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.

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 transcriptionally 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 Wrap53γ, 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.

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 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    |
| Age (years) >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.
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), 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
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-III A 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 \([\text{log-rank } P (P_{\text{LR}})=0.01]\) (Table III and Fig. 3). When stratified according to clinicopathological characteristics of patients, Wrap53α 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=0.01, 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 \([\text{adjusted } \text{HR} (\text{adj} \text{HR})=2.44, 95\% \text{ CI}=0.98-6.04, 0.05]\).
P=0.05]. Notably, *Wrap53α* promoter methylation significantly associated with poor OS in *p53* mutation-negative patients (adjHR=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 (adjHR=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*, *RBI*, 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 methylation 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 Wrap53a 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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