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Clinical Study

Implication of DNA Methylation Profiling in Oral Epithelium for Lung Cancer Screening

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In lung cancer, the roles of molecular alterations in blood, sputum, bronchial brushing, and exhaled gas samples, which are relatively easy to obtain, have been evaluated for clinical availability. This study was based on the hypothesis that similar molecular alterations occur in the lung and oral cavity because both are exposed to the same environmental or tobacco-derived carcinogens. Because epigenetic alterations due to exposure to carcinogens are thought to play a major role in the development of lung cancer, the DNA methylation status of 11 genes in the oral epithelium was analyzed in lung cancer patients ($n = 16$) and control individuals without lung cancer ($n = 32$). DNA methylation profiling revealed that *GDNF*, *RARB*, and *HS3ST2* were methylated more frequently in cancer patients than in the control participants ($P = 0.0017$, 0.0062, and 0.0193, resp.). Combined analysesindicated that 6 of 16 cancer patients (37.5%), but only 1 of 32 control individuals (3.1%) showed DNA methylation in 2-3 of these 3 genes ($P = 0.0015$). These combined analyses showed the high specificity and positive predictive value in total and subgroup analyses. Our data suggest that DNA methylation profiling using oral epithelium may help in the identification of individuals with a high risk of lung cancer.

1. Introduction

Lung cancer is the leading cause of cancer-related death in many countries [1]. A major factor contributing to this high mortality is that most tumors are not diagnosed until an advanced stage [2]. Although a variety of investigations and screening programs for early detection of lung cancer have been conducted, each approach has lacked diagnostic specificity [3, 4]. Recent reports indicated that screening using low-dose computed tomography (CT) showed encouraging results in reducing lung cancer mortality in patients with a history of cigarette smoking of at least 30 packs per year

[5]. However, the frequency of lung cancer in that screening program was considerably low, although the participants were limited to individuals with a heavy smoking history. These results indicate that identification of individuals with a high risk of lung cancer is important for improving the specificity and reliability of lung cancer screening programs.

Inhaled carcinogens from tobacco smoke, workplace agents, and other environmental factors such as air pollution are well known to be among the main etiological factors that cause molecular alterations in lung cancer pathogenesis [6], and the predisposition of smokers to epigenetic alterations has been characterized [7–9]. Because gene promoter methylation is a particularly significant epigenetic modification in lung cancer initiation and progression [10], DNA methylation patterns are potentially useful biomarkers for assessing the risk of lung cancer and for predicting the outcome of disease [11, 12]. Hypermethylation of several gene promoters in lung cancer patients has been detected in tumors as well as in blood samples, sputum samples, bronchial brushings, and exhaled gas, which are relatively easier to obtain than from lung tissue [13–15]. Although previous reports have shown that epigenetic alterations in gastric mucosa or washes are promising biomarkers for gastric cancer screening [16, 17], the clinical value of epigenetic alterations in surrogate tissues and fluids in lung cancer screening remains unclear.

In this study, we used oral epithelium as a surrogate tissue for lung tissue because the entire airway from the oral cavity to the lungs is exposed to the same inhaled carcinogens. By comparing the DNA methylation profiles in the oral epithelium of patients with lung cancer with that of controls without lung cancer, we examined whether the DNA methylation profile of the oral epithelium is a potential biomarker for risk assessment and early detection of lung cancer.

2. Materials and Methods

2.1. Study Population. Sixteen lung cancer patients and 32 control individuals without lung cancer were included in this study. All cancer patients had undergone lung resection at Kure Medical Center and Chugoku Cancer Center; none had received chemotherapy or radiation therapy prior to the collection of oral epithelial samples. The control group comprised 8 patients with nonmalignant lung diseases treated at Kure Medical Center and Chugoku Cancer Center and 24 healthy volunteers who were participants in a health screening program at Hitoyoshi General Hospital. None of the controls had a history of cancer. Written informed consent was obtained from all individuals, and the study protocol was approved by the Institutional Review Board.

2.2. Sample Collection and Preparation. Oral epithelium was collected by gently brushing the insides of both cheeks with a cotton swab for 1 min each. Genomic DNA was extracted from the cotton swabs by incubation in 500 μL of proteinase K solution for 8 h at 55°C, followed by phenol-chloroform extraction and ethanol precipitation, as previously described [18].

