

Advances in Brief

Correlation between Insulin-like Growth Factor-binding Protein-3 Promoter Methylation and Prognosis of Patients with Stage I Non-Small Cell Lung Cancer¹

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Abstract

Purpose: The activities of insulin-like growth factors (IGFs) in regulating cell proliferation, differentiation, and apoptosis are modulated by a family of high-affinity specific IGF-binding proteins (IGFBPs), especially IGFBP-3, the most abundant IGFBP in circulation. Hypermethylation of the promoter represses the expression of the *IGFBP-3* gene. The purpose of this study was to determine whether the methylation status of IGFBP-3 promoter influences the prognosis of non-small cell lung cancer (NSCLC).

Experimental Design: Eighty-three patients with pathological stage I NSCLC who had undergone curative surgery were investigated for promoter hypermethylation of IGFBP-3 by methylation-specific PCR. Statistical analyses, all two-sided, were performed to determine the prognostic effect of methylation status of the IGFBP-3 promoter on various clinical parameters. IGFBP-3 was the only molecular parameter tested on these tissues in this study.

Results: Hypermethylation of the IGFBP-3 promoter was found in 51 (61.5%) of the 83 tumors. The clinicopathological factors, such as age, histological type, histological grade, gender, and smoking status, of corresponding patients, did not exhibit statistically significant association with the methylation status of IGFBP-3 promoter. However, patients with a hypermethylated IGFBP-3 promoter had a significantly lower 5-year disease-specific, disease-free, and overall survival rate than did those without a methylated

IGFBP-3 promoter (53.1% versus 86.1%, $P = 0.006$; 36.5% versus 76.2%, $P = 0.007$; and 38.9% versus 64.0%, $P = 0.022$, respectively). Moreover, multivariate analysis indicated that hypermethylation of the IGFBP-3 promoter was the only independent predictor for disease-free and disease-specific survival among the clinical and histological parameters tested.

Conclusions: Hypermethylation of the IGFBP-3 promoter, as measured by methylation-specific PCR, is a frequent phenomenon and strongly associated with poor prognosis among patients with stage I NSCLC.

Introduction

NSCLC³ accounts for ~75–80% of lung cancer cases, and its dismal survival rate has improved only marginally in the past 2 decades. Surgical resection is the treatment of choice for patients with early-stage disease. Patients with advanced disease are treated with surgery, chemotherapy, radiation therapy, or combinations of the above, all of which have substantial side effects. Despite these treatments, the 5-year survival rate of patients with NSCLC remains low (1), illustrating the need for more effective strategies for early diagnosis and chemoprevention. To identify useful biomarkers for early detection and chemoprevention strategies in lung cancer, it is important to understand the genetic abnormalities that occur during lung tumorigenesis.

Tumorigenesis is a multistep process that results from the accumulation and interplay of genetic and epigenetic changes (2). Overexpression of dominant oncogenes and inactivation of tumor-suppressor genes by genetic changes, such as mutations in *p53*, occur frequently in lung cancer patients and generally are poor prognostic factors for this disease. Epigenetic changes, including aberrant methylation of normally unmethylated CpG islands in the promoter region, are also implicated in lung tumorigenesis (reviewed in Ref. 3). Aberrant methylation of CpG islands in the promoter region, the covalent addition of a methyl group to the 5 position of cytosine, has been associated with transcriptional inactivation of gene expression by alterations of chromatin structure that directly prevent the binding of transcription factors (4). An increasing number of genes have been found to play important roles in cell-cycle control, DNA damage repair, the inhibition of apoptosis, the invasion of tumors, and growth factor response (3, 5). Thus, methylation of DNA involved in these pathways may have a critical role in lung tumor progression (5–7).

Received 5/31/02; revised 7/18/02; accepted 7/28/02.

