

Wif1 Hypermethylation as Unfavorable Prognosis of Non-Small Cell Lung Cancers with *EGFR* Mutation

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Lung cancer is a leading cause of cancer-related mortality across the world and tobacco smoking is the major risk factor. The Wnt signaling pathway is known to be involved in smoke-induced tumorigenesis in the lung. Promoter hypermethylation of Wnt inhibitory factor 1 (*Wif1*) has become a common event in a number of human tumors. Using a methylation-specific PCR, hypermethylation of the *Wif1* gene promoter was evaluated in 139 primary non-small cell lung cancers (NSCLCs) and its correlation with clinicopathological and prognostic parameters was evaluated. Methylation of *Wif1* was observed in 47.5% and 20.9% of neoplastic and adjacent normal lung tissues, respectively. Its methylation rate tended to be higher in stage I than stages II-IIIa. Results of Kaplan-Meier analysis showed no significant difference in overall survival according to *Wif1* methylation status. However, *Wif1* methylation showed an association with unfavorable prognosis of adenocarcinoma (AC) patients with *EGFR* mutation. According to our current findings, *Wif1* promoter methylation is an early, frequent event as an epigenetic field manner and could be considered as a useful prognostic marker for AC patients with *EGFR* mutation. Further investigation into the therapeutic potential of this finding is warranted.

INTRODUCTION

Lung cancer continues to be a major public health problem worldwide. Late diagnosis and lack of effective treatments for lung cancer are associated with unsatisfactory prognosis and high mortality (Jemal et al., 2010). In addition, lung cancer is a heterogeneous group of diseases made up of entities characterized by distinctive clinical, pathological, morphologic, and genetic features (Minna et al., 2002). However, the molecular basis of these variations in behavior and epidemiology is not well known. Of interest, growing evidence has demonstrated a remarkable difference in genetic and epigenetic factors leading to this neoplasm between East Asian and Western populations (Gazdar et al., 2004; Toyooka et al., 2003). In Korea, prevalence of male smoking is among the highest in the world, and

mortality rates from smoking-related cancers, particularly lung cancer, are escalating (Jee et al., 2004). Therefore, advances in understanding of the molecular pathogenesis of lung cancer are critical to conquering lung cancer. Transcriptional silencing of genes by CpG island (CGI) methylation is now recognized as a critical component in initiation and progression of lung cancer (Kerr et al., 2007; Kim et al., 2007; Na et al., 2010; Toyooka et al., 2001). In addition, variation in methylation status has been associated with tobacco smoking (Liu et al., 2010).

Dysregulation of Wnt signaling is common in several types of cancer, including colon, lung, breast, and prostate (Mazieres et al., 2005; Paul et al., 2008). In particular, mutations of key Wnt signaling genes, such as *APC*, *β-catenin*, or *Axin* appear to be rare in lung cancer (Mazieres et al., 2005). The Wnt pathway is highly regulated and very complex, with different Wnt inhibitors, which consistently inhibit or facilitate Wnt signaling depending on molecular, cellular, and tissue context (Rubin et al., 2006). The Wnt pathway has been associated with normal lung development and homeostasis as well as chronic lung diseases such as fibrosis and asthma (Konigshoff et al., 2010; Van et al., 2008). However, there are contradictory findings indicating that the Wnt pathway is activated or down-regulated by cigarette smoke in bronchial epithelial cells (Lemjabbar-Alaoui et al., 2006; Wang et al., 2011). Wnt inhibitory factor 1 (*Wif1*), an extracellular antagonist that acts by binding to Wnt ligands, is a frequent target for epigenetic silencing in various human cancers (Ying et al., 2009). Of particular interest, *Wif1* promoter hypermethylation is common in Western patients with lung cancer; however, it is rare in Japanese cases (Licchesi et al., 2008; Mazieres et al., 2004; Tang et al., 2006; Yoshino et al., 2009), which have been conducted in small numbers to draw definitive conclusions. We have recently found that *DKK1* methylation is common in NSCLC and is a good prognostic indicator for a certain group of patients (Na et al., 2012). Herein, in order to further understand the molecular pathogenesis and clinical relevance of the Wnt pathway in lung cancer, we performed methylation-specific PCR (MSP) in order to determine the methylation status of the *Wif1* gene promoter in Korean patients with NSCLC and investigated the relationship between methylation status and clinicopathological factors.

