Association Between Insulin-Like Growth Factor Binding Protein-3 Promoter Polymorphism of –1590 C>A and Lung Cancer Susceptibility in a Chinese Han Population

Xiaofang Dai, Zhao Liu, Jinghua Ren, and Gang Wu

Recent studies have identified a negative correlation between serum levels of insulin-like growth factor binding protein-3 (*IGFBP-3*) and the risk of lung cancer. In this study, polymorphisms present at the –1590 site of the *IGFBP-3* promoter were evaluated in relation to lung cancer risk in a Chinese Han population. A total of 248 nonsmall cell lung cancer (NSCLC) cases and 29 small cell lung cancer cases were compared with 252 matched, healthy controls. Polymerase chain reaction-based restriction fragment length polymorphism assays were used to detect polymorphisms present. The A/A genotype and an A allele were both associated with an increased risk of NSCLC after being adjusted for age and gender (adjusted odds ratio = 2.296, 95% confidence interval = 1.133–4.655; and adjusted odds ratio = 1.390, 95% confidence interval = 1.042–1.854, respectively). In conclusion, the A/A genotype and A allele of the *IGFBP-3* promoter –1590 site may represent a genetic risk factor for NSCLC, with the A/A genotype being associated with a higher risk for squamous cell carcinoma than adenocarcinoma.

Introduction

IN CHINA, LUNG CANCER is the most common cancer diagnosed in men, and the second most common cancer diagnosed in women (Yang *et al.*, 2005; Jemal *et al.*, 2009). Moreover, despite the availability of surgery, chemotherapy, and radiotherapy for the treatment of lung cancer, only small improvements in the survival rates for patients with lung cancer have been observed (Spiro and Silvestri, 2005). However, growing evidence suggests that both genetic and environmental factors play important roles in the biology of lung cancer, and progress continues to be made in understanding the exact mechanisms involved. For example, both *in vitro* and *in vivo* studies have shown that insulin-like growth factor-I (IGF-I) is an effective mitogen in normal epithelial cells and mediates strong antiapoptotic effects in lung cancer cells (Furstenberger and Senn, 2002; Hofmann and Garcia, 2005; Tao *et al.*, 2007). Further, in the presence of high levels of circulating IGF-I, an increased risk of breast cancer (Hankinson *et al.*, 1998), prostate cancer (Chan *et al.*, 1998), colorectal cancer (Ma *et al.*, 1999; Giovannucci *et al.*, 2000), and lung cancer (Yu *et al.*, 1999) have been identified. In contrast, insulin-like growth factor binding protein-3 (*IGFBP-3*) is a major serum carrier protein for IGFs and is able to reduce the binding of IGFs to the IGF-I receptor to inhibit the mitogenic and antiapoptotic actions of IGF-I (Jones and Clemmons,

1995). *IGFBP-3* has also been shown to induce apoptosis and inhibit cell growth (Oh *et al.*, 1993) independent of IGF-I and the IGF-I receptor, based on its interactions with a number of other proteins such as retinoid X-receptor-alpha (Liu *et al.*, 2000) and transferrin (Weinzimer *et al.*, 2001). Correspondingly, overexpression of *IGFBP-3* has been shown to reduce the growth of a subset of nonsmall cell lung cancer (NSCLC) cells (Lee *et al.*, 2002). In addition, recent studies (Yu *et al.*, 1999; London *et al.*, 2002) have identified a negative correlation between serum *IGFBP-3* levels and the risk of lung cancer (Lee *et al.*, 2002; London *et al.*, 2002), indicating that *IGFBP-3* may have a protective role against lung cancer.

