

Aberrant Methylation of the *Adenomatous Polyposis Coli* (APC) Gene Promoter 1A in Breast and Lung Carcinomas¹

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ABSTRACT

The *adenomatous polyposis coli* (APC) gene is a tumor suppressor gene associated with both familial and sporadic cancer. Despite high rates of allelic loss in lung and breast cancers, point mutations of the APC gene are infrequent in these cancer types. Aberrant methylation of the APC promoter 1A occurs in some colorectal and gastric malignancies, and we investigated whether the same mechanism occurs in lung and breast cancers. The methylation status of the APC gene promoter 1A was analyzed in 77 breast, 50 small cell (SCLC), and 106 non-small cell (NSCLC) lung cancer tumors and cell lines and in 68 nonmalignant tissues by methylation-specific PCR. Expression of the APC promoter 1A transcript was examined in a subset of cell lines by reverse transcription-PCR, and loss of heterozygosity at the gene locus was analyzed by the use of 12 microsatellite and polymorphic markers. Statistical tests were two-sided. Promoter 1A was methylated in 34 of 77 breast cancer tumors and cell lines (44%), in 56 of 106 NSCLC tumors and cell lines (53%), in 13 of 50 SCLC cell lines (26%), and in 3 of 68

nonmalignant samples (4%). Most cell lines tested contained the unmethylated or methylated form exclusively. In 27 cell lines tested, there was complete concordance between promoter methylation and silencing of its transcript. Demethylation with 5-aza-2'-deoxycytidine treatment restored transcript 1A expression in all eight methylated cell lines tested. Loss of heterozygosity at the APC locus was observed in 85% of SCLCs, 83% of NSCLCs, and 63% of breast cancer cell lines. The frequency of methylation in breast cancers increased with tumor stage and size. In summary, aberrant methylation of the 1A promoter of the APC gene and loss of its specific transcript is frequently present in breast and NSCLC cancers and cell lines and, to a lesser extent, in SCLC cell lines. Our findings may be of biological and clinical importance.

INTRODUCTION

The protein product of the APC³ TSG at chromosome 5q21 is an important component of the Wnt signaling pathway (1), which binds to and inactivates β -catenin. Biallelic inactivation of the gene in familial adenomatous polyposis and most sporadic colorectal tumors promotes tumorigenesis (1). Inactivation of TSGs may occur via multiple mechanisms, including allelic loss, gene mutation, or by methylation of CpG sites in promoter regions. Germ-line or somatic mutations of APC are present in most colorectal carcinomas (2, 3), and >60% occur within the mutation cluster region, a small region of exon 15 between codons 1286 and 1513 (4). Whereas 18% of breast cancers have somatic mutations (5), mostly outside the mutation cluster region, mutations are rare or absent in other cancers, including NSCLCs (5–7). However, allelic losses at 5q21 are frequent in breast and lung carcinomas (6, 8, 9), suggesting that mechanisms other than mutation may inactivate the other allele.

Two promoters (1A and 1B) of the large APC gene initiate transcription from distinct sites (exons 1A and 1B), and multiple transcripts are generated by alternative splicing (10). Aberrant methylation of the 1A promoter occurs in some colorectal and gastrointestinal malignancies and is accompanied by loss of expression of its specific transcript (11–13). To determine whether this epigenetic phenomenon occurs in breast and lung cancers, we determined the methylation status of the 1A promoter in breast and lung tumors and cell lines and in nonmalignant tissues. We analyzed gene expression from this promoter in cell lines before and after treatment with a demethylating

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³ The abbreviations used are: APC, adenomatous polyposis coli; NSCLC, non-small cell lung cancer; SCLC, small cell lung cancer; MSP, methylation-specific PCR; RT-PCR, reverse transcription-PCR; Aza-CdR, 5-aza-2'-deoxycytidine; LOH, loss of heterozygosity; TSG, tumor suppressor gene.

agent. We associated our findings with allelic losses at chromosome 5q21.

