFBP-3 expression and other clinicopathological parameters, the level of IGFBP-3 expression remained as an independent factor for predicting a shorter disease-specific survival probability ($P = 0.020$). Our work demonstrates that down-regulation of IGFBP-3 is a frequent event in stage I NSCLC and correlates with the disease-specific survival probability of patients with stage I NSCLC. These results suggest that IGFBP-3 functions as a tumor suppressor and plays an important role in determining biological aggressiveness in early NSCLC.

INTRODUCTION

NSCLC³ comprises a spectrum of phenotypic subtypes, including large cell, squamous carcinoma, and adenocarcinoma, and accounts for more than 80% of lung cancer. Surgical resection is the treatment of choice for patients with stage I and II disease, whereas the adjuvant chemotherapy and radiation therapy with their attendant toxicities have not contributed any survival advantage for these groups of patients as a whole (1). Nevertheless, up to 20% of stage I and 30% of stage II patients develop recurrent cancer, which is often incurable at the time of discovery. New biomarkers are needed to predict which patients have more aggressive disease because these patients could benefit from adjuvant radiation therapy or chemotherapy and close monitoring for disease recurrence. In this way, biomarkers that are predictive of aggressive disease could improve the survival of early-stage NSCLC. Furthermore, markers of aggressive cancer behavior can identify new mechanisms for the biological therapy of cancer.

The development of NSCLC involves numerous abnormal cellular activities, involving cell differentiation, transformation, proliferation, and apoptosis, that are maintained and controlled by a large number of peptide growth factors (2). Among the growth factors, IGFs play a crucial role in regulating cell proliferation and differentiation. IGFs including IGF-I and IGF-II are peptide hormones with strong mitogenic effects on both normal and cancer cells, including lung cancer cells (2–4). In addition to stimulating cell proliferation, IGFs also suppress apoptosis (5). The effects of the IGFs on cell proliferation and apoptosis are mediated by a specific cell-membrane receptor, IGF-1R, which has been involved in cell transformation (6, 7).

These activities of IGFs are modulated by a family of IGFBPs, especially by IGFBP-3, a major serum carrier protein for IGFs (8, 9). Outside the circulation, IGFBP-3 has been found to be a negative regulator of cell proliferation in lung and other tissues (10, 11). This growth inhibition has been attributed not only to the reduction of bioactivity of IGFs, but also to an IGF-independent antiproliferative activity that appears to in-

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³ The abbreviations used are: NSCLC, non-small cell lung cancer; IGFBP-3, insulin-like growth factor-binding protein-3.

Table 1 Expression status of IGFBP-3 according to the clinicopathological features of patients with stage I NSCLC

	Loss of expression (42/74)	Without loss of expression (32/74)	Total	<i>P</i>
Age, yr, mean \pm SD	63.3 \pm 9.93	63.1 \pm 9.02	63.2 \pm 9.48	0.951
Sex, <i>n</i> (%)				0.796
Male	30 (40.5)	24 (32.4)	54	
Female	12 (16.2)	8 (10.8)	20	
Pathology, <i>n</i> (%)				0.083
Adenocarcinoma	20 (27.0)	18 (24.3)		
Squamous carcinoma	16 (21.6)	14 (18.9)		
Others	6 (8.1)	0 (0.0)		
Histological grade, <i>n</i> (%)				0.186
Well differentiated	2 (2.7)	0 (0.0)	2	
Moderately differentiated	14 (18.9)	13 (17.5)	27	
Poorly differentiated	18 (24.3)	11 (14.9)	29	
Undifferentiated/Anaplastic	3 (4.1)	0 (0.0)	3	
Unclassified	5 (6.8)	8 (10.8)	13	
Smoking status, <i>n</i> (%)				0.43
Current; pack-year	37 (50.0); 62.2 \pm 39.53	30 (40.5); 69.6 \pm 36.04	67 (90.5); 62.7 \pm 39.41	0.32
Nonsmoker	3 (4.1)	1 (1.4)	4 (5.4)	
Unknown	2 (2.7)	1 (1.4)	3 (4.1)	
5-year survival probability (%)				
Overall	51.3	71.0		0.0876
Disease specific	64.6	85.7		0.0193
Disease free	54.4	71.4		0.1025

