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Promoter methylation of *Wrap53α*, an antisense transcript of p53, is associated with the poor prognosis of patients with non-small cell lung cancer

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Abstract. Lung cancer, of which non-small cell lung cancer (NSCLC) accounts for ~85% of cases, remains a leading cause of cancer-associated mortality and morbidity worldwide. Tumor suppressor p53 is a master regulator of diverse cellular processes and is a therapeutic target in cancer. However, many aspects of its transcriptional regulation are still not well defined. WD repeat containing antisense to TP53α (*Wrap53α*) a newly identified natural antisense transcript of p53, can regulate p53 expression following DNA damage. The present study determined the methylation status of the *Wrap53α* promoter in primary lung tissues using methylation-specific polymerase chain reaction and evaluated its associations with clinicopathological features and survival in patients with NSCLC. The *Wrap53α* promoter was methylated in 12 (8.2%) of 146 malignant tissues. Its methylation was associated with the downregulation of its transcription and was frequently detected in patients with stages II-IIIA (P=0.03), and *p53* mutation-negative cases (P=0.08). Methylation of *Wrap53α* promoter was associated with worse overall survival of total patients with a borderline significance [adjusted Hazard Ratio (HR)=2.44, 95% Confidence Interval (CI)=0.98-6.04, P=0.05]. Notably, *Wrap53α* promoter methylation significantly associated with poor overall survival in *p53* mutation-negative patients (log-rank P=0.01, adjusted HR=2.92, 95% CI=1.00-8.60, P=0.05), but not in patients with p53 mutations. The results of the present study suggest that *Wrap53α* may serve a role in the pathogenesis of a subset of lung cancer, and its methylation may be considered to be a prognostic marker for surgically resected NSCLC patients. However, further studies with a larger sample size are required to confirm this finding.

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Introduction

Lung cancer is the leading cause of cancer-related mortality in many countries, called 'a global scourge' with a dismal prognosis. Diagnosis is frequently made at an advanced stage when prognosis is poor and therapeutic options are limited (1). The molecular mechanisms underlying global variations in lung cancer biology remains poorly understood (2). Therefore, it is necessary to identify multiple biomarkers for early detection and prognosis. Transcriptional silencing of genes by CpG islands (CGIs) methylation is now recognized as a crucial component in lung cancer initiation and progression (3). In addition, gene-specific hyper-methylation has emerged as an important factor in the earliest stages of preinvasive lung cancer related to tobacco smoking, a major etiological factor (4).

Tumor suppressor p53 is a cellular gatekeeper that guards against genetic instability and abnormality by sensing multiple stress signals, including DNA damage, oncogene activation, and hypoxia (5). Lung cancer has a higher *p53* mutation rate compared to other kinds of cancer (6). Expression of *p53* is tightly controlled through multiple regulatory layers, but limited information is available on how *p53* is transcriptional and epigenetically regulated (7). Recently, WD40 repeat containing antisense to p53 (Wrap53) (also known as WDR79/TCAB1) was found to be a natural antisense transcript (NAT) of p53 that regulates endogenous p53 mRNA levels by targeting the 5'untranslated region (UTR) (8). Transcripts initiated from exon 1α, 1β, and 1γ are called *Wrap53α*, *Wrap53β*, and *Wrap53*γ, respectively. Exon 1α directly overlaps the first exon of *p53* in an antisense fashion and forms an RNA-RNA hybrid with *p53* mRNA to protect it from degradation (8). Interestingly, *Wrap53α* transcript is upregulated by cancer therapeutic drugs and miR-4732-5p has a binding site in the 5' UTR of the *Wrap53α* transcript (9-11). However, overexpression of the WRAP53 protein, mainly produced from the *Wrap53β* transcript, is linked to progression of several types of tumors, including lung cancer (12-15). Importantly, neither

β- or γ-transcripts, nor WRAP53 protein, has any effects on p53 when overexpressed or knocked down. Therefore, it is conceivable that dysfunction of *Wrap53α* could contribute to tumorigenesis by failing to sustain *p53* expression and function in wild-type (WT) *p53*-carrying tumors. In order to test this hypothesis and understand the biological role of *Wrap53α* in lung cancer, we investigated the methylation status of the *Wrap53α* promoter in resected primary non-small cell lung cancers (NSCLC) using methylation-specific polymerase chain reaction (MSP) and assessed the correlation of these results with clinicopathological characteristics.

