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Advances in Brief

Quantitative Adenomatous Polyposis Coli Promoter Methylation Analysis in Tumor Tissue, Serum, and Plasma DNA of Patients with Lung Cancer[1]

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Abstract

The serum of cancer patients often harbors increased free DNA levels, which can potentially be used for cancer detection. Because genetic and epigenetic alterations of the adenomatous polyposis coli (*APC*) gene are common events in gastrointestinal tumor development, we sought to investigate the frequency and level of aberrant *APC* promoter methylation in primary tumors and paired preoperative serum or plasma samples of lung cancer patients by semiquantitative methylation-specific fluorogenic real-time PCR. We detected methylation of *APC* in 95 of 99 (96%) primary lung cancer tissues. Forty-two of 89 (47%) available serum and/or plasma samples from these cases carried detectable amounts of methylated *APC* promoter DNA. In contrast, no methylated *APC* promoter DNA was detected in serum samples from 50 healthy controls. A highly elevated *APC* methylation level in lung cancer tissue was the only independent factor predicting inferior survival in this cohort (*P* = 0.015). *APC* methylation analysis appears to be promising as a prognostic factor in primary lung cancer and as a noninvasive tumor marker in plasma and/or serum DNA.

Introduction

Lung cancer is the leading cause of cancer-related death in men in the United States (1). Surgery is the most effective treatment for lung cancer, but this modality is limited because >60% of patients have advanced stages of the disease at the time of diagnosis (2). In addition, many of the patients who undergo curative surgical resection will ultimately die of recurrent lung cancer depending mainly on the initial stage of the disease (3). Thus, earlier detection of lung cancer would improve the efficacy of treatment and management of this disease. Therefore, it is vitally important to identify and develop reliable diagnostic and prognostic markers of early stage lung cancer. Recent reports show that tumors can release DNA into the circulation, and this DNA is often detectable in the serum and plasma of cancer patients. The serum of cancer patients contains, on average, ~4–40 times more free DNA, compared with normal individuals (4). We and others have shown that it is possible to identify tumor-specific micro-satellite alterations, *p53,* and *ras* gene mutations in the serum and/or plasma DNA of patients with different cancers (5, 6–8).

Silencing of tumor suppressor- or other cancer-associated genes by methylation of CpG islands, located in the promoter and/or 5′-regions of many genes, is a common feature of human cancer (9–12). CpG island methylation is often associated with a transcriptional block and loss of the relevant protein (13). In addition to functional implications of gene inactivation in tumor development, these aberrant methylation patterns represent excellent targets for novel diagnostic approaches based on methylation sensitive PCR techniques.

Recently, aberrant promoter methylation of at least one of the genes *p16, O*[6]*-*methylguanine-DNA methyltransferase, glutathione S-transferase 1, and death-associated protein kinase was identified in 68% of NSCLC.[3] The identical alterations were detectable in the serum of 73% of patients positive for methylated DNA in the primary tumor (14). These experiments were carried out using MSP. In this approach, DNA can be amplified using primer pairs designed to distinguish methylated from unmethylated DNA by taking advantage of sequence differences as a result of sodium-bisulfite treatment, where unmethylated Cytosines are converted to Uracil, and methylated Cytosines remain unconverted (15). An advancement of this technique permits reliable quantification of methylated DNA and is called real-time quantitative MSP. The method is based on the continuous optical monitoring of a fluorogenic PCR. This PCR approach is more sensitive (16) than conventional PCR and more specific because of the use of an internally binding, fluorogenic hybridization probe. This PCR reaction provides additional quantitative information about the number of target sequences within a sample. Using EBV DNA as the target genome, real-time PCR has been shown to be useful for monitoring the progress of nasopharyngeal cancer and to assess the effects of treatment (17). Moreover, these approaches are amenable to high-throughput techniques allowing the analysis of 96 samples in ~2 h without requirement for gel-electrophoresis.

