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Adenomatous polyposis coli gene promoter hypermethylation in non-small cell lung cancer is associated with survival

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Methylation of 5′ CpG islands in promoter and upstream coding regions has been identified as a mechanism for transcriptional inactivation of tumor suppressor genes. The purpose of this study was to determine whether hypermethylation of the adenomatous polyposis coli (*APC*) gene promoter occurs in primary non-small cell lung cancer (NSCLC), and whether hypermethylated *APC* has any relationship with survival. *APC* promoter 1A methylation was determined in normal and corresponding tumor tissue from 91 NSCLC patients and in a control group of 10 patients without cancer, using a quantitative fluorogenic real-time PCR (Taqman®) system. *APC* promoter methylation was detectable in 86 (95%) of 91 tumor samples, but also in 80 (88%) of 91 normal samples of NSCLC patients, and in only two (20%) of 10 normal lung tissues of the control group. The median level of *APC* promoter methylation was 4.75 in tumor compared to 1.57 in normal lung tissue ($P<0.001$). Patients with low methylation status showed significantly longer survival than did patients with high methylation status ($P=0.041$). In a multivariate analysis of prognostic factors, *APC* methylation was a significant independent prognostic factor ($P=0.044$), as were pT ($P=0.050$) and pN ($P<0.001$) classifications. This investigation shows that *APC* gene promoter methylation occurs in the majority of primary NSCLCs. High *APC* promoter methylation is significantly associated with inferior survival, showing promise as a biomarker of biologically aggressive disease in NSCLC. *Oncogene* (2001) **20,** 3528–3532.

Keywords: APC; methylation; neoplasia; NSCLC

Introduction

Lung cancer is the leading cause of cancer-related deaths among both males and females in the United States (Landis *et al*., 1998). Each year, approximately 171 000 new cases of lung cancer will be diagnosed, and 160 000 individuals will die from the disease. Despite improvements in the detection and treatment of lung cancer in the past two decades, the overall survival remains less than 15% (Ginsberg *et al*., 1997). Significant progress has been made towards understanding the genetic changes associated with lung cancer. It is still important to investigate additional molecular alterations that emphasize our knowledge regarding tumorigenesis in non-small cell lung NSCLC.

Methylation is a main epigenetic modification in humans and changes in methylation patterns play an important role in tumorigenesis (Baylin *et al*., 1998). In particular, methylation of normally unmethylated CpG islands has been identified as an alternative mechanism for tumor suppressor gene inactivation in human cancer cells (Baylin *et al*., 1998; Merlo *et al*., 1995; Jones and Laird, 1999). In fact, a recent publication reports an association between adenomatous polyposis coli (*APC*) gene promoter 1A methylation and silencing of its expression in gastric cancer (Tsuchiya *et al*., 2000). Several studies report aberrant promoter methylation in various genes in human NSCLC, for example the putative metastasis suppressor gene death-associated protein (*DAP*) kinase (Esteller *et al*., 1999; Tang *et al*., 2000), the detoxification gene glutathione S-transferase P1 (*GSTP1*) (Esteller *et al*., 1999), and the DNA repair gene 06-methylguanine-DNA-methyltransferase (*MGMT*) (Esteller *et al*., 1999). Hypermethylation of the *DAP* kinase promoter was associated with poor survival in patients with stage I NSCLC (Tang *et al*., 2000). In addition, we recently reported an association between hypermethylated *APC* DNA in plasma and prognosis of patients with esophageal adenocarcinoma (Kawakami *et al*., 2000),

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suggesting hypermethylation of these genes as markers for biological aggressiveness in these diseases.

The purpose of this study was to determine whether CpG island hypermethylation of the *APC* promoter 1A occurs in primary NSCLC, and whether the presence or quantity of hypermethylated APC has any relationship with clinical outcome in patients with primary NSCLC.