Materials and methods

Patients and tissue samples. Tumor and corresponding non-malignant lung tissue specimens (n=146) were provided by the National Biobank of Korea, Kyungpook National University Hospital (KNUH; Daegu, Korea), which is supported by the Ministry of Health, Welfare, and Family Affairs. The present study was conducted with the approval of the Ethics Committee of KNUH (no. 2014-04-210) and written informed consent was obtained from all of the participants prior to obtaining the samples. The clinicopathological characteristics of the patients are summarized in Table I.

Genomic DNA isolation and methylation analysis. Genomic DNA was extracted using a QIAamp DNA Mini kit (QIAGEN, Valencia, CA). After treatment of the genomic DNA with sodium bisulfite, the methylation status of *Wrap53α* promoter encompassing the transcription start site was analyzed using MSP with primers specific for either unmethylated or methylated alleles. The primer sequences for *Wrap53α* were described in Table II. All polymerase chain reaction (PCR) amplifications were carried out using reagents supplied in a GeneAmp DNA Amplification kit with AmpliTaq Gold as the polymerase (PE Applied Biosystems, Foster City, CA, USA) on a PTC-100 thermal cycler (MJ Research, Watertown, MA, USA). CpGenome™ Universal methylated and unmethylated DNA (Chemicon, Temecula, CA, 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 to confirm the results.

Cell culture, total RNA isolation, and semi-quantitative (sq)-PCR. Ten human NSCLC cell lines (A549, HCC827, H23, H358, H520, H522, H1299, H1703, H2009 and PC9) were obtained from the American Type Culture Collection (ATCC, Manassas, VA). All cells were propagated according to instructions from the ATCC. HCC827 cells were treated with 20 μM 5-AzadC for 3 days and the culture media was changed daily. Total RNA was extracted from cultured cells and primary tumor tissues using TRIzol (Invitrogen; Thermo Fisher Scientific, Inc., Waltham, MA, USA). After removal of residual DNA, first-strand cDNA was synthesized from total RNA using SuperScript preamplification (Invitrogen; Thermo Fisher Scientific, Inc.) according to the manufacturer's instructions. The resulting cDNA was amplified with *Wrap53α*- and p53-specific primers as previously described (8). The following thermocycling conditions were applied: 95˚C for 2 min, then 30 cycles of 95˚C for 1 min, 58˚C for 1 min and 72˚C for 1 min, and a final extension at 72˚C for 10 min. Amplification of *GAPDH* was used as an internal loading control. All primer sequences were described in Table II.

Table I. Correlation between *Wrap53α* promoter methylation status and characteristics of non-small cell lung cancer patients.

Variables	Methylation, n (%)	P-value
All subjects (n=146)	12 (8.2)	
Age (years)		
≤64 (n=76)	7 (9.2)	0.66
>64 (n=70)	5 (7.1)	
Sex		
Men (n=98)	8 (8.2)	0.97
Women (n=48)	4 (8.3)	
Smoking status		
Ever (n=101)	8 (7.9)	0.84
Never (n=45)	4 (8.9)	
Histological types		
SQC (n=43)	2 (4.7)	0.31
ADC (n=103)	10 (9.7)	
Pathologic stage		
Stage I (n=91)	4 (4.4)	0.03
Stage II-IIIA (n=55)	8 (14.6)	
p53 mutations		
Negative (n=87)	10 (11.5)	0.08
Positive (n=59)	2 (3.4)	

SQC, squamous cell carcinoma; ADC, adenocarcinoma; *Wrap53α*, WD repeat containing antisense to TP53α.