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MATERIALS AND METHODS

Patients and tissue samples

Tumor and corresponding non-malignant lung tissue specimens (n = 139) were provided by the National Biobank of Korea - Kyungpook National University Hospital (KNUH), which is supported by the Ministry of Health, Welfare and Family Affairs. All materials derived from the National Biobank of Korea - KNUH were obtained under institutional review board approved protocols. A summary of the clinicopathological characteristics of the patients which had previously been examined in another study (Lee et al., 2012) is provided in Table 1.

Cell culture and 5-aza-2'-deoxycytidine (5-Aza-dC) treatment

A normal human bronchial epithelial cell (NHBE) and 4 human NSCLC cell lines (A549, H460, H1703, and H2009) were obtained from the American Type Culture Collection (ATCC, USA). All cells were propagated with the instructions from the ATCC. 5-Aza-dC treatment was performed as described previously (Lee et al., 2012).

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

Total RNA was extracted from the cultured cells using TRIzol (Invitrogen, 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 of total RNA in a total volume of 20 µl using oligo (dT) and a SuperScript preamplification kit (Invitrogen). The resulting cDNA was amplified by forward (5'-CCGAAATGGAGGCTTTTGTAT-3') and reverse (5'-TGGTTGAGCAGTTTGCTTTG-3') primers. Amplified products were separated on 2% agarose gels, visualized using ethidium bromide, and photographed.

Genomic DNA isolation and methylation analysis

Genomic DNA was extracted using a QIAamp DNA Mini Kit (QIAGEN, USA). Bisulfite modification of genomic DNA was carried out by using an EZ DNA methylation kit (Zymo Research Co, USA) according to the manufacturer's protocol. Bisulfite-treated genomic DNA was amplified using either a methylation-specific or an unmethylation-specific primer set; for the methylated reaction 5'-GGGCGTTTTATTGGCGTAT-3' (forward) and 5'-AAACCAACAATCAACGAAAC-3' (reverse), and for the unmethylated reaction 5'-GGGTGTTTTATTGGG TGTAT-3' (forward) and 5'-AAACCAACAATCAACAAAAC-3' (reverse). All PCR amplifications were performed using reagents supplied in a GeneAmp DNA Amplification Kit with AmpliTaq Gold as the polymerase (PE Applied Biosystems, USA) on PTC-100 (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 gel, stained with ethidium bromide, and visualized under UV light. Each MSP was repeated at least once in order to confirm the results.

Statistical analysis

The relationship between the methylation status and the clinicopathological characteristics was analyzed using a chi-square test or Fisher's exact test for categorical variables. A *P* value of

Table 1. Correlation between *Wif1* methylation and the clinicopathological features of NSCLCs

Feature	<i>Wif1</i> Methylation (%)	<i>P</i>
All subjects (n = 139)	66 (47.5)	
Age (years)		
≤ 63 (n = 67)	30 (44.8)	0.328
> 63 (n = 72)	36 (50.0)	
Gender		
Male (n = 101)	48 (47.5)	0.570
Female (n = 38)	18 (47.4)	
Smoking status		
Ever (n = 104)	51 (49.0)	0.331
Never (n = 35)	15 (41.9)	
Histologic types		
Squamous cell carcinoma (n = 60)	30 (50.0)	0.364
Adenocarcinoma (n = 79)	36 (45.6)	
<i>EGFR</i> mutations in Adenocarcinoma		
Absent (n = 46)	24 (52.2)	0.122
Present (n = 33)	12 (36.4)	
<i>TP53</i> mutations*		
Absent (n = 54)	25 (46.3)	0.470
Present (n = 49)	24 (49.0)	
Pathologic stages		
Stage I (n = 85)	45 (52.9)	0.068
Stage II-IIIa (n = 54)	21 (38.9)	