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¹ Supported in part by M. D. Anderson Cancer Center Institutional Grant RP33763 (to H-Y. L.), National Cancer Institute Grant U19 CA 68437 (to W. K. H.), and the Tobacco Research Fund from the State of Texas (to M. D. Anderson Cancer Center). W. K. H. is an American Cancer Society Clinical Research Professor.

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³ The abbreviations used are: NSCLC, non-small cell lung cancer; IGFBP-3, insulin-like growth factor-binding protein 3; MSP, methylation-specific PCR; DAP, death-associated protein; CI, confidence interval.

IGFBP-3, the most abundant IGFBP in circulation, regulates the role of IGFs in cellular growth, differentiation, transformation, and apoptosis (8–11). The finding of a negative correlation between serum IGFBP-3 levels and lung cancer risk (12) suggests that IGFBP-3 protects against the effects of systemic IGF-I. IGFBP-3 also has IGF-I-independent antiproliferative and proapoptotic effects, as shown by the finding that IGFBP-3 overexpression inhibits the growth of fibroblasts that are IGF-1 receptor (IGF-1R) null (13, 14). Overexpression of IGFBP-3 has significant growth-inhibitory effects on NSCLC *in vitro* and *in vivo*, indicating the potential of IGFBP-3 as a tumor suppressor in lung cancer (15). Expression of IGFBP-3 is induced by other growth-inhibitory (and apoptosis-inducing) agents, such as tumor growth factor- β 1 (16), tumor necrosis factor- α (17), retinoic acid (18), anti-estrogen ICI 182780 (19), vitamin D and its analogues EB1089 and CB1093 (20, 21), and transcription factor p53 (22), raising the possibility that these agents mediate their cellular effects through IGFBP-3. It has been shown that the expression of IGFBP-3 was repressed by the polymorphism or hypermethylation in the promoter region of the gene (23, 24).

In the present study, we sought to elucidate whether hypermethylation of the IGFBP-3 promoter is associated with the prognosis of patients. We analyzed surgically resected primary tumor specimens from 83 patients with pathological stage I NSCLC for the methylation status of CpG sites in the 5' end of the *IGFBP-3* gene. Other molecular parameters have been tested in previous study using the same tissues (*e.g.*, the expression of PTEN, cyclooxygenase-2, cyclin B1, RAR β , Fhit, and E-cadherin and type IV collagenase, the hypermethylation of the DAP kinase promoter, and microsatellite alteration profiles; Refs. 25–32). In this study, we have tested IGFBP-3 as a molecular parameter specifically. In addition, statistical analysis was performed to determine the prognostic effect of the hypermethylation status of IGFBP-3 on various clinical parameters.

Materials and Methods

Clinical Samples. Formalin-fixed, paraffin-embedded tissue blocks of lung cancer were obtained from surgical specimens from 83 patients who were diagnosed with pathological stage I NSCLC and who had undergone curative surgical removal of a primary lesion at The University of Texas M. D. Anderson Cancer Center from 1975 through 1998. Tissue sections (4 μ m thick) were obtained from each block, stained with H&E, and reviewed by a pathologist to confirm the diagnosis and the presence or absence of tumor cells in these sections. All of the information, including clinical, pathological, and follow-up data, was 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 all pertinent information.

Microdissection and DNA Extraction. DNA was extracted from microdissected tumor specimens as described previously (33, 34). Briefly, tumor parts in sections from formalin-fixed and paraffin-embedded tissue blocks were dissected under a stereomicroscope. Dissected tissues were digested in 200 μ l of digestion buffer containing 50 mM Tris-HCl (pH 8.0), 1% SDS, and proteinase K (0.5 mg/ml) at 42°C for 36 h. The purification of digested products was performed by phenol/chloroform extraction. DNA

was then precipitated by the ethanol precipitation method in the presence of glycogen (Boehringer Mannheim Biochemicals, Indianapolis, IN) and recovered in distilled water.