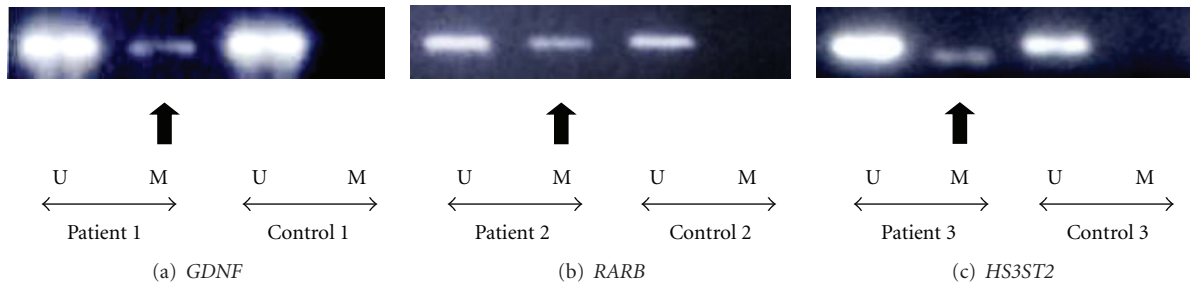
2.3. Bisulfite Modification and Methylation-Specific Polymerase Chain Reaction (PCR). DNA was digested with *Bam*HI (New England Biolabs, MA, USA), and 1 μg of the digested DNA was denatured in NaOH (0.3 N; 37°C for 15 min). Bisulfite modification was performed; 15 cycles of denaturation (95°C for 30 s) followed by incubation in sodium bisulfite (3.1 N, 500 μM, pH 5.0; 50°C for 15 min) and hydroquinone (0.5 mM). Samples were then desalted using the Wizard DNA cleanup system (Promega, WI, USA), desulfonated in NaOH (0.6 N), precipitated with ethanol, and dissolved in 20 μL of TE buffer. Methylation-specific PCR (MSP) was performed for 37 cycles using 1 μL of bisulfite-modified DNA solution, as previously described [19–21]. All procedures were repeated at least thrice for each sample. Fifteen microliters of the PCR reaction were separated on 2% agarose gel, stained with ethidium bromide, and visualized under ultraviolet illumination.

2.4. Statistical Analysis. Comparisons were performed using Fisher's exact test and the χ^2 test. For subgroup analyses, the population was divided according to smoking history and participants more than 50 years of age were compared. Differences were considered statistically significant at $P < 0.05$. Statistical analysis was performed using JMP for Windows version 9 statistical software package (SAS Institute, NC, USA).

3. Results

3.1. Difference in the Methylation Profile of Oral Epithelium between Patients and Control Individuals. Table 1 shows the patient demographics of our retrospective, nonmatched study population. Twelve of 16 patients had adenocarcinoma, and 13 of 16 patients were diagnosed with stage I lung cancer. We evaluated the promoter methylation profile of 11 genes: adenomatous polyposis coli (*APC*), glial cell line-derived neurotrophic factor (*GDNF*), retinoic acid receptor beta (*RARB*), glutathione *S*-transferase P 1 (*GSTP1*), tissue inhibitor of metalloproteinase-3 (*TIMP3*), *O*6-methylguanine-DNA methyltransferase (*MGMT*), ras association domain family 1 (*RASSF1A*), serpin peptidase inhibitor (*MASPIN*), cadherin 1 (*CDH1*), heparan sulfate 3-*O*-sulfotransferase 2 (*HS3ST2*), and cholinergic receptor, muscarinic 1 (*CHARM1*) (Table 1). These genes were selected because they had shown a relatively high frequency of methylation in cancer tissue from patients with early-stage lung cancer in our preliminary analysis (data not shown). Of these 11 genes, the methylation frequency of *GDNF*, *RARB*, and *HS3ST2* was apparently higher in the oral epithelium of lung cancer patients than in the oral epithelium of the controls (P = 0.0017, 0.0062, and 0.0193, resp.; Table 2), although the P-values adjusted for the number of comparisons performed for *HS3ST2* showed nonsignificant difference. Figure 1 shows representative electrophoretic banding patterns from MSP for *GDNF*, *RARB*, and *HS3ST2*.

3.2. Differences in Methylation Frequency by Subgroup Analysis. We conducted subgroup analyses according to the



(a) *GDNF* (b) *RARB* (c) *HS3ST2*

Figure 1: Methylation of *GDNF*, *RARB*, and *HS3ST2* genes in oral epithelium. Methylation-specific PCR amplification of the *GDNF* (a), *RARB* (b), and *HS3ST2* (c) genes using unmethylation-specific (U) and methylation-specific (M) primers were performed with DNA isolated from oral epithelium. Aberrant methylation was detected in patients 1, 2, and 3 (arrows).

Table 1: Demographic and clinicopathological features of the patient and control groups.

