Identification of Novel Methylation Markers in Cervical Cancer Using Restriction Landmark Genomic Scanning

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Abstract

Aberrant methylation of CpG islands in gene promoters often represents an early clonal event in carcinogenesis. Accordingly, defining methylation profiles may be useful for developing marker panels for early detection or predicting the risk of cancer precursors. To identify specific genes frequently methylated in cervical cancer, we conducted methylation profiling of 20 primary human cervical cancers using *Not*I-based restriction landmark genomic scanning (RLGS). Of 2,172 RLGS fragments analyzed (average, 1,753 CpG islands per patient), 186 RLGS fragments were lost in at least one tumor and 40 were lost in three or more. Methylation was identified in 19 (95%) of 20 tumor samples compared with normal DNA. Bisulfite sequencing was conducted to confirm RLGS results. Of the confirmed markers frequently methylated, we developed Methylight assays for two corresponding genes, nucleolar protein 4 (*NOL4*), and lipoma HMGIC fusion partner–like protein 4 (*LHFPL4*), which were methylated in 85% and 55% of cancers, respectively. Using these assays, we further confirmed frequent CpG island methylation in the original cancers and in another independent series of 15 cervical cancers. We also showed methylation at a reduced frequency in a set of carefully reviewed cytology specimens demonstrating cells exfoliated from cancer precursor lesions. In summary, we identified, for the first time, *NOL4* and *LHFPL4* as novel methylation targets specific for cervical cancer. Inclusion of *NOL4* and *LHFPL4* in evaluating methylation panels for early detection, risk prediction, and etiologic research on cervical cancer is warranted. [Cancer Res 2008;68(7):2489–97]

Introduction

Infection with oncogenic human papillomaviruses (HPV) represents a necessary cause of invasive cervical cancer and its precursor. Although HPV infections are extremely common among young women, most regress spontaneously. Nonetheless, concerns about missing a clinically occult cancer precursor often prompt early treatment of mild lesions, resulting in morbidity and costs that can be avoided if the natural history of infection could be predicted (1). Therefore, the development of a clinical test to distinguish women with HPV infections who require immediate treatment from those who can be managed conservatively would be useful. Although many molecular markers associated with cervical cancer have been identified, markers that can predict the future risk of progression among women with HPV infection have not been widely implemented in clinical practice.

Methylation of CpG islands in gene promoter regions is a common, nonrandom epigenetic event that is associated with silencing of tumor suppressor genes and the development of cancer. Similar to patterns of somatic genetic alterations, hypermethylation is a clonal, somatically heritable event that has been identified in cancer precursors and seems to involve a greater number of regions as lesions progress to invasion. Specifically, hypermethylation of several candidate genes have been identified in cervical cancer and its precursors (2–12), suggesting that assays for these molecular events might be clinically useful. However, data from methylation profiling studies performed to identify novel markers are limited (13).

In this study, we performed restriction landmark genome scanning (RLGS) to identify novel methylation targets in a discovery set of 20 cervical cancers and, then, retested a subset of promising markers using Methylight assays (14) in a second set of 15 cases. RLGS permits the evaluation of hypermethylation in target DNA at >2,000 loci, mostly occurring within CpG islands of DNA promoter regions (15, 16). Previous studies have shown the utility of RLGS for identifying frequently methylated DNA targets for many tumor types (17–19).

Materials and Methods

Cervical specimen collection. We evaluated a discovery set of 20 tissues samples of primary cervical squamous cell cancers collected from the Washington University School of Medicine. Sample collection was approved by Washington University in St. Louis Human Studies Committee and under Institutional Review Board (IRB) exempt protocols at the National Cancer Institute. We compared RLGS patterns for tumor DNA to normal DNA extracted from 10 mL peripheral blood lymphocytes (PBL) of the same subjects in 15 cases or to DNA extracted from normal adjacent cervical tissue (n = 2) or to normal cervical tissue from another individual (n = 3). Tissue specimens weighing at least 50 to 100 mg and blood samples containing at least 10^7 cells that had been snap frozen in liquid nitrogen and stored at −80°C were used. Specimens were shipped to The Ohio State University on dry ice and kept at −80°C. A second test set of 15 tumors collected and stored in a similar manner was tested using quantitative methylation-specific PCR-based assays (Methylight) for selected promising markers.