The *APC* gene, mapped to chromosome 5q21 (18), plays a prominent role in the development of colorectal cancer both in the autosomal dominant inherited familial APC syndrome and in sporadic colorectal cancer (19–21). An impaired function of APC, most often attributable to mutations within the coding sequence of the gene, leads to a lack of degradation and nuclear accumulation of β-catenin which acts as a transcriptional activator, causing loss of cell growth control (22). Although previous investigations reported loss of heterozygosity frequencies of ~40% at 5q21 within or nearby the *APC* gene in NSCLCs, inactivating point mutations, at least in the mutation cluster region of the *APC* gene, were not detected in human lung cancer (23–26). Therefore, as described in a subset of colorectal and other gastrointestinal cancers (27–30), inactivation by promoter methylation

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[3] The abbreviations used are: NSCLC, non-small cell lung cancer; APC, adenomatous polyposis coli; MSP, methylation-specific PCR.

could represent a putative mechanism of impairing *APC* gene function in lung cancer.

In the present study, we show a high frequency of promoter 1 A methylation of the *APC* gene in lung cancer. This *APC* promoter methylation was also detectable in matching serum and/or plasma samples from almost half of the individuals within our study population, providing a new approach for early diagnostic testing and precise monitoring of lung cancer. Moreover, we found that high levels of methylated *APC* DNA in tumor tissue are an independent predictive factor of poor survival.

Materials and Methods

Sample Collection and DNA Preparation. We evaluated tissue samples of 99 patients with lung cancer, who underwent curative surgery between 1995 and 1999 at the Johns Hopkins University, School of Medicine. The demographic characteristics of the patient population are depicted in Table 1. The tumor staging was performed according to the UICC Classification (Ed. 4, 2nd revision, 1992). Obtained tissue specimen were immediately snap frozen in liquid nitrogen and stored at $-80°C$. H&E-stained sections were histologically examined every 20 sections for the presence or absence of tumor cells, as well as for tumor density. Only sections that showed >70% of tumor cells were used for DNA extraction. Microdissection was performed to reach that standard.

Matching serum ($n = 71$) and/or plasma samples ($n = 33$) of 89 of the lung cancer patients were collected in tubes with K^+-EDTA or no additives before surgery or other diagnostic procedures. DNA from tumor tissue and from 0.2 to 1 ml of serum or plasma was extracted as described previously (5). Purification was carried out by phenol-chloroform extraction and ethanol precipitation. In 15 of these lung cancer patients, both serum and plasma samples were available for comparison and collected at the same time before surgery. The serum controls were taken from 50 healthy volunteers, 32 men and 18 women, in a stochastic manner and were processed in the same way as the patient samples. The median age of the control population was 72 years (range: 49–94 years) and 64.2 years (range: 40–84 years) in the lung cancer patient population.

Methylation-specific Real-time PCR. Real-time quantitative PCR is based on the continuous monitoring of a progressive fluorogenic PCR by an optical system (31). This PCR system uses two amplification primers and an additional, amplicon-specific, and fluorogenic hybridization probe whose target sequence is located within the amplicon. The probe is labeled with two fluorescent dyes. One serves as a reporter on the 5′-end (FAM). Its emission spectrum is quenched by a second fluorescent dye at the 3′-end (TAMRA). If amplification occurs, the 5′ to 3′ exonuclease activity of the Taq DNA polymerase cleaves the reporter from the probe during the extension phase, thus releasing it from the quencher (32). The resulting increase in fluorescence emission of the reporter dye is monitored during the PCR process (Fig. 1).

Chemical modification of unmethylated but not of methylated Cytosines to Uracil within CpG islands using sodium-bisulfite treatment was performed as described previously (15). The protocol was modified by using 2 μg of serum/plasma DNA and 2 μg of tumor DNA for the sodium-bisulfite treatment. After bisulfite modification, the DNA was eluted in 30 μl of H_2O.
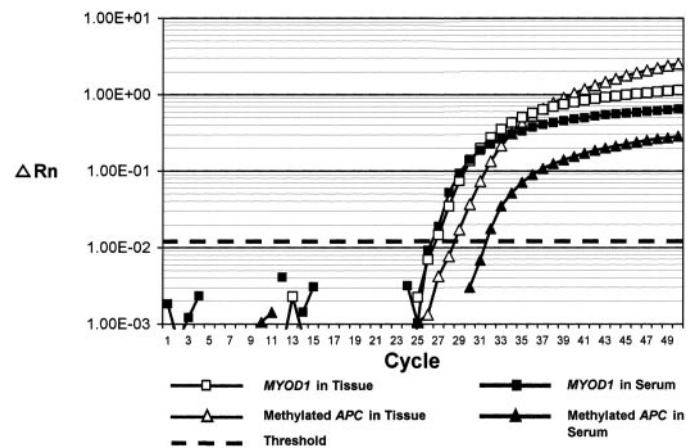