MATERIALS AND METHODS

Clinical Samples. Surgically resected specimens from 42 primary breast tumors and 28 corresponding nonmalignant breast tissues from these patients were obtained from the Tumor and Tissue Repository at the Hamon Center (Dallas, TX). Tumor samples from 48 primary NSCLCs and 18 corresponding nonmalignant lung tissues were obtained from surgical resections performed at the M. D. Anderson Cancer Center (Houston, TX). For gene expression studies, six nonmalignant tissue samples (two breast, two peripheral lung tissues, and one sample each of bronchial and colonic mucosa) were obtained as far from the tumor tissue as possible. Epithelial cells from buccal swabs of 12 healthy nonsmoking volunteers and peripheral blood lymphocytes from 10 other healthy volunteers were also obtained. Appropriate Institutional Review Board permission was obtained from both participating centers, and written informed consent was obtained from all subjects. Tissues were stored at -80°C for up to 3 years before testing.

Cell Lines. Human tumor cell lines (35 breast lines, 53 SCLC lines, and 58 NSCLC lines) and B-lymphoblastoid lines ($n = 47$) were either established by us (14, 15) or, in a few cases, obtained from the American Type Culture Collection (Manassas, VA). Most breast and NSCLC lines were established from primary tumors, and most SCLC lines were established from metastases. Cell cultures were grown in RPMI 1640 (Life Technologies, Inc., Rockville, MD) supplemented with 5% fetal bovine serum and incubated in 5% CO_2 .

Nucleic Acid Purification. Genomic DNA was extracted from cell pellets and tissue homogenates suspended in digestion buffer containing 50 mM Tris-HCl (pH 8.0), 100 mM NaCl, 10 mM EDTA, 1% SDS, and 200 $\mu\text{g}/\text{ml}$ proteinase K (Life Technologies, Inc.). Digestion was carried out at 50°C for 1 day, and then two extractions with phenol-chloroform (1:1; Ref. 16) were performed. Total RNA was extracted from 27 cell lines and from the six nonmalignant tissues using the Trizol Kit (Life Technologies, Inc., Rockville MD; Ref. 17).

MSP. The methylation status of the *APC* gene promoters 1A and 1B was determined by MSP (18). One μg of genomic DNA was treated with 0.2 M NaOH for 10 min at 37°C . Aliquots of 10 mM hydroquinone (30 μl) and 3 M sodium bisulfite (pH 5.0; 520 μl ; Sigma Chemical Co., St. Louis, MO) were added, and the solution was incubated at 50°C for 16 h. Treated DNA was purified by the use of a Wizard DNA purification system (Promega Corporation, Madison WI). Modified DNA was stored at -70°C until used. Bisulfite treatment converts unmethylated cytosines to uracils while leaving the methylated cytosines unaffected. PCR was performed using primer sequences essentially as described (12, 13). The primers used for amplification of the methylated form of the *APC* gene promoter 1A (13) were 5'-TATTGCGGAGTGCGGGTC-3' (sense) and 5'-TCGAC-GAATCCCGACGA-3' (antisense); and the primers used for amplification of the unmethylated form of the *APC* promoter 1A were 5'-GTGTTTTATTGTGGAGTGTGGGTT-3' (sense) and 5'-CCAATCAACAACTCCCAACAA-3' (anti-

sense). Amplifications of 35 cycles were performed using HotStarTaq DNA Polymerase (Qiagen Inc., Valencia, CA) at annealing temperatures of 64°C (for the primer pair that detects the methylated sequence) and 62°C (for the primer pair that detects the unmethylated sequence). PCR products were analyzed on 2% agarose gels.

A positive control for each MSP reaction was provided by the use of normal lymphocyte DNA that had been methylated by treatment with Sss I DNA methyltransferase (New England Biolabs, Inc., Beverly, MA) before bisulfite modification (19). A water blank was used as a negative control in each set of PCR reactions. To confirm methylation status, MSP products of six cell lines were purified on agarose gels, precipitated with ethanol, and sequenced (PE Biosystems Model 377; Norwalk, CT) using big dye chemistry.

RT-PCR. *APC* transcripts from exons 1A, 1B, and 6–10 were analyzed by RT-PCR of RNA from 27 tumor cell lines (16 breast lines and 11 lung lines) and from 6 nonmalignant tissue samples (2 breast, 1 bronchial mucosa, 2 peripheral lung, and 1 colonic mucosa). One μg of total RNA treated with 1 unit of DNase I (Life Technologies, Inc.) was reverse-transcribed into DNA using SuperScript II First Strand Synthesis System (Life Technologies, Inc.) at 42°C for 52 min using oligo-dT primer according to the manufacturer's instructions. The resulting cDNA was then subjected to PCR using the conditions and primers for exons 1A, 1B, and 6–10 as described (12). To confirm the integrity of the RNA preparation, RT-PCR was performed using primers for the housekeeping gene β -actin (12).