Based on sequencing studies of the human genome project, 99% of DNA between individuals was found to be identical, with only 1% of genomic DNA representing variants. Despite such a small percentage of diversity, significant variations in the susceptibility of various individuals to cancer have been observed. For *IGFBP-3*, a single-nucleotide polymorphism (SNP) in the *IGFBP-3* promoter has been recently shown to correlate with an increased risk for a number of cancers. Specifically, the –202 locus and the –1590 locus have been associated with lung cancer (Moon *et al.*, 2006; Han *et al.*, 2008), whereas the –202 locus has been associated with both breast cancer (Ren *et al.*, 2004) and prostate cancer (Hernandez *et al.*, 2007). In addition, an SNP at the –1590 site of the *IGFBP-3* promoter has been shown to exist at a higher frequency in a

Korean population (Han *et al.*, 2008). Therefore, in this case-control study, the frequency of an SNP at the -1590 site of the *IGFBP-3* promoter and lung cancer susceptibility were evaluated in a Chinese population.

Materials and Methods

Study population

Patients enrolled in this study were selected from an ethnic Han Chinese population living in the Hubei Province and its surrounding regions in China. Written informed consent was obtained from all participating subjects, and this study was approved by the bioethics committee of the Tongji Medical College of Huazhong University of Science and Technology (Wuhan, China).

A total of 277 patients with newly diagnosed and histologically confirmed primary lung cancer were registered at the Union Hospital of Huazhong University of Science and Technology between June 2008 and July 2009. These included 248 cases of NSCLC and 29 cases of small cell lung cancer (SCLC). Tumor, node, metastasis (TNM) stage designations were determined using the 2002 American Joint Committee on Cancer clinical staging criteria.

Control subjects included 252 unrelated, healthy individuals who visited the General Health Check-up Division of Union Hospital. Control subjects were eliminated from the study if they had been diagnosed with any type of cancer, or if they had a family history of cancer. As the incidence of lung cancer has been shown to increase with age in China and to have a difference in incidence between men and women (Yang *et al.*, 2005), control subjects were frequency matched to the NSCLC cases included in this study according to patient age, gender, and ethnic background. Because of the low frequency of SCLC cases, the frequency of matching control subjects with SCLC cases was 1:3.

IGFBP-3-1590 C>A genotype

Genomic DNA was extracted from peripheral blood samples using a blood genomic DNA miniprep kit (Axygen Biosciences, Union City, CA), according to the manufacturer's instructions. Using the polymerase chain reaction (PCR)-based restriction fragment length polymorphism method, SNPs present at the *IGFBP-3*-1590 site were detected. PCR was performed using a thermocycler (Biometra, Göttingen, Germany) and PCR mixture (35 μ L) included ~80 ng of genomic DNA, 12 pmol of each primer (forward: 5'-GCA GACCTGGGACCTCAA-3'; reverse: 5'-CGATCTCTCGCC CAGTGT-3') (Han *et al.*, 2008), 0.2 mM of each dNTP, 1 $\times$ PCR

buffer (50 mM KCl, 10 mM Tris-HCl, and 0.08% Nonidet P40), 1.0 mM MgCl₂, and 1.5 U Taq DNA polymerase (Fermentas, Vilnius, Lithuania). Reaction conditions involved an initial denaturation step for 10 min at 95°C, followed by 34 cycles of 95°C for 30 s, 57°C for 30 s, and 72°C for 30 s, and a final elongation step of 72°C for 10 min. A 548-bp product represented a successful amplification and was confirmed using 2% agarose gel electrophoresis and ethidium bromide staining. The 548-bp PCR product was then digested with 5 U *Apa*I (Fermentas) at 37°C overnight. Restriction fragments were separated by gel electrophoresis on a 2% agarose gel and then imaged on a UV transilluminator. The presence of homozygous C alleles was represented by two DNA bands at 227 and 321 bp, and an uncut fragment of 548 bp indicated the presence of homozygous A alleles. A heterozygous genotype correlated with the presence of bands at 548, 227, and 321 bp (Fig. 1). Five percent of samples were randomly selected for direct sequencing, and 100% concordance was confirmed.

