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Communication

Molecules and Cells

Hypermethylation of Growth Arrest DNA-Damage-Inducible Gene 45 in Non-Small Cell Lung Cancer and Its Relationship with Clinicopathologic Features

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The growth arrest DNA-damage-inducible protein 45 (GADD45) can serve as a key coordinator of the stress response by regulating cell cycle progression, genomic stability, DNA repair, and other stress-related responses. Although deregulation of *GADD45* expression has been reported in several types of human tumors, its role in lung cancer is still unknown. DNA hypermethylation of promoter CpG islands is known to be a major mechanism for epigenetic inactivation of tumor suppressor genes. We investigated the methylation status of *GADD45* family genes (*GADD45A, B,* and *G*) in 139 patients with non-small cell lung cancer (NSCLC) using methylation-specific PCR (MSP) and correlated the results with clinicopathologic features of the patients. Methylation frequencies in tumors were 1.4% for *GADD45A,* 7.2% for *GADD45B*, and 31.6% for *GADD45G*. RT-PCR and MSP analysis showed that promoter methylation of the *GADD45G* gene resulted in down-regulation of its mRNA expression. *GADD45G* methylation was significantly more frequent in female patients than male patients ($P = 0.035$). This finding suggests that methylation-associated down-regulation of the *GADD45G* gene may be involved in lung tumorigenesis.

INTRODUCTION

Lung cancer is the leading cause of cancer deaths worldwide, with over 1 million deaths each year (Jemal et al., 2008). The poor prognosis is largely attributable to lack of efficient diagnostic methods for early detection. Thus, there is an increasing demand for molecular markers useful in detecting early cancer (Sidransky, 2002). Epigenetic gene silencing, which is associated with aberrant methylation of the promoter region of genes and transcriptional repression, is the major mechanism for the loss of tumor suppressor gene function in human cancer, along with genetic alterations such as mutations and deletions (Baylin and Herman, 2000). Several studies have shown that many cancer-related genes are inactivated by promoter methylation in a number of human cancers, including lung cancer (Belinsky, 2004; Tsou et al., 2002), suggesting that cancer-specific aberrant promoter methylation may be useful as a diagnostic and prognostic marker for lung cancer (Belinsky, 2004; Safar et al., 2005; Tsou et al., 2002).

The growth arrest DNA damage-inducible gene (*GADD45*) family is composed of *GADD45A*, *GADD45B*, and *GADD45G* (originally termed *GADD45*, *MYD118*, and cytokine response gene 6 [*CR6*], respectively). The GADD45 proteins encoded by these genes, GADD45α, GADD45β and GADD45γ, function as stress sensors in signaling responses to various physiological or environmental stresses and mediate their activity via interactions with other cellular proteins, including proliferating cell nuclear antigen, p21, cyclin-dependent kinase 1 (cdc2)/cyclin B1, and the p38 and JNK stress response kinases, resulting in cell cycle arrest, DNA repair, genomic stability, and apoptosis (Cretu et al., 2009; Hoffman and Liebermann, 2008). Individual members of the GADD45 family are differentially induced by a variety of genotoxic and environmental stresses, indicating that each gene is induced by a distinct subset of environmental stresses (Shaulian and Karin, 1999; Zhang et al., 1999). In addition, GADD45 proteins have been shown to play similar but not identical roles, depending on the particular stress response pathway activated (Guillouf et al., 1995; Yoo et al., 2003).

It has been documented that GADD45 proteins modulate tumor development in response to oncogenic stress (Hollander et al., 1999; Tront et al., 2006). In addition, several studies have shown that *GADD45* genes are frequently down-regulated due to aberrant promoter methylation in various human cancers, such as nasopharyngeal, breast, and prostate cancers (Cretu et al., 2009; Ying et al., 2005). Taken together, these observations suggest that *GADD45* genes play an important role in carcinogenesis as tumor suppressors. However, although there has been one report evaluating *GADD45G* methylation status in five lung cancer cell lines (Ying et al., 2005), the biological role of aberrant promoter methylation of *GADD45* genes in lung cancer remains unclear. Therefore, we investigated the methylation status of the promoter regions of *GADD45A*, *GADD45B*, and *GADD45G* genes in non-small cell lung cancers (NSCLCs)

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and correlated the results with clinicopathologic characteristics.