Mutational analysis of p53 gene. *P53* mutational analysis of the entire coding regions (exons 2-11), including exon/intron boundaries, was performed by PCR-based direct sequencing. The primers and conditions for PCR reactions were described previously (16). Sequencing was done using an ABI Prism 3100 Genetic Analyzer (PE Applied Biosystems). All sequence variants were confirmed by sequencing the products of independent PCR amplifications in both directions.

Statistical analysis. The associations between methylation status and clinicopathological characteristics were analyzed using a chi-square test for categorical variables. Logistic regression analysis was conducted to estimate the association between methylation status and the covariates of age, sex, exposure to tobacco smoke, and histology. The overall survival (OS) of NSCLC patients according to methylation status of the *Wrap53α* promoter was compared using the Kaplan-Meier method and the log-rank test. Hazard ratios (HRs) and 95% confidence intervals (CIs) were estimated using a multivariate Cox proportional hazard model. Data were analyzed using SAS v9.4 software (SAS Institute, Inc., Cary, NC, USA).

Table II. Primer sequences used for MSP and sqPCR.

Primer	Forward primer (5' to 3')	Reverse primer (5' to 3')
MSP		
U-MSP	AATATATGGAGTTGAGAGTTT	AAAAACATACTTTCCACAACA
M-MSP	AATATACGGAGTCGAGAGTTC	AAAAACGTACTTTCCACGACG
sqPCR		
Wrap53α	CGGAGCCCAGCAGCTACC	TTGTGCCAGGAGCCTCGCA
Wrap53β	GTCCCGGCTCCGCGGGTTC	GGCTGAGGACATCAGAGAATACCAGC
P53	GACGGTGACACGCTTCCCTGGAT	CGTGCAAGTCACAGACTTGGCTGTC
GAPDH	CATGACAACTTTGGTATCGTG	GTGTCGCTGTTGAAGTCAGA

MSP, methylation-specific polymerase chain reaction; sqPCR, semi-quantitative polymerase chain reaction; M-MSP, MSP for the methylated allele; U-MSP, MSP for the unmethylated allele.

