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Promoter methylation of APC and RAR-β genes as prognostic markers in non-small cell lung cancer (NSCLC)

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ABSTRACT

Aberrant promoter hypermethylations of tumor suppressor genes are promising markers for lung cancer diagnosis and prognosis. The purpose of this study was to determine methylation status at APC and RAR-β promoters in primary NSCLC, and whether they have any relationship with survival. APC and RAR-β promoter methylation status were determined in 41 NSCLC patients using methylation specific PCR. APC promoter methylation was detectable in 9 (22.0%) tumor samples and 6 (14.6%) corresponding non-tumor samples (P = 0.391). RAR-β promoter methylation was detectable in 13 (31.7%) tumor samples and 4 (9.8%) corresponding non-tumor samples (P = 0.049) in the NSCLC patients. APC promoter methylation was found to be associated with T stage (P = 0.046) and nodal status (P = 0.019) in non-tumor samples, and with smoking (P = 0.004) in tumor samples. RAR-β promoter methylation was found associated with age (P = 0.031) in non-tumor samples and with primary tumor site in tumor samples. Patients with APC promoter methylation in tumor samples showed significantly longer survival than patients without it (Log-rank P = 0.014). In a multivariate analysis of prognostic factors, APC methylation in tumor samples was an independent prognostic factor (P = 0.012), as were N1 positive lymph node number (P = 0.025) and N2 positive lymph node number (P = 0.06). Our study shows that RAR-β methylation detected in lung tissue may be used as a predictive marker for NSCLC diagnosis and that APC methylation in tumor sample may be a useful marker for superior survival in NSCLC patients.

1. Introduction

Lung cancer is the leading cause of cancer-related deaths in the world. Lung cancer survival tends to be poorer in developing countries as a result of increasing use of tobacco product compared with developed countries (Jemal et al., 2011; Molina et al., 2008). Non-small cell lung cancer (NSCLC) comprises the majority (85–90%) of lung cancer (Zhang et al., 2011b). Despite advances in the detection and treatment of NSCLC, the overall 5-year survival rate remains under 15% (Kim et al., 2006). The poor outcome of NSCLC patients may be attributed partially to late diagnosis and tumor recurrence (Henschke et al., 2006). Thus, developing new markers for early diagnosis and prognostic prediction may help to improve survival rate and life quality of patients with NSCLC.

DNA methylation is an epigenetic modification causing the silence of target genes, and can be induced by environmental factors such as exposure to smoking (Salskov et al., 2011). The silencing of tumor suppressor genes by promoter methylation is a common mechanism in the genesis of human cancers (Cheishvili et al., 2014). As promising biomarkers for early detection of NSCLC, methylation of multiple genes has been studied, including CADM1, CDH1, CDH13, p16, GSTP1, MGMT, and RASSF1A (Zhang et al., 2011b; Ji et al., 2011; Anglim et al., 2008). Moreover, methylation of some tumor suppressor genes has also been studied for prognosis in different cancers. For example, Henrique et al. (2007) reported that high promoter methylation levels of Adenomatous polyposis coli (APC) gene predict poor prognosis in prostate cancer patients, and Chen and Chiu (2009) showed that methylation in the APC promoter may serve as a predictor for the prognosis of Taiwanese colorectal cancer patients. In patients with stage I NSCLC, Kim et al. (2006) suggested that cohypermethylation of p16 and FHIT genes may be a valuable biomarker for predicting the recurrence-associated prognosis of the disease. Saito et al. (2010) reported that LINE-1 hypomethylation was an independent marker of poor prognosis in stage IA NSCLC.

APC and retinoic acid receptor-beta (RAR-β) are two important tumor suppressor genes (Houle et al., 1993; Virmani and Gazdar, 2003). However, Khuri et al. (2000) demonstrated that strong expression of RAR-β correlated with worse outcome of early-stage NSCLC. In addition, Brabender et al. (2001) reported that high APC promoter methylation is associated with inferior survival whereas Safar et al. (2005) showed that patients whose tumors were hypermethylated at APC enjoyed substantially longer 1- and 2-year survival using recursive

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partitioning. Additional studies are needed to elucidate the prognostic values of the methylation status of these two genes. Here we examined the methylation status of APC and RAR-β promoters in tumor tissue and corresponding non-tumor lung tissue in a cohort of 41 Chinese patients with NSCLC, and analyzed whether the methylation of APC or RAR-β, combined with or without other clinicopathological variables, could be used to predict the clinical outcomes of patients with primary NSCLC after surgical resection.

2. Materials and methods

2.1. Patients

Clinicopathological characteristics of the participated patients are presented in Table 1. All tumors were completely resected (R0 category). Patients with histopathological stage IIIa tumors received postoperative chemotherapy. The median follow-up was 18 months (min. 0.2; max. 60 months) and no patient was lost to follow-up. Histologically normal lung tissue specimens obtained at surgery from 8 patients with no evidence of cancer were included as a control group. All patients provided written informed consent, and the protocol was approved by Ethics Committee of China–Japan Friendship Hospital.