MATERIALS AND METHODS

Patients and tissue samples

Tumor and corresponding non-malignant lung tissue specimens were obtained from 139 Korean NSCLC patients who underwent curative resection at the Kyungpook National University Hospital (Korea) between January 2002 and July 2006. None of these patients received chemotherapy and radiotherapy before the surgery. Informed consent was obtained from each patient before surgery. This study was approved by the Institutional Review Board of the Kyungpook National University Hospital. The clinicopathologic characteristics of the patients have been described elsewhere (Kim et al., 2009). All of the tumor and macroscopically normal lung tissue samples were obtained at the time of surgery, and were rapidly frozen in liquid nitrogen and stored at -80°C until genomic DNA preparation. Only tumors with > 80% tumor component were sent for DNA extraction and methylation analysis. The macroscopically normal lung tissues were confirmed to be normal by hematoxylin-eosin staining. Genomic DNA was extracted using a QIAamp DNA Mini Kit (QIAGEN, USA).

Methylation analysis

The methylation status of the *GADD45* genes was determined using methylation-specific PCR (MSP) with primers specific for the methylated and unmethylated alleles of each gene after treatment of the genomic DNA with sodium bisulfite. The primer sequences and annealing temperatures are summarized in Table 1. All PCR amplifications were carried out using reagents supplied in a GeneAmp DNA Amplification Kit with AmpliTaq Gold as the polymerase on a PTC-100 thermal cycler (MJ Research, USA). CpGenome™ Universal methylated and unmethylated DNA (Chemicon, USA) was used as a positive control for the methylated and unmethylated genes, respectively. Negative control samples without DNA were included for each set of PCR. PCR products were analyzed on 2% agarose gels, stained with ethidium bromide, and visualized under UV light. Each MSP was repeated at least once to confirm the results.

Total RNA isolation and reverse transcription-polymerase chain reaction (RT-PCR)

Total RNA was extracted from lung cancer tissues and corresponding non-malignant lung tissues (n = 15) and from cultured NSCLC cell lines using TRIzol (Invitrogen, Mt Waverly, VIC, Australia) according to the manufacturer's instructions. The structural integrity of the total RNA was confirmed by electrophoresis on 1.2% agarose-formaldehyde gels. Residual genomic DNA was digested with RNase-free DNase (Invitrogen). First strand cDNA was reverse-transcribed from 2 μg total RNA in a total volume of 20 μl using oligo(dT) and a SuperScript preamplification kit (Invitrogen). The resulting cDNA was amplified using primers specific to the *GADD45* genes with AmpliTaq Gold (PE Applied Biosystems, USA). The primer sequences and annealing temperatures were described previously (Ying et al., 2005). PCR was performed in a PTC100 and amplified products were separated on 2% agarose gels, visualized using ethidium bromide, and photographed.

Statistical analysis

The relationship between methylation and clinicopathologic characteristics was analyzed using a Chi-square test or Fisher's exact test for categorical variables. A P value < 0.05 was considered to be statistically significant. A logistic regression test was conducted to estimate the relationship between methylation and the covariates of age, gender, smoking history, tumor histology, and pathologic stage. The overall survival times of NSCLC patients with or without methylation of *GADD45* genes were compared using the Kaplan-Meier method and the log-rank test. All analyses were performed using the Statistical Analysis System for Windows, version 9.1 (SAS Institute, USA).