*The mutation of TP53 was studied in 103 of the 139 NSCLCs.

less than 0.05 was considered statistically significant. A logistic regression test was performed in order to estimate the relationship between methylation and the covariates of age, gender, exposure to tobacco smoke, and histology. The overall survival (OS) of NSCLC patients according to methylation status of the *Wif1* gene was compared using the Kaplan-Meier method and the log-rank test. Hazard ratios (HR) and 95% confidence interval (CI) were estimated using a multivariate Cox proportional hazard model, with adjustment for age (≤ 63 versus > 63 years), gender (male versus female), smoking status (never- versus ever-smoker), and pathologic stage (I versus II-IIIa). The homogeneity test was performed for comparison of the difference between HR of different groups. The Statistical Analysis System for Windows, version 9.1 (SAS Institute, USA) was used in performance of all analyses.

RESULTS AND DISCUSSION

Methylation status of the *Wif1* gene in tissues and inverse correlation with its mRNA expression

The methylation status of the human *Wif1* gene was analyzed in 139 primary NSCLCs and matched nonmalignant lung tissues using the MSP method. Methylated and unmethylated allele-specific primers yielded a single band of the expected size; representative examples of the MSP analysis are shown in Fig. 1A. Unmethylated bands were detected in most of the nonmalignant and malignant tissues, thus confirming the integrity of the DNA in these samples. Bisulfite-sequencing of the representative PCR products confirmed their methylation status

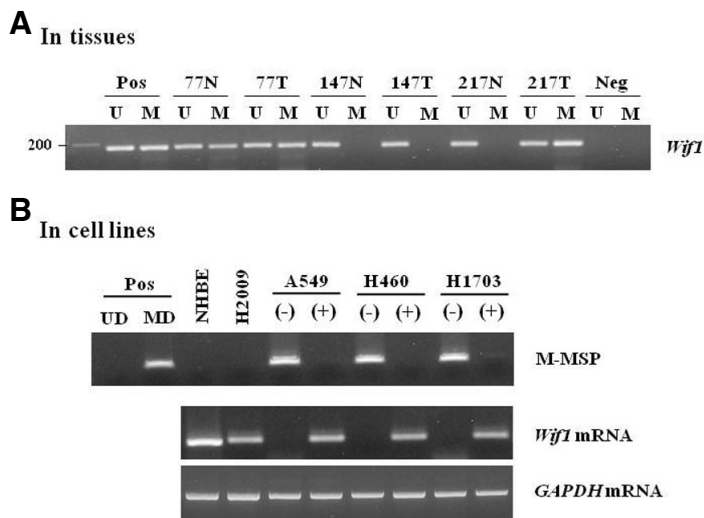


Fig. 1. Hyperethylation of *Wif1* in NSCLC patients (A) and correlation of its methylation with silencing in cell lines (B). (A) Promoter methylation of the *Wif1* gene was analyzed using MSP. CpGenome™ Universal methylated DNA (MD) or unmethylated DNA (UD) was used as a positive control for the methylated or unmethylated products, respectively. Water was used as a negative control. N, non-malignant tissue; T, tumor tissues; U and M, amplified product with primers that recognize the unmethylated or methylated sequences. (B) Expression of *Wif1* mRNA and its methylation status were determined in 5 cell lines by RT-PCR and MSP, respectively. Amplified product was visible at 188-bp for *Wif1*. Amplification of *GAPDH* was used as an internal loading control. The symbol (-) indicated vehicle alone treatment, whereas (+) indicated the 5-AzadC treatment for 3 days.