Fig. 1. Amplification plots of the real-time MSP from paired tumor and serum samples of a patient with an adenocarcinoma of the lung. Tumor and serum show strong amplification of the internal reference gene *MYOD1* and the gene of interest *APC*. The relative amount of methylated *APC* promoter DNA is much higher in tumor tissue compared with the serum sample. ΔRn is defined as the cycle-to-cycle change in the reporter fluorescence signal normalized to a passive reference fluorescence signal (log scale). Calculation of the *APC:MYOD1* ratios are based on the cycle number where fluorescence of each reaction passes the threshold, which is set to the geometrical phase of the amplification above the background.

Primers and probes were designed to specifically amplify either bisulfite-converted *APC* promoter 1 A DNA, in our case the gene of interest, or the internal reference gene *MYOD1* (27). Primers and probes of the *MYOD1* gene were located in an area without CpG nucleotides; thus, amplification of *MYOD1* by MSP occurs independently of a CpG island methylation status, whereas the amplification of *APC* is proportional to the degree of cytosine methylation within the *APC* promoter. The methylation ratio was defined as the ratio of the fluorescence emission intensity values for the *APC* PCR products to those of the *MYOD1* PCR products obtained by TaqMan analysis, multiplied by 100. This ratio ($APC:MYOD1 \times 100$) was used as a measure for the relative level of methylated *APC* DNA in the particular sample (Fig. 1). PCR was performed in separate wells for each primer/probe set.

The amplicon size of the *APC* promoter MSP assay was 74 bp (position 761–834, deposited at GenBank as accession no. U02509). Primers and probes covered 8 CpG dinucleotides within this CpG island. 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 sequences were the following: (*a*) *APC* methylation-specific, 5′-GAA CCA AAA CGC TCC CCA T-3′, 5′-TTA TAT GTC GGT TAC GTG CGT TTA TAT-3′, 6FAM5′-CCC GTC GAA AAC CCG CCG ATT A-3′TAMRA; and (*b*) *MYOD1,* 5′-CCA ACT CCA AAT CCC CTC TCT AT-3′, 5′-TGA TTA ATT TAG ATT GGG TTT AGA GAA GGA-3′, 6FAM5′-TCC CTT CCT ATT CCT AAA TCC AAC CTA AAT ACC TCC-3′TAMRA.

Fluorogenic PCRs were carried out in a reaction volume of 25 μl using components of the TaqMan PCR Buffer A Pack (Perkin-Elmer Applied Biosystems, Foster City, CA). Fluorogenic probes were custom synthesized by Perkin-Elmer Applied Biosystems. PCR primers were synthesized by Life Technologies, Inc. (Gaithersburg, MD). Each PCR reaction mixture consisted of 600 nM each primer; 200 nM probe; 5 units of AmpliTaq Gold polymerase; 200 μM each dATP, dCTP, and dGTP; 400 μM dUTP; 5.5 mM $MgCl_2$; and 1 $\times$ TaqMan Buffer A. Three μl of treated DNA solution (200 ng) was used in each real-time MSP reaction. Thermal cycling was initiated with a first denaturation step of 95°C for 10 min. The thermal profile for the PCR was 95°C for 15 s, and 60°C for 1 min. Data obtained during 50 cycles of amplification were analyzed.

Amplifications were carried out in 96-well plates in a 7700 Sequence detector (Perkin-Elmer Applied Biosystems). Each plate consisted of patient samples and multiple water blanks, as well as a positive and negative control. Serial dilutions of the *APC* promoter methylation-positive lung cancer cell line H157 were used for constructing the calibration curve on each plate. Dilution experiments showed linearity of amplification down to a dilution of 1:10.000 for methylated *APC* promoter DNA, as well as for unmethylated *MYOD1* DNA representing 20 pg of target DNA. All data presented are within this linear

