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Advances in Brief

Detection of Aberrant Promoter Hypermethylation of Tumor Suppressor Genes in Serum DNA from Non-Small Cell Lung Cancer Patients[1]

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Abstract

Recent evidence suggests that tumor cells may release DNA into the circulation, which is enriched in the serum and plasma, allowing detection of *ras* and *p53* mutations and microsatellite alterations in the serum DNA of cancer patients. We examined whether aberrant DNA methylation might also be found in the serum of patients with non-small cell lung cancer. We tested 22 patients with non-small cell lung cancer using methylation-specific PCR, searching for promoter hypermethylation of the tumor suppressor gene *p16*, the putative metastasis suppressor gene *death-associated protein kinase*, the detoxification gene *glutathione S-transferase P1*, and the DNA repair gene *O^6-methylguanine-DNA-methyltransferase*. Aberrant methylation of at least one of these genes was detected in 15 of 22 (68%) NSCLC tumors but not in any paired normal lung tissue. In these primary tumors with methylation, 11 of 15 (73%) samples also had abnormal methylated DNA in the matched serum samples. Moreover, none of the sera from patients with tumors not demonstrating methylation was positive. Abnormal promoter methylation in serum DNA was found in all tumor stages. Although these results need to be confirmed in larger studies and in other tumor types, detection of aberrant promoter hypermethylation of cancer-related genes in serum may be useful for cancer diagnosis or the detection of recurrence.

Introduction

Lung cancer is one of the most common malignancies in the world and is the leading cause of cancer-related deaths in the United States (1). The most effective treatment for NSCLC[3] is surgical resection, but this modality is limited by the fact that 65% of patients have advanced disease at the time of diagnosis (2). Many patients undergoing surgical resection will ultimately die of recurrent NSCLC, suggesting the frequent presence of occult metastatic disease (2). This failure rate is dependent on the stage of the disease, however. For example, the 5-year survival rate following surgical resection is ~70% for patients with stage I NSCLC but drops to only 30% in patients with stage IIIa disease (3). Thus, earlier detection of lung cancer would facilitate more effective management of the disease. Standard diagnostic techniques rely on radiological images with a tumor size detection limit of ~1 cm, which represents 10^9 cells or a mass of 1 g (4). Conventional sputum cytology and bronchoalveolar lavage have not been proven effective as screening tools. Therefore, it is important to develop new methods that provide sensitive and reliable diagnostic and prognostic markers of NSCLC.

Nanogram quantities of DNA circulating in blood are present in healthy individuals (5, 6). Previous studies have proposed that tumor DNA is also released in plasma and serum (7, 8). However, the serum of cancer patients is enriched in DNA, containing, on average, ~4 times the amount of free DNA, compared to normal controls (9). On the basis of these observations, several studies have shown that is possible to identify microsatellite alterations in the plasma and serum DNA of patients with head and neck carcinoma (10) and small cell lung carcinoma (11). In addition, *p53* and *ras* gene mutations have been detected in the plasma and serum of patients with colorectal (12–14) and pancreatic (15, 16) carcinomas and hematological malignancies (17).

In addition to the molecular genetic alterations described above, silencing of tumor suppressor genes by promoter hypermethylation is a common feature in human cancer (18). Hypermethylation of normally unmethylated CpG islands in the promoter regions of many cancer key genes, including *p16*, *p15*, *E-cadherin*, *VHL*, and *hMLH1*, correlates with its loss of transcription in human tumors (19, 20). In primary lung carcinomas, the inactivation of the tumor suppressor gene *p16* (18, 21), the DNA repair gene *MGMT* (22), and the detoxifying gene *GSTP1* (23) by promoter hypermethylation have been well described. In addition, loss of expression of the *DAP kinase* gene, reactivated by the demethylating agent 5′-azadeoxycytidine (24), has also been correlated with metastatic potential in experimental lung cancer models (25). On the basis of these observations, here, we examined 22 NSCLC patients using the sensitive MSP technique for abnormal promoter hypermethylation in *p16*, *DAP kinase*, *GSTP1*, and *MGMT*, in primary tumors and paired normal tissue and serum samples.