2.2. Tissue acquisition and nucleic acid isolation

Immediately after lung resection and before starting mediastinal lymphadenectomy, tissues were obtained from the tumor and uninvolved lung tissue from the greatest distance to the tumor, and were frozen in liquid nitrogen. 10-mm frozen sections were cut from blocks of tumor tissue and numbered. Every 5th section was stained with H&E and histopathologically evaluated. Sections were pooled for analysis from areas of estimated 75% malignant cells. DNA was isolated by standard methods of proteinase K digestion and phenol-chloroform extraction using Tiangen Cells and Tissue DNA Isolation Kit (Tiangen Biotech Co., LTD, Beijing, China) according to the instructions of the manufacturer.

Table 1
Clinicopathological characteristics of enrolled 41 NSCLC patients and 8 non-tumor patients.

Variables		N (%)
NSCLC patients		
Gender	Male	25 (61.0)
	Female	16 (39.0)
Age[a]	<60	20 (48.8)
	≥60	21 (51.2)
Smoking	Never	17 (41.5)
	Ever	24 (58.5)
Primary tumor site	Left lung	21 (51.2)
	Right lung	20 (48.8)
Pathologic classification	Squamous cell carcinoma	15 (36.6)
	Adenocarcinoma	21 (51.2)
	Others	3 (7.3)
Differentiation degree	Undifferentiated/poorly	25 (61.0)
	Moderately/well	16 (39.0)
TNM stage	I/II	26 (63.4)
	III/IV	15 (36.6)
T stage	T1/2	28 (68.3)
	T3/4	13 (31.7)
Nodal status	N 0	18 (43.9)
	N +	23 (56.1)
Non-tumor patients		
Sex	Male	5(62.5)
	Female	3(37.5)
Age	<60	6(75.0)
	≥60	2(25.0)
Smoking	Never	7(87.5)
	Ever	1(12.5)
Sample site	Left lung	5(62.5)
	Right lung	3(37.5)

T, tumor size; N, total number of patients.
[a] Age according to median value of 60 years (range 36–78).

2.3. Detection of APC and RAR-β methylation using methylation-specific PCR (MSP)

500 ng of DNA extracted from tissue samples was modified by sodium bisulfite with DNA Methylation Detection Kit (BioChain,Newark, CA, USA) according to the manufacturer's instructions. 7 μl of the modified DNA was used for PCR test in a total reaction volume of 25 μl for a program of 35 cycles of melting (30 s at 95 °C), annealing (30 s at 55 °C) and extension (30 s at 72 °C). The reaction mixture contained 1 × PCR Buffer (Mg^{2+} Plus), 200 μM of each dNTP, 0.5 μM of forward primer,0.5 μM of reverse primer, 0.5 unit of GoTaq® Hot Start Polymerase (Promega, USA). PCR products were separated in 3% agarose gel supplemented with ethidium bromide staining. MSP products were visualized under UV illumination. APC and RAR-β were recorded as methylated if MSP amplification products were detected in both reactions with unmethylation and methylation primers, or with methylation primers only as described elsewhere (3).The primer sequences are: (a) APC unmethylation-specific primer, GTGTTTATTGTGGAGTGT GGGTT (forward), CCAATCAACAAACTCCCAACAA (reverse); methylation-specific primer, TATTGCGGAGTGCGGGTC (forward), TCGACGAA CTCCCGACGA (reverse) (b) RAR-β unmethylation-specific primer, TTGGGATGTTGAGAATGTGAGTGATTT (forward), CTTACTCAACCAATCC AACCAAAACAA (reverse); methylation-specific primer, TGTCGAGAAC GCGAGCGATTC (forward), CGACCAATCCAACCGAAACGA (reverse).

2.4. Statistical analysis

Statistical comparisons were performed using either the χ2-test or Fisher's exact test, as appropriate. Student's *t*-test was performed to assess the statistical significance between the mean values. Overall survival was calculated from the date of surgery until death, or the date of last follow-up. Overall survival was analyzed using the Kaplan–Meier method and differences between two groups were compared using the Log-rank test. For multivariate analysis, independent prognostic actors were assessed by using the Cox proportional hazards model with enter method. All data were analyzed with using SPSS software (SPSS Statistics 19, Rel. 19.0. 2010. Armonk, NY: IBM Corp.).

3. Results

3.1. DNA methylation profile of APC and RAR-β

We determined the methylation status of the promoters of two genes, APC and RAR-β, in tumor tissue and corresponding non-neoplastic lung tissue of 41 NSCLC patients, and in 8 non-tumor patients (Table 2). Based on the DNA methylation profile of APC and RAR-β, we found that while there was no obvious relationship between APC methylation and tumor tissue, there was a significant correlation between RAR-β methylation status and tumor tissue, with a P-value of 0.049. In addition, the methylation of these genes occurred more frequently in NSCLC patients than patients without cancer. Methylation of APC and RAR-β is detected in only 1 and 0 of 8 non-cancer participants, respectively.

3.2. Association of DNA methylation with clinicopathological variables in NSCLC patients

We analyzed the association between DNA methylation status of APC or RAR-β and the clinicopathological variables in NSCLC patients using Fisher or χ^2-test (Table 3). The methylation of APC appears more commonly associated with several clinicopathological characters