involve cell surface receptors for IGFBP-3 (9, 12). The expression of IGFBP-3 is increased by growth-inhibiting agents such as transforming growth factor- β 1 (13), vitamin D-related compounds (14), retinoic acid (15), antiestrogens (16), antiandrogens (17), the *p53* gene (18), and tumor necrosis factor- α (19), suggesting that these agents may mediate their cellular effects through IGFBP-3. Additionally, it has been recently demonstrated that sodium butyrate and trichostatin A also up-regulate IGFBP-3 synthesis and secretion (20). The dual regulatory effects of the IGFBPs are further modulated by many factors including the IGFBP proteases, which include prostate-specific antigen and Cathepsin D (4, 21, 22). Cell culture experiments (10, 23–25) have demonstrated that most lung cancer cell lines are able to express IGFs and their binding proteins. Although IGFs are known to be potent mitogens for lung cancer cells, the level of IGFBP-3 in NSCLC tissue and its significance remain undetermined.

In this study, we investigated the IGFBP-3 expression in NSCLC patients using NSCLC samples from 74 patients with stage I NSCLC, and correlated the results with the clinicopathological parameters. Statistical analysis was performed to determine the prognostic effect of the IGFBP-3 expression in primary NSCLC on various clinical parameters.

MATERIALS AND METHODS

Study Population. Tissue specimens were obtained at surgery from a total of 74 patients whose diagnosis revealed NSCLC and who had undergone curative surgical removal of a primary lesion at The University of Texas M. D. Anderson Cancer Center from October 1975 through April 1993. Pathological evaluation established the histological classification and staging in all of the patients. None of the patients had either

radiotherapy or chemotherapy before or after surgery until the disease recurred. Patients' ages ranged from 37.8 to 82.7 years, with a mean age of 63.2 \pm 9.48 years, which is similar to the age distribution in the large database of patients with stage I NSCLC from our institution (data not shown). Fifty-four (73%) of the patients were men and 20 (27%) were women. All of the clinical and pathological information and follow-up data were based on reports from our tumor registry service. The study was reviewed and approved by the institution's Surveillance Committee to allow us to obtain tissue blocks and other pertinent information from the patients' files. The general clinical characteristics of the patients are shown in Table 1.

Immunohistochemical Staining for IGFBP-3. Paraffin-embedded, 4- μ m-thick tissue sections from 74 primary NSCLC samples were stained for the IGFBP-3 protein using a rabbit polyclonal antibody against human IGFBP-3 (Diagnostic Systems Laboratories, Inc., Webster, TX). All of the sections were deparaffinized in a series of xylene baths and then rehydrated using a graded alcohol series. The sections were then immersed in methanol containing 0.3% hydrogen peroxidase for 20 min to block endogenous peroxidase activity and then incubated in 2.5% blocking serum to reduce nonspecific binding. Sections were incubated overnight at 4°C with primary anti-IGFBP-3 antibody (1:100). The sections were then processed using standard avidin-biotin immunohistochemical techniques according to the manufacturer's recommendations (Vector Laboratories, Burlingame, CA). Diaminobenzidine was used as a chromogen, and commercial hematoxylin was used for counterstaining. Three human NSCLC cell lines, H1944, H460, and A549, which have intrinsic IGFBP-3 expression, were stained at the same time to serve as positive controls. Adjacent normal-appearing bronchial epithelium within each tissue section served as an internal reference. Representative areas of each tissue