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Residual liquid-based cytology specimens collected in PreservCyt (Cytyc) from 12 clinical specimens were collected at the Northwestern University Feinberg School of Medicine and at the University of Rochester Medical Center. Expert cytopathologists confirmed the cytologic interpretations of high-grade squamous intraepithelial lesions equivalent to histologic CIN3. PreservCyt specimens were anonymized, stored at −80°C, and subsequently tested under an IRB exemption granted by participating institutions.

RLGS. RLGS spot loss has previously been shown to be indicative of hypermethylation of the *Not*I site (18). High molecular weight DNA from patient samples was isolated according to published protocol (18, 20). Standard RLGS gels were run according to previously published protocols using the enzyme combination *Not*I-*Eco*RV-*Hinf*I, with digested (unmethylated) NotI sites being labeled and serving as the landmarks (20, 21). For each patient, the two-dimensional gels for normal DNA (2 tissue; 15 PBL) and tumor DNA were visually compared to identify lost spots, presumptively reflecting loci of relative DNA methylation. For the three patients who lacked paired samples of normal DNA, we compared the profile of the tumor to that of another normal profile derived from the present group. RLGS spot loss frequencies were recorded according to the coordinate naming system for the Master Profile derived from PBL DNA from a single healthy female adult as previously described (18).

Cloning and characterizing RLGS fragments. RLGS spots of interest were cloned using the *Not*I-*Eco*RV boundary library cloning method previously described (22) and were confirmed by single clone mixing gels. The BLAT search tool[8] (March 2006 assembly) was used to analyze the genomic context of the RLGS spot clone sequences to identify associated CpG islands, gene homologies, and location relative to the transcriptional start sites. Southern hybridization to confirm methylation were performed as previously described (22).

Bisulfite sequencing and development of Methylight assays for validation. To confirm methylation at specific tumor DNA sequences and to identify CpG dinucleotides best distinguishing normal from tumor DNA, we performed bisulfite sequencing on tumor and matched peripheral blood lymphocyte DNA for RLGS loci of interest. When possible, bisulfite PCR assays were targeted to the CpG island in a region surrounding the transcriptional start sites of genes. Bisulfite PCR products were subcloned, and multiple clones were sequenced. Methylation data for each CG dinucleotide from 50 normal alleles (5 individuals, 10 alleles each) and 50 tumor alleles (5 individuals, 10 alleles each) for each locus were collected. We determined CG dinucleotides that best distinguished normal from tumor. For two RLGS loci, 2C58 and 2C24, Methylight assays were developed with the primer and probe design aimed at targeting CpG dinucleotides that best distinguished between normal and tumor DNA as determined by the bisulfite sequencing described above. In addition, Methylight assays for seven leading candidate genes found methylated in recently reported cervical cancer series, including *DAPK1, CDH13, CDKN2A, TIMP3, SOCS2, hTERT*, and *CDH13* were performed as previously described (Appendix A; refs. 11, 14, 23–26). All Methylight assays were performed on the second set of 15 cervical cancer tissues and the 12 CIN3 cytology specimens.

Statistical methods. The total number of hypermethylation events and the total number of loci analyzed by tumor were calculated, thus enabling us to determine the percentage of loci methylated for each tumor. We assessed the median number of events for clones generated from RLGS fragments. Number and percentage of each individual gene methylated and the combination of all 9 genes evaluated were calculated.

