Promoter -202 *A/C* Polymorphism of Insulin-like Growth Factor Binding Protein-3 Gene and Non-small Cell Lung Cancer Risk

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인슐린양 성장 인자 결합 단백-3 유전자 -202 좌위의 다형성에 따른 비소세포폐암의 위험도

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인슐린양 성장 인자 결합 단백-3(Insulin-like growth factor (IGF) binding protein-3 (IGFBP-3))는 혈액 내에서 IGF와 결합하여 복합체 혹은 저장소로 작용함으로써, IGF가 수용체에 결합하는 것을 방해하여 IGF의 항세포사멸(anti-apoptosis) 및 세포분열 촉진의 기능을 억제한다. 하지만, 특정 상황에서는 도리어 IGFBP-3가 IGF의 파괴를 억제하여 IGF에 의한 암세포의 분화 및 성장을 촉진할 수도 있다는 것이 알려져 있다. 대부분의 환자에서 혈액내 IGFBP-3 수치는 IGFBP-3 유전자의 -202 좌위(locus)의 다형성(polymorphism)에 의해 크게 영향을 받는다. 따라서, 저자 등은 제한 효소(restriction enzyme)를 이용하여 비소세포폐암 환자의 IGFBP-3 유전자 -202 좌위의 다형성을 분석함으로써, 이 좌위의 다형성이 비소세포폐암의 위험도와 연관되어 있는지 조사하였다. 본 연구는 104명의 비소세포폐암 환자군과, 연령, 성별, 흡연력이 비슷한 104명의 대조군을 비교 분석하였다. 대조군에서 -202 좌위 유전자 다형성의 빈도는 *AA*형 48명 (46.2%), *AC*형 45명 (43.3%), *CC*형 11명 (10.5%)이었고, 비소세포폐암 환자군에서 -202 좌위 유전자 다형성의 빈도는 *AA*형 67명 (64.4%), *AC*형 35명 (33.7%), *CC*형 2명 (1.9%)이었다. -202 좌위의 유전자 다형성에 있어서 대조군과 비소세포폐암 환자군 사이에 유의한 빈도 차이가 있었으며 ($p < 0.05$, Pearson's χ^2-test), 비소세포폐암의 위험도는 -202 좌위의 *AA*형에서 가장 높고 *CC*형에서 가장 낮았다. *CC*형을 기준으로 하면 *AC*형의 비교 위험도는 2.60 (95% 신뢰구간: 0.89 - 8.60)이었으며 *AA*형의 비교 위험도는 5.89 (95% 신뢰구간: 1.92 - 21.16)이었다. 본 연구 결과는, IGFBP-3 유전자의 -202 좌위(locus)의 다형성(polymorphism)이 비소세포폐암의 위험인자 중의 하나일 가능성을 제시하며, 따라서 비소세포폐암에 대한 항암치료 개발에 있어서 새로운 표적이 될 가능성을 시사한다. *(Tuberc Respir Dis 2005; 58: 359-366)*

Key words : Insulin-like growth factor (IGF), Insulin-like growth factor binding protein-3 (IGFBP-3), Non-small cell lung cancer (NSCLC), Promoter -202 A/C polymorphism, Restriction fragment length polymorphism (RFLP)

Introduction

Insulin-like growth factor (IGF) is a pair of secreted proteins which exhibit a variety of effects on growth, development, and metabolism[1]. IGFs including IGF-I and IGF-II are peptide hormones which exert strong mitogenic effects on both normal and cancerous cells[2,3]. In addition to stimulating cell proliferation, IGFs also suppress the cellular apoptotic pathways, thereby facilitating the cell growth[4,5]. The IGFs' effects on the cell proliferation and the apoptosis are mediated via a specific cell-membrane receptor, insulin-like growth factor-I receptor (IGF-IR), which has been demonstrated to be involved in the cell transformation[6] and also exhibit the tyrosine kinase activity.