Table 1 *Demographic characteristics of the patient population*

	Histology			
Characteristics	Squamous cell carcinoma	Adenocarcinoma	Other	Total
Patients, no. (%)	35 (35)	47 (48)	17 (17)	99 (100)
Gender, no. (%)				
Female	11 (31)	26 (55)	13 (76)	50 (50)
Male	24 (69)	21 (45)	4 (24)	49 (50)
Age, y, mean $\pm$ SD	66.1 $\pm$ 8.0	63.7 $\pm$ 10.6	61.6 $\pm$ 9.6	64.2 $\pm$ 9.6
Smoking status, no. (%)				
Smoker	34 (97)	44 (94)	16 (94)	94 (95)
Nonsmoker	1 (3)	3 (6)	1 (6)	5 (5)
Stage, no. (%)				
I	22 (63)	22 (47)	9 (52)	53 (54)
II	7 (20)	14 (30)	2 (12)	23 (23)
III	5 (14)	8 (17)	4 (24)	17 (17)
IV	1 (3)	3 (6)	2 (12)	6 (6)

range of amplification. The *APC* promoter methylation-negative fetal human epithelial lung cell line L132 was used as a negative control. To further verify the specificity of this methylation-specific real-time PCR assay, we treated 100 µg DNA of the *APC* promoter methylation-negative control cell line L132 *in vitro* with Sss I Methyltransferase, according to the manufacturer's protocol (New England Biolabs, Inc., Beverly, MA). This enzyme adds methyl-groups to all CpG dinucleotides within the genomic DNA. Sodium-bisulfite treated DNA of *in vitro* methylated L132 DNA revealed positivity for *APC* promoter methylation compared with untreated cells (data not shown). All of the assays were performed at least twice.

Statistical Analysis. Real-time PCR analyses yield values that are expressed as ratios between two absolute measurements (gene of interest:internal reference $\times$ 100). Medians and ranges were calculated for the methylation values of each sample. Associations between variables were tested by using the Wilcoxon signed rank test or the Mann-Whitney *U* test. The significance of rank ordering between variables was tested by using the Kruskal-Wallis ANOVA for ordinal data. The maximal χ^2 method was adapted to determine which methylation value best segregated patients into poor and good prognosis subgroups (in terms of likelihood of surviving; Refs. 33 and 34). Survival was estimated according to Kaplan and Meier (35). 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).

Results

We collected tissue samples of primary lung cancers from 99 patients who underwent curative resection (Table 1). Methylation of the *APC* promoter 1 A was detected by real-time quantitative PCR in 95 (96%) of 99 lung cancer tissues (Fig. 2). The median methylation level (*APC:MYOD1* $\times$ 100) was 7.33 (range: 0.001–346.8; Fig. 2). Because we detected *APC* methylation in most lung tumors, we went on to investigate whether this epigenetic alteration was also specifically detectable in matching serum and/or plasma from these patients. Methylated *APC* promoter 1 A DNA was detected in 42 (47%) of 89 matched serum or plasma samples. The median level of methylated *APC* promoter DNA in the positive samples was 0.36 (range: 0.01–11.5; Fig. 2). *APC* methylation in serum or plasma DNA was never detected if this alteration was not present in the primary tumor tissue. Moreover, methylated *APC* DNA was not detected in 50 control serum samples from healthy individuals, whereas robust amplification of the *MYOD1* control for input DNA was documented.
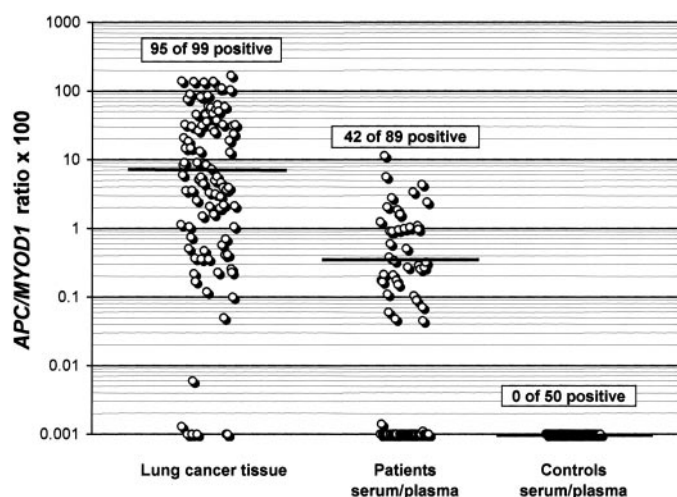
We compared the detectability, as well as the levels of detected methylated *APC* DNA, in between serum and plasma samples taken before surgery from 15 individuals with lung cancer. Methylated APC-DNA was detectable in 14 of 15 (93%) plasma samples and in 6 of 15 (40%) serum samples. The median of the *APC:MYOD1* ratio in the positive samples was 0.35 (range: 0.015–1.6) for serum samples and 0.21 (range: 0.015–1.6) for plasma samples, respectively. The ratio differences and the distribution differences in these samples and in all of the serum and plasma samples of the study population were not statistically significant, but we observed a trend toward higher levels of methylated *APC* DNA in plasma *versus* serum in both groups ($P = 0.08$). Although this observation suggests that larger quantities of tumor DNA are present in plasma, further validation is needed in a larger series.

