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Aberrant Promoter Methylation of the Transcription Factor Genes PAX5 α and β in Human Cancers[1]

William A. Palmisano, Kevin P. Crume, Marcie J. Grimes, Sally A. Winters, Minoru Toyota, Manel Esteller, Nancy Joste, Stephen B. Baylin, and Steven A. Belinsky[2]

Lovelace Respiratory Research Institute, Lung Cancer Program, Albuquerque, New Mexico 87108 [W. A. P., K. P. C., M. J. G., S. A. W., S. A. B.]; First Department of Internal Medicine, Sapporo Medical University, Sapporo 060-8543, Japan [M. T.]; Cancer Epigenetics Laboratory, Molecular Pathology Program, Spanish National Cancer Center, Majadahonda 28220, Spain [M. E.]; Department of Pathology, University of New Mexico, Albuquerque, New Mexico 87131 [N. J.]; and Johns Hopkins University Medical Institutions, The Johns Hopkins Comprehensive Cancer Center, Baltimore, Maryland 21231 [S. B. B.]

ABSTRACT

Aberrant methylation of 5′CpG islands is a key epigenetic event in many human cancers. A PCR-based technique of methylated CpG island amplification followed by representational difference analysis was used to identify genes methylated in cancer. Two of the CpG islands identified mapped to the 5′ untranslated region of the *PAX5* α and β genes. These genes, located on chromosome 9p13, are transcribed from two distinct promoters and form two alternative first exons that are subsequently spliced to the common exons 2–10. The resulting splice variants encode two distinct transcription factors important in cell differentiation and embryonic development. Examination of the methylation status of each gene using methylation-specific PCR revealed that both genes are methylated in ~65% of breast and lung tumors. Bisulfite sequencing revealed dense methylation patterns within each 5′CpG island, strongly correlating with transcriptional silencing. Expression in cell lines with dense methylation of either the PAX5 α or β promoter region was restored after treatment with the demethylating agent 5-Aza-2′-deoxycytidine. The *PAX5* β gene encodes for the transcription factor B cell-specific activating protein that, in turn, directly regulates *CD19*, a gene shown to negatively control cell growth. A strong association was observed between PAX5 β methylation and loss of expression of the *CD19* gene demonstrating that inactivation of the *PAX5* β gene likely contributes to neoplastic development by inhibiting growth regulation through effects on *CD19* gene expression. Recent studies have demonstrated the importance of *PAX5* gene alterations in human cancer. Our results are the first to identify aberrant promoter methylation as a common mechanism for dysregulation of these genes in solid tumors.

INTRODUCTION

Breast cancer is the second leading cause of cancer-related death in the United States, and the number of cases is increasing each year. Mortality from this disease could be reduced greatly through an improved understanding of the molecular alterations that contribute to disease initiation and progression. A common mechanism in many sporadic cancers, aberrant promoter hypermethylation, is an epigenetic event involving the methylation of normally unmethylated cytosines within the promoter region of genes (1, 2). This change in methylation pattern leads to transcriptional silencing and serves as an alternative to coding region mutation (1, 2). In breast cancer, aberrant methylation inactivates numerous genes functioning in key cellular pathways (3–7). It is likely that additional novel genes inactivated by methylation will also prove to be pivotal in cancer development.

Several cancer genome-wide screening approaches have been developed to identify genes inactivated by promoter hypermethylation. These approaches include methylation-sensitive arbitrarily primed PCR (8, 9), restriction landmark genomic scanning (10), CpG microarrays (11, 12), methyl-CpG binding domain chromatography (13), and MCA[3] coupled with RDA (14). The MCA/RDA approach has identified several methylated genes involved in colorectal (15, 16) and pancreatic cancers (17). MCA/RDA is a PCR/subtraction hybridization-based assay that allows for the rapid amplification and selection of densely methylated CpG-rich regions ranging in size from 200 bp to 2 kb. The purpose of the current study was to use the MCA/RDA technique to identify novel genes inactivated by promoter hypermethylation in breast cancer and to determine the commonality for gene inactivation in lung cancer, another solid tumor.

Using amplicons from the breast cancer cell line MCF7 as the tester, and amplicons from normal breast tissue as the driver, one region identified was the 5′ untranslated region and exon 1 of the transcription factor PAX5. The *PAX5* gene plays an important role in cell differentiation and embryonic development, and is located on chromosome 9p13 (18). This locus is associated frequently with chromosomal translocations and contains two distinct promoters resulting in two alternative 5′ exons (α and β) that are spliced to common coding sequences of exons 2–10 (19). The current studies demonstrate that the *PAX5* α and β genes are both frequent targets for aberrant methylation in tumor cell lines, as well as primary tumors from breast and lung.