The interactions between IGFs and IGF-IR are regulated by six IGF-binding proteins (IGFBPs)

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whose pronounced affinities for IGFs have been previously identified and characterized[3]. IGFBP-3, the major IGF-binding protein in serum, serves as a reservoir for IGF in circulation, and is a glycoprotein which forms a 140-kD complex with IGF and an acid-labile component. The cell culture studies indicate that the antiproliferative effects of both retinoic acid (a metabolite of vitamin A) and wild-type *p53* protein are mediated via the increased expression of IGFBP-3, which in turn inhibits IGFs' mitogenic effects on the cell proliferation[7-10]. The IGFBP-3 expression levels are affected by the growth-inhibiting agents including transforming growth factor-ß1[11], vitamin D-related compounds[12], retinoic acid[7], antiestrogens[13], antiandrogens[14], *p53* gene[9], and tumor necrosis factor-α[15], which suggests that the cellular effects of these agents may be mediated by IGFBP-3. Additionally, it has been recently demonstrated that sodium butyrate and trichostatin A also induce the increased synthesis and secretion of IGFBP-3[16]. The epigenetic changes in the IGFPB-3 gene further modulate the dual regulatory effects of IGFPBs, as do many other factors including IGFBP protease, prostate specific antigen (PSA), and cathepsin D[3,17-19] .

Twin studies and multiethnic epidemiological studies indicate that about half of the interindividual variabilities in the levels of circulating IGF-I and IGFBP-3 are genetically determined, and that the polymorphic variations at the -202 locus (relative to the mRNA CAP site) of the IGFBP-3 gene mediate the age-adjusted levels of circulating IGFBP-3[20-23].

In the recent epidemiologic studies, the high IGF-1 and the low IGFBP-3 levels were independently associated with a greater risk of common cancers, including prostate cancer[24], premenopausal breast cancer[25-27], colorectal cancer[28], and lung cancer[29].

Therefore, under the hypothesis that the genotype of IGFBP-3 gene might affect the prevalence of NSCLC, we attempted to characterize the relationship between the *A-202C* polymorphism of IGFBP-3 gene and the risk of NSCLC.

Materials and Methods

Study Population and Samples

We used the genomic DNAs from 104 patients who had been diagnosed with NSCLC and had undergone surgery for the management of the primary tumors at the Severance Hospital, between 1997 and 1999. The NSCLC cases included 82 male and 22 female patients, and the mean age of the patients was 60.8 ± 9.9 years. The age-, gender-, and smoking status-matched control subjects' blood samples were randomly selected from a blood bank comprising 1038 subjects who visited the Yong-in Severance Hospital in 2003 for an annual health examination conducted by the National Health Insurance Institute. The matched control subjects included 82 male and 22 female participants with the mean age of 61.0 ± 9.6 years (Table 1).

Table 1. The baseline characteristics of the study population.

	NSCLC group (n=104)	Control group (n=104)
Age (years old)		
mean±SD	60.8±9.9	61.0±9.6
range	32-78	40-83
No. of < 65 yr.	62 (59.6%)	60 (57.7%)
Smoking (pack years) (mean±SD)	29.0±24.9	23.5±22.0
No. of females	22 (21.2%)	22 (21.2%)

PCR Restriction Fragment Length Polymorphism (RFLP) Analysis

The genomic DNAs were extracted from the blood samples using QIAamp Blood Kit (Qiagen, Hilden, Germany) or by the standard method with proteinase-K digestion followed by phenol/chloroform extraction. The 168-base-pair (bp) fragment encompassing the *A-202C* polymorphic site in the IGFBP-3 gene was amplified using the specific primers: 5'-CTGAGTTGGCCAGGAGTGACT-3' in sense and 5'-CGAGCTCGGGGGCGTGCA-3' in antisense. The PCR reactions were performed in a 25μl volume containing 20 ng of genomic DNA, 10X PCR buffer supplied by a manufacturer, 0.2 mM of each deoxyribonucleotide triphosphate (dATP, dCTP, dGTP, and dTTP), 1 mM of $MgCl_2$, 50 pM of each primer, and 1 unit of Ampli-Taq Gold DNA polymerase (Perkin-Elmer, Branchburg, NJ). After an initial denaturation step for 10 minutes at 95°C, 40 cycles of PCR reactions consisting of 95°C for 30 seconds, 66°C for 1 minute, and 72°C for 1 minute were carried out, which were followed by a final extension step for 15 minutes at 72°C in a thermal cycler (GeneAmp PCR System 9700; Perkin-Elmer). After confirming the successful PCR amplification by 2% agarose gel electrophoresis, each PCR product was digested overnight with 5 units of *Fsp*I enzyme at 37°C (New England Biolabs, Inc., Beverly, MA) and electrophoresed on 2.5% agarose gel. When the *Fsp*I site was present, the 168-bp PCR fragment was divided into 116- and 52-bp fragments. The allele was designated either *C* or *A* depending on whether the *Fsp*I restriction site was present or absent, respectively (Figure 1). The validity of the PCR-RFLP analysis was verified by the direct sequencing of several PCR samples of each genotype using the BigDye FN Sequencing kit (PE Applied Biosystems, Foster City, CA).