Bisulfite Modification and MSP. The entire DNA from the microdissected tumor samples was mixed with 1 μ g of salmon sperm DNA (Life Technologies, Inc., Gaithersburg, MD) and submitted for chemical modification as described by Herman *et al.* (35). Briefly, DNA was denatured with 2 M NaOH, followed by treatment with 10 mM hydroquinone and 3 M sodium bisulfite (Sigma Chemical Co., St. Louis, MO). After it was purified in a Wizard SV Plus kit column (Promega, Madison, WI), the DNA was treated with 3 M NaOH and precipitated with three volumes of 100% ethanol, and a one-third volume of 10 M NH₄OAc at room temperature over 30 min. The precipitated DNA was washed with 70% ethanol and dissolved in 20 μ l of distilled water. PCR was conducted with primers specific for either the methylated or the unmethylated sequence of the IGFBP-3 promoter. Methylated primers consisted of BP-3 sense 5'-CGAAGTACGGGTTTCGTAGTCG-3' and BP-3 antisense 5'-CGACCCGAACGCGCCGACC-3'; unmethylated primers consisted of BP-3 sense 5'-TTGGTTGTTTAGGGTGAAGTATGGGT-3' and BP-3 antisense 5'-CACCCAACCACAATACTCACATC-3'. The 25- μ l total-reaction volume contained 2 μ l of modified DNA, 1% DMSO, all four deoxynucleoside triphosphates (each at 200 μ M), 1.5 mM MgCl₂, 0.4 μ M PCR primers, and 0.25 units of HotStar TaqDNA polymerase (Qiagen, Valencia, CA). Negative control samples without DNA were included for each set of PCR. For methylated PCR, DNA was amplified by an initial cycle at 95°C for 15 min as required for enzyme activation, followed by 40 cycles of 94°C for 30 s, 66°C for 1 min, and 72°C for 1 min and ending with a 5-min extension at 72°C for 1 min in a thermocycler (Applied Biosystems, Foster City, CA). For unmethylated PCR, the annealing temperature was 64°C. PCR products were separated on 2.5% agarose gels and visualized after staining with ethidium bromide.

Statistical Analysis. In univariate analysis, independent sample *t* and χ^2 tests were used to analyze continuous and categorical variables, respectively. Survival probability as a function of time was computed by the Kaplan-Meier estimator. The log-rank test was used to compare patient survival time between groups. Overall survival, disease-specific survival (from the date of diagnosis to date of death specifically from cancer-related causes), and disease-free survival time (from the date of completion of surgery to the date of relapse or death of cancer-related causes) were analyzed. Cox regression was used to model the influence of promoter hypermethylation on survival time, with adjustment for clinical and histopathological parameters (age, sex, tumor histology subgroup, and smoking status). The two-sided test was used to test equal proportion between groups in two-way contingency tables. All of the statistical tests were two-sided. *P* < 0.05 was considered to be statistically significant.

Results

Hypermethylation of IGFBP-3 Promoter in Stage I NSCLC and Clinicopathological Characteristics. A total of 83 patients with pathologically confirmed stage I NSCLC were evaluated for the methylation status of the IGFBP-3 promoter.

Table 1 Analysis of the patients with stage I NSCLC according to the methylation status of IGFBP-3 promoter