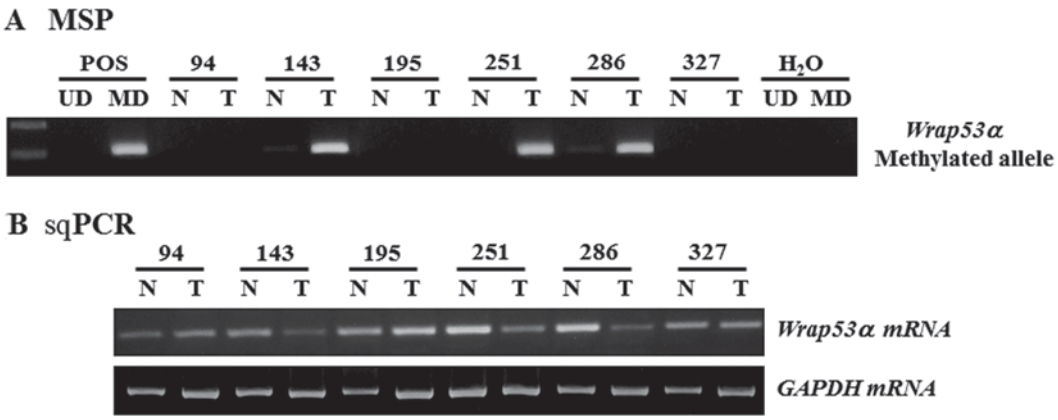

Figure 1. Representative results of MSP and sqPCR analysis of *Wrap53α* gene in NSCLC patients. (A) The methylation status of the *Wrap53α* promoter in NSCLCs was analyzed by MSP. CpGenome™ Universal MD or UD was used as a POS for the methylated or unmethylated products, respectively. Water was used as a negative control. (B) Expression of *Wrap53α* mRNA was measured in primary tissues from NSCLC patients by sqPCR. Amplified products were run on 2% agarose gel and appeared at positions corresponding to the expected base pair lengths. Amplification of *GAPDH* was used as an internal loading control. sqPCR, semi-quantitative polymerase chain reaction; N, non-malignant tissue; T, tumor tissues; M-MSP, amplified product with primers that recognize the methylated sequences; *Wrap53α*, WD repeat containing antisense to TP53α; NSCLC, non-small cell lung cancer; MD, methylated DNA; UD, unmethylated DNA; POS, positive control.

P<0.05 was considered to indicate a statistically significant difference.

Results

Methylation status and expression of Wrap53α gene in NSCLC samples. We have analyzed the methylation status of the human *Wrap53α* gene in 146 primary NSCLCs and their corresponding nonmalignant lung tissues using MSP. There were no classical CGIs in the 5'-flanking region of the human *Wrap53α* gene, including the first exon, but 13 CGIs were found from -150 to +30 bp upstream of the transcription start site. Thus, we designed the MSP primer pairs to cover this region. Methylated alleles of representative samples were shown as in Fig. 1A. Unmethylated bands were detected in most of the nonmalignant and malignant tissues (data not shown), thus confirming the integrity of the DNA in those samples. Bisulfite-sequencing of the representative PCR products confirmed the assigned methylation status and showed that all cytosines at non-CpG sites were converted to thymine (data not shown), ruling out the possibility of incomplete bisulfite conversion. *Wrap53α* methylation was exclusively detected in malignant tissues at a frequency of 8.2% (12/146), suggesting that *Wrap53α* promoter methylation may be a tumor-associated event during NSCLC tumorigenesis.

To determine whether CpG methylation was involved in the regulation of *Wrap53α* expression, we analyzed *Wrap53α* mRNA levels in representative tissue specimens. sqPCR analysis showed low or undetectable levels of *Wrap53α* transcripts in tumor tissues with a methylated allele, whereas high levels were detected in tumor and non-tumor lung tissues with an unmethylated allele (Fig. 1B). We have further confirmed these results in 10 human NSCLC cell lines. Comparison of methylation status with sqPCR findings demonstrated that *Wrap53α* mRNA was present in all examined cell lines except a HCC827 cell line that had methylated alleles (Fig. 2A). After treatment with the demethylating agent 5-AzadC for 3 days, HCC827 cells exhibited the disappearance of methylated alleles and induced the re-expression of *Wrap53α* mRNA, resulting in increased *p53* mRNA levels (Fig. 2B). These

Table III. Overall survival according to methylation of the *Wrap53α* promoter in non-small cell lung cancer patients.

					Crude			Adjusted		
Methylation negative/positive	Cases (n)	Mortality (%)[a]	5 SYR[b]	P_{LR}	HR (95% CI)	P-value	P_{HT}	HR (95% CI)[c]	P-value	P_{HT}
All subjects	146									
Negative	134	35 (26.1)	58		1			1		
Positive	12	6 (50.0)	34	0.01	2.88 (1.19-6.96)	0.02		2.44 (0.98-6.04)	0.05	
p53-mutation negative										
Negative	77	13 (16.9)	65		1			1		
Positive	10	5 (50.0)	38	0.01	3.59 (1.26-10.20)	0.02		2.92 (0.99-8.60)	0.05	
p53-mutation positive										
Negative	57	22 (38.6)	53		1			1		
Positive	2	1 (50.0)	0	0.32	2.73 (0.35-21.19)	0.34	0.81	1.90 (0.21-16.86)	0.57	0.73

[a]Row percentage; [b]5 SRY, proportion of survival derived from Kaplan-Meier analysis; [c]HRs, 95% CIs and their corresponding P-values were calculated using multivariate Cox proportional hazard models, adjusted for age, sex, smoking status, histology, and pathological stage. HR, hazard ratio; CI, confidence interval; 5 SRY, 5 year survival rate; P_{LR}, log-rank P-value; P_{HT}, test for homogeneity P-value; *Wrap53α*, WD repeat containing antisense to TP53α.