Results

APC promoter methylation was detectable in 86 (94.5%) of 91 tumor samples, 80 (87.9%) of 91 normal tissue samples of the NSCLC group, and two (20%) of 10 normal lung specimens of the control group. In 69 (75.8%) of 91 patients the APC promoter methylation level, expressed as the ratio between methylated *APC* and *MYOD1* PCR product, was elevated in tumor compared to matching normal tissue (the T/N ratio was higher than 1.0). The median level of *APC*/*MYOD1* was 4.75 (range: 0.00–240.73) in tumor compared to 1.57 (range: 0.00–73.90) in matching normal tissue ($P<0.001$; Wilcoxon test). However, as shown in Figure 1, the extreme value in this range (73.9) was due to only one patient. All the rest of the *APC*/*MYOD1* ratios were within the range of 0–19. The median *APC* methylation level in normal lung tissue of the control group was 0.00 (range: 0.00–5.09), and significantly lower compared to the *APC* methylation in normal tissue of the NSCLC group ($P=0.003$; Mann–Whitney test).

Table 1 shows the clinicopathological data and the median *APC* promoter methylation in tumor tissues of patients with NSCLC. There were no statistical differences between *APC* promoter methylation in tumor tissue and clinicopathological factors, such as age, sex, etc. observable. In addition, there was no significant association between smoking history and *APC* methylation in normal lung tissues from NSCLC patients. The median *APC* methylation in normal lung tissue was 0.80 (range: 0.15–8.0) for non-smokers and 2.03 (range: 0.00–73.90) for smokers ($P=0.253$; Mann–Whitney test).

To determine whether there was any prognostic significance attached to quantitative differences in *APC* methylation levels, we used the maximal chi-square method to determine a cut-off value to segregate patients into groups with low and high *APC* methylation status (Miller and Siegmund, 1982; Halpern, 1982). Obtaining a T/N *APC* methylation ratio of 14 as a cut-off, 68 (75%) of patients had a low *APC* methylation status and 23 (25%) had a high *APC* methylation status. The median survival was not reached in the low methylation group at the time we did these studies, whereas it was reached in 24.7 months (95% C.I.:17.86; 31.54) in the high methylation group. The observed Tarone-Ware test statistic was 4.05. To determine a *P* value, we used bootstrap-like simulations to estimate the distribution of a maximal chi-square statistic, since the cut-off point of 14 had been chosen after examining the data. The resulting *P* value was 0.041. The respective survival

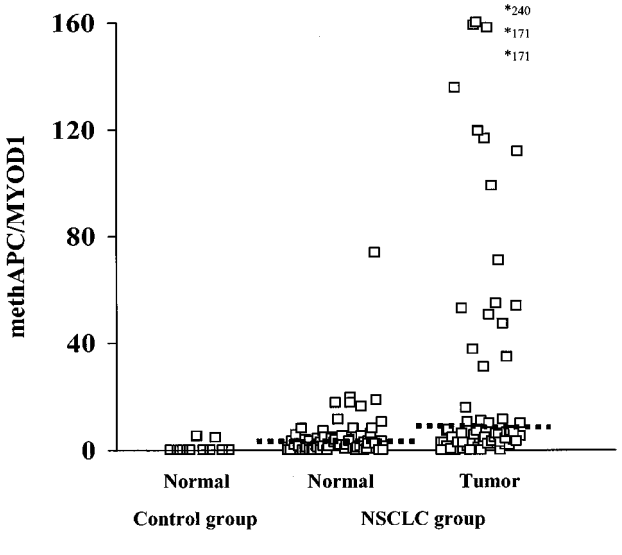

Figure 1 Methylated APC DNA in patients with NSCLC. Shown is a linear plot of *APC* promoter methylation, relative to unmethylated *MYOD1* levels, in normal lung tissues of a control group without cancer, and matching normal and tumor tissues from 91 patients with NSCLC. Far outlying values, are indicated at the top of the figure. The median *APC* methylation levels are shown as horizontal bars within each group

Table 1 Association of *APC* gene promoter methylation in tumor tissue and clinico-pathological data

Variable	n	*Median* APC *meth/MYOD1*	P-*value*
Age			
<65	49	3.62 (0.00–240.73)	0.286
⩾65	42	5.17 (0.00–135.71)	
Sex			
Male	69	4.24 (0.00–240.73)	0.630
Female	22	6.42 (0.004–171.79)	
Smoking			
Smoker	81	4.69 (0.00–240.73)	0.980
Non-Smoker	10	3.52 (0.04–135.71)	
pT			
pT1	20	3.93 (0.00–170.59)	0.397
pT2	58	5.03 (0.00–240.73)	
pT3	13	3.51 (0.00–47.03)	
pN			
pN0	50	5.37 (0.00–170.59)	0.089
pN1	25	3.16 (0.00–240.73)	
pN2	16	5.96 (0.00–171.79)	
UICC Stage			
I	45	5.79 (0.00–171.59)	0.160
II	19	3.16 (0.00–240.73)	
IIIa	27	4.64 (0.00–171.79)	
Histology			
Squamous Cell Carcinoma	43	5.08 (0.00–240.73)	0.215
Adenocarcinoma	33	4.32 (0.00–170.59)	
Large Cell Carcinoma	15	3.16 (0.00–135.71)	
Grading			
Well differentiated	1	3.05 (3.05–3.05)	0.848
Moderately differentiated	19	4.97 (0.00–240.73)	
Poorly differentiated	71	4.76 (0.00–171.79)	