MATERIALS AND METHODS

Tissue Samples and Cell Lines. Lung squamous cell carcinomas and adjacent tissue were obtained from patients previously enrolled in a Lung Cancer Surveillance Study conducted through St. Mary's Hospital (Grand Junction, CO). Lung adenocarcinomas were acquired from the Johns Hopkins Lung Spore Repository (Baltimore, MD). Breast tumors and adjacent tissue were collected from women enrolled in a New Mexico Women's Health Study being conducted within the Epidemiology and Cancer Control Program at the University of New Mexico. Nonmalignant bronchial epithelial cells and blood lymphocytes were obtained from veterans who use the Multispecialty Chest Clinic at the New Mexico Veterans Health Care System for their primary care. Bronchial epithelial cells were collected through diagnostic bronchoscopy and expanded in short-term tissue culture (20). All of the subjects gave informed consent according to institutional guidelines. Tumor-derived cell lines were obtained from the American Type Culture Collection and cultured according to their conditions.

MCA/RDA. MCA/RDA was performed as described by Toyota *et al.* (14). Briefly, 5 μg of DNA from the cell line MCF7 was used as the tester, and a mixture of DNA from normal breast tissue of five women (1 μg each) was used as the driver. MCA amplicons were produced using adaptors, and two rounds of competitive hybridizations were performed. The resulting RDA products were digested with the restriction endonuclease *Xma*I (New England Biolabs) and subsequently cloned into pBluescript KS+.

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[2] To whom requests for reprints should be addressed, at Lovelace Respiratory Research Institute, 2425 Ridgecrest Drive SE, Albuquerque, NM 87108-5127. Phone: (505) 348-9465; Fax: (505) 348-4990; E-mail: sbelinsk@LRRI.org.

[3] The abbreviations used are: MCA, methylated CpG island amplification; RDA, representational difference analysis; DAC, 5-aza-2′-deoxycytidine; RT-PCR, reverse transcription-PCR; MSP, methylation-specific PCR; BSAP, B-cell-specific activating protein; COBRA, combined bisulfite restriction analysis.

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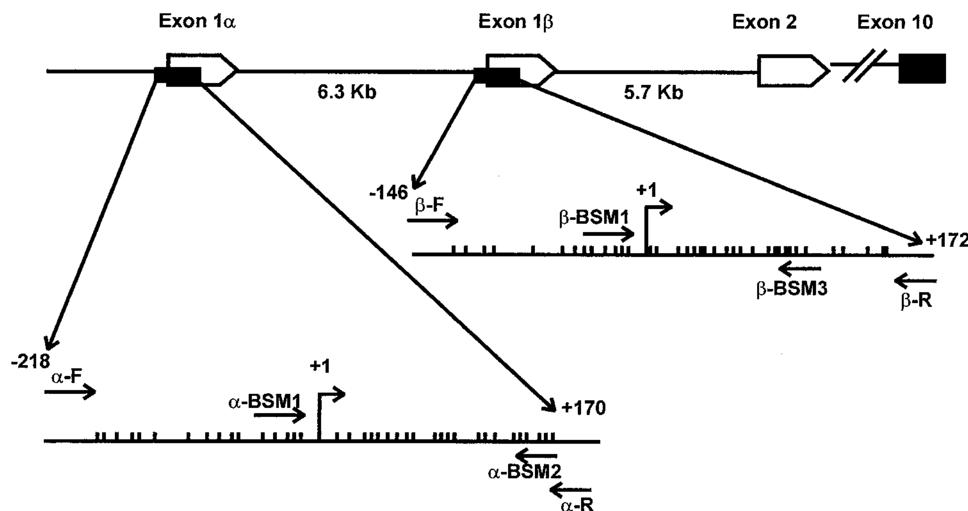

Fig. 1. Genomic structure and localization of the PAX5 CpG islands evaluated for methylation. The location of PAX5 α and β exon 1 sequences with respect to common exons 2–10 is depicted in this line drawing. In addition, an expansion of the CpG island assayed for methylation is shown. The transcriptional start site is indicated for both genes as +1. The forward (*F*) and reverse (*R*) primers for stage 1 amplification for the PAX5 α and β genes begin at −218; +170 and −146; +172, respectively. The location of the methylation-specific stage 2 primers (*BSM*) is also indicated, and the *vertical ticks* represent CpG sites within the region.