Results

RLGS analysis. Results of RLGS analysis of 20 cervical cancers using NotI as the "landmark" are presented in Table 1 In the current study, an average of 1,753 RLGS spots were assessed for methylation in each tumor with variations in the number of spots analyzed depending on DNA quality and gel resolution (range, 824–2,172). One cervical cancer showed 0 RLGS spots lost with >2,000 spots analyzed and compared against normal DNA. For the 19 other cancers, RLGS spot loss (hypermethylation) was observed with percentages ranging from 0.12% (2 spots lost) to 4.0% (69 spots lost), with a median of 0.5% (Table 1). Thirteen of the twenty tumor samples showed hypermethylation levels below 1% (14 or less spots lost); 4 of 20 patients possessed levels of CpG island methylation above 2% (range of spots lost, 44–69).

Of the total 2,172 RLGS fragments analyzed, 187 spots were lost in at least one tumor and 40 were lost in 3 or more of the 20 tumors evaluated. Of the 40 fragments lost in 3 or more tumors, 25 were successfully cloned, and their sequence characteristics are shown in Table 2. The remaining 15 are not yet known. Of the 25 fragments methylated in at least 3 tumors, 23 *Not*I sites were found to be located within CpG islands, thus confirming the bias of RLGS toward detecting methylation in CpG islands. Of the 20 CpG islands with identified homology to genes, 14 are located upstream, in the 5′end of the gene. The remaining six are located in the body of the gene. Notably, methylation in 2C58, a fragment corresponding to nucleolar localization protein or nucleolar localized protein (NOL4), was identified in 17 (85%) of 20 squamous cell cancers.

Confirmation of DNA methylation. RLGS spot loss has been shown to be indicative of hypermethylation of the *Not*I site previously (18). For selected RLGS spots of interest from the above analysis, we confirmed that RLGS spot loss was indeed indicative of DNA methylation in this data set by bisulfite sequencing and Southern blot analysis. Figure 1*A* shows RLGS profiles for two fragments: 2C12 and 2C24 where spot loss is shown. Loss is clearly seen in the cervical tumor for patient 2 but not patient 5. We used *Not*I/*Eco*RV plasmid clone library mixing gels to locate the clone and confirm that the correct fragment was cloned. We then performed bisulfite sequencing on tumor and matched peripheral

Table 1. Summary of RLGS spots analyzed in 20 cervical cancers

Patient	No. spots lost	No. analyzed	% methylated RLGS spots
8	69	1,728	3.99
7	61	1,733	3.52
1	60	2,102	2.85
6	44	1,753	2.51
5	39	2,077	1.88
2	29	1,719	1.69
11	9	824	1.09
9	14	1,593	0.88
10	13	1,776	0.73
4	8	1,514	0.53
14	8	1,557	0.51
3	10	2,013	0.50
13	9	1,879	0.48
17	5	1,071	0.47
12	9	2,142	0.42
15	7	2,031	0.34
16	6	1,848	0.32
18	3	1,818	0.17
19	2	1,707	0.12
20	0	2,172	0.00%

[8] http://genome.ucsc.edu/cgi-bin/hgBlat

Table 2. Sequence characteristics of cloned RLGS spots lost 3 or more times in 20 cervical cancers

Spot*	Methylated (n = 20)	NotI +/− 200 bp †	% GC ‡	O:E	Cytogenetic mapping	Gene homology§	Context
2C58	17	chr18:30058291-30058691	60	0.83	18q12.1	NOL4	5′ end‖
2C12	15	chr2:186311808-186312208	71	0.77	2q32.1	BC039382	5′ end
2D45	13	chr19:36534086-36534486	72	0.95	19q12	ZNF537	5′ end
4C46	13	chr4:166097583-166097983	63	0.76	4q32.3	FLJ31659	5′ end
2C24	11	chr3:9570143-9570543	78	0.88	3p25.3	LHFPL4	5′ end
2F70	9	chr2:161809737-161810137	64	0.82	2q24.2	AK027541	Body
3D35	5	chr6:85529922-85530322	63	0.92	6q14.3	TBX18	5′ end
3D40	5	chr18:4445197-4445597	77	0.94	