Statistical analysis

Student's *t*-test and chi-square tests were performed according to the differences in demographic characteristics compared between patients with lung cancer versus healthy controls. The chi-square test was also applied to Hardy-Weinberg equilibrium calculations. Binary logistic regression analysis was performed to evaluate the association between genotype and the risk of lung cancer (including NSCLC and SCLC), with odds ratio (OR) and 95% confidence interval (CI) also calculated. Wild-type homozygotes were used as references. Multivariate logistic regression analysis also evaluated the association between genotype and different pathologies associated with NSCLC cases, the types of pathology observed, and the smoking habits of NSCLC patients. A *p*-value of <0.05 was considered significant. All of these statistical analyses were performed using the statistical analysis system software SPSS 15.0 (SPSS, Chicago, IL).

Results

Population characteristics

The study population examined included 248 cases of NSCLC, 29 cases of SCLC, and 252 cancer-free controls (90 of them were also controls for SCLC patients). The mean age of the NSCLC cases versus the controls was 54.46 ± 10.92 and 54.19 ± 12.72 years ($p = 0.803$). For the SCLC cases, the mean age of the patients was 57.00 ± 8.67 years and of the corresponding controls was 56.60 ± 12.01 years ($p = 0.869$). Cases of NSCLC or SCLC and the corresponding controls did not

FIG. 1. Polymorphism analysis of the -1590 locus in the *IGFBP-3* gene. Polymerase chain reaction followed by specific restriction enzyme digestion was performed, and the presence of an *Apa*I site at the -1590 locus results in the production of two fragments, 321 and 227 bp. Therefore, the homozygous wild-type (C/C) locus is detected by the presence of two bands of 321 and 227 bp, the heterozygous type (C/A) locus is detected by the presence of three bands of 548, 321, and 227 bp, and a homozygous variant type (A/A) locus is detected by the presence of only one band of undigested DNA of 548 bp.

exhibit any statistically significant difference in age or gender, indicating that the frequency-matching performed was adequate (Table 1).

A diagnosis of adenocarcinoma, squamous cell carcinoma, or another type of cancer represented 57.3%, 36.7%, and 6.0% of the NSCLC cases analyzed, respectively. Further, 64.5% of NSCLC patients were staged as IIIB-IV at diagnosis, 50.4% had distant metastasis detected at diagnosis, and 16.1% of patients had a family history of malignant tumors. Female patients were associated with a higher proportion of adenocarcinoma cases (74.2%), whereas male patients were associated with a greater proportion of squamous cell carcinoma cases (43.5%). Cumulative cigarette dose (pack-years) was calculated using the following formula: pack-years = [(pack per day) × (years smoked)], and patients were separated into three categories as follows: heavy smokers (>400 pack-years), mild smokers (<400 pack-years), and nonsmokers. For the NSCLC cases, their distribution among these categories was 41.9%, 12.1%, and 46.0%, respectively. In addition, the majority of male patients were found to be heavy smokers (61.5%) or mild smokers (18.6%), whereas the majority of female patients were nonsmokers (94.3%) (data not shown).

IGFBP-3-1590 genotype and clinicopathologic factors

The SNP detected for the NSCLC patients, SCLC patients, and controls were found to be in Hardy-Weinberg equilibrium ($\chi^2 = 3.828$, $p = 0.051$; $\chi^2 = 2.778$, $p = 0.095$; and $\chi^2 = 0.081$, $p = 0.776$, respectively). For the NSCLC cases, the proportion of homozygous wild (C/C), heterozygous (C/A), and homozygous variant (A/A) types were 54.0%, 35.5%, and 10.5%, respectively. Compared with the C/C genotype, patients with the A/A genotype were associated with an increased risk of NSCLC after adjusting for patient age and gender (adjusted OR = 2.296, $p = 0.021$). The C/A genotype also had a higher frequency with an OR of 1.189, although the difference was not statistically significant ($p = 0.370$). An analysis of the alleles also showed that persons carrying the A allele exhibited a higher risk of NSCLC (adjusted OR = 1.390, $p = 0.024$). When the NSCLC cases were stratified according to gender, male individuals carrying the A/A genotype had nearly a threefold increase in the risk of NSCLC than normal control persons (adjusted OR = 2.755, $p = 0.031$). In contrast, women with NSCLC showed no significant difference in their risk for NSCLC when they carried the A/A genotype. When the pathologies associated with the NSCLC cases were compared with the normal controls, the A/A genotype was found to be

associated with a greater than threefold increase in the risk of squamous cell carcinoma ($p = 0.005$). In contrast, the C/A and A/A genotypes were associated with a 1.5-fold increase in the risk of adenocarcinoma ($p = 0.055$) (Table 2).