DNA Sequencing and Analysis. Plasmid DNA containing the RDA products was prepared using the QIAprep Spin Miniprep according to the manufacturer's instructions (Qiagen). Virco (Cambridge, United Kingdom) analyzed the sequences using an automated DNA sequencer (Applied Biosystems). Sequence homologies were determined using the Blast program of the National Center for Biotechnology Information.[4]

MSP and Bisulfite Sequencing. DNA was isolated by standard phenol-chloroform extraction and ethanol precipitation. Genomic DNA was modified by treatment with sodium bisulfite that converts only unmethylated cytosines to uracil. The methylation status of the *PAX5* α and β genes was determined using a nested, two-stage method described previously (21). Primer sequences used in the stage-1 amplification of each gene are as follows: α-Forward, (5′-GGGTTTGTATATGGAGATGTTATAGG-3′); α-Reverse, (5′-CAACATCACAAAATATCCCCAAACAC3′); β-Forward, (5′-AGTTTGTGGGTTGTTTAGTTAATGG-3′); and β-Reverse, (5′-CAAAAAATCCCAACCACCAAAACC-3′). All of the PCR amplifications were performed using a Biometra T3 thermocycler and Taq Gold polymerase (Perkin-Elmer). The cycling parameters for stage-1 α were as follows: 94°C for 10 min; then 40 cycles of 94°C for 1 min, 60°C for 1 min, and 72°C for 1 min; and a 5-min final extension at 72°C. Stage-1 β conditions were identical except the annealing temperature was reduced to 54°C, and all of the cycling times were performed for 30 s. The size of the stage-1 α and β PCR products was 389 and 328 bp, respectively.

Primer sequences used in the stage-2 amplification of each gene are as follows: α-BSM1, (5′-ATAAAAGTTTGGGGCGGCGC-3′); α-BSM2, (5′-GCGCCCCCAACGCGCCG-3′); β-BSM1, (5′-GAGTTGAGTTTCGGGCGGC-3′); and β-BSM3, (5′-GCCGCCGCCGCCGTCG-3′). The cycling parameters for stage-2 α were as follows: 94°C for 10 min; then 40 cycles of 94°C for 15 s, 66°C for 15 s, and 72°C for 15 s; and a 5-min final extension at 72°C. Stage-2 β conditions were identical except the annealing temperature was decreased to 64°C. The size of the stage 2-α and β PCR products was 166 and 124 bp, respectively. DNA isolated from cell lines MDA-MB-231 and NCI-H2009 served as positive and negative controls, respectively, for both genes. All of the assays were conducted in at least duplicate, and positive methylation was confirmed by restriction digestion with *Bst*UI or by DNA sequencing.

Bisulfite-modified DNA from the T47D and MCF-7 cell lines was amplified using the stage-1 primers for the *PAX5* α and β genes. Stage-1 and methylation-specific stage-2 primers were used to amplify modified DNA from three breast tumors shown to be methylated for the *PAX5* α or β gene. The PCR products were ligated into the PCR II vector using the TA cloning kit (Invitrogen, San Diego, CA). Three to five clones from each sample were sequenced.

DAC Treatment and RT-PCR. Re-expression studies for PAX5 α and β were performed using three breast cancer cell lines (MCF7, MDA-MB-231, and T47D) and one lung cancer cell line (Calu6). Cell lines were treated for 3 days in culture medium with 1 μM DAC (Sigma Chemical Co.). Total RNA was prepared using TRIzol (Life Technologies, Inc.), and 3 μg aliquots were reverse-transcribed using the Superscript kit (Life Technologies, Inc.). The expression of the PAX5 α and β transcripts was determined by RT-PCR using the exon 1α forward primer (5′-CCTGTCCATTCCATCAAGTCCTG-3′) and the exon 1β forward primer (5′-CCCGATG GAAATACACTGTAAGCAC-3′) with an exon 2 reverse primer (5′-TTTTGCTGACA CAACCATGGCTGAC-3′). PCR amplification was performed at 94°C for 10 min; then 40 cycles of 94°C for 30 s, 64°C for 30 s, and 72°C for 30 s; and a 5-min final extension at 72°C.