Characteristics	Patients ($n = 16$)	Controls ($n = 32$)
Gender		
Male	8	18
Female	8	14
Age		
Range	58–82	34–72
Median	64	54
Histology		
Adenocarcinoma	12	
Squamous cell carcinoma	4	
Pathological stage		
IA, IB	13	
IIA, IIB	3	
Smoking history		
Current and former	11	17
Never	5	15

smoking history and age. Table 3 shows the methylation frequency of *GDNF*, *RARB*, and *HS3ST2* relative to different demographic parameters. In current and former smokers, *GDNF* methylation frequency was significantly higher in patients than in controls ($P = 0.0072$), whereas the *RARB* and *HS3ST2* methylation frequency did not differ significantly ($P = 0.1142$ and 0.1142, resp.). Among the never smokers, *GDNF* and *RARB* methylation was seen more frequently in the cancer patients than in the controls ($P = 0.0369$ and 0.0098, resp.), whereas the difference between patients and controls with regard to *HS3ST2* methylation frequency was not statistically significant ($P = 0.0756$). Because the control group included a higher proportion of young individuals than the patient group, we conducted subgroup analysis limited to the population aged more than 50. In this setting, *GDNF* methylation frequency was significantly higher in the patients than in the controls ($P = 0.0143$), whereas *RARB* and *HS3ST2* methylation was not significantly different between the 2 groups ($P = 0.1025$ and 0.1441, resp.).

3.3. Sensitivity and Specificity of Detecting Specific DNA Methylation by Using Combined Analysis. Table 4 shows the combined analysis of *GDNF, RARB,* and *HS3ST2*. Six of the 16 patients showed methylation of 2-3 genes, compared with 1 of 32 individuals in the control group ($P = 0.0015$). Among current and former smokers, 3 of 11 patients, whereas only 1 of 17 controls showed methylation of 2-3 genes ($P = 0.1142$). Combined analysis indicated a statistically significant difference for never smokers ($P = 0.0011$) as well as for participants more than 50 years of age ($P = 0.0143$). These combined analyses demonstrated the high specificity and positive predictive value for comparisons between lung cancer patients and controls (Table 5)

4. Discussion

To improve the clinical outcome of lung cancer, a variety of screening approaches for its early detection have been investigated; however, most of them lack sensitivity and may involve invasive techniques. Therefore, the identification of specific biomarkers using samples that are easy to collect may provide further refinement of lung cancer screening programs. Gene silencing through epigenetic alterations has been shown to be a major mechanism underlying tumor initiation and progression [12]; therefore, promoter's hypermethylation has become a target for developing strategies to enable molecular screening for the early detection of cancer [22]. Because previous studies demonstrated that promoter methylation patterns in the oral epithelium correlated with those in the lungs [23], we evaluated DNA methylation profiling in oral epithelium for the risk assessment and early detection of lung cancer.

We found a significant difference in the methylation profiles of oral epithelium between lung cancer patients and controls. When the methylation profiles of *GDNF*, *RARB*, and *HS3ST2* were combined, this method showed high specificity and positive predictive value for the risk assessment of lung cancer. These results indicated that the assessment of epigenetic alterations using oral epithelium may help to identify a high risk of lung cancer in individuals who would most benefit from intensive screening, for example, by low-dose CT. Similarly, identifying individuals at extremely low risk of lung cancer helps to reduce the number

Table 2: Frequency of promoter methylation in oral epithelium from lung cancer patients and controls.

Gene		Number (%)		
Symbol	Description	Patients ($n = 16$)	Controls ($n = 32$)	P
APC	Adenomatous polyposis coli	0 (0)	0 (0)	N/A
GDNF	Glial cell line-derived neurotrophic factor	7 (43.8)	2 (6.3)	0.0017
RARB	Retinoic acid receptor beta	6 (37.5)	2 (6.3)	0.0062
GSTP1	Glutathione *S*-transferase P 1	0 (0)	0 (0)	N/A
TIMP3	Tissue inhibitor of metalloproteinase-3	0 (0)	0 (0)	N/A
MGMT	*O*6-methylguanine-DNA methyltransferase	0 (0)	0 (0)	N/A
RASSF1A	Ras association domain family 1	0 (0)	0 (0)	N/A
MASPIN	Serpin peptidase inhibitor	16 (100)	32 (100)	N/A
CDH1	Cadherin 1	0 (0)	0 (0)	N/A
HS3ST2	Heparan sulfate 3-*O*-sulfotransferase 2	4 (25.0)	1 (3.1)	0.0193
CHARM1	Cholinergic receptor, muscarinic 1	15 (93.8)	28 (87.5)	n. s.

Table 3: Methylation frequencies of *GDNF*, *RARB*, and *HS3ST2* according to subgroup analysis.

	GDNF	*P*	*RARB*	*P*	*HS3ST2*	*P*
Current and former smokers						
Patients ($n = 11$)	4	0.0072	3	0.1142	3	0.1142
Controls ($n = 17$)	0		1		1	
Never smokers						
Patients ($n = 5$)	3	0.0369	3	0.0098	1	0.0756
Controls ($n = 15$)	2		1		0	
Age > 50 years						
Patients ($n = 16$)	7	0.0143	6	0.1025	4	0.1441
Controls ($n = 16$)	1		2		1	

of unnecessary further examinations as well as increase the power of the early detection of lung cancer.