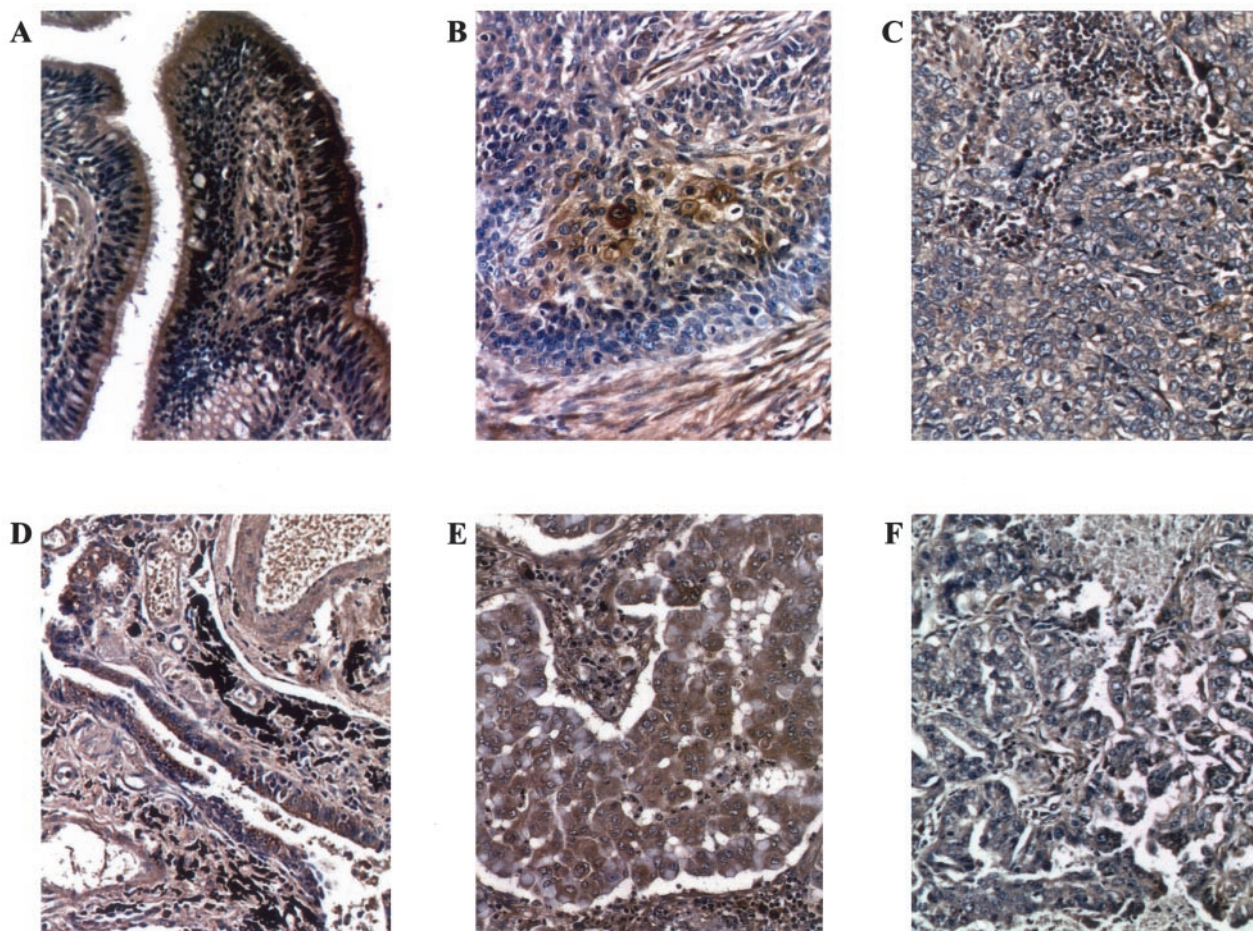


Fig. 1 Immunohistochemical analysis of IGFBP-3 expression in bronchial epithelium and primary NSCLC tissues. The staining for IGFBP-3 was prominent in the cytoplasm of histologically normal bronchial epithelial cell layers. Frequent nuclear staining was also observed in the basal, parabasal, and ciliated cells (A). The bronchial epithelial cells comprising small airways revealed similar staining patterns (D). Normal bronchial epithelial layers showed a homogeneous staining pattern for IGFBP-3, whereas, in the majority of tumor tissues, a heterogeneous pattern of negative staining (C and F), scattered focal positive staining (B), and positive staining (E) were seen. But we could not find IGFBP-3 expression in the nuclei of tumor cells examined in this study (B, C, E, and F).

section were selected, and cells were counted in at least four fields (at $\times 200$). IGFBP-3 labeling index was defined as the percentage of tumor cells displaying membranous, cytoplasmic, or nuclear immunoreactivity; and it was calculated by counting the number of IGFBP-3-stained tumor cells among more than 1000 tumor cells from representative areas of each tissue section. In this study, we used a 5% labeling index as a cutoff point. On the basis of the results of the immunohistochemical staining, tissue sections showing less than 5% of positive staining were considered as down-regulation of IGFBP-3. All of the slides were evaluated and scored independently by two pathologists (A. K. E-N. and K. G.) who were blinded to the clinical information of the subjects.

Statistical Analysis. In univariate analysis, independent sample *t* tests and χ^2 tests were used for continuous and categorical variables, respectively. The Kaplan-Meier estimator was used to compute survival probability as a function of time. The log-rank test was used to compare patients' survival time be-