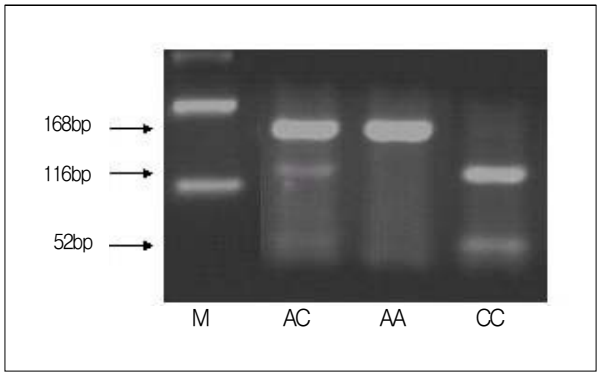

Figure 1. The representative result of RFLP according to the IGFBP-3 promoter genotype.
The patterns for the genotype *AC*, *AA*, and *CC* (Lane 2-4) in the PCR-RFLP analyses are shown. The numbers in the left indicate the size of each DNA fragment (bp: base-pairs). M: DNA size marker (Lane 1)

Table 2. The frequencies of the -202 site IGFBP-3 genotype in the NSCLC and control group.

Genotype	NSCLC group (n=104)	Control group (n=104)
AA	67 (64.4%)	48 (46.2%)
AC	35 (33.7%)	45 (43.3%)
CC	2 (1.9%)	11 (10.5%)

$p<0.05$, Pearson's χ^2 - test was employed

Statistical Analysis

Pearson's χ^2 - test was employed in the comparison of genotypic frequencies between the NSCLC and

Table 3. The relationship between the -202 site IGFBP-3 genotype and the pathologic stage at the time of the diagnosis of NSCLC.

Pathologic Stage	Genotype		
	AA (n=67)	*AC* (n=35)	*CC* (n=2)
I (IA + IB)	29 (27.9%)	11 (10.5%)	0 (0.0%)
II (IIA + IIB)	10 (9.6%)	8 (7.7%)	1 (1.0%)
III (IIIA + IIIB)	28 (26.9%)	16 (15.4%)	1 (1.0%)

$p>0.05$, Fisher's exact test was employed

Table 4. The relationship between the -202 site IGFBP-3 genotype and the histologic subtype of NSCLC.

Histologic Subtype	Genotype		
	AA (n=67)	*AC* (n=35)	*CC* (n=2)
Adenocarcinoma	29 (27.9%)	15 (14.4%)	2 (1.9%)
Squamous cell carcinoma	34 (32.7%)	19 (18.3%)	0 (0.0%)
Other cell type*	4 (3.8%)	1 (1.0%)	0 (0.0%)

$p>0.05$, Fisher's exact test was employed
* Other cell types include anaplastic, giant, and large cell carcinomas

the control group (Table 2). The odds ratio (*OR*) and the 95% confidence intervals (CI) with regard to the IGFBP-3 genotypes were calculated using multiple logistic regression analysis adjusted for age. The statistical modeling was performed on the relative risk of *AA* or *AC* genotype against *CC* genotype. The relationship of the genotype with the pathologic stage (Table 3) and the histologic subtype (Table 4) was also assessed, using Fisher's exact test.

Results

Control Subjects' Characteristics and IGFBP-3 Genotypes

The age (mean ± SD) was 61.3 ± 9.9 years for the 82 males and 59.8 ± 8.7 for the 22 females. The smoking status (mean ± SD) was 29.7 ± 20.8 pack years for the male subjects and 0.5 ± 1.7 for the female subjects, respectively (Table 1). The frequencies of each polymorphic variation at the -202 locus in the control population were as follows: *AA* = 48 (46.2%), *AC* = 45 (43.3%), and *CC* = 11 (10.5%) (Table 2). The allelic frequencies of the Korean control subjects (*A*=0.68, *C*=0.32) were significantly different from those of the multi-ethnic North American survey reported by the Physicians' Health Study (*A*=0.47, *C*=0.53; $p<0.05$)[21,23]. However, the allelic frequencies of the Korean control subjects were comparable to those of Japanese male control subjects (*A*=0.75, *C*=0.25; $p=0.45$)[30].