Eight of the 27 tumor cell lines (four each of lung and breast) in which the *APC* promoter 1A had been demonstrated to be methylated were treated with the demethylating agent Aza-CdR. One million cells were seeded into T175 flasks and incubated in culture medium with and without Aza-CdR (2 $\mu\text{g}/\text{ml}$) for 7 days with culture medium changes on days 1, 3, and 5 (20). Cells were harvested at the end of day 7 for RNA extraction and RT-PCR analysis.

Analysis of LOH. Ten microsatellite markers flanking the *APC* gene on chromosome 5q21 analyzed are D5S656, D5S658, D5S489, D5S346, D5S1468, D5S404, D5S494, D5S639, D4S429, D5S471, and two restriction fragment-length polymorphic markers within exons 11 and 15 of the *APC* gene (21) were used for LOH analysis. DNA from 47 paired tumor and B-lymphoblastoid cell lines (13 SCLC, 18 NSCLC, and 16 breast) were analyzed for LOH as described previously (22). Briefly, 20 ng of genomic DNA were amplified by PCR in the presence of ^{32}P - α -labeled deoxycytidine-5'-triphosphate using primers for the microsatellite markers. The PCR products were separated by electrophoresis in 6% polyacrylamide gels containing 7 M urea and visualized by autoradiography. The polymorphic markers in exons 11 and 15 were analyzed in the same way, except that the PCR products were separated on a nondenaturing 6% polyacrylamide gel using the conditions for single-strand conformational polymorphism analysis and not by restriction analysis (23). Markers that amplified two distinguishable bands of different size but similar intensity in the lane having B-lymphoblastoid (constitutional) DNA were termed informative (*i.e.*, heterozygous). LOH (in tumor cell line DNA) was defined

Table 1 Frequency of methylation of the *APC* gene promoter 1A in breast and lung cancers, cancer cell lines, and control tissues

The differences in methylation frequencies between SCLC and NSCLC lines were significant (two-sided $P = 0.001$; Fisher's exact test) for all samples.

Samples	No. tested	No. methylated (%) ^a
Breast carcinoma		
Primary breast cancers	42	19 (45)
Breast cancer cell lines	35	15 (42)
Total breast cancer samples	77	34 (44)
SCLC		
Cell lines	50	13 (26)
NSCLC		
Primary tumors	48	22 (46)
Cell lines ^a	58	34 (59)
Total NSCLC samples	106	56 (53)
Nonmalignant tissues		
Peripheral blood lymphocytes ^b	10	0 (0)
Buccal swabs ^b	12	0 (0)
Nonmalignant breast ^c	28	3 (11)
Nonmalignant peripheral lung ^c	18	0 (0)
Total nonmalignant samples	68	3 (4)

^a The *APC* gene methylation frequency by tumor subtype was 72% (18 of 25) in adenocarcinomas, 50% (3 of 6) in squamous cell carcinomas, and 48% (13 of 27) in other subtypes (large cells, carcinoids, and mixed tumors).

^b From healthy volunteers.

^c From resections for breast and lung cancers.

as the loss of a band corresponding to one of the two alleles present in informative cases.

Statistical Analysis. Statistical differences between groups were examined by using Fisher's exact test with continuity correction. The association of methylation frequency with clinicopathological features was analyzed using the Mantel-Haenszel χ^2 test, two-sided. P s < 0.05 were considered statistically significant.

RESULTS

Methylation of the *APC* Gene Promoters 1A and 1B in Breast and Lung Cancers. We tested breast and lung cancer cell lines and tumors and adjacent tissues for *APC* promoter methylation (Table 1). The first 47 malignant breast and lung samples tested for promoter 1B methylation were negative, and additional studies were limited to promoter 1A. (13). *APC* promoter 1A was methylated in 44% of breast and 53% of NSCLC tumors and tumor cell lines analyzed and in 26% of the SCLC cell lines. Frequencies of methylation in NSCLC tumors and cell lines were statistically significantly higher than in SCLC cell lines ($P = 0.001$). The methylation frequencies of breast or NSCLC tumors and cell lines were similar. *APC* promoter 1A was not methylated in lymphocytes or epithelial cells from healthy volunteers, or in nonmalignant peripheral lung tissues. A low frequency of methylation (11%) was seen in nonmalignant breast tissues. Representative examples of the MSP products of bisulfite-treated samples using primers for specific unmethylated and methylated sequences are shown in Fig. 1A (see legend to Fig. 1 for details).