The expression of the *CD19* gene was examined in 6 bronchial epithelial cell lines, in 2 breast cancer cell lines (SKBR3 and MDA-231), and in 3 lung cancer cell lines (NCI-H23, A549, and Calu6). Cell lines were treated with DAC and RNA isolated as described above. CD19 expression was determined after RT-PCR using the forward primer (5′-CTCAGCCAGGACCTCACCATG-3′) and reverse primer (5′-CATAGTACTGGCCGAGCA GTGA-3′). PCR amplification was performed at 94°C for 10 min; then 40 cycles of 94°C for 30 s, 60°C for 30 s, and 72°C for 30 s; and a 5-min final extension at 72°C. β-Actin was also amplified as a control for RNA integrity. PCR amplification was performed at 94°C for 10 min; then 30 cycles of 94°C for 30 s, 60°C for 30 s, and 72°C for 30 s; and a 5-min final extension at 72°C. PCR products for all of the genes were analyzed on a 3% agarose gel containing ethidium bromide and visualized under UV illumination.

Data Analysis. The proportion of tumors positive for methylation of PAX5 α or β was compared among cancer types with the Fisher's exact test. The association between PAX5 α and β methylation for each cancer type was also assessed by the Fisher's exact test.

RESULTS

Identification of PAX5 α and β Hypermethylation by MCA. The MCA/RDA technique developed by Toyota *et al.* (14–16) was used to identify genes methylated in breast cancer. A subtractive library was constructed, and 100 clones were analyzed by DNA sequencing. Comparison of these clones revealed 50 unique sequences, and a Blast search of each clone confirmed that 48 of 50 clones were homologous to GenBank sequences located in the high-throughput genomic sequence database. Seven of these clones corresponded to known genes. One of these clones was identical to the 5′ flanking region and exon 1 β of the transcription factor PAX5 (GenBank accession no. AF074913). We decided to focus on characterization of the *PAX5 β* gene for several reasons. Analysis of the genomic structure of this gene revealed the presence of two distinct promoters resulting in two alternative 5′ exons (1 α and 1 β) that are spliced to common exons 2–10 resulting in the translation of two unique proteins because of a frameshift (Fig. 1; Ref. 19). This allowed

[4] Internet address: http://www.ncbi.nlm.nih.gov/BLAST/.

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us to address coordinate aberrant regulation of two genes that share common exons, a scenario identical to the $p16^{INK4a}$ and $p14^{ARF}$ genes. The region identified by the MCA/RDA approach was localized in the 5′ flanking region and exon 1, the site for most CpG islands. Inspection of the PAX5 α and β promoters revealed that each region is representative of a CpG island (22, 23). The GC content was 0.68 and 0.6 with a CpG:GpC ratio of 0.70 and 0.73; 53 and 162 CpG sites were found in a 520-bp and 1780-kb region, respectively.

MSP Analysis of the *PAX5* α and β Genes. COBRA (8) was initially conducted on DNA from 10 breast cell lines, 5 lung cancer cell lines, and 5 normal lymphocytes to screen for aberrant methylation of the *PAX5* α and β genes. After bisulfite modification, PCR products were produced using stage-1 primers that do not discriminate between methylated and unmethylated alleles. Methylated alleles were detected in the PAX5 α (389 bp) and β (328 bp) products after digestion with the restriction enzyme *BSTU1*, which specifically cleaves at CGCG sites that are retained after bisulfite modification because of the presence of methylated CpGs. Complete digestion of the PCR products indicative for methylation of the *PAX5* α gene was observed in 7 breast and 5 lung cancer cell lines. The *PAX5* α PCR product was partially digested (10–80%) for 2 breast and lung cancer cell lines. Similarly, the *PAX5* β gene was completely digested in 5 breast and 3 lung cancer cell lines. The PAX5 β PCR product was also partially digested (10–80%) for 1 lung and 2 breast cancer cell lines. PCR products from normal lymphocytes remained undigested, indicating a lack of methylation (data not shown).

The frequency of PAX5 α and β methylation was then characterized in a panel of primary tumors (breast and lung) using our two-stage MSP approach (21). The results are summarized in Table 1, and a representative MSP analysis is shown in Fig. 2. In both breast and lung tumors, the frequency of PAX5 α methylation approximated 70% and tended to be greater than that observed for the *PAX5* β gene (54%). Methylation of the β gene was strongly associated ($P = 0.05$) with methylation of the α gene in lung tumors and cell lines suggesting that some selective advantage to target both genes for inactivation in this cancer type may exist (Table 1). This association was not observed in breast tumors.

MSP analysis for methylation in cell lines corroborated that seen by the COBRA assay. Overall, 90 and 60% of breast cancer-derived cell lines were methylated for the *PAX5* α and β genes, respectively. Eleven lung tumor-derived cell lines were analyzed by MSP, and 82 and 55% were methylated for the *PAX5* α and β genes, respectively.