Materials and Methods

Sample Collection and DNA Preparation. Normal lung, primary NSCLC, and corresponding serum were obtained from each of the 22 patients included in the study. Serum samples were obtained prior to surgery or at the time of surgery but before the chest incision was made. All patients were diagnosed at Germans Trias i Pujol Hospital (Badalona, Spain). DNA from normal and tumor tissue was isolated using previously published methods (26). H&E-stained sections from each tumor sample were histologically examined, and only those that were verified to contain >50% neoplastic cells by section evaluation were selected for the study. To obtain serum, blood specimens from each patient were incubated at 36°C overnight and then centrifuged at low speed for 20 min. The serum was stored at −80°C before DNA extraction. Serum DNA was partially purified by phenol-chloroform extraction and ethanol precipitation (10). After purification, 1 ml of serum yielded an average of 50 ng of DNA (10).

MSP. DNA methylation patterns in the CpG island of the *MGMT* gene were determined by chemical modification of unmethylated but not the methylated cytosines to uracil and subsequent PCR using primers specific for either

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[3] The abbreviations used are: NSCLC, non-small cell lung carcinoma; MGMT, O^6-methylguanine-DNA-methyltransferase; GSTP1, glutathione S-transferase P1; DAP kinase, death-associated protein kinase; MSP, methylation-specific PCR.

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methylated or the modified unmethylated DNA (27). Primer sequences of *MGMT* were for the unmethylated reaction 5′-ATG GTA TGG ATT TTA GTG TTG GTT-3′ (sense) and 5′-ACT ATA CCT AAA CTC AAC AAA CAC CA-3′ (antisense) and for the methylated reaction 5′-GTA TGG ATT TTA GCG TCG GTC-3′ (sense) and 5′-TAA ACT CGA CGA ACG CCG-3′ (antisense). Primer sequences of *DAP kinase* were for the unmethylated reaction 5′-GGA GGA TAG TTG GAT TGA GTT AAT GTT-3′ (sense) and 5′-CAA ATC CCT CCC AAA CAC CAA-3′ (antisense) and for the methylated reaction 5′-GGA TAG TCG GAT CGA GTT AAC GTC-3′ (sense) and 5′-CCC TCC CAA ACG CCG A-3′ (antisense). Primer sequences of *GSTP1* were for the unmethylated reaction 5′-GAT GTT TGG GGT GTA GTG GTT GTT-3′ (sense) and 5′-CCA CCC CAA TAC TAA ATC ACA ACA-3′ (antisense) and for the methylated reaction 5′-TTC GGG GTG TAG CGC TCG TC-3′ (sense) and 5′-GCC CCA ATA CTA AAT CAC GAC G-3′ (antisense). Primer sequences for *p16* have been described previously (27). The annealing temperatures were 60°C for *p16* and *DAP kinase* and 59°C for *MGMT* and *GSTP1*. PCR conditions for the four genes were as follows: 95°C for 5 min; then 35 cycles of 95°C for 30 s, the specific annealing temperature for 30 s, and 72°C for 30 s; and a final extension of 4 min at 72°C. All PCR amplification was performed using an Omnigene thermocycler (Hybaid) with tube control for accurate annealing temperatures. Hot start was used. Human placental DNA was treated *in vitro* with excess *Sss*I methyltransferase (New England Biolabs), to generate completely methylated DNA at all CpGs and was used as positive control for methylated alleles of each gene. DNA from normal lymphocytes was used as the control for unmethylated alleles. Some samples were analyzed for *p16* methylation using 40 cycles of amplification, with concordant results.

Briefly, 1 μg of DNA was denatured by NaOH and modified by sodium bisulfite. DNA samples were then purified using Wizard DNA purification resin (Promega), again treated with NaOH, precipitated with ethanol, and resuspended in water. Controls without DNA were performed for each set of PCR. Ten μl of each PCR were directly loaded onto nondenaturing 6% polyacrylamide gels, stained with ethidium bromide, and visualized under UV illumination.

Results

We found that 68% (15 of 22) of NSLCC primary tumors exhibited abnormal promoter hypermethylation in at least one gene (Table 1). *p16* was hypermethylated in 41% (9 of 22) of the cases, a similar percentage to that previously described in lung cancer (18). The presence of abnormal methylation of *p16* was found in all of the stages, consistent with the finding that *p16* aberrant methylation is an early event in lung carcinogenesis (21). The incidence of *DAP kinase* promoter hypermethylation was 23% (5 of 22 cases), suggesting that this is a common epigenetic alteration in lung cancer. Consistent with this data, the treatment with the demethylating agent 5′-azadeoxycytidine restores the expression of *DAP kinase* in some cancer cell lines (24), strongly suggesting the methylation-mediated inactivation of *DAP kinase* in human neoplasia. Abnormal *GSTP1* promoter methylation was found in 9% (2 of 22) of cases, consistent with previous findings (23). Finally, *MGMT* promoter hypermethylation was present in 27% (6 of 22) of the cases. We recently analyzed a wide spectrum of tumor types and found a similar incidence of *MGMT* hypermethylation in primary NSCLC (22). Thus, the incidence of hypermethylation of each gene was consistent with previous work in NSCLC. Moreover, none of the 22 paired lung normal tissues exhibited abnormal promoter hypermethylation of any gene.