	Methylated group (51/83)	Unmethylated group (32/83)	<i>P</i>
Age, yr, mean \pm SD	64.7 \pm 8.83	66.2 \pm 8.92	0.461
Gender, <i>n</i> (%)			
Male	33 (39.8)	22 (26.5)	0.704
Female	18 (21.7)	10 (12.0)	
Pathology, <i>n</i> (%)			
Adenocarcinoma	28 (33.7)	12 (14.5)	0.101
Squamous carcinoma	21 (25.3)	15 (18.1)	
Others	2 (2.4)	5 (6.0)	
Histologic grade, <i>n</i> (%)			
Well-differentiated	3 (3.6)	1 (1.2)	0.913
Moderately differentiated	20 (24.1)	11 (13.3)	
Poorly differentiated	20 (24.1)	16 (19.3)	
Undifferentiated/Anaplastic	2 (2.4)	1 (1.2)	
Unclassified	6 (7.2)	3 (3.6)	
Smoking status, <i>n</i> (%)			
Current, pack-year	43 (51.8); 67.6 \pm 40.89	27 (32.5); 63.2 \pm 34.19	0.643
Ex-smoker	2 (2.4)	0 (0.0)	
Nonsmoker	2 (2.4)	1 (1.2)	
Unknown	4 (4.8)	4 (4.8)	
5-yr survival <i>P</i> (%) (95% CI)			
Overall	38.9 (27.3–55.4)	64.0 (48.9–83.7)	0.022
Disease specific	53.1 (40.0–70.6)	86.1 (74.3–99.8)	0.006
Disease free	36.5 (24.3–55.0)	76.2 (62.3–93.3)	0.007



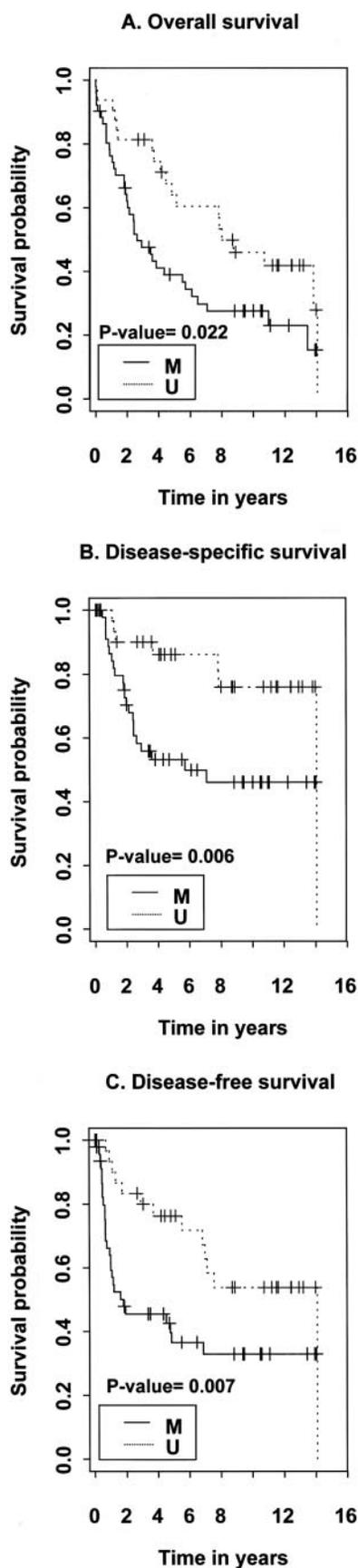
Fig. 1 MSP analysis performed on stage I NSCLC samples. Representative results using tumor tissues from seven stage I NSCLC patients are shown. Lanes U, MSP product with primers recognizing unmethylated IGFBP-3 promoter; Lanes M, MSP product with primers recognizing methylated IGFBP-3 alleles. Numbers above each gel, the primary tumor analyzed. Among the tumor data shown, methylation was observed in tumors T69, T75, T76, T79, and T81.

The general clinical characteristics of the patients are shown in Table 1. The frequencies of IGFBP-3 promoter hypermethylation in microdissected primary NSCLC samples and the corresponding nonmalignant adjacent lung tissues were determined by MSP using the primers designed specifically for methylated and unmethylated sequences. The IGFBP-3 promoter was methylated in 51 (61.4%) of 83 stage I NSCLC samples. The representative results of 7 tumor samples are shown in Fig. 1. Most of the tumor samples showed the bands that correspond to either unmethylated (158-bp) or methylated (129-bp), or to both. PCR products obtained from both methylated and unmethylated primer in selected cases were directly analyzed and verified for the expected methylated or unmethylated status by sequencing (data not shown).