Methylation of the *PAX5* genes was also examined in tissue adjacent to the tumors using our sensitive two-stage MSP approach to determine the extent of the tumor spread within the resected tissue. PAX5 α methylation was seen in 4 of 8 and 6 of 10 adjacent tissues from breast and squamous cell tumor cases, respectively (Table 1). PAX5 β methylation was not seen in any adjacent tissue from breast cancers, but was seen in 5 of 10 adjacent tissue from squamous cell tumor cases. Methylation was only detected in adjacent tissue from breast tumors that were also positive for methylation. However, one squamous cell tumor negative for PAX5 α and another tumor negative for β had faint methylation signals in their respective adjacent lung tissue. The detection of PAX5 methylation in tissue adjacent to breast and squamous cell carcinomas probably reflects tumor spread, because 80% of the cases evaluated had lymph nodes positive for malignancy or field cancerization commonly seen in bronchial epithelium from smokers (20). The detection of epigenetic and genetic changes in tissue adjacent to tumors is quite common and has important implication for predicting disease recurrence. For example, patients with squamous cell carcinoma of the head and neck with cytologically negative tumor margins but positive for p53 mutation had a substantial risk for local recurrence (24, 25). To determine whether methylation of the *PAX5* genes was specific to cancer, we examined bronchial epithelial cells from 35 cancer-free heavy smokers and peripheral blood mononuclear cells from 20 never-smokers. Methylation of the *PAX5* α and β genes was detected in bronchial epithelial cells from only 1 of 35 smokers (Table 1). Neither *PAX* gene was methylated by MSP in any blood lymphocytes from never-smokers.

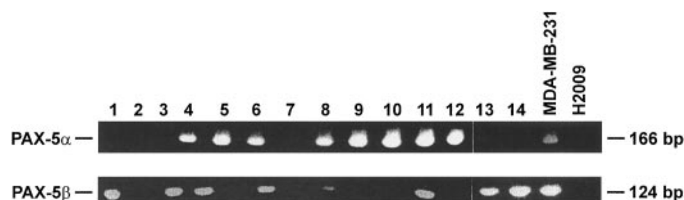

Fig. 2. MSP analysis of the *PAX5* α and β genes in a panel of primary breast tumors. Bisulfite-modified DNA was amplified with stage-2 primers specific for methylated alleles as described in "Materials and Methods." The MDA-MB-231 and NCI-H2009 represent positive and negative cell lines for methylation of both the *PAX5* α and β genes, respectively.

Table 1 *Frequency of PAX5 α and β methylation in tumor cell line primary tumors and nonmalignant specimens*

Sample type	Samples assayed	Number methylated (%)		
		α	β	$\alpha + \beta$
Breast				
Tumor cell lines	10	9 (90)	6 (60)	6 (60)
Tumors	30	21 (70)	15 (50)	11 (37)
Adjacent tissue	8	4 (50)	0 (0)	0 (0)
Lung				
Tumor cell lines	11	9 (82)	6 (55)	6 (55)
Tumors				
ADC[a]	25	16 (64)	13 (52)	10 (40)[b]
SCC	23	17 (74)	14 (61)	12 (52)[c]
Adjacent tissue	10	6 (60)	5 (50)	4 (10)
NHBE[d]	35	1 (2.9)	1 (2.9)	0 (0)
Blood lymphocytes[e]	20	0 (0)	0 (0)	0 (0)

[a] ADC, adenocarcinoma; SCC, squamous cell carcinoma; NHBE, normal human bronchial epithelial cell.
[b] $P = 0.05$ for association of PAX5 α methylation to β methylation in lung tumors.
[c] $P < 0.0001$ as compared with methylation in lung tumors.
[d] Cancer-free heavy smokers.
[e] Cancer-free nonsmokers.

Re-Expression of the *PAX5* α and β Genes after DAC Treatment. The expression of the PAX5 α and β transcripts was determined by RT-PCR on cDNA from three normal lung tissues adjacent to squamous cell tumors that were negative for PAX5 methylation. Expression of both transcripts was readily detected in all three of the adjacent lung tissues (Fig. 3*A*). RT-PCR was also conducted on cDNA from three breast cancer cell lines (MCF7, MDA-MB 231, and T47D) and one lung cancer cell line (Calu6) to determine the relationship between methylation of the *PAX5* α and β genes, and transcription. COBRA analysis demonstrated complete digestion for the *PAX5* α gene in the MDA-MB-231 and T47D cell lines and partial digestion for MCF7 (20%) and Calu6 (80%). For the *PAX5* β gene, complete digestion was seen in the MCF7, MDA-MB 231, and Calu6 cell lines, but no digestion in T47D. These methylation changes were corroborated by MSP (Fig. 3*B*). PAX5 α transcript was abundant in MCF7 and to a lesser extent present in Calu6, whereas no transcript was present in the MDA-MB 231 and T47D cell lines (Fig. 3*C*). Treatment of these cell lines with 1 μM DAC, an inhibitor of DNA methyltransferase, increased expression in Calu6 and restored expression in MDA-MB231 and T47D. PAX5 β transcript was only detected in T47D; however, treatment with DAC restored expression in the other three cell lines. Treatment with DAC did not affect the expression of the housekeeping gene β-actin.