Abbreviations: pT (tumor infiltration), pN (lymph node involvement)

curves are shown in Figure 2 and show 5-year survival rates of $50.8 \pm 0.06\%$ for low-methylated and $37.7 \pm 0.1\%$ for high-methylated NSCLC ($P = 0.04$ Wilcoxon-Gahan).

The importance of hypermethylated *APC* DNA as a prognostic factor was next determined by the Cox's proportional hazards model analysis. The logistic regression model included the parameters gender, age, histopathological type, pT (tumor infiltration) and pN (lymph node involvement) categories, grade of differention of the primary tumor, and *APC* methylation status. Significant independent prognostic factors were shown to be the pT ($P = 0.050$) and the pN categories ($P < 0.001$), and *APC* methylation status ($P = 0.044$). Table 2 shows the statistically significant parameters in the regression model.

Discussion

In this study, we measured methylation within the promoter 1A of the *APC* gene at nine CpG sites in 182 lung tissues from NSCLC patients, and 10 normal lung tissues from patients without cancer. Methylated *APC* genes were found in a very high percentage (95%) of NSCLC, suggesting a fundamental role for *APC* methylation in tumorigenesis. However, the intratumoral content of *APC* methylation at this site varied among the tumors over a range of 240-fold. This observation of seemingly variable amounts of methylated *APC* among the tumors may have several possible explanations. One is that the tumor consists of a mixture of cells having either methylated or unmethylated *APC*. In that case, the monoclonal tumor may be heterogeneous, with different degrees of *APC* inactivation among different subclones. Another explanation might be heterogeneity of methylation patterns within individual cells at CpG sites. It is possible that transcription of the *APC* gene could be suppressed by any number of different methylation patterns. In that case, the methylation at any specific site within the promoter could be variable and the amount of PCR product from that site will vary accordingly. Finally, specimen heterogeneity in terms of stromal cell contamination may be a contributor to the apparent variation in methylation, although it probably does not account for most of the difference in methylated *APC* content among the tumor specimens. As we have reported here, the specimens that we obtained for analysis were specifically selected to contain a large percentage of malignant cells.

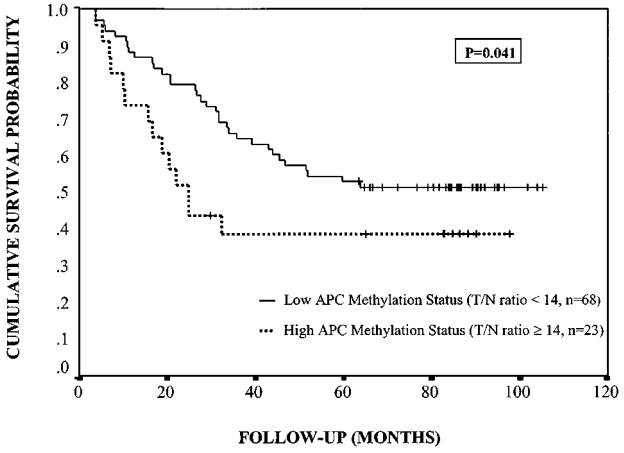

Figure 2 Survival in patients with NSCLC. Kaplan-Meier survival curve for non-small cell lung cancer patients with high and low *APC* promoter methylation status. The probability of survival was significantly decreased for the high *APC* methylation group ($P = 0.041$)

Table 2 Cox-proportional hazard regression model

Parameter	*Odds ratio*	*CI 95%*	P *value*
pT			0.050
pT_1/pT_3	0.28	0.10–0.78	0.015
pT_2/pT_3	0.55	0.26–1.19	0.129
pN			<0.001
pN_0/pN_2	0.11	0.05–0.24	<0.001
pN_1/pN_2	0.27	0.13–0.59	0.033
APC meth.	2.01	1.02–3.96	0.044