The unmethylated amplicon product of promoter 1A was always present in tumor tissues, whether or not the methylated

form was present. However, 91% of tumor cell lines had either the methylated or unmethylated *APC* promoter 1A sequences, whereas 9% had both. Tumor specimens always contain non-malignant cells, presumably the source of the unmethylated sequences in methylated tumor samples.

To confirm the findings, the laboratories of Adi F. Gazdar (UT Southwestern Medical Center, Dallas, TX) and James Herman (The Johns Hopkins Oncology Center, Baltimore, MD) exchanged 3 DNA samples from lung and breast carcinoma cell lines. The samples were analyzed in a blinded fashion in both laboratories using the same primer pairs, and similar PCR steps. The methylation-positive rates from the two laboratories were 11 of 23 (48%) and 12 of 23 (52%), with a concordant rate of 20 of 23 (87%).

Direct sequencing of methylated DNA PCR products from six cell lines (breast and NSCLC) confirmed that all cytosines not at CpG sites were converted to uracils by bisulfite treatment, whereas the cytosines at all seven CpG sites remained unchanged.

***APC* Gene Expression in Breast and Lung Cancer Cell Lines.** We studied 27 of the tumor cell lines for gene expression of specific transcripts from exons 1A and 1B as well as from exons 6–10 (which are spliced transcripts originating from exons 1A or 1B). The exon 1A transcript was not expressed in any of the 8 cell lines (4 breast, 3 NSCLC, and 1 SCLC) in which the promoter was methylated, but it was expressed in all 19 unmethylated cell lines (12 breast and 7 NSCLC). Thus, expression of the exon 1A transcript showed complete concordance with the methylation status of its promoter. Of the eight methylated cell lines, three also contained an unmethylated allele. All of these three lacked expression. Presumably, heterogeneous amounts of promoter methylation in these lines significantly reduces expression, or the unmethylated allele is silenced by another mechanism. In contrast, transcripts from exons 1B and 6–10 were expressed in all cell lines tested. Transcripts from exons 1A and 1B were present in all nonmalignant tissues examined (two breast, two peripheral lung, and one each of bronchial and colonic mucosa). Treatment with the demethylating agent Aza-CdR of all eight methylated cell lines tested restored expression of transcript 1A. Fig. 1B shows representative examples of RT-PCR analysis of *APC* gene expression using primers corresponding to exon 1A, exon 1B, and exons 6–10.

LOH Analysis of Tumor Cell Lines. Of the 143 breast and lung cancer cell lines studied for methylation status, paired B-lymphoblastoid cell lines were available from 47. These 47 lines were tested for LOH at 5q21–22 using 12 polymorphic markers (Table 2). They included 21 of the 27 lines examined for gene expression. LOH involving one or more markers was present in 10 of 15 (67%) methylated cell lines and in 27 of 32 (84%) unmethylated cell lines. The differences between methylated and unmethylated cell lines were not significant.

Clinical Associations. Frequencies of methylation in breast tumors and cell lines were similar and therefore their combined results were correlated with patient age, race, or nodal status and with tumor histology, size, or stage. The frequency of promoter 1A methylation in breast cancers was associated with increased tumor stage ($P = 0.01$) and tumor size ($P = 0.05$;