For the SCLC cases, the C/C, C/A, and A/A genotypes represented 58.6%, 27.6%, and 13.8% of the 29 cases, respectively. When the C/C genotype was used as a reference, there was no significant association found between any of the genotypes and an increased risk for SCLC. There was also no significant difference in the risk of SCLC for male or female SCLC patients versus normal controls (data not shown).

Pathology of lung cancer and smoking habits

For patients with squamous cell carcinoma, their frequency of heavy smoking was greater than that for cases of adenocarcinoma (adjusted OR = 2.551, $p = 0.024$). Further, the number of heavy and mild smokers among SCLC patients was found to be greater than the number of heavy or mild smokers diagnosed with adenocarcinoma (adjusted OR = 10.311, $p = 0.009$; and adjusted OR = 7.605, $p = 0.040$, respectively) (data not shown).

Discussion

Previous studies have reported an increased risk of cancer for persons with a polymorphism present in the *IGFBP-3* promoter. Specifically, the C allele of the -202 A>C locus was found to correlate with a decline in serum levels of *IGFBP-3* and an increased risk of lung cancer (Han *et al.*, 2008), breast cancer (Ren *et al.*, 2004), and prostate cancer (Hernandez *et al.*, 2007). Similarly, Han *et al.* (2008) identified the -1590 locus of *IGFBP-3* to be associated with a higher risk of lung cancer in a Korean population. In the present study, a variant A/A genotype and an A allele of the *IGFBP-3* promoter were found to correlate with the risk of developing NSCLC in a Chinese Han population (Table 2). These results suggest that the A allele of *IGFBP-3*-1590 may represent a marker for susceptibility to NSCLC.

Expression of *IGFBP-3* has been shown to be affected by polymorphisms present in the promoter region, as the A allele of the -202 locus is associated with a higher serum level of *IGFBP-3* (Deal *et al.*, 2001). The promoter activity of *IGFBP-3* can also be influenced by hypermethylation of the upstream stimulatory factor binding sites (Paquette *et al.*, 2007). As the -1590 site is located in the promoter region of the *IGFBP-3* gene, we hypothesize that the A allele of this site may impair

TABLE 1. BASELINE CHARACTERISTICS OF STUDY SUBJECTS

Variables	NSCLC cases	Controls	p-Value	SCLC cases	Controls	p-Value
No. of cases	248	252		29	90	
Age at diagnosis, years						
Mean	54.46	54.19	0.803 ^a	57.00	56.60	0.869 ^a
SD	10.92	12.72		8.67	12.01	
Gender, <i>n</i> (freq.)						
Male	161 (64.9)	164 (65.1)	0.970 ^b	26 (89.7)	82 (91.1)	0.814 ^b
Female	87 (35.1)	88 (34.9)		3 (10.3)	8 (8.9)	

^aIndependent-sample *t*-test.

^bChi-square test.

NSCLC, nonsmall cell lung cancer; SCLC, small cell lung cancer; *n*, number; freq., frequency.

TABLE 2. GENOTYPE DISTRIBUTION AND NONSMALL CELL LUNG CANCER RISK IN OVERALL POPULATION AND SUBPOPULATION STRATIFIED BY GENDER AND PATHOLOGY TYPES