Statistical analyses of the data using the Mann-Whitney *U* test revealed no significant differences between *APC* promoter methylation levels in tissue, serum and plasma, and common clinical or pathological parameters, such as histological subtype of lung cancer, grade, age, gender, smoking history, tumor size, lymph node status, and overall stage.

To determine whether different methylation levels in tumor tissue have prognostic value, we analyzed the available survival data for 93 lung cancer patients. Twenty-four patients (26%) died of recurrent disease, 4 (4%) from other causes. The median overall survival of the entire study population was 51.8 months. A methylation ratio of 32.7 in lung cancer tissue best segregated patients into good and bad prognosis subgroups. By this criterion, 70 of 93 (75%) patients had a low ($\leq$32.7) and 23 of 93 (25%) a high *APC* methylation level in lung cancer tissue. The median overall survival for patients with tissue *APC* methylation levels >32.7 was 32.4 months (95% confidence interval: 14.9 and 42.6), whereas the median survival for those with an *APC* methylation $\leq$32.7 was not reached. The resulting adjusted *P* was 0.006 for overall survival (Log-rank test; Fig. 3*A*).

For serum- and plasma-methylated *APC* levels, survival data were analyzed for 83 of the 89 patients. A methylation ratio of 0.45 best segregated patients into good and bad prognosis subgroups. Forty-six of 83 patients (55%) had a low and 37 of 83 (45%) had a high methylation level. In this subgroup, the median survival was not reached for both, and no significant differences in survival could be observed ($P = 0.386$). The respective survival curves are shown in Fig. 3*B*.

The importance of *APC* methylation in tumor tissues as a prognostic factor was next determined by the Cox proportional hazards model analysis. The logistic regression model included common clinical and pathological parameters, such as age, gender, histopathological type, tumor stage, grade of differentiation of the primary tumor, and *APC* methylation status. A very strong trend for stage as an independent prognostic factor was observed ($P = 0.052$), but only a high *APC* methylation level in tumor tissue was of independent prognostic importance ($P = 0.015$).

Discussion

Detection of Aberrant Methylation of the APC Promoter 1 A in Primary Lung Cancer. We detected methylation of the *APC* promoter 1 A in virtually all studied lung cancer tissues (96%). Our current observation supports a role for *APC* promoter methylation in the development and progression of lung cancer and is consistent with the results of our previous investigation where *APC* promoter methylation was observed in 94% of 91 NSCLC tissues (36). On the other hand, there are data in other investigations where either 53% of 106 NSCLC samples (37) showed *APC* methylation, or no methylation of the *APC* promoter was detectable in 17 lung cancer samples (29). These variant results are most likely attributable to increased sensi-