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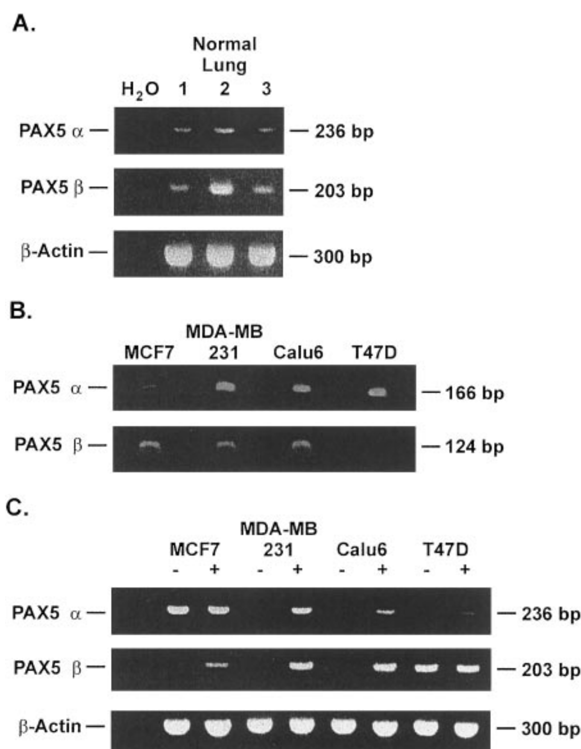

Fig. 3. *A,* RT-PCR analysis for expression of the *PAX5 α* and β genes in adjacent normal lung tissue. Expression of both transcripts was detected in all three adjacent normal lung tissues. Expression of the *β-actin* gene was determined as a control for RNA integrity. *B,* MSP analysis of breast and lung cancer cell lines. Bisulfite-modified DNA was amplified with stage-2 primers specific for methylated alleles as described in "Materials and Methods." *C,* RT-PCR analysis for expression of the *PAX5 α* and β genes in breast and lung cancer cell lines, which were grown in the presence (+) and absence (−) of 1 μM DAC for 72 h. Expression of the *PAX5 α* and β genes was restored in nonexpressing cell lines after DAC treatment. Expression of the *β-actin* gene was determined as a control for RNA integrity.

Methylation Density of PAX5 α and β Promoters. Methylation density within the PAX5 α and β promoter regions amplified by the MSP primers was determined for the MCF-7 and T47D cell lines, and three primary breast tumors. The PCR primers used for the COBRA analysis were also used to amplify the modified DNA from the cell lines, whereas methylation-specific primers were used to detect methylated sequences in the primary tumors where contaminating stromal and inflammatory cells are present. Sixteen and 14 CpG sites spanning the 116- and 86-bp regions between the MSP primers for the PAX5 α and β sequence, respectively, were evaluated to compare methylation density among cell lines and tumors. The sequencing results corroborated the expression studies with 78% of sites methylated within the PAX5 α promoter in the T47D cell line, and 100% of sites methylated within the PAX5 β promoter in the MCF-7 cell line (Figs. 3 and 4). The CpG methylation density in all three of the primary tumors was very similar to the patterns observed in the methylated cell lines for each gene, consistent with these regions being important sites for methylation and silencing of the *PAX5 α* and β genes (Fig. 4).

Effect of PAX5 β Methylation on Expression of the *CD19* Gene. The *PAX5 β* gene encodes for the transcription factor BSAP (26). Previous studies have demonstrated that the promoter of the *CD19* gene, a negative regulator of cell proliferation, contains a high-affinity BSAP-binding site instead of a TATA sequence to directly regulate its transcription (26). Studies in PAX5-proficient and -deficient (knockout) cells corroborated this regulation with loss of CD19 expression correlating directly with gene status (27). Thus, we evaluated expression of CD19 in replicating nonmalignant bronchial epithelial cells and also determined whether there was a relationship between the PAX5 β methylation state and CD19 expression in breast and lung cancer-derived cell lines. Abundant expression of the *CD19* gene was observed in all of the bronchial epithelial cultures (Fig. 5*A*). In addition, CD19 expression was observed in cell lines SKBR3 and NCI-H23 that lack methylation of the *PAX5 β* gene (Fig. 5*B*). In contrast, no CD19 expression was detected in the Calu6, MDA-MB-231, and A549 cell lines, all of which are methylated for PAX 5 β. Treatment of these cell lines with DAC restored expression of the *CD19* gene (Fig. 5*B*).