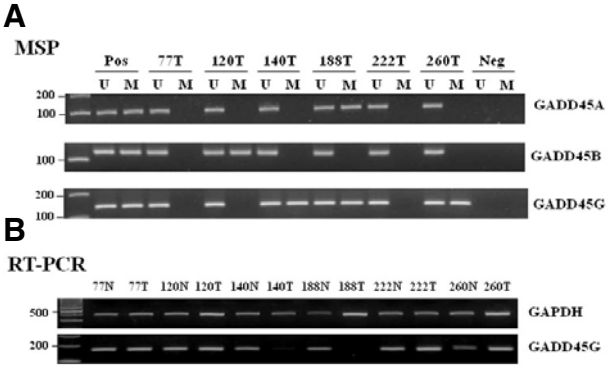

Fig. 1. Representative data of MSP analysis of the *GADD45* genes and RT-PCR analysis of *GADD45G* mRNA in NSCLC patient tissues. (A) The methylation status of *GADD45A, GADD45B*, and *GADD45G* genes was analyzed in tumor and non-malignant lung tissues. MSP was carried out using unmethylation-specific (U) and methylation-specific (M) primers as described in "Materials and Methods." CpGenome™ Universal methylated and unmethylated DNA (Chemicon) was used as a positive control for the M and U forms. Water was used as a negative control. Lane U, amplified product obtained using U primers; Lane M, amplified product obtained using M primers; Pos, positive control; Neg, negative control. (B) Expression of *GADD45G* mRNAs was measured in a subset of primary lung tissues by RT-PCR. Amplified products were run on 2% agarose gel and were visible at 178-bp for *GADD45G*. Amplification of GAPDH was used as an internal loading control.

RESULTS AND DISCUSSION

The methylation status of *GADD45* genes (*GADD45A*, *GADD45B*, and *GADD45G*) was determined in 139 surgically-resected NSCLCs and their corresponding non-malignant lung tissues using MSP. Complete CpG islands (CGIs) were detected in the 5′ flanking region of the genes including the first exon, which exactly covered the putative active promoter regions (ENSG 116717, 99860, 130222). For the *GADD45B* gene, the previously described MSP primer was used (Qui et al., 2004), and for *GADD45A* and *GADD45G* genes, novel MSP primers were designed to cover the same regions that were previously evaluated by the bisulfite genomic sequencing method (Schulz et al., 2007; Ying et al., 2005). Each specific primer set yielded a single band of the expected size, and representative examples of the MSP analysis are illustrated in Fig. 1A. For all genes, unmethylated bands were detected in both non-malignant and malignant tissues, thus confirming the integrity of the DNA in these samples. Because the tumor specimens represented macroscopically isolated samples that contained both tumor and non-malignant tissue, this was expected. Bisulfite-sequencing of the representative PCR products confirmed their methylation status and showed that all cytosines at non-CpG sites were converted to thymine (data not shown). This excluded the possibility that successful amplification could

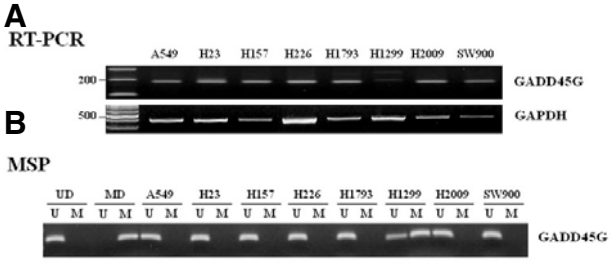

Fig. 2. RT-PCR and MSP analysis of the *GADD45G* gene in NSCLC cell lines. Expression of *GADD45G* mRNA (A) and its methylation status (B) were determined in various human NSCLC cell lines by RT-PCR and MSP, respectively. The experimental details are the same as in Fig. 1.

be attributable to incomplete bisulfite conversion. Methylation of *GADD45A*, *GADD45B*, and *GADD45G* genes was observed in 1.4, 7.2 and 31.7% of the tumors, respectively. At least one of the three genes was methylated in 52 (34.7%) of the tumors. However, no aberrant promoter methylation of *GADD45* genes was detected in the corresponding non-malignant lung tissues, indicating that methylation of *GADD45* genes is tumor specific. These results represent the first demonstration of aberrant methylation of *GADD45* genes in primary tumors of NSCLC patients, and are consistent with a previous report demonstrating frequent methylation of the *GADD45G* gene promoter in lung cancer cell lines (Ying et al., 2005).

To determine whether promoter methylation was associated with transcriptional silencing of the *GADD45G* gene, the mRNA level and methylation status were investigated in tumor and corresponding non-malignant lung tissues (n = 15), as well as

Table 1. Primer sets for MSP

Primers	Sequences	Size (bp)	Annealing temp (°C)
Methylated			
GADD45A (F)	5′-GTTTGAGTTGTCGGAGCGGC-3′	101	63
GADD45A (R)	5′-CCCAAAAAAAAAACGCGACGTACG-3′		
GADD45B (F)	5′-GAAAGTTCGGGTCGTTTCGCGC-3′	133	55
GADD45B (R)	5′-AAAACCGAATAAATAACCGCG-3′		
GADD45G (F)	5′-GCGGGTGTCGGTTAATAGGCGC-3′	130	68
GADD45G (R)	5′-CAACCAAAACTCAACACGACGCG-3′		
Unmethylated			
GADD45A (F)	5′-GATTTTTGTAATAATTTGTTGTGTT-3′	101	55
GADD45A (R)	5′-TAACTCATACCCAACCAACA-3′		
GADD45B(F)	5′-GAAAGTTTGGGTTGTTTTGTGT-3′	133	50
GADD45B (R)	5′-AAAACCAAATAAATAACCACA-3′		
GADD45G (F)	5′-GTGGGTGTTGGTTAATAGGTGT-3′	130	58
GADD45G (R)	5′-CAACCAAAACTCAACACAACACA-3′		