In univariate analysis, no statistically significant association was found between the hypermethylation of IGFBP-3 and clinicopathological factors, such as age, histological type, histological grade, sex, and smoking status, of corresponding patients. The age of patients with stage I NSCLC ranged from 39 to 83 years (mean, 65.3 \pm 8.84 years), a range that is similar to the age distribution in the large database of NSCLC patients from our institution during the period from 1975 to 1998 (data not shown). Fifty-five (66.3%) of the patients were men, and 28 (33.4%) were women. This distribution also is similar to the

gender distribution from our institution and is comparable with the gender distribution of the disease in the 1970s and the 1980s (36). Of these patients, all except five had a smoking history. Two patients had stopped smoking before diagnosis, but 70 patients had continued to smoke until the time of diagnosis. The mean number of pack-years of the 70 current smokers at the time of diagnosis was 63.8 \pm 39.42. We also found no differences in the distribution of smoking status or pack-years between the methylated and unmethylated groups.

Methylation Status of IGFBP-3 Promoter and Prognosis. Kaplan-Meier estimate was used to examine the relationship between methylation status of IGFBP-3 and patient survival. The probability of 5-year overall survival among all of the patients evaluated was 56.7%, similar to that reported in a previous study with a large number of patients from our institution (37). Of the 83 patients with stage I NSCLC, 49 died, and 34 were still alive at the time of the last follow-up report. Of the 49 patients who died, 29 died of lung cancer and 20 died of other causes. The median follow-up duration is 10.3 years among the patients who remain alive. None of the stage I NSCLC patients received chemotherapy or radiation therapy before or after surgery. We found that patients whose primary tumors exhibited hypermethylation at the CpG sites of the IGFBP-3 gene had a statistically significant poorer overall survival probability ($P =$



0.022, log-rank test). Furthermore, these patients had a poorer disease-specific survival probability ($P = 0.006$) and disease-free survival probability ($P = 0.007$) compared with the group with the unmethylated promoter at 5 years after diagnosis (Fig. 2). We also analyzed potential associations between the methylation status and disease-specific and disease-free survival probability in histological subgroups. We found that promoter hypermethylation was associated with a statistically significant poorer disease-specific and disease-free survival for squamous cell carcinoma ($P = 0.048$ and $P = 0.021$, respectively; Fig. 3). A similar trend in disease-specific and disease-free survival was observed with other histological types (*i.e.*, adenocarcinoma, large-cell carcinoma, and unclassified tumors; $P = 0.0497$ and $P = 0.055$, respectively). To determine whether hypermethylation of the IGFBP-3 promoter is an independent factor in predicting survival duration for patients with pathological stage I NSCLC, we performed multivariate analysis using the Cox model. The factors used in the multivariate analysis include age, gender, histological type, histological grade, and smoking status that are considered to be associated with poor prognosis of early stage NSCLC. Promoter methylation status was the only independent predictor for disease-free and disease-specific survival among the clinical and histological parameters tested.

Discussion

DNA methylation, the major form of epigenetic information in mammalian cells, has profound effects on the mammalian genome, including transcriptional repression, chromatin structure modulation, X-chromosome inactivation, genomic imprinting, and suppression of the detrimental effects of repetitive and parasitic DNA sequences on genome integrity (38–40). A recent study using a monoclonal antibody specific for 5-methylcytosine to evaluate the status of global DNA methylation suggests that alteration in DNA methylation is an important epigenetic difference in susceptibility for the development of lung cancer (41).

Genomic methylation patterns are frequently altered in tumor cells, with global hypomethylation accompanying region-specific hypermethylation events. Most often, the CpG islands are located at the promoter and first exon of the associated gene. Although these sequences are usually unmethylated, the hypermethylation of CpG islands in specific regions of the promoter has been described for common epigenetic mechanism for the silencing of the tumor suppressor gene in cancer and a regulator of growth during cancer (3).