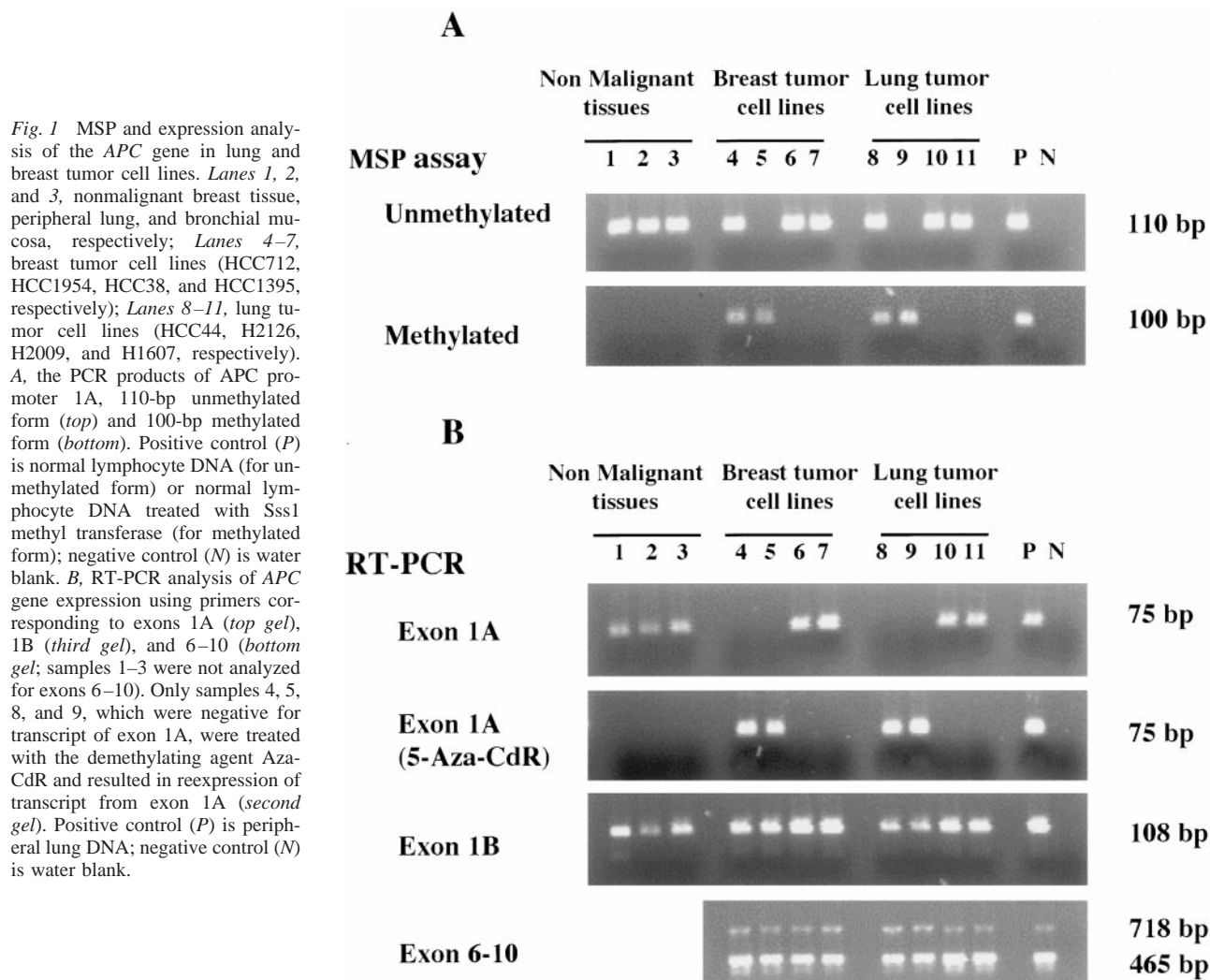


Fig. 1 MSP and expression analysis of the *APC* gene in lung and breast tumor cell lines. Lanes 1, 2, and 3, nonmalignant breast tissue, peripheral lung, and bronchial mucosa, respectively; Lanes 4–7, breast tumor cell lines (HCC712, HCC1954, HCC38, and HCC1395, respectively); Lanes 8–11, lung tumor cell lines (HCC44, H2126, H2009, and H1607, respectively). A, the PCR products of *APC* promoter 1A, 110-bp unmethylated form (*top*) and 100-bp methylated form (*bottom*). Positive control (P) is normal lymphocyte DNA (for unmethylated form) or normal lymphocyte DNA treated with *Sss1* methyl transferase (for methylated form); negative control (N) is water blank. B, RT-PCR analysis of *APC* gene expression using primers corresponding to exons 1A (*top gel*), 1B (*third gel*), and 6–10 (*bottom gel*); samples 1–3 were not analyzed for exons 6–10). Only samples 4, 5, 8, and 9, which were negative for transcript of exon 1A, were treated with the demethylating agent *Aza-CdR* and resulted in reexpression of transcript from exon 1A (*second gel*). Positive control (P) is peripheral lung DNA; negative control (N) is water blank.