Variable	NSCLC	Controls	Crude ^a			Adjusted for gender and age ^a		
	n	n	OR	95% CI	p-Value	OR	95% CI	p-Value
Genotype								
C/C	134	154	1			1		
C/A	88	85	1.190	0.816–1.735	0.367	1.189	0.814–1.739	0.370
A/A	26	13	2.299	1.136–4.651	0.021 ^b	2.296	1.133–4.655	0.021 ^b
C allele	356	393	1			1		
A allele	140	111	1.392	1.045–1.856	0.024 ^b	1.390	1.042–1.854	0.025 ^b
Patient sex								
Male								
C/C	87	96	1			1		
C/A	56	61	1.013	0.637–1.612	0.957	0.982	0.615–1.571	0.941
A/A	18	7	2.837	1.131–7.120	0.026 ^b	2.755	1.095–6.929	0.031 ^b
C allele	230	253	1			1		
A allele	92	75	1.349	0.948–1.921	0.097	1.319	0.925–1.882	0.126
Female								
C/C	47	58	1			1		
C/A	32	24	1.645	0.855–3.165	0.136	1.684	0.871–3.255	0.121
A/A	8	6	1.645	0.534–5.074	0.386	1.763	0.566–5.492	0.328
C allele	126	140	1			1		
A allele	48	36	1.481	0.903–2.429	0.119	1.530	0.929–2.518	0.095
Pathology types								
Adenocarcinoma								
C/C	74	154	1			1		
C/A	57	85	1.396	0.903–2.157	0.133	1.466	0.943–2.280	0.089
A/A	11	13	1.761	0.753–4.118	0.192	1.808	0.769–4.253	0.175
C/A+ A/A	68	98	1.444	0.953–2.188	0.083	1.510	0.991–2.302	0.055
Squamous cell carcinoma								
C/C	53	154	1			1		
C/A	23	85	0.786	0.451–1.372	0.397	0.722	0.410–1.269	0.257
A/A	15	13	3.353	1.498–7.504	0.003 ^b	3.214	1.423–7.260	0.005 ^b
C/A+ A/A	38	98	1.127	0.692–1.835	0.632	1.046	0.638–1.716	0.859
Other cell types ^c								
C/C	7	154	1			1		
C/A	8	85	2.071	0.726–5.907	0.174	2.098	0.725–6.066	0.172
A/A	—	13	—	—	—	—	—	—
C/A+ A/A	8	98	1.796	0.631–5.109	0.272	1.833	0.636–5.285	0.262

^aORs, 95% CI, and *p*-values were calculated using a logistic regression model (binary or multinomial).

^bStatistically significant (*p* < 0.05).

^cIncluding alveolar cell carcinoma, adenosquamous carcinoma, and large cell carcinoma. OR, odds ratio; CI, confidence interval.

the transcriptional activity of the *IGFBP-3* promoter, resulting in a decrease in levels of *IGFBP-3* and an increased risk for lung cancer. Further, male patients were found to have a significant difference in genotype distribution for the *IGFBP-3*-1590 locus, which was in contrast with the results of Han *et al.* (2008), which showed that female patients and non-smokers had significant differences in genotype. Further studies will be needed to investigate this difference.

As mentioned earlier, the A allele of the –1590 locus is a possible risk factor for NSCLC. However, it is not clear what determines whether adenocarcinoma, squamous cell carcinoma, or another type of NSCLC could develop. Using multivariate logistic regression analysis, the A/A genotype was found to be associated with a significantly higher risk of developing squamous cell carcinoma, and persons with the A/C genotype had a higher frequency of developing adenocarcinoma (Table 2). We hypothesized that smoking habits would

also be a factor on the basis of previous studies that have shown that the incidence of squamous carcinoma and small cell carcinoma is higher in patients with a history of smoking, compared with cases of adenocarcinoma (Barbone *et al.*, 1997). Our results are consistent with these results, because the squamous cell carcinoma patients in this study were found to be heavier cigarette smokers than the adenocarcinoma patients. Further, a Chinese epidemiological study drew a similar conclusion (Tse *et al.*, 2009). In combination, these results emphasize the close relationship between cigarette smoking and the development of squamous cell carcinoma.