Seventy-three % (11 of 15) of the patients showing hypermethylation in tumor DNA also demonstrated abnormal methylation in serum DNA. Representative MSP analyses for *p16*, *DAP Kinase*, *GSTP1*, and *MGMT* in tumor and serum DNA, as compared with paired normal lung are shown in Fig. 1. Promoter hypermethylation in serum *DNA* was 33% (3 of 9 cases) for *p16*, 80% (4 of 5 cases) for *DAP kinase*, 50% (1 of 2 cases) for *GSTP1*, and 66% (4 of 6 cases) for *MGMT* (Table 2). Each of the 11 patients who had abnormal promoter hypermethylation in the serum DNA demonstrated identical alterations in the primary tumor DNA (Table 1). Importantly, only patients whose tumors harbored a hypermethylated marker showed aberrant methylation of the same genes in serum. After abnormal promoter hypermethylation analysis of all specimens was completed, clinical data were correlated with the results. The clinical stage, histology, follow-up (development of recurrence), and clinical outcome in the 22 patients and the methylation status of each gene studied are listed in Table 1. The presence of hypermethylation occurred with all stages, including stage I. Detection of abnormal methylation in serum DNA did not appear to be related to an early recurrence in this small series.

Table 1 *Detection of aberrant methylation of p16, DAP kinase, GSTP1, and MGMT in serum and tumor DNA from lung cancer patients*[a]

Patient no.	Tumor DNA/Serum DNA				Stage (TNM)	Histology	Follow-up[b]	Outcome[b]
	p16	*DAP*	*GSTP1*	*MGMT*				
1	U/U	M/M	U/U	U/U	IIIA ($T_2N_2M_0$)	SCC	15 met^{bone}	17.DOD
42	M/U	U/U	U/U	U/U	IIIA ($T_1N_2M_0$)	SCC	22 met^{lung}	29+.AWD
52	M/U	U/U	M/M	U/U	II ($T_1N_1M_0$)	SCC	27+	27+.NED
63	M/M	U/U	U/U	U/U	I ($T_2N_0M_0$)	SCC	25+	25+.NED
64	M/U	M/M	U/U	M/U	II ($T_2N_1M_0$)	SCC	25+	25+.NED
68	M/U	U/U	U/U	U/U	IIIA ($T_3N_0M_0$)	AC	8	10.DOD
76	U/U	U/U	U/U	M/M	I ($T_2N_0M_0$)	SCC	26+	26+.NED
78	M/M	U/U	U/U	M/M	I ($T_2N_0M_0$)	SCC	6 met^{lung}	24.DOD
83	U/U	U/U	U/U	M/M	I ($T_2N_0M_0$)	SCC	23+	23+.NED
84	U/U	M/M	M/U	U/U	I ($T_2N_0M_0$)	AC		LTF
94	M/M	U/U	U/U	U/U	IIIA ($T_3N_0M_0$)	SCC	15 met^{bone}	19+.AWD
95	M/U	U/U	U/U	U/U	IIIA ($T_2N_2M_0$)	AC	5 met^{brain}	22.DOD
98	U/U	U/U	U/U	M/M	I ($T_2N_0M_0$)	AC	21+	21+.NED
106	U/U	M/U	U/U	U/U	I ($T_2N_0M_0$)	LCC		LTF
112	M/U	M/M	U/U	M/U	II ($T_1N_1M_0$)	SCC	19+	19+.NED
36	U/U	U/U	U/U	U/U	IIIA ($T_3N_0M_0$)	AC	12 met^{bone}	16.DOD
49	U/U	U/U	U/U	U/U	IIIA ($T_3N_2M_0$)	SCC	11 met^{bone}	31+.AWD
66	U/U	U/U	U/U	U/U	I ($T_2N_0M_0$)	bAC	9 met^{lung}	18+.DOD
74	U/U	U/U	U/U	U/U	I ($T_2N_0M_0$)	SCC	12 met^{liver}	14.DOD
80	U/U	U/U	U/U	U/U	IV ($T_1N_2M_1$)	AC	6 met^{bone}	8.DOD
108	U/U	U/U	U/U	U/U	I ($T_2N_0M_0$)	SCC	2 met^{bone}	3.DOD
110	U/U	U/U	U/U	U/U	IIIA ($T_1N_2M_0$)	SCC	8 met^{lung}	24+.AWD