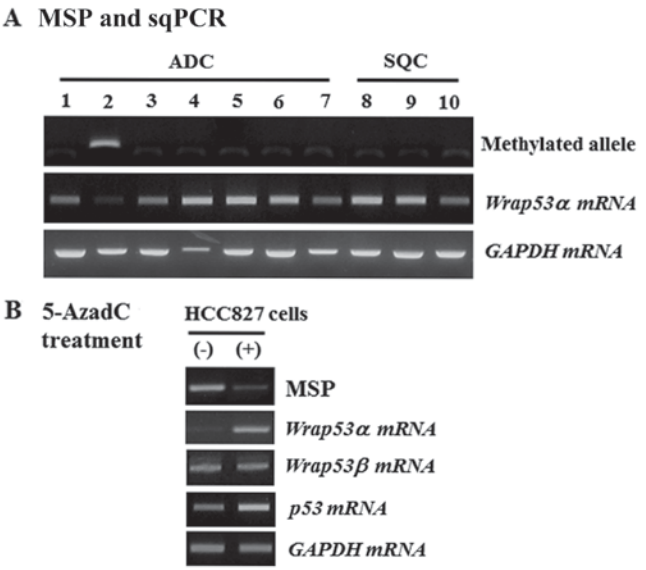

Figure 2. MSP and sqPCR analysis of *Wrap53α* gene in NSCLC cell lines. (A) The methylation status of the *Wrap53α* promoter was analyzed in 10 cell lines by MSP. Expression of *Wrap53α* mRNA was performed on the same cell lines by sqPCR. Lane 1, A549; Lane 2, HCC827; Lane 3, H23; Lane 4, H358; Lane 5, H522; Lane 6, H1299; Lane 7, PC9; Lane 8, H520; Lane 9, H1703; Lane 10, H2009. Lanes 1-7, ADC; Lanes 8-10, SQC. (B) Methylation status and expression of *Wrap53α* was analyzed in HCC827 cells following 20 μM 5-AzadC treatment for 3 days. Simultaneously, *Wrap53α* and *p53* mRNA levels were measured. *GAPDH* was amplified as an internal loading control. (-), vehicle alone; (+), 5-AzadC addition; MSP, methylation-specific polymerase chain reaction; sqPCR, semi-quantitative polymerase chain reaction; Wrap53α, WD repeat containing antisense to TP53α; NSCLC, non-small cell lung cancer; ADC, adenocarcinoma; SQC, squamous cell carcinoma.

results suggest that CpG island methylation may be a mechanism for downregulating the expression of *Wrap53α* gene.

Association of Wrap53α promoter methylation with clinicopathological parameters and clinical outcomes. *Wrap53α* promoter methylation was significantly more frequent in stages II-IIIA tumors than stages I tumors (P=0.03) (Table I). In addition, its methylation was more frequently detected in *p53* mutation-negative cases than in *p53* mutation-positive cases with borderline significance (P=0.08). However, no significant correlation was observed between its methylation and any other factors, such as age, sex, or smoking status (Table I). Next, Kaplan-Meier survival analysis was carried out to determine the prognostic potential of *Wrap53α* promoter methylation. Interestingly, the patients with the methylation had worse OS compared to those without *Wrap53α* methylation [log-rank *P* ($P_{L\text{-}R}$)=0.01] (Table III and Fig. 3). When stratified according to clinicopathological characteristics of patients, *Wrap53* promoter methylation was significantly associated with an unfavorable survival in a subset of patients including younger, female, never-smoker, squamous cell carcinoma, and *p53* mutation-negative ($P_{L\text{-}R}$=0.0003, 0.03, 0.02, 0.01, and 0.01, respectively) (data not shown). To evaluate the *Wrap53α* promoter methylation as an independent prognostic factor in NSCLC, we further analyzed the data using the Cox proportional hazards regression adjusting for possible confounders of survival. Methylation of *Wrap53α* promoter was significantly associated with worse OS of total patients [adjusted HR ($_{adj}$HR)=2.44, 95% CI=0.98-6.04,