and showed that all cytosines at non-CpG sites were converted to thymine (data not shown), ruling out the possibility of incomplete bisulfite conversion. *Wif1* hypermethylation was detected in 47.5% (66/139) of cancerous tissues and in 20.9% (29/139) of adjacent normal tissues ($P < 0.0001$), suggesting that its methylation may be not an intrinsic, developmentally programmed event, but a tumor-associated *de novo* event. Our data is similar to recent finding that *Wif1* are methylated in 31.1% of normal adjacent specimens and 68.9% of AC samples (Licchesi et al., 2008), although there was a difference of methylation prevalence due to MSP assay sensitivity (conventional vs nested MSP). Notably, *Wif1* methylation was observed in a subset of nonmalignant lung tissues of the patients. Methylation of nonmalignant lung tissues concurred predominantly with that in the corresponding cancer tissues (data not shown), therefore, it is likely that the presence of *Wif1* methylation in histologically nonmalignant tissues may be due to unavoidable contamination of nonmalignant cells with methylated cancer cells. Alternatively, it is possible that even phenotypically normal lung tissues can already harbor epigenetic alterations because the entire field of the lung was exposed to carcinogenic insult, such as cigarette smoking. Frequent occurrence of aberrant promoter methylation in histologically normal-appearing lung tissues has been reported, representing a field defect of widespread epigenetic change in lung tissues (Belinsky et al., 2002; Guo et al., 2004). Considering that methylation of the *Wif1* gene is nearly as frequent in normal lung parenchyma as in low grade-atypical adenomatous hyperplasia (Licchesi et al., 2008), our findings suggest that *Wif1* promoter hypermethylation may be a common and tumor-associated event as a field-cancerization manner.

To determine whether promoter methylation was associated with transcriptional silencing of the *Wif1* gene, the mRNA level and methylation status were investigated in NSCLC cell lines using RT-PCR and MSP analysis. *Wif1* mRNA was found in NHBE and H2009 cells lacking methylated alleles, while its expression was hardly detected in the cell lines (A549, H460, and H1703) with methylated ones (Fig. 1B). Moreover, treatment of DNA demethylating agent 5-AzadC dramatically restored *Wif1* mRNA expression in these cell lines. These results are agreement with previous finding by Mazieres et al. (2004), indicating an inverse correlation between *Wif1* promoter methylation

and its mRNA expression.

Correlation between methylation status and clinicopathological characteristics

Wif1 methylation was more frequent in patients with stage I disease than those with stages II-IIIa with a borderline significance ($P = 0.068$). However, no correlations with other clinicopathological profiles, such as age, sex, smoking status, tumor histology, *EGFR* and *TP53* mutations were found (Table 1). In addition, no significant difference in overall survival (OS) of total patients was observed according to *Wif1* methylation status (Log-Rank $P [P_{L-R}] = 0.965$, Fig. 2A). Of particular interest, when ACs were categorized according to *EGFR* mutation, *Wif1* methylation showed an association with a worse OS in AC patients with *EGFR* mutation ($P [P_{L-R}] = 0.035$, Figs. 2B and 2C). Multivariate analysis showed that *Wif1* methylation exhibited a trend toward worse OS in AC patients with *EGFR* mutation (adjusted HR = 3.85, 95% CI = 0.79-18.72, $P = 0.075$) (Table 2), being comparable with a recent observation that in ACs with *EGFR* mutation group, patients with methylated Wnt antagonist *SFRP5* has a significantly shorter progression free survival than those with unmethylated *SFRP5* (Zhu et al., 2012). Crosstalk between Wnt and EGFR signaling in cancers has been well documented (Hu et al., 2010). Wnt binds to Frizzled receptor, leading to transactivation of EGFR signaling by matrix metalloproteinase-mediated release of soluble EGFR ligands. Upon activation, EGFR could transactivate β -catenin through the receptor tyrosine kinase-PI3K/Akt pathway, and β -catenin might also form a heterodimer with EGFR, leading to activation of the EGFR pathway. A meta-analysis has demonstrated a frequent association of EGFR overexpression in NSCLC with poor prognosis, even in the presence of contradicting results (Nakamura et al., 2006). In addition, somatic mutations of the *EGFR* gene hyperactivate the downstream pro-survival pathways and consequently confer oncogenic shock (Sharm et al., 2007). Similarly, the EGFR kinase domain mutation is associated with overexpression of EGFR (Ohtsuka et al., 2006). Taken together, it is tempting to speculate that synchronous alterations of *Wif1* and *EGFR* might act synergistically in enhancement of Wnt and EGFR signaling, tumor formation, and progression, contributing to a poor prognosis. However, further investigation with large numbers of patients is needed in order to confirm this clinical