[a] TNM, tumor-node-metastasis; M, methylated; U, unmethylated; SCC, squamous cell carcinoma; AC, adenocarcinoma; bAC, bronchoalveolar adenocarcinoma; LCC, large cell carcinoma; met^{bone}, bone metastasis; met^{brain}, brain metastasis; met^{liver}, metastasis; met^{lung}, lung metastasis; +, alive; AWD, alive with disease; DOD, died of disease; LTF, lost to follow-up; NED, no evidence of disease.

[b] The follow-up and outcome are indicated in months.

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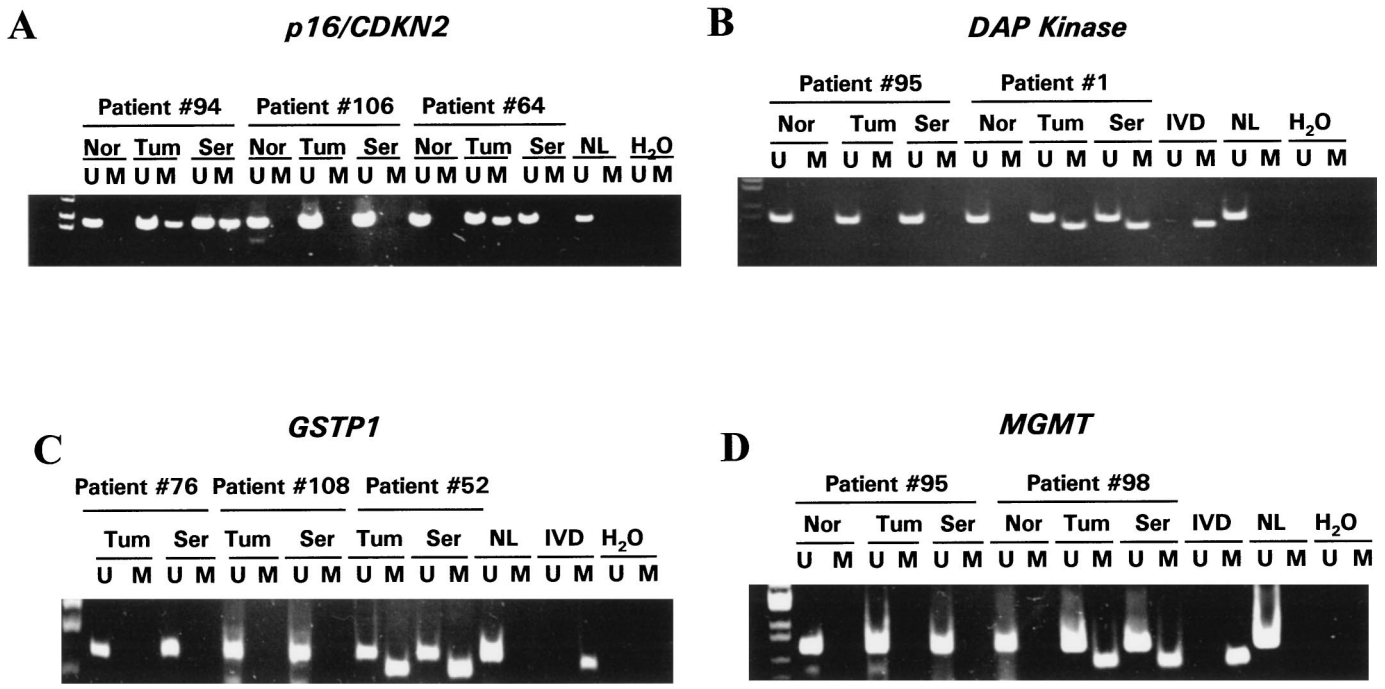