Fig. 2. *APC:MYOD1* ratios $\times$ 100 on a log scale. Lung cancer tissue and paired serum and plasma samples from patients with lung cancer positive for methylated *APC* Promoter 1 A DNA. *APC:MYOD1* ratios of serum from healthy control individuals are negative. *Boxes,* the number of samples positive for methylated *APC* promoter DNA. *Bars,* the median *APC*-methylation level within a sample type. Values diagrammed at 0.001 are zero values, which cannot be plotted correctly on a log scale.
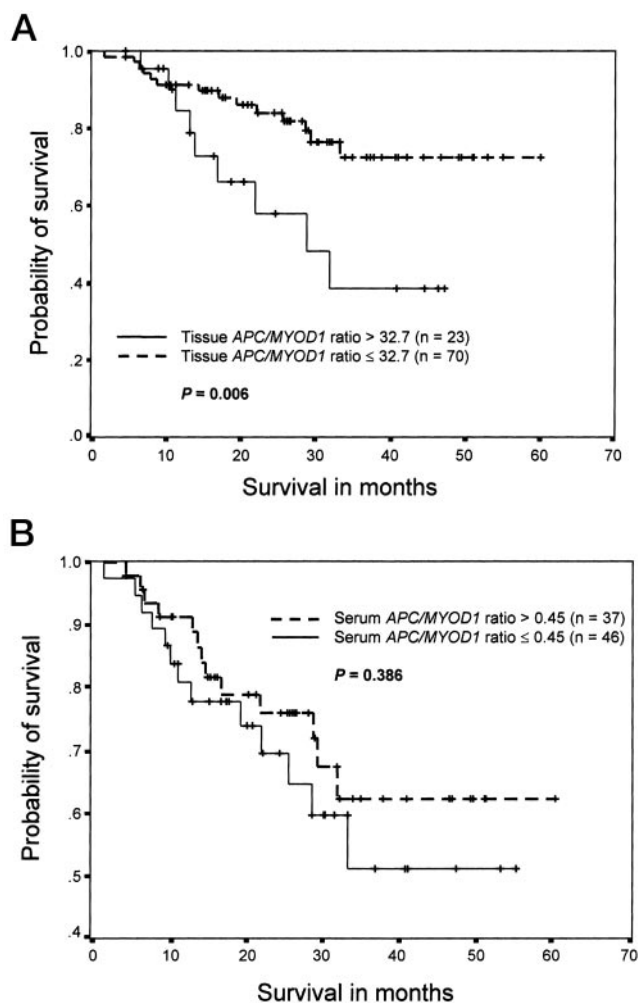
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Fig. 3. Overall survival in patients with lung cancer. *A,* Kaplan-Meier survival curves for lung cancer patients with high and low *APC* promoter methylation status in tumor tissue. The probability of survival was significantly decreased for the group of patients with high levels of methylated *APC* promoter 1 A DNA ($P = 0.006$). *n,* the number of samples analyzed in each methylation group. *B,* Kaplan-Meier survival curves for patients with high and low levels of methylated *APC* promoter DNA in serum or plasma samples. No significant difference in the probability of survival was observed ($P = 0.386$).

tivity of fluorogenic real-time MSP compared with traditional MSP, as well as to differences with respect to coverage of different CpG dinucleotides within the promoter (Ref. 29: nt 702–799; present study: nt 761–834; GenBank accession no.: U02509). A 10-fold greater sensitivity was recently reported for fluorogenic MSP in comparison with traditional MSP (16). We confirmed these differences in sensitivity by comparing the real-time *APC* MSP assay with the traditional MSP using the same primer sets (data not shown). In addition, the reporter hybridization probe used in the real-time MSP adds greater specificity to this assay.

The wide range of the amounts of methylated *APC* detectable in tumors is remarkable (Fig. 2). This variation may be attributable to the heterogeneity of tumors harboring cells with either methylated or unmethylated *APC,* as well as to differences in the stromal cell contamination within the tumors. Furthermore, we observed previously that *APC* promoter methylation is present in 87% of 91 matching histopathologically nonmalignant tissue samples from lung cancer patients (36). These methylation levels were significantly lower compared with the tumor tissues. Methylation of the *APC* promoter 1 A could be a tissue-specific epigenetic alteration, which occurs in normal lung tissues, like in other tissue types, *i.e.,* normal gastric epithe-

lium, or in lung tissue damaged by environmental factors, such as cigarette smoke (36, 38).

It is not clear if methylation levels in normal lung tissue or tumors lead to inactivation of *APC*. To better understand the relationship between different *APC* methylation levels and protein expression, quantitative real-time reverse transcriptase PCR of *APC* mRNA, as well as immunohistochemical studies, might uncover the distribution of cells, which do not express the APC protein within a lung tumor and the matching nonmalignant tissue. This approach would also lighten up the degree of heterogeneity within a single tumor.