Cigarette smoking has also been shown to have a dose-dependent correlation with lung DNA methylation (Toyooka *et al.*, 2004), suggesting that methylation may serve as a biomarker of exposure to cigarette smoking (Phillips and Goodman, 2009). Additional studies have also revealed that silencing of tumor suppressor genes can occur following DNA

hypermethylation events (Esteller, 2007), suggesting that the transcriptional activity of the *IGFBP-3* promoter could be affected (Paquette *et al.*, 2007) and *IGFBP-3* expression reduced (Chang *et al.*, 2002a; Hanafusa *et al.*, 2002). In studies of lung squamous cell carcinoma, the detection of global DNA methylation suggests that the methylation status of genomic DNA may affect the development, aggressiveness, and prognosis of this type of cancer (Piyathilake *et al.*, 2003). Therefore, cigarette smoking may contribute to the development of squamous carcinoma due to an increased number of methylation events, which are further facilitated by the presence of an A allele at the -1590 locus. Further studies of the molecular details involved will be needed to confirm this hypothesis.

In this study, the presence of the *IGFBP-3*-1590 C>A polymorphism, clinical characteristics of NSCLC, and the risk of SCLC risk, were not found to be associated (data not shown). Previously, serum levels of *IGFBP-3* were found to correlate with the probability of disease-specific survival (Chang *et al.*, 2002b). Therefore, *IGFBP-3* would be anticipated to play an important role in the biological aggressiveness exhibited in the early stages of NSCLC. However, in the NSCLC cases evaluated, gender, cancer stage, family history, and metastasis present at diagnosis were factors that were not found to significantly correlate with the *IGFBP-3*-1590 C>A polymorphism. However, it is possible that this polymorphism may still represent a risk factor for lung cancer, have the capacity to affect serum levels of *IGFBP-3*, and/or be a predictive factor for NSCLC. Additional follow-up of the enrolled subjects will be needed to further address these considerations. For the SCLC cases, no correlation between the risk of SCLC and the polymorphisms present was detected. However, this may be due to the small sample size of SCLC patients that was available to be enrolled in this study, which did not allow a statistically significant evaluation of genotype distribution to be performed. In addition, it is possible that variants at this locus of *IGFBP-3* do not affect SCLC to the same extent as they affect NSCLC.

As a potential risk factor for NSCLC, persons with an A allele at the *IGFBP-3*-1590 locus should be proactive in reducing their risk of lung cancer by reducing, or eliminating, their habit of smoking. In addition, detection of the A allele could represent an opportunity to employ novel antineoplastic therapies and/or preventative strategies aimed at treating high-risk groups. For example, gene-related antibodies could be designed to target the A allele, and demethylating agents could restore the expression of *IGFBP-3*. Treatment with cell factors to stimulate the formation of *IGFBP-3* may also be possible.

There were also several limitations present in our study. For example, the Union Hospital is one of the best known hospitals in the Hubei Province. As a result, severe cases would have a higher probability of being transferred to this hospital, which would bias the cases available for recruitment. Second, the results were not adjusted for other probable risk factors (e.g., occupational factors, environmental pollution, and dietary habits) of lung cancer, because the medical records available for each patient did not include this information. Third, serum levels of *IGFBP-3* were not tested in this study. Therefore, any further analysis of a genotype-phenotype correlation was unable to be per-

formed. Lastly, additional studies that have a larger cohort and that include other ethnic populations are needed to clarify the role of the -1590 locus in relation to lung cancer.

In conclusion, the *IGFBP-3*-1590 C>A polymorphism may affect an individual's susceptibility to NSCLC, and for the Chinese Han population, the A allele appears to be a risk factor for NSCLC, particularly in men. Further, those homozygous for A/A were found to be predisposed to lung squamous cell carcinoma, and there was no correlation between the clinical characteristics of NSCLC patients and the *IGFBP-3*-1590 polymorphism identified. For the SCLC patients examined, the risk of SCLC did not correlate with the presence of polymorphisms at the *IGFBP-3*-1590 site. In combination, these results suggest that polymorphisms of the *IGFBP-3* promoter at -1590 may represent important risk factors for NSCLC as well as potential targets for novel antineoplastic therapies.

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Disclosure Statement

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Address correspondence to:

Gang Wu, Ph.D.

Cancer Center

Union Hospital

Tongji Medical College

Huazhong University of Science and Technology

Wuhan 430023

China

E-mail: wugangzr2010@163.com