IGFBP-3 has IGF-dependent and -independent antiproliferative and proapoptotic effects on a variety of cancer cells including NSCLC cells (8–15). Recently, we observed the hy-

Fig. 2 Methylation status of the IGFBP-3 promoter and probability of survival in patients with stage I NSCLC. A, probability of overall survival for patients with promoter hypermethylation of IGFBP-3 (M) versus patients without it (U). B, probability of disease-specific survival for patients with hypermethylation of IGFBP-3 promoter (M) versus patients without it (U). C, probability of disease-free survival for patients with hypermethylation of IGFBP-3 promoter (M) versus patients without it (U).

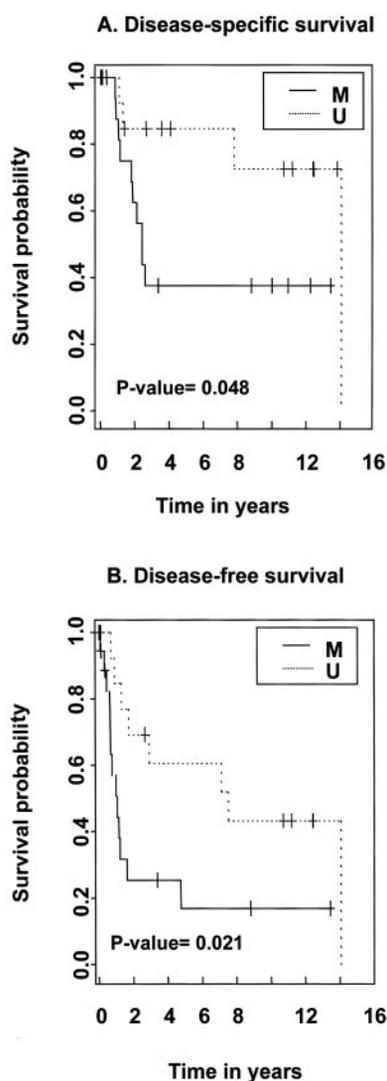


Fig. 3 Probability of disease-specific and disease-free survival at various times for patients with squamous cell carcinoma. **A**, probability of disease-specific survival at various times for patients with squamous cell carcinoma and IGFBP-3 promoter hypermethylation versus patients with squamous cell carcinoma but without promoter hypermethylation. At year 5, for the group with squamous cell carcinoma and IGFBP-3 promoter hypermethylation, the 95% CI is 14.5–56.2; for the group with squamous cell carcinoma but without promoter hypermethylation, the 95% CI is 1.4–84.5. **B**, probability of disease-free survival at various times for patients with squamous cell carcinoma and IGFBP-3 promoter hypermethylation versus patients with squamous cell carcinoma but without promoter hypermethylation. At year 5, for the group with squamous cell carcinoma and IGFBP-3 promoter hypermethylation, the 95% CI is 5.3–54; for the group with squamous cell carcinoma and without IGFBP-3 promoter hypermethylation, the 95% CI is 38.7–94.7.

permethylation of CpG islands in a promoter region of the *IGFBP-3* gene in NSCLC tumor samples. Previous studies demonstrated that hypermethylation at the CpG sites of *IGFBP-3* promoter can repress expression of the gene (24). In fact, we found that down-regulation of IGFBP-3 is a frequent event in stage I NSCLC (42). These findings suggest that

hypermethylation of IGFBP-3 promoter provides the cells with a growth advantage in a manner akin to deletions or mutations.