Abbreviations: CI 95% (confidence interval for odds ratio), pT (tumor infiltration), pN (lymph node involvement); Parameter section: e.g. pT_1/pT_3 means pT_1 compared to pT_3

Unexpectedly, we also observed detectable *APC* methylation in the majority (88%) of non-cancerous tissue taken from the cancer patients at the time of surgery, although at a significantly lower median level than in the tumor tissue. There are several possible explanations for this observation. The low level methylation may reflect the infiltration of neoplastic cells in histologically 'normal' surrounding mucosa. It is also possible that the non-malignant tissue, even though it appeared to be histologically normal, has been damaged by environmental factors such as cigarette smoke. The finding that there was no significant association between *APC* promoter methylation and smoking history in the normal lung tissues of patients with NSCLC suggests, however, that this factor does not account for this epigenetic alteration in these tissues. Alternatively, some of the early molecular events of tumorigenesis may have already occurred and indeed, it may be the presence of these changes that predispose this apparently normal tissue to start to undergo a further chain of events leading ultimately to tumor development. From previous studies with gastrointestinal cancers, it is generally agreed that alterations in the *APC* gene are early events in tumorigenesis. In fact, we have observed methylated *APC* in Barrett's esophagus, a precancerous condition arising in tissue exposed to chronic acid reflux from the stomach (Kawakami *et al*., 2000). A recent publication reports aberrant *APC* methylation in nine of 48 (18%) benign colorectal adenomas, a frequency similar to invasive carcinomas (Esteller *et al*., 2000).

It is also possible that methylated *APC* constitutes an age-related event in normal lung tissue. To test this

hypothesis, we analysed histological normal lung tissues from a significantly younger group of patients without cancer, and detected methylated *APC* in only 20% of these patients. These data suggest that *APC* promoter 1A methylation may constitute an age-related event in the normal lung, as it does in noncancerous gastric mucosa (Tsuchiya *et al*., 2000), and that this event has already occurred in the two normal tissues of the control group. Methylation of genes is known to occur in other normal tissues, such as *APC* in normal colon mucosa (Hiltunen *et al*., 1997), and in normal gastric mucosa, but not in normal esophageal epithelium (Kawakami *et al*., 2000), and at the *D17S5* locus in non-cancerous lung tissues (Eguichi *et al*., 1997). These findings suggest that methylation of certain genes may be a much more common occurrence than previously thought, and while tissue-specific, may not always be tumor-specific.

A recently published study reported *APC* promoter methylation in 0 of 17 (0%) lung tumor specimens analysed (Esteller *et al*., 2000). There are several possible explanations for these apparently discrepant results. First, primers used in the latter study did not cover the same sites in the *APC* promoter as did ours, and therefore might have amplified sites undergoing infrequent methylation in NSCLC. Secondly, Esteller *et al*. (2000) determined *APC* hypermethylation using methylation-specific PCR (MSP), whereas quantitative real-time PCR (Taqman®) was used in our study. The reported 10-fold greater sensitivity of the Taqman® method may account for these discrepant results (Eads *et al*., 2000).

The most striking finding in the current study was that high levels of methylated *APC* were significantly associated with worse clinical outcome. The extent of change in methylated *APC* during the normal to tumor tissue transition could be an indicator of the overall methylation propensity of the tumor. A greater propensity for methylation may result in a more aggressive tumor because of increased capability for inactivation of undesirable tumor suppressor genes. This hypothesis is strengthened by the results of a recently published study reporting a correlation between *DAP kinase* gene methylation and inferior survival in patients with stage I NSCLC (Tang *et al*., 2000). Additional studies need to be conducted that will help in assessing the clinical importance of the present findings and to understand possible underlying mechanisms. In particular, the importance of hyper-methylated *APC* DNA as a prognostic marker in the plasma of NSCLC patients has yet to be determined.

In conclusion, this investigation shows that promoter 1A of *APC* is methylated in the majority of NSCLCs. High levels of *APC* promoter methylation are significantly associated with inferior survival, showing potential as a marker of biologically aggressive disease. Quantitation of methylated *APC* may have value in identifying NSCLC patients at high risk of early disease recurrence after surgery, and in selecting patients who will benefit from intensive adjuvant therapy.