DISCUSSION

The *PAX* gene family consists of nine members, each of which shares a common motif called the paired box that displays DNA-binding properties (28). PAX proteins function as nuclear transcription factors important for cellular differentiation, migration, and proliferation (29). The fact that these genes are strong transcriptional

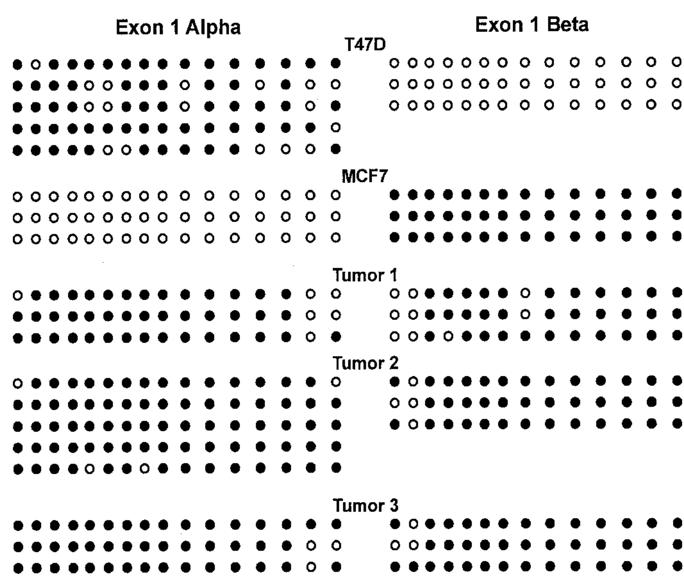

Fig. 4. Methylation density of the *PAX5 α* and β genes. Genomic DNA from breast cancer cell lines T47D and MCF-7, and three primary breast tumors were treated with sodium bisulfite. PAX5 α and β PCR products containing 16 and 14 CpG sites, respectively, were generated. The PCR products were cloned, and 3–5 individual clones/sample were sequenced. ○ and ● indicate that the CpG site is unmethylated or methylated, respectively. Each *row* represents one clone.

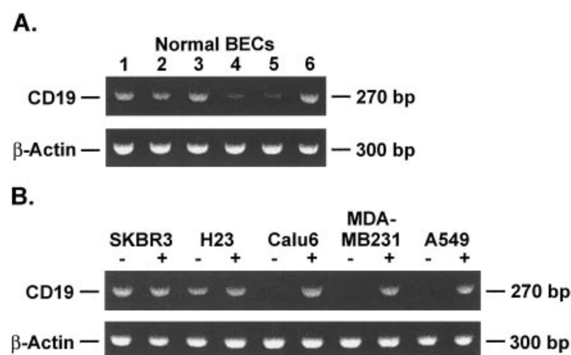

Fig. 5. *A,* RT-PCR analysis for expression of the *CD19* gene in normal bronchial epithelial cells (*BECs*). Expression of the CD19 transcripts was detected in all six BECs. Expression of the *β-actin* gene was determined as a control for RNA integrity. *B,* RT-PCR analysis for expression of the *CD19* gene in breast and lung cancer cell lines, which were grown in the presence (+) and absence (−) of 1 μM DAC for 72 h. Expression of the *CD19* gene was restored in nonexpressing cell lines after DAC treatment. Expression of the *β-actin* gene was determined as a control for RNA integrity.

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regulators makes them likely targets for disruption in oncogenesis. Consistent with this premise, inappropriate expression of the *PAX5* gene has been implicated in the pathogenesis of small lymphocytic lymphoma cancer and advanced-stage glioblastoma (29). DNA methylation was linked recently to the transcriptional silencing of both *PAX5* genes in murine myeloma cells (30). Our results implicate the *PAX5* α and β genes as common targets for inactivation by aberrant promoter hypermethylation in lung and breast tumors. Both genes exhibited homogeneous and dense methylation patterns that correlated with transcription loss. Silencing of the *PAX5* β gene was associated directly with loss of *CD19* gene expression in lung and breast cancer cells. A similar finding was observed in murine myeloma cells (30). Thus, the tight regulation of *CD19* gene transcription by the PAX5 β-encoded BSAP protein likely contributes to the selective growth advantage displayed by both solid and liquid tumors. CD19 antibodies provided a positive proliferation signal for early precursor B cells indicating that CD19 is involved in the negative control of proliferation (31).