The personal medical histories including diabetes, ischemic heart disease, hypertension, liver disease, lung disease, and cerebrovascular accident were not affected by the genotype in the control subjects (data not shown).

NSCLC Subjects' Characteristics and IGFBP-3 Genotypes

The age (mean ± SD) was 61.0 ± 10.0 years for the 82 male patients and 59.9 ± 9.8 for the 22 female patients. The smoking status (mean ± SD) was 35.1 ± 22.5 pack years for the males and 6.1 ± 19.9 for the females, respectively (Table 1). The frequencies of each polymorphic variation at the -202 locus in the NSCLC population were as follows: *AA* = 67 (64.4%), *AC* = 35 (33.7%), and *CC* = 2 (1.9%) (Table 2). Significant differences in the allele frequency were apparent when the NSCLC patients (*A*=0.81, *C*=0.19) were compared with the gender-, age-, and smoking status-matched control subjects (*A*=0.68, *C*=0.32) ($p<0.05$).

A-202C Polymorphism of IGFBP-3 Gene and Risk of NSCLC

In order to determine whether the NSCLC risk is related to the genotype, the logistic regression analyses were conducted with adjustments for the age at the time of diagnosis. Compared with the *CC* genotype

subjects, the subjects with *AA* or *AC* genotype harbored a significantly higher risk of NSCLC. Using *CC* genotype as a reference (*OR*=1.0), the *OR* for *AC* genotype was 2.60 (95% CI: 0.89 - 8.60), and the *OR* associated with *AA* genotype was 5.89 (95% CI: 1.92 - 21.16).

Regarding the tumor stage, our study included 40 patients with pathologic stage (*p*stage) I (IA and IB), 19 with *p*stage II (IIA and IIB), and 45 with *p*stage III (IIIA and IIIB). The genotype of the IGFBP-3 gene -202 locus had no appreciable influence on the tumor *p*stage at the time of initial diagnosis (Table 3). 46 subjects (44.2%) were diagnosed as adenocarcinoma, 53 (51.0%) as squamous cell carcinoma, and 5 (4.8%) as other cell types including anaplastic and large cell carcinomas. Neither did the polymorphic distribution of each genotype differ with regard to the histologic subclassification (Table 4).

Discussion

The reports based on the multiethnic population residing in the Montreal metro area demonstrated that *C* allele (53.5%) was slightly more frequent than *A* (46.5%) allele at the *A-202C* polymorphic site of IGFBP-3 gene in the general population. However, the study of Japanese male adults, the only previous study of an ethnic Oriental population, indicated that *A* allele (75.0 %) was appreciably more frequent than *C* allele (25.0 %). The genotypic distribution of our control population was similar to that of the Japanese study, which suggests the differences in the genotypic distribution between Caucasians and Asians with regard to the -202 site of IGFBP-3 gene.

A recent report on the polymorphic variations at the -202 site of IGFBP-3 gene indicated that *AA* genotype was associated with higher circulating levels of IGFBP-3[21], which had been previously shown to correlate with a reduced cancer risk. Initially, therefore, we hypothesized that *A* allele at the -202 site of IGFBP-3 gene might be associated with the reduced risks of lung cancer. On the contrary, however, our results suggested that *A* allele actually was related with an increased NSCLC risk. Using *CC* genotype as a reference (*OR*=1.0), the *OR* for *AC* genotype was 2.60 (95% CI: 0.89 - 8.60) and the *OR* for AA genotype was 5.89 (95% CI: 1.92 - 21.16).

We did not measure the serum levels of IGF and IGBBP-3, and hence could not confirm the correlation between the genotype and the level of IGF and IGFBP-3 in this study population. However, our another study including 85 subjects in their 40's, demonstrated that the serum level of IGFBP-3 was significantly lower in the subjects with *CC* genotype than in the ones with other genotypes (p=0.005, One way ANOVA) (data not shown). According to Deal *et al.,*[21] too, *AA* genotype was associated with higher circulating levels of IGFBP-3.