Materials and methods

Patients

Ninety-one patients were included in this study, consisting of 69 (76%) men and 22 (24%) women, with a median age of 63.3 years (range, 34–82). Forty-three (47%) patients had squamous cell carcinomas, 33 (36%) had adenocarcinomas, and 15 (16%) had large cell carcinomas. The primary tumors were graded histopathologically as well-differentiated (G1, one patient), moderately-differentiated (G2, 19 patients), and poorly-differentiated (G3, 71 patients). Tumor staging was performed according to the International Union Against Cancer (UICC) TNM classification: Forty-five (49%) had stage I tumors, 19 (21%) had stage II tumors, and 27 (30%) had stage IIIa tumors. All tumors were completely resected (R0 category). Patients with histopathological stage IIIa tumors received postoperative chemotherapy. The median follow-up was 85.9 months (min. 63.3; max. 105.2 months) and no patient was lost to follow-up. Ten histologically normal lung tissue specimen, obtained at surgery or autopsy, from patients with no evidence of cancer were used as a control group. Nine (90%) men and one (10%) woman, with a median age of 46.0 years (range, 1–64) were included in this group.

Tissue acquisition and nucleid acid isolation

Tissue for DNA analysis was obtained immediately after lung resection before starting mediastinal lymphadenectomy and was frozen in liquid nitrogen. Tissue was analysed from the following two locations: tumor and uninvolved lung tissue taken from the greatest distance to the tumor. Six μm frozen sections were taken from blocks of tumor tissue and starting with the first section every 5th was routinely stained with HE and histopathologically evaluated. Sections were pooled for analysis from areas of estimated 75% malignant cells. Genomic DNA was isolated from frozen tissue by standard methods of proteinase K digestion and phenol-chloroform extraction using the GenomicPrep® Cells and Tissue DNA Isolation Kit (Amersham Pharmacia Biotech Inc., Piscataway, NJ, USA) according to the instructions of the manufacturer.

Bisulfite treatment

Sodium bisulfite conversion of genomic DNA was performed as previously described (Olek *et al*., 1996; Eads *et al*., 1999).

Methylation analysis

Templates were amplified by a fluorescence based, real-time PCR (Taqman®) as previously described (Heid *et al*., 1996). In brief, primers and probes were designed to specifically amplify bisulfite-converted promoter 1A *APC* DNA, as well as the internal reference gene, *MYOD1*. The ratios between the values of the gene of interest versus the internal reference gene obtained by the Taqman® analysis were used as a measure for representing the relative level of methylated *APC* DNA in the particular sample. The primer and probe sequences are listed below. In all cases, the first primer is the forward PCR primer, the second is the reverse PCR primer, and the third is the Taqman® probe.

The primer and probe sequences are as follows: (a) *APC* methylation, TTATATGTCGGTTACGTGCGGTTTATAT, GAACCAAAACGCTCCCCAT; 6FAM5′- CCCGTCGAA-AACCCGCCGATTA-3′TAMRA; (b) *MYOD1*, CCAACTC-

CAAATCCCCTCTCTAT, TGATTAATTTAGATTGGGT-TTAGAGAAGGA; 6FAM5′- TCCCTTCCTATTCCTAAA-TCCAACCTAAATACCTCC-3′TAMRA.

Statistical analysis

Taqman® analyses yield values that are expressed as ratios between two absolute measurements ([gene of interest/internal reference] × 100). Medians and ranges were calculated for the methylation values and the T/N ratio (tumor/normal) for each patient. Associations between variables were tested by either using Wilcoxon signed rank test or Mann – Whitney *U*-test. The significance of rank ordering between variables was tested by using the Kruskal-Wallis analysis of variance for ordinal data. Hazards ratios were used to calculate the relative risks of death. These calculations were based on the Pike estimate, with the use of the observed and expected number of events as calculated in the Tarone-Ware test statistic (Pike, 1972). The maximal chi-square method was adapted to determine which methylation value best segregated patients into poor- and good prognosis subgroups (in terms of likelihood of surviving), with the Tarone-Ware test as the statistics used to measure the strength of the grouping (Miller and Siegmund, 1982; Halpern 1982). Multivariate analysis was performed with the Cox proportional hazards regression model. The level of significance was set to $P < 0.05$. Analyses were carried out using the SPSS software package (Chicago, IL, USA).

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