tween groups. Overall, disease-specific, event-free, and disease-free survival times were analyzed. Cox regression was used to model the risk of the loss of expression on survival time, with adjustment for clinical and histopathological parameters (age, sex, tumor histology subgroup, grade of differentiation, and smoking status). All of the statistical tests were two-sided. $P < 0.05$ was considered to be statistically significant.

RESULTS

Expression of IGFBP-3 in Histologically Normal Lung Tissues and NSCLC. The staining for IGFBP-3 was prominent in the cytoplasm of histologically normal bronchial epithelial cell layers. The intensity of the staining was moderate to strong, and the distribution was homogeneous (Fig. 1A). The expression of IGFBP-3 was also noted in the epithelium of small airways (Fig. 1D). The staining pattern of normal bronchial epithelium was consistent and was used as a reference. In

addition, frequent nuclear staining was also observed in the basal, parabasal, and ciliated cells. The human NSCLC cell lines, H1944, H460, and A549 NSCLC cells, which exhibited strong IGFBP-3 expression by Western blot analysis, were used as a positive control, and H226Br cells, which showed no IGFBP-3 expression, were used as a negative control (data not shown). Normal cells showed a homogeneous staining pattern for IGFBP-3, but in the majority of tumor tissue, a heterogeneous pattern of negative staining (Fig. 1, C and F), scattered positive staining (Fig. 1B), and positive staining was seen (Fig. 1E). In contrast to the previous reports of nuclear staining of the IGFBP-3 in NSCLC cell lines (10), we observed that IGFBP-3 expression was localized mainly in the cytoplasm and was not detected in the nuclei of tumor cells (Fig. 1, B and E). In addition, histologically well-differentiated tumors showed more frequent and intense IGFBP-3 staining, although it was not statistically significant ($P = 0.186$; Table 1). Five cases showed typical membranous staining that was not related to histological subtype or grade; two cases were adenocarcinoma, two cases were squamous cell carcinoma, and the other was diagnosed as a large cell carcinoma.