IGFBPs are known to function normally as the inhibitors of the IGFs' action by blocking the binding of IGFs to their receptors. However, under certain circumstances, they can enhance the IGFs' action by protecting them from degradation[31-33]. This might explain why the NSCLC risk was more strongly associated with *A* allele.

The multiethnic population-based North American study previously reported no difference in the genotypic distribution at the IGFBP-3 gene -202 site between the breast cancer patients and the control population. Another study, involving the prostate cancer patients and the control male subjects from an ethnic Oriental population, showed a slightly increased frequency of *A* allele in the prostate cancer patients. Our results were in accordance with these ones.

We were unable to prove that the tumor aggressiveness was associated with the IGFBP-3 gene

A-202C polymorphism. In order to prove this in the NSCLC subpopulations, it will be required to conduct an analysis of long-term follow-up data regarding overall, disease-free and disease-specific survivals.

The factors modulating the level of IGFBP-3, besides the epigenetic change in IGFBP-3 gene[17], include the proteases such as catheptin D[3], PSA[18,19], and various drugs such as retinoic acid[7], antiestrogen[13], antiandrogen[14], sodium butyrate[16]. Therefore, further studies regarding the relationships between the IGF/IGFBP-3 levels, the cancer risk, and the factors mentioned above will be required in the future.

As the serum level of IGFBP-3 has also been implicated in hypertension, atherosclerosis[34], heart remodeling after acute myocardiac infarction[35], diabetic nephropathy, and glucose homeostasis[36], we also evaluated the relationship between the IGFBP-3 gene polymorphism and the diseases mentioned above. Fasting blood glucose level, history of ischemic heart disease, history of cerebrovascular accidents, blood pressure, and urinalysis did not show statistically significant differences among the three genotypes (data not shown). These findings might be attributable to the selection bias in the control subjects, who were matched against the NSCLC patients with regard to age, gender, and smoking status.

In conclusion, the genotypic frequency of -202 site polymorphism of IGFBP-3 gene in the Korean population differs from that in the North American multiethnic population. Also, *A* allele at the -202 site of IGFBP-3 gene was associated with a greater risk of NSCLC than was *C* allele. To our knowledge, this is the first study of the relationship between the -202 site polymorphism of IGFBP-3 gene and the lung cancer risk. However, as we mentioned above, much larger studies including the measurements of the IGF and IGFBP-3 levels are warranted.

The alterations of IGF-axis in the patients suffering from common medical diseases including diabetic nephropathy, acute myocardiac infarction, and atherosclerosis, make it clear that the further investigations of the effects of the IGFBP-3 genotype on these common diseases are required in the large general populations[34,37,38].

Summary

Background :

IGFBP-3 inhibits the mitogenic and anti-apoptotic activity of IGF by blocking the binding of IGF to its receptor. However, under certain circumstances, IGFBP-3 can enhance the activity of IGF by protecting IGF from its degradation. More than half of the inter-individual variations in IGFBP-3 levels are known to be genetically determined by the polymorphism at -202 locus of IGFBP-3 gene.

Method :

We attempted to ascertain whether *A-202C* polymorphic variation of IGFBP-3 gene constitutes a risk factor for non-small cell lung cancer (NSCLC), using PCR-restriction fragment length polymorphism (RFLP). Our study included 104 NSCLC patients and 104 age-, gender-, and smoking status-matched control subjects.

Result :

In the 104 NSCLC subjects, the genotypic frequencies at the -202 site were as follows: *AA* = 67 (64.4%), *AC* = 35 (33.7%), and *CC* = 2 (1.9%). We did detect significant differences in the genotypic distribution between the NSCLC and the control subjects (p <0.05), and the NSCLC risk correlated significantly with *AA* genotype at the -202 locus (*AA*>*AC*>*CC*). Using *CC* genotype as a reference, the odds ratio (*OR*) for the subjects with *AC* genotype was 2.60 (95% CI: 0.89 - 8.60), and the *OR* associated with *AA*

genotype was 5.89 (95% CI: 1.92 - 21.16).

Conclusion :

These results indicate that the dysregulation of IGF axis should now be considered as another important risk factor for NSCLC, and a potential target for novel antineoplastic therapies and/or preventative strategies in high-risk groups.

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