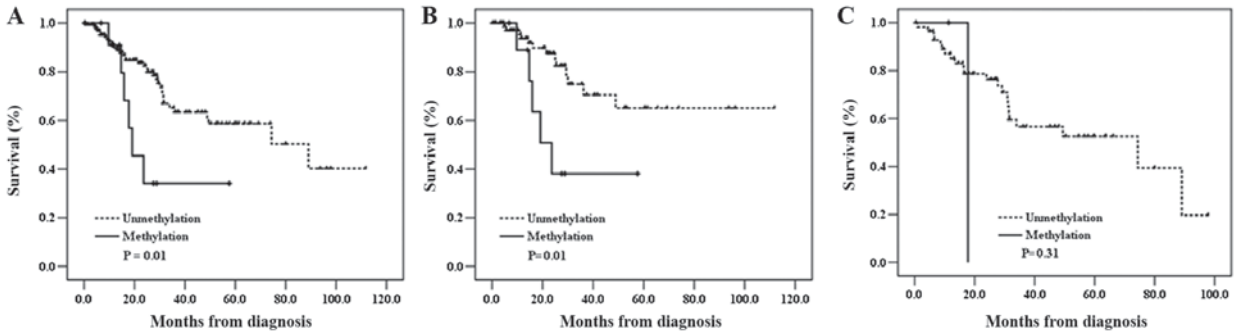

Figure 3. Kaplan-Meier survival curves of non-small cell lung cancer patients according to *Wrap53α* promoter methylation status. (A) Overall patients, (B) patients without *p53* mutations and (C) patients with *p53* mutations. P-values were calculated via a log-rank test. *Wrap53α*, WD repeat containing antisense to TP53α.

P=0.05]. Notably, *Wrap53α* promoter methylation significantly associated with poor OS in *p53* mutation-negative patients ($_{adj}$HR=2.92, 95% CI=1.00-8.60, P=0.05; Table III) but not in patients with p53 mutations. Moreover, *Wrap53α* promoter methylation exhibited a trend toward worse OS in patients with stages II-IIA ($_{adj}$HR=2.76, 95% CI=0.93-8.22, P=0.07) (data not shown). These results suggest that *Wrap53α* may play an important role in lung cancer pathogenesis and its methylation could be considered as a prognostic marker for NSCLC patients.

Discussion

Although the majority of investigations concerned with P53 have focused on coding regions, recent studies have highlighted the significant roles that regulatory elements located in *p53* mRNA play, particularly the 5'UTR that displays high conservation and immutability (17,18). *Wrap53* antisense RNA targets *p53* mRNA via the 5'UTR and increases p53 protein levels, indicating that dysfunction of *Wrap53* itself may be a separate cause of cancer. *Wrap53* has three different start exons: Exon 1α, 1β, and 1γ. Exon 1α and 1γ match the first exon and intron, respectively, of p53 in a *cis*-antisense manner. Exon 1β does not produce transcripts that are complementary to any section of *p53* (8). Moreover, knockdown of *Wrap53α* reduces *p53* abundance (8). There are several studies focusing on the function and expression of *Wrap53β* transcript in tumor progression (12-15), however, the exact function of have no information about is available. Thus, we focused on the methylation status of the *Wrap53α* promoter. The discovery of *Wrap53α* would elucidate the role of NAT-mediated gene regulation in the P53 pathway. NATs, as a member of long non-coding RNAs, occur ubiquitously in mammals and are crucial players in carcinogenesis, invasion, and metastasis (19). These RNAs regulate gene expression through direct interaction with sense transcripts or indirect interactions with other targets, such as DNA methyltransferases (DNMTs), histone acetylases and histone deacetylases. Many NATs interact with cancer relevant genes such as *p53*, *p15*, *p21*, *RB1*, and *PTEN* (20). Taken together, our findings provide new insights that NATs could be a potential rich sources of biomarkers for use in diagnosis and prognosis of cancer.