Table 2. Association of *Wif1* methylation with overall survival in NSCLC patients

Variables	P_{L-R}	Crude HR (95% CI)	P	P_H	Adjusted HR (95% CI)	P	P_H
Overall subjects (n = 139)	0.965	0.96 (0.58-1.58)	0.857		1.13 (0.68-1.89) [‡]	0.644	
<i>EGFR</i> mutations in AC							
Absent (n = 46)	0.632	0.82 (0.35-1.89)	0.633	0.054	1.00 (0.43-2.35) [¶]	0.968	0.141
Present (n = 33)	0.035	4.21 (0.99-17.98)	0.052		3.85 (0.79-18.72) [¶]	0.075	

[‡] Hazard ratios (HRs), 95% confidence intervals (CIs), and their corresponding P -values were calculated using multivariate Cox proportional hazard models, adjusted for age, gender, smoking status, tumor histology, and pathologic stage

[¶] HRs, 95% CIs, and their corresponding P -values were calculated using multivariate Cox proportional hazard models, adjusted for age, gender, smoking status, and pathologic stage

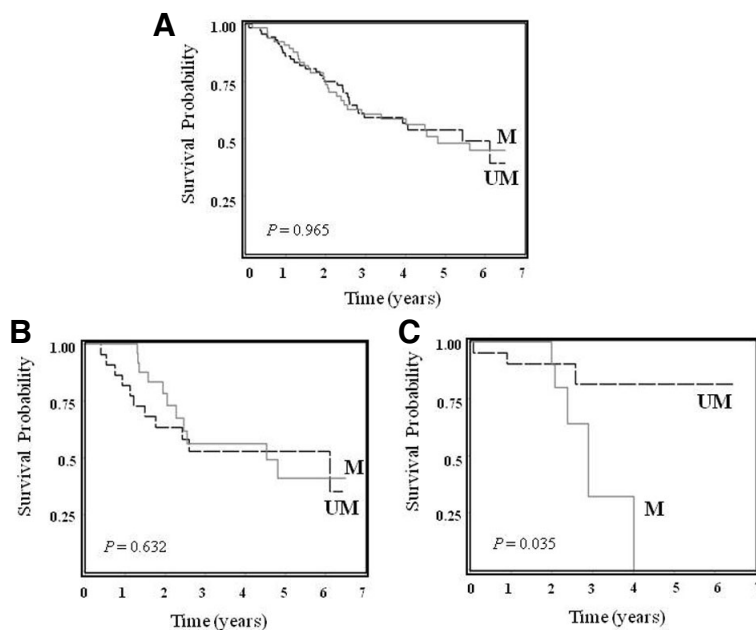


Fig. 2. Kaplan-Meier survival curves of NSCLC patients according to *Wif-1* methylation status. Kaplan-Meier survival curve for all patients (A), AC patients without *EGFR* mutations (B), and AC patients with *EGFR* mutations (C). P -values from log-rank test. UM denotes patients with unmethylation, while M indicates patients with methylation.

implication.

Collectively, although the present study is limited by the small number of samples and the virtual lack of information on protein, it shows that *Wif1* promoter is frequently methylated in NSCLCs to down-regulate its mRNA expression. In addition, *Wif1* methylation is significantly associated with unfavorable prognosis of AC patients with *EGFR* mutation.

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