Clinicopathological Parameters Associated with Loss of IGFBP-3 Expression. To this date, there has been no available labeling index for the staining of IGFBP-3 in NSCLC; and, therefore, we applied a 5% labeling index as a cutoff point for the down-regulation of IGFBP-3. On the basis of this criterion, 42 (56.8%) of the 74 stage I NSCLC specimens showed a loss of IGFBP-3 expression. Table 1 summarizes the associations between the IGFBP-3 expression status and the clinicopathological parameters. The IGFBP-3 expression status did not differ significantly with respect to age, gender, smoking status, pack-years, or histological grade of differentiation. There were no differences in the frequency of loss of expression between adenocarcinoma and squamous carcinoma, but a loss of IGFBP-3 expression was, however, frequent in large cell and unspecified carcinomas. The smoking status of 71 of 74 patients was known; 69 patients had been smokers, and 67 were current smokers at the time of diagnosis. The mean number of pack-years for these 67 people was 62.7 ± 39.41 . There was also no difference in the distribution of smoking status or pack-years between groups that showed down-regulation of IGFBP-3 and those that did not show down-regulation. Three patients had a history of exposure to asbestos, but the associations between IGFBP-3 expression and this parameter are not provided because of the small sample size.

Down-Regulation of IGFBP-3 Expression in NSCLC Related to Patients' Prognosis. We analyzed the relationship between IGFBP-3 expression and patients' clinical outcomes. The probability of 5-year overall survival in this study population was 59.8% (95% confidence interval, 49.5–72.3), which is similar to the probabilities reported in a previous study with a large number of cases from our institution (26). Of the 74 patients, 49 patients died, and 25 patients were still alive at the time of the last follow-up report. Of the 49 patients who died, 20 died of lung cancer, and 29 patients died of other causes. The median follow-up duration among the patients who remained alive was 10.5 years. Thirty (71.4%) of the 42 patients whose

tumors showed loss of IGFBP-3 expression were dead, whereas 19 (59.4%) of the 32 patients whose tumors showed IGFBP-3 expression were dead during the follow-up time. However, the difference did not reach statistical significance ($P = 0.0876$ by log-rank test; Fig. 2A). Of the 42 patients whose tumors lost expression of IGFBP-3, 15 (35.7%) patients died of cancer or a cancer-related cause; only 5 (15.6%) of the 32 patients whose tumors showed IGFBP-3 expression died of cancer or a related cause. Patients who have tumors with low IGFBP-3 expression showed significantly shorter disease-specific survival ($P = 0.0193$ by log-rank test; Fig. 2B). The 5-year disease-free survival probability for patients whose tumors showed loss of IGFBP-3 expression was 54.4% as compared with 71.4% in patients whose tumors showed IGFBP-3 expression ($P = 0.1025$ by log-rank test; Fig. 2C). In a multivariate analysis using IGFBP-3 expression and other clinicopathological parameters, IGFBP-3 remained an independent prognostic factor for disease-specific survival time ($P = 0.0124$).

DISCUSSION

IGFs are potent mitogens in several types of cancer, including NSCLC and SCLC (24, 27), and IGFBP-3, which is a major serum carrier protein for the IGFs, regulates cell growth by sequestering IGFs away from its receptors in the extracellular milieu and thereby inhibiting the mitogenic and antiapoptotic action of IGF-I.

There is growing evidence demonstrating the growth-regulatory effects of IGFBP-3 on several types of cancer cells (8–12). We have previously shown that overexpression of IGFBP-3 reduces the growth of a subset of NSCLC cells *in vitro* and *in vivo* by inducing apoptosis (28). An inverse relationship exists between serum or plasma levels of IGFBP-3 and the risk of death or metastases from many cancers including breast, colon and rectum, prostate, and childhood leukemia as well as lung cancer. Additionally, Yu *et al.* (2) showed a negative correlation between serum IGFBP-3 levels and lung cancer risk, indicating a protective role of IGFBP-3 against lung cancer. These results strongly support the function of IGFBP-3 as a tumor suppressor.