Although the molecular mechanisms contributing to promoter methylation of *Wrap53α* remain elusive, there is evidence that malignant transformation associated with chronic inflammation, persistent viral infection, cigarette smoking, and oxidative stress can upregulate the expression and activity of DNMTs through transcriptional and post-translational regulation (21-24). Interestingly, Lin *et al* (25) have shown that dysregulation of p53 control leads to *DNMT1* and *DNMT3A* overexpression, resulting in promoter hypermethylation of multiple tumor suppressor genes in NSCLC patients (26). Thus, it is likely that the overexpressed DNMT can induce *Wrap53α* hypermethylation.

It is noteworthy that *Wrap53α* methylation was significantly associated with unfavorable survival in a subset of NSCLC patients, especially for *p53* mutation-negative patients. The downregulation of *Wrap53α* transcripts by promoter methylation could destabilize *p53* mRNA to reduce tumor suppressor activity of the WT P53, contributing to poor prognosis. Smoking causes a high percentage of missense mutations in the DNA-binding domain of *p53*, producing a striking gain-of function phenotype (6). In addition, mutant P53 can drive cancer by subverting multiple tumor suppression pathways independent of WT *p53* (27). Mutations of the *p53* gene usually but not always lead to an increased half life of the *p53* protein, and result in a nuclear accumulation of protein (27). Consequently, p53 alteration that can be detected as either protein overexpression or mutation makes the problem more complicated. Alternatively, alterations in regulators of P53 provide an alternative way to deregulate P53 function in p53 WT tumors, but are likely redundant in tumors that already have a dysfunctional P53 protein. Indeed, we found that *Wrap53α* methylation showed a tendency for *p53* mutation-negative patients, indicating the absence of concomitant alterations in *p53* and *Wrap53α*. Mutual exclusive alterations is frequently observed in cancer, being believed to occur between functionally related genes (28). Taken together, it could be speculated that the effect of *Wrap53α* promoter methylation on the clinical outcome could be more noticeable in patients with WT P53. Therefore, our findings suggest that the close interplay between the *Wrap53α* and *p53* might be involved in the pathogenesis of NSCLC. However, further studies with larger sample sizes are required to establish that *Wrap53α* methylation is a useful prognostic indicator for patients with NSCLC.

The present study has shown that the *Wrap53α* promoter was methylated in a subset of NSCLCs, and its methylation was significantly associated with unfavorable OS of patients, particularly in patients with p53 mutation negative, suggesting that *Wrap53α* methylation status could be informative for

prediction of NSCLC prognosis. Although the current study did not have a full viewpoint because of the small sample size and no information regarding P53 protein expression, it is the first report to demonstrate an aberrant methylation of the *Wrap53α* promoter in NSCLC and may provide new pieces in the P53 targeting puzzle for cancer therapy.

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Availability of data and materials

The analyzed datasets generated during the study are available from the corresponding author on reasonable request.

Authors' contributions

DSK contributed to the experimental design and implementation, performed the experiments and data analysis, and drafted the manuscript. WKL performed the statistical analyses. JYP contributed to experimental implementation, interpreted the patient data and modified the manuscript. All authors have read and approved the final manuscript.

Ethics approval and consent to participate

The present study was approved by the Ethics Committee of KNUH (no. 2014-04-210). Written informed consent was obtained from all participants or their families prior to obtaining the samples.

Patient consent for publication

All participants provided written informed consent for the publication of any associated data and accompanying images.

Competing interests

The authors declare that they have no competing interests.

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