In this study, we assembled a panel of 83 tumors from patients with stage I NSCLC. This allowed us to determine the rate of IGFBP-3 hypermethylation and to investigate the correlation between the methylation status of IGFBP-3 promoter in patients diagnosed with stage I NSCLC and clinicopathological characteristics and prognostic factors. We found hypermethylation of the IGFBP-3 promoter in 51 (61.4%) of 83 patients who were diagnosed with stage I NSCLC. The frequent hypermethylation of IGFBP-3 promoter in early stage of lung cancer indicates the role of epigenetic abnormality in IGFBP-3 in lung cancer development. Interestingly, the frequency of IGFBP-3 promoter hypermethylation did not differ by histology, although there are reports that the activity of methyltransferase can be modulated differently depending on the cell types. In addition, exposure to carcinogens, such as tobacco and asbestos, was not correlated with hypermethylation of IGFBP-3. Hence, the mechanisms that mediate the hypermethylation of IGFBP-3 promoter in NSCLC are related neither to histology nor to prototypic carcinogens for lung cancer. Three of 83 patients were non-smokers, and 2 of them had IGFBP-3 promoter methylation. However, the small number of samples from non-smokers analyzed in this study made it impossible to determine an accurate rate of methylation and the role of IGFBP-3 methylation in multistep lung tumorigenesis and chemoprevention.

The most interesting finding of this study is that patients with promoter hypermethylation had significantly poorer overall, disease-specific, and disease-free survival probability compared with those without hypermethylation. According to the multivariate analysis, methylation status was the only independent factor that predicted poorer overall, disease-specific, and disease-free survival probability among patients diagnosed with pathological stage I NSCLC. The promoter hypermethylation of cancer-related genes has been reported as an indicator of patient prognosis in resected primary NSCLC. Recently, Tang *et al.* (32) reported that the promoter hypermethylation of DAP-kinase is one indicator of poorer overall and disease-specific survival probability in early-stage NSCLC. *p16* promoter hypermethylation was also an independent risk factor predicting significantly shorter postsurgical survival in patients with stage I adenocarcinoma of the lung (43). The frequency of hypermethylation in these molecules in primary NSCLC varies from 7% to 44% (32, 43–45). The association between IGFBP-3 hypermethylation and poor survival rates suggests that IGFBP-3 plays an important role in the invasion and metastasis of lung cancer, consistent with previous findings showing the significance of IGF-mediated signaling pathways in anchorage-independent cell growth and tumorigenicity (47–50).

In conclusion, we have demonstrated for the first time that hypermethylation of the IGFBP-3 promoter is strongly associated with the survival of stage I NSCLC patients, indicating the protective role of IGFBP-3 against lung cancer, which is consistent with the previous finding, by Yu *et al.* (12), of a negative correlation between serum IGFBP-3 levels and lung cancer risk. The mechanism responsible for the hypermethylation of IGFBP-3 promoter in NSCLC is not histologically specific and is not likely to be directly related to tobacco carcinogen dose. Our findings have significant clinical implications. Detection of

methyated IGFBP-3 may be a potential biomarker for early detection of lung cancer and for monitoring chemoprevention efforts. The combination of the previous observations on other molecular parameters (*e.g.*, the expression of PTEN, cyclooxygenase-2, cyclin B1, RAR β , Fhit, and E-cadherin and type IV collagenase, the hypermethylation of the DAP-kinase promoter, and microsatellite alteration profiles) and our new findings on IGFBP-3 expression may have potential importance because none of these parameters have been, to date, validated as predictive or prognostic factors and it is likely a combination of various molecular factors that will truly contribute to carcinogenesis and prognosis in lung cancer. In addition, activation of IGFBP-3 expression in NSCLC patients by the treatment of demethylation agents, which have been under clinical evaluation, can be considered as a novel therapeutic approach. This approach may even lead to a survival advantage in stage I NSCLC through critical regulatory function of IGFBP-3 on cell growth and apoptosis. Additional studies on the mechanism through which IGFBP-3 is targeted for methylation and on the enzyme involved in this methylation merit special attention, and the answers should be adjusted to the clinical strategy using *IGFBP-3* as a target gene.

Acknowledgments

We thank Georgia O. Lange at Institutional Tumor Registry for her dedication in providing clinical information.

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Clin Cancer Res 2002;8:3669-3675.

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