In the present study, we focused on the expression level of IGFBP-3 in patients with stage I NSCLC and its significance in clinical outcome. Our results showed that IGFBP-3 expression is down-regulated in a significant fraction of patients with stage I NSCLC. Compared with the staining pattern seen in the normal-appearing bronchial epithelium, a large subset of stage I NSCLC tumors showed down-regulation of IGFBP-3 expression. Overall, 42 (56.8%) of 74 tumors expressed IGFBP-3 in <5% of the tumor cells examined. Five (6.8%) of 74 cases showed a typical membranous staining pattern, which was not related to histological subtype or grade of differentiation. Interestingly, the expression of IGFBP-3 was localized in the cytoplasm rather than in the nucleus of NSCLC cells. The significance of this phenomenon is unclear, but one explanation could be the presence of a signaling mechanism that induces the degradation of IGFBP-3 in the nucleus and inhibits the activity of IGFBP-3 as a transcriptional factor. Our data showed that low IGFBP-3 expression is an unfavorable prognostic factor in patients

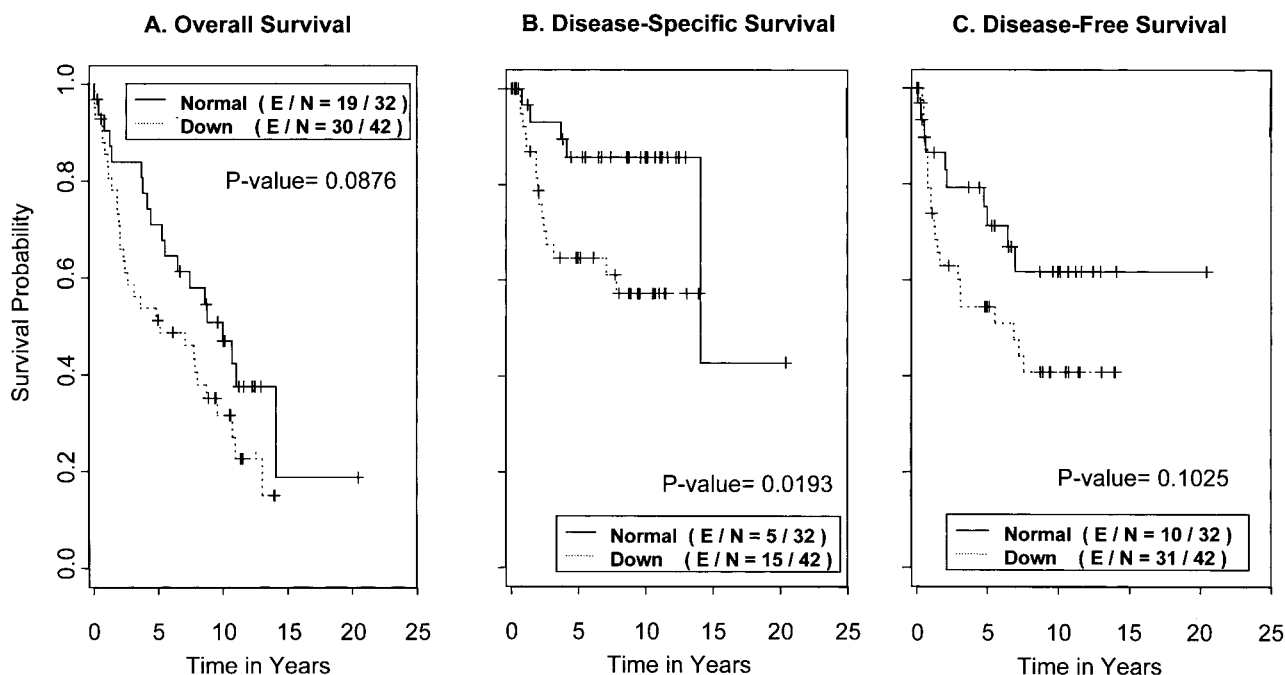


Fig. 2 The Kaplan-Meier curves of patients with stage I NSCLC stratified by IGFBP-3 expression. The probability of 5-year overall survival of patients whose tumors showed loss of IGFBP-3 expression was 51.3% as compared with 71.3% for patients whose tumors showed IGFBP-3 expression (A). The probability of 5-year disease-specific survival of patients whose tumors showed loss of IGFBP-3 expression was 64.3% as compared with 85.7% in patients whose tumors showed IGFBP-3 expression (B). The 5-year disease-free survival rate for patients whose tumors showed loss of IGFBP-3 expression was 54.4% as compared with 71.4% for patients whose tumors showed IGFBP-3 expression (C).