Previous studies using MCA and RDA identified the *PAX6* gene that is involved in eye, nose, pancreas, and brain development as a common target for aberrant methylation in colonic mucosa during aging (14, 15). The CpG island identified was localized within an enhancer present in the 5′ region of the *PAX6* gene. A CpG island within the exon 5 coding region of the *PAX6* gene was also hypermethylated in bladder and colon tumors (32). Interestingly, whereas methylation of the promoter region and exon 5 was common in cell lines, methylation in primary tumors was largely confined to exon 5 and did not affect gene transcription. This was not the case for the *PAX5* α and β genes where dense methylation was seen in both promoter regions in derived cell lines, and primary lung and breast cancers that correlated directly with loss of transcription. The difference seen in primary tumors for silencing the *PAX5* and *6* genes may be linked to their functions in the respective tissues. No expression of the *PAX6* gene was seen in normal colonic mucosa, thereby negating any selective advantage for methylation of the promoter region, whereas we and others observed abundant expression of both PAX5 and CD19 transcripts in normal lung tissue and bronchial epithelial cells (33).

A critical role for the *PAX5* gene in development is evident from knockout studies in mice. Homozygous mutant mice were born alive, but their growth was retarded, and they usually died within 3 weeks. Developmental defects include alteration of the posterior midbrain morphogenesis and a complete arrest of B-cell development at early precursor stages (34). Gene targets regulated directly or indirectly by the *PAX5* β gene have been identified (27). For example, the BSAP protein can regulate *p53* and *IgHC* genes, in addition to CD19 (28, 35). The BSAP protein can bind directly to a sequence in the 5′ region of the *p53* gene to inhibit expression, and dysregulation of this process has been correlated with p53 protein levels in astrocytomas (28, 35). Whereas this paradigm appears limited to the brain, it does speak to a critical role for the BSAP transcription factor in modulating proteins involved in basal cellular activity. Our studies now indicate that the BSAP protein directly regulates CD19 expression in both lung and breast tumors. There is precedent for inactivating genes that code for transcription factor binding proteins by promoter hypermethylation. Hypermethylation in cancer (*HIC-1*) is a zinc-finger transcription factor gene that is commonly expressed in normal tissues, but inactivated by promoter hypermethylation in lung, breast, colon, and hematopoietic tumors (36, 37). Similar to the *PAX5* gene, *HIC-1* is also important in development with knockout mice dying perinatally and exhibiting gross developmental defects involving the brain, cleft palate, and limbs (38). As downstream targets are identified for the PAX5 α protein, the impact of silencing this gene on normal cellular function will be realized.

Our recent studies suggest that changes in gene-specific methylation could serve as intermediate biomarkers for cancer detection, risk assessment, and monitoring disease in sputum and blood (21, 39). Our group has focused largely on lung cancer where the aberrant methylation of the p16 and/or O^6-methylguanine-DNA methyltransferase promoters was detected in DNA from sputum of patients with squamous cell carcinoma up to 3 years before clinical diagnosis (21). The development of risk models for predicting lung cancer through marker detection in biological fluids such as sputum will necessitate the conduct of longitudinal studies in high-risk populations and the development of a panel of markers that are inactivated throughout the progressive phases of cancer development. The fact that methylation of the *PAX5* α and β genes was found only in tumors and surrounding tissue, and only rarely in normal epithelial cells, albeit from a heavy smoker, supports their evaluation as intermediate markers in our model. Other groups have also demonstrated the utility of plasma or serum for the detection of circulating aberrantly methylated DNA in people with colorectal, head and neck, lung, and breast cancers (39–41). Thus, the inclusion of the *PAX5* genes into molecular marker panels for lung and breast cancer may improve the sensitivity and specificity for developing risk models for detecting these cancers through analysis of sputum and blood in high-risk subjects.

Our studies identify a new class of genes, those that code for transcription factor binding proteins, which are methylated at high prevalence in the two most common human cancers. The silencing of the *PAX5* β gene is linked functionally to *CD19*, a gene already established as a key regulator of the cell cycle. This link implicates the targeting of another pathway that controls the cell cycle for inactivation in liquid and now solid tumors.

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