NSCLC cell lines, using RT-PCR and MSP analysis. In a subset of tissue samples (n = 15), *GADD45G* mRNA expression was lost or reduced in tumor tissues concordant with its methylation status, except for two tumors (Fig. 1B). We also found that *GADD45G* mRNA expression was concordant with its methylation status in all corresponding normal tissue samples (Fig. 1B). Moreover, *GADD45G* mRNA was expressed at high levels in the cell lines with unmethylated alleles, while its expression was remarkably reduced in the H1299 cell line with a methylated allele (Fig. 2). These data indicate that there is a

Table 2. Relationship of methylation of *GADD45* family genes with clinicopathologic features

Variables	Methylation, no (%)			
	GADD45A	*GADD45B*	*GADD45G*	*GADD45A*, *B*, or G
All subjects (n =139)	2 (1.4)	10 (7.2)	44 (31.7)	52 (37.4)
Age (years)				
≤ 62 (n = 60)	2 (3.3)	5 (8.3)	17 (28.3)	21 (35.0)
> 62 (n = 79)	0 (0.0)	5 (6.3)	27 (34.2)	31 (39.2)
Gender				
Male (n = 101)	1 (1.0)	8 (7.9)	27 (26.7)*	33 (32.7)*
Female (n = 38)	1 (2.6)	2 (5.3)	17 (44.7)	19 (50.0)
Smoking status				
Ever (n = 104)	1 (1.0)	8 (7.7)	30 (28.8)	36 (34.6)
Never (n = 35)	1 (2.9)	2 (5.7)	14 (40.0)	16 (45.7)
Histological types				
Squamous cell ca. (n = 60)	0 (0.0)	7 (11.7)	19 (31.7)	23 (38.3)
Adenoca. (n = 79)	2 (2.5)	3 (3.8)	25 (31.6)	29 (36.7)
Pathologic stage				
I (n = 85)	0 (0.0)	5 (5.9)	30 (35.3)	34 (40.0)
II-III (n = 54)	2 (3.7)	5 (9.3)	14 (25.9)	18 (33.3)
EGFR mutations in Adenoca.				
Absent (n = 46)	1 (2.2)	1 (2.2)	16 (34.8)	18 (39.1)
Present (n = 33)	1 (3.0)	2 (6.1)	9 (27.3)	11 (33.3)
Tp53 mutation[a]				
Absent (n = 54)	2 (3.8)	2 (3.7)	16 (29.6)	19 (35.2)
Present (n = 39)	0 (0.0)	5 (12.8)	16 (32.7)	20 (40.8)

*$P < 0.05$
[a]*TP53* mutations were studied in 93 of the 139 NSCLCs

direct correlation between *GADD45G* promoter methylation and mRNA expression.

The clinicopathologic features were compared to determine the methylation status of the *GADD45A*, *GADD45B* and *GADD45G* genes according to patient profiles. *GADD45G* methylation was significantly more frequent in female patients than in male patients ($P = 0.035$). However, the methylation of *GADD45* genes was not significantly correlated with other clinicopathologic factors, such as age, gender, smoking status, histologic types, *TP53* mutation status, or *EGFR* mutation status in adenocarcinomas (Table 2). In addition, methylation of *GADD45* genes was not significantly associated with survival outcomes of the patients (data not shown). Thus, it would be interesting to investigate the mechanisms underlying the female-prevalent hypermethylation of *GADD45G* gene.

Collectively, the present study showed that the promoter region of GADD45G gene was frequently methylated in NSCLCs and its methylation was related to reduced or lost mRNA expression. These results suggest that methylation-associated downregulation of GADD45G plays an important role in the pathogenesis of NSCLC, particularly in females. However, further studies with a large number of patients are needed to confirm these findings.

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