Recent evidence suggests that CpG island methylation of the promoter region of certain genes in normal-appearing tissues may also be associated with aging (39). In fact, higher *APC* methylation levels were observed in nonmalignant lung tissue from older lung cancer patients compared with normal lung tissue from a younger group of individuals without lung cancer (36).

All these data suggest that *APC* promoter methylation plays a role in lung cancer but does not ultimately clarify if this alteration has more relevance in the initiation or in the progression of lung cancer.

Detection of APC Promoter Methylation in Serum and Plasma of Lung Cancer Patients. Methylated *APC* promoter 1 A DNA was detected in about half of the patients with lung cancer from whom serum and/or plasma samples were available. None of the 50 serum controls showed *APC* methylation. The control population used in our investigation was not significantly different compared with our population of lung cancer patients. The age distribution of controls (median: 72 years) and lung cancer patients (median: 64.2 years) was comparable. The number of smokers was lower in the control group (62%) compared with the population of lung cancer patients. However, our results are consistent with a previous report in which none of 54 control serum samples from either healthy individuals or patients with gastritis or Barrett's esophagus were positive for methylated *APC* DNA (30).

Our detection rate in serum/plasma parallels another investigation in which the frequency of detected methylated DNA for different genes in plasma was ∼50% of the detection rate found in the primary tumors (14). We found methylated *APC* DNA in the serum/plasma samples at all stages and primary tumor sizes of individuals with lung cancer. Therefore, this quantitative serum assay could potentially be used as a marker for early detection of lung cancer. In fact, we were able to detect methylated *APC* DNA in the serum from a patient with a primary tumor size of only 1 cm (*APC*:*MYOD1* ratio × 100 = 0.51). We found no correlation between methylated *APC* DNA levels in the tumor tissue and corresponding levels in serum/plasma (correlation coefficient = 0.2). There are a number of possible reasons for this result, including specific physiological characteristics in the progression of each tumor, *e.g.,* angiogenesis capacity or the ability to cause local thrombosis and necrosis. In addition, the quantity and quality of DNA template extracted from serum and/or plasma is likely to differ from the quality of the original tumor tissue, based on time of collection, the content of DNase, and other factors.

One important aspect of a quantitative analysis is the ability to follow changes longitudinally for each patient. We had the opportunity to collect plasma samples from 25 lung cancer patients during their clinical follow-up. Ten of these individuals were initially positive for methylated *APC* DNA in serum or plasma before surgery, and all turned negative after curative surgery. Four of these 25 developed recurrence, which was detected by rising levels of methylated *APC* in plasma. Three were initially negative in plasma before surgery, but all of the primary tumors were positive. Furthermore, *APC* methylation levels in one of these recurrent patients accurately accompanied repeated remissions and recurrence during different treatment approaches (data not shown).

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Association of APC Promoter Methylation Levels with Survival. We demonstrate here that the level of methylated *APC* promoter DNA in lung cancer tissue is predictive with regard to overall survival. Patients with high levels of methylated *APC* DNA (*APC*: *MYOD1* ratio × 100 > 32.7) had the worst prognosis. This observation was not only significant in the Kaplan-Meier analysis (Fig. 3*A*) but also turned out to be the only independent, predictive indicator of inferior survival. Tumors with the highest methylation levels may be those likely to inactivate *APC* resulting in loss of cellular growth control via the *β-catenin* pathway. In particular, the striking consistency of the association of high *APC* methylation levels with inferior survival in our previous work (36) suggests that *APC* promoter methylation has either functional effects or is at least influential within the tumor environment providing a powerful indicator of patients at high risk of recurrence.

We did not observe a correlation between levels of methylated *APC* in serum/plasma and survival (Fig. 3*B*). This result may be partially attributable to our choice of samples, because we extracted and analyzed free DNA within the circulation intentionally and not DNA from circulating tumor cells with a high potential for metastasis.

We have detected methylated *APC* promoter DNA in the serum/plasma of patients with lung cancer. Because this epigenetic alteration is ubiquitous in lung cancer cells and is amenable to quantification, it represents a novel approach for the diagnosis and monitoring of this disease. Large case-control studies are under way to determine the value of quantitative detection of methylated *APC* DNA in serum for the early detection and follow-up of lung cancer.

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