with stage I NSCLC; a significant inverse relationship between the loss of IGFBP-3 expression and the disease-specific survival probability was observed. According to a multivariate analysis, IGFBP-3 was an independent prognostic factor for disease-specific survival. We found that loss of IGFBP-3 expression was closely associated with hypermethylation of IGFBP-3 promoter and that IGFBP-3 methylation status was strongly correlated with poor prognosis among patients with stages I NSCLC (29). Aberrant methylation of CpG islands in the promoter region has been associated with transcriptional inactivation of gene expression (30). Therefore, expression of IGFBP-3 in NSCLC cells containing hypermethylation in its promoter was likely suppressed. These findings suggest that IGFBP-3 is a potential prognostic marker as well as a therapeutic target in NSCLC cells. However, two case-control studies in breast cancer (31) and prostate cancer (32) showed increased risk in association with higher levels of IGFBP-3, although these findings were not statistically significant. This interesting phenomenon could be explained by the ability of IGFBP-3 to potentiate IGF-I bioactivity in several different cell types (33–34). Although the mechanism is largely unknown, enhancement of the actions of IGF-I is thought to occur after the association of IGFBP-3 with the cell membrane and thereby facilitating the binding of IGF-1 to its receptor (35). Alternatively, posttranslational mechanisms such as phosphorylation, glycosylation, and proteolysis are important in determining the regulatory impact of IGFBPs on IGF action (33). Prostate-specific antigen, Cathepsin D, and matrix metalloproteinases including interstitial collagenase, gelatinase A, stromelysin 1, gelatinase B, and

disintegrin metalloproteinase and other proteolytic enzymes such as plasmin, thrombin, and pregnancy-associated plasma protein-A are involved in the proteolysis of IGFBPs, whereby the binding affinity of IGFBP to IGF-I is reduced, and the mitogenic activity of IGF-I is restored (21, 36–41). It has also been shown that IGFs modulate the proteolytic activities of IGFBPs, suggesting that IGFs have an autocrine regulatory loop to control their own action (42). Whether IGFBP-3 assumes an inhibitory or enhancing role may depend on the cell type and the compound that induces its expression, and additional investigations to address the function of IGFBP-3 in cancer development are required. Nevertheless, our observations in this study are compatible with previous results from *in vitro* and *in vivo* studies demonstrating antimitogenic and anti-apoptotic effects of IGFBP-3 on lung cancer cells (28) through the regulation of IGF-I binding to IGF-I receptor as well as through an IGF-I-independent growth-inhibitory mechanism.

In summary, this study has shown by using a homologous population of 74 patients with stage I NSCLC that a loss of IGFBP-3 expression is frequent in stage I NSCLC and that such a loss was associated with how long patients lived. To our knowledge, this is the first report that correlates a loss of IGFBP-3 expression with clinical outcomes in stage I NSCLC. More comprehensive studies involving the mechanism that induces the loss of IGFBP-3 expression in NSCLC cells are necessary to define the role of IGFBP-3 in lung carcinogenesis.

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Correction

In the article by Yoon Soo Chang *et al.*, which appeared in the December 2002 issue of *Clinical Cancer Research* (pp. 3796–3802), the author “Koo Gong” was misspelled and listed

incorrect affiliations. The correct author name and affiliation are: Gu Kong, Department of Pathology, College of Medicine, Hanyang University, Seoul, Korea 133-791.

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