Table 2 LOH at chromosome locus *5p21* in breast and lung cancer cell lines

Marker ^a	Marker type	No. with LOH / No. of informative cases (% LOH)			<i>P</i> ^b
		Breast (<i>n</i> = 14)	SCLC (<i>n</i> = 13)	NSCLC (<i>n</i> = 18)	
D5S656	Microsatellite	4/6 (67)	4/7 (57)	1/10 (10)	0.1
D5S658	Microsatellite	8/11 (73)	6/8 (75)	3/12 (25)	0.07
D5S489	Microsatellite	4/9 (44)	5/6 (83)	1/10 (10)	0.01
D5S346	Microsatellite	10/12 (83)	8/10 (80)	3/12 (25)	0.03
APC exon 11	RsaI	3/5 (60)	6/6 (100)	2/5 (40)	0.07
APC exon 15	AspHI	5/7 (71)	6/6 (100)	2/5 (40)	0.07
D5S1468	Microsatellite	5/8 (62)	3/6 (50)	2/6 (33)	1.0
D5S404	Microsatellite	7/10 (70)	7/10 (70)	4/12 (33)	0.19
D5S494	Microsatellite	7/9 (78)	7/12 (80)	2/12 (17)	0.09
D3S639	Microsatellite	7/10 (70)	6/8 (75)	2/3 (67)	1.0
D5S429	Microsatellite	8/15 (53)	7/7 (100)	2/12 (17)	0.01
D5S471	Microsatellite	6/8 (75)	7/9 (78)	5/12 (42)	0.18
Any marker		11/16 (69)	11/13 (85)	15/18 (83)	

^a Although the precise order of the markers is controversial, the markers are arranged, as best we could determine, in order from centromeric (D5S656) to telomeric (D5S471).

^b Comparison of LOH frequencies between SCLC and NSCLC lines was performed using Fisher's exact test, two-sided. Statistically significant values are shown in boldface.

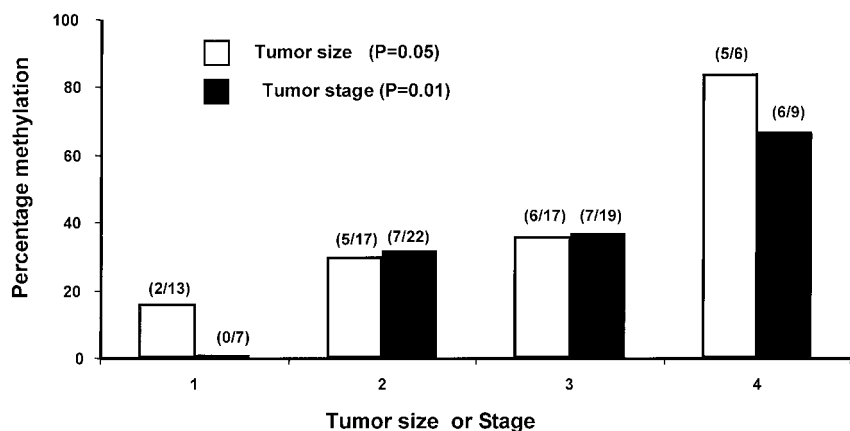


Fig. 2 Relationship between APC promoter 1A methylation of breast tumors and cell lines with breast tumor size and stage. Tumor size and stage categories were assigned using the criteria of the American Joint Committee on Cancer (43). There is a statistically significant trend of increasing methylation with tumor size ($P = 0.05$) and with tumor stage ($P = 0.01$).

Fig. 2). There was no association with the other patient and tumor characteristics in both breast and lung tumors.

DISCUSSION

The *APC* gene has been convincingly linked to the development of colorectal cancer. Whereas mutations within the mutation cluster region of the *APC* gene are rare in lung and breast cancers (7), functional somatic mutations outside the cluster region have been described in 18% of breast cancers (5). High rates of allelic loss at chromosome locus *5q21* and other findings suggest that *APC* inactivation may also play a role in lung and breast cancer pathogenesis. Moreover, female mice carrying a germ-line *APC* mutation develop both intestinal and mammary tumors (24). Decreased expression of *APC* and up-regulation or cellular relocation of β -catenin have been described in human lung and breast cancers and cell lines and in their animal models (25–29). *APC* and β -catenin mutations appear during the multistage development of carcinogen-induced rat lung tumors (30). Occasional somatic mutations of the *PP2A* gene (31), whose protein product interacts with β -catenin (32), are additional evidence of disruption of the Wnt signaling pathway in lung cancers.