Fig. 1. MSP of tumor suppressor genes in normal lung, primary lung carcinoma, and serum DNA from NSCLC patients. PBR322/Msp digest are shown at left as molecular weight markers. The presence of a visible PCR product in *Lanes U* indicates the presence of unmethylated genes; the presence of product in *Lanes M* indicates the presence of methylated genes. In each case, normal lymphocytes (*NL*) were used as negative controls for methylation, *in vitro* methylated DNA (*IVD*) was used as a positive control for methylation, and water (H_2O) was used as a negative PCR control. *A*, MSP of *p16*. *Lanes* were assigned as follows: *Patient #94*, normal tissue, primary tumor, and serum; *Patient #106*, normal tissue, primary tumor, and serum; *Patient #64*, normal tissue, primary tumor, and serum. *B*, MSP of *DAP kinase*. *Lanes* were assigned as follows: *Patient #95*, normal tissue, primary tumor, and serum; *Patient #1*, normal tissue, primary tumor, and serum. *C*, MSP of *GSTP1*. *Lanes* were assigned as follows: *Patient #76*, primary tumor and serum; *Patient #108*, primary tumor and serum; *Patient #52*, primary tumor and serum. *D*, MSP of *MGMT*. *Lanes* were assigned as follows: *Patient #95*, normal tissue, primary tumor, and serum; *Patient #98*, normal tissue, primary tumor, and serum.

Discussion

Previous studies have identified several tumor-specific genetic alterations in the plasma and serum DNA of cancer patients. Microsatellite shifts (low-level instability) and loss of heterozygosity has been detected in plasma or serum DNA from patients with small cell lung carcinoma (11) and head and neck carcinoma (10) but not in serum of colorectal cancer patients (14). Mutant K-*ras* and *p53* DNA have also been identified in plasma or serum of patients with colorectal, pancreatic, and hematological neoplasms (12–17). Our study demonstrates, for the first time, that it is possible to detect abnormal promoter hypermethylation in serum DNA from cancer patients.

Methylation is the main epigenetic modification in humans (18), and changes in methylation patterns play an important role in tumorigenesis. In particular, hypermethylation of normally unmethylated CpG islands in many tumor suppressor genes correlates with loss of expression (18). The recent development of the MSP technique (27) has simplified the study of genes inactivated by promoter hypermethylation in human cancer and its high sensitivity, approaching 1 methylated gene copy in 1000 unmethylated copies in dilution experiments (27), support the potential power of this approach for molecular detection. Moreover, the MSP technique has demonstrated great specificity in our study, because we did not find any abnormal methylation in serum DNA if this alteration was not present in the primary tumor (Table 1). In other studies, genetic alterations were sometimes detected in the serum, whereas they were absent in the primary specimens (11, 13). Our approach with MSP avoids the use of expensive sequencing reagents and radioactivity, allowing the study of multiple markers by rapid analysis. Unlike some tumor suppressor gene mutations (*i.e.*, *p53*), MSP allows the detection of aberrant DNA methylation as a molecular detection method without prior analysis of the primary tumor. Furthermore, the analysis of abnormal promoter methylation status of several genes in a timely and economic fashion, combined with the study of previously described genetic alterations, may allow the detection of almost all patients with circulating tumor DNA.

Table 2 *Epigenetic markers for the detection of lung cancer cells in the serum*

Gene aberrantly methylated	Occurrence in tumor, no./total no. (%)	Serum positive, no./total no. (%)
p16	9/22 (41)	3/9 (33)
DAP kinase	5/22 (23)	4/5 (80)
GSTP1	2/22 (9)	1/2 (50)
MGMT	6/22 (27)	4/6 (66)

Some markers in serum DNA, such as *p16* methylation, may be helpful in the monitoring of multiple tumor types because inactivation of *p16* by methylation is a common feature in human neoplasia (18). However, depending upon the particular tumor type, other markers may also be detected in serum DNA. For example, promoter hypermethylation of *hMLH1*, present in the majority of sporadic colon and endometrial carcinomas with microsatellite instability (19, 20), and *GSTP1*, aberrantly methylated in ~90% of prostate carcinomas (18), may be ideal markers for these particular tumor types. In addition, the particular spectrum of genes with promoter hypermethylation in the serum DNA may provide us clues about the biological behavior of the primary tumor. For example, the finding of the inactivation of a putative metastasis tumor suppressor gene like *DAP kinase* may suggest the metastatic potential of a tumor (25). The detection of the methylation-mediated silencing of the *MGMT* gene (22), which provides resistance to alkylating agents, may modify the chemotherapeutic treatment.

Our results indicate that abnormal promoter hypermethylation of tumor suppressor genes is readily detectable in the serum DNA of

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cancer patients using MSP analysis. By using the right combination of target genes, MSP may detect aberrant methylated DNA across a broad spectrum of neoplasms. This approach allows sensitive and accurate detection of circulating tumor DNA and may have multiple applications in the follow-up and management of cancer patients.

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