GDNF, which encodes glial cell line-derived neurotrophic factor, is involved in brain development and neuronal differentiation [24]. Recent reports have demonstrated that *GDNF* plays a role in the progression of pancreatic and lung cancers [25, 26], and Garnis et al. reported that *GDNF* may contribute to lung tumorigenesis at an early stage [26]. In addition, *GDNF* has been reported to show a high frequency of methylation in both lung cancer tissue and adjacent nontumor tissues [27], indicating that similar epigenetic alteration of *GDNF* may occur in other airway epithelia. *RARB*, which encodes retinoic acid receptor beta and is known to regulate cell differentiation [26], is also frequently methylated in lung tumors [28]. Chung et al. demonstrated that aberrant methylation of *RARB* occurred in preinvasive lesions of lung adenocarcinoma [29]; however, Topaloglu et al. demonstrated that no aberrant methylation was evident in bronchoalveolar lavage samples from lung cancer patients [14]. *HS3ST2*, which encodes an *O*-sulfotransferase involved in the modification of heparin sulfate proteoglycans, is silenced in a wide range of human cancers [18]. Shivapurkar et al. observed hypermethylation of *HS3ST2* in 93% of cervical tumors and in 70% of cytology specimens from cervical intraepithelial neoplasias [30]. To date, the possible role of *HS3ST2* in lung cancer pathogenesis has not been investigated in detail. Taken together, although the influence of environmental carcinogens on the molecular alteration of *GDNF*, *RARB*, and *HS3ST2* has not been evaluated in detail, this is the first characterization of the methylation status of these 3 genes in the oral epithelia of lung cancer patients.

Although promoter methylation in oral epithelium was observed predominantly in lung cancer patients compared with controls, several individuals in the control group showed a positive result for promoter methylation. Because previous studies demonstrated that promoter methylation was detected in the sputum of lung cancer patients up to 3 years before clinical diagnosis [31], long-term followup is required. Additionally, a growing body of evidence shows that promoter's methylation is also initiated by a variety of diseases other than cancer. Therefore, we performed combined analysis, which showed an improved accuracy of the test.

This study has several limitations that should be noted when assessing the results. This study included a small number of patients, the study participants in the 2 groups were not matched with regard to clinical demographics, and reproducibility tests using oral epithelium collected at different timepoints were not performed even though methylation-specific PCR was performed at least 3 times for each sample. Moreover, the relatively low sensitivity of the combined DNA methylation pattern may limit its suitability

Table 4: Difference in the number of gene methylation sites (*GDNF*, *RARB*, and *HS3ST2)* according to total population and subgroup analysis.

Number of DNA methylation	Number (%) Patients	Controls	*P*
Total participants	16	32	
0-1	10 (62.5)	31 (96.9)	0.0015
2-3	6 (37.5)	1 (3.1)	
Current and former smokers	11	17	
0-1	8 (72.7)	16 (94.1)	0.1142
2-3	3 (27.3)	1 (5.9)	
Never smokers	5	15	
0-1	2 (40.0)	15 (100)	0.0011
2-3	3 (60.0)	0 (0)	
Age > 50 years	16	16	
0-1	9 (56.3)	15 (93.8)	0.0143
2-3	7 (43.7)	1 (6.2)	

Table 5: Diagnostic value of the combined analysis.

	Sensitivity	Specificity	PPV	NPV
Total participants	37.5%	96.8%	85.7%	75.6%
Current and former smokers	27.3%	94.1%	75.0%	66.7%
Never smokers	60.0%	100%	100%	88.2%
Age > 50 years	43.7%	93.8%	88.2%	62.5%

PPV: positive predictive value; NPV: negative predictive value.

for future clinical application. Although further prospective longitudinal investigations using larger number of patients are needed to evaluate the clinical effectiveness of oral epithelium epigenetic analysis in lung cancer screening, the results of this study suggest that analysis of DNA methylation in oral epithelium is a promising noninvasive tool to increase the specificity and sensitivity of lung cancer screening. Long-term followup of individuals in whom specific genes are methylated is also needed. Additionally, although current smokers and former smokers were evaluated as 1 group in this study because of the small size of the cohort, the possible role of smoking should be considered as a confounding factor in future studies.

In conclusion, considering the high mortality rate of lung cancer and the low sensitivity of current screening programs, there is a need for effective and noninvasive risk assessment tools for lung cancer screening. The results of this study demonstrated that assessing epigenetic alterations in oral epithelium is a potential tool for risk assessment of lung cancer and for designing improved screening strategies for the early detection of lung cancer.

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