Aberrant methylation of CpG-rich sites in gene promoter regions is recognized as an alternate mechanism to gene mutations for the transcriptional silencing of many TSGs (33). We and others have demonstrated that the promoter regions of several genes are aberrantly methylated in lung and breast cancers (20, 34–39). In this report we demonstrate that promoter 1A of *APC* was hypermethylated in 44% of breast cancer tumors and cell lines, in 53% of NSCLC tumors and cell lines, and in 26% of SCLC cell lines. One of us (J. H.) has previously reported a low frequency of *APC* promoter methylation in NSCLC and breast cancers (13). For this reason, the Gazdar and Herman laboratories exchanged samples of breast and lung cancer cell line DNAs and analyzed them in a blinded manner. Both laboratories obtained similar frequencies of methylation and the concordance between their results was 87%. The reasons for the previous low positive frequency from the Herman laboratory are not known, but may reflect the smaller number of samples tested. In colorectal carcinomas, the *APC* gene promoter 1A is

methylated in about 18% of sporadic tumors but not in adjacent nonmalignant mucosa (11, 13). In gastric cancer, selective methylation of promoter 1A and silencing of its transcript (but not of promoter 1B) is frequently present both in cancerous tissue and in adjacent nonmalignant mucosa (12). Our findings indicate that only occasional methylation was present in nonmalignant samples adjacent to tumor, and it was absent in other normal tissues from healthy subjects.

Our results indicate that most cell lines tested contained either the unmethylated or the methylated form of promoter 1A, and only occasional cell lines contained both forms. By contrast, the unmethylated form was always present in tumors, presumably reflecting the presence of nonmalignant cells. Tumor cell lines (which represent pure populations of malignant cells) having both methylated and unmethylated forms lacked expression. Whether this reflects heterogeneity of methylation or whether the unmethylated allele is silenced by another mechanism is not known. There was complete concordance between promoter 1A methylation and the loss of its transcript. Demethylation Aza-CdR treatment restored transcript 1A, and there was expression in all eight-methylated cell lines. LOH at the *APC* locus (*5q21–22*) was observed in a high proportion of SCLC, NSCLC, and breast cancer cell lines. A high frequency of LOH at *5q21–22* was present in most of the methylated (10 of 15; 67%) cell lines, suggesting a mechanism for biallelic inactivation. However a high frequency of LOH was also found in unmethylated cell lines. Thus, although *APC* remains a likely target, the allelic loss in this region could be targeting other genes. The frequency of methylation in breast cancers increased with tumor stage and size, suggesting that methylation may be associated with poor prognosis. Our results and those of others (40) indicate that the *APC* gene product and the 1A transcript are expressed in normal bronchial and breast epithelial cells.

In lung and breast cancers, as with colorectal (13) and gastric cancers (12), methylation and the lack of expression of *APC* are limited to the 1A promoter and its transcript. The consequences of *APC* gene promoter 1A methylation and the loss of expression of its specific transcript are not entirely clear. Breast and lung tumors frequently have weaker *APC* gene immunostaining than their adjacent nonmalignant epithelial

cells.⁴ Loss of expression was observed in tumors irrespective of APC promoter 1A methylation. Similar results have been described for BRCA1 promoter methylation in the majority of sporadic breast and ovarian tumors that had unmethylated, non-mutant *BRCA1* genes (41), suggesting that additional mechanisms may be responsible for reduced expression of these genes in the various tumors studied. Methylation of a single promoter has been described for other genes having multiple promoters, including *RARβ* (20) and *RASSF1* (35, 42). In these genes, as with *APC*, methylation and loss of transcript expression are highly selective and always involve only a single specific promoter—the other promoter is never methylated. Esteller *et al.* (13) correlated the methylation status of the 1A promoter with mutations of the *APC* gene in 66 colorectal cancers. Aberrant methylation was present in 5 of 19 (26%) cases with wild-type *APC*, but only in 3 of 47 (6%) tumors with a mutant gene ($P = 0.04$). Thus methylation was biased toward tumors with a genetically intact gene. We noted a significant trend between tumor size or stage and methylation frequency in breast cancers. Both tumor size and stage are negative prognostic factors for breast cancer, suggesting that aberrant methylation of the *APC* promoter 1A is associated with breast cancer progression.

In summary, aberrant methylation of the 1A promoter of the *APC* gene and loss of its specific transcript is frequent in breast and NSCLC cancers and cell lines and, to a lesser extent, in SCLC cell lines. Strong circumstantial evidence indicates that these findings may be of biological and clinical importance.

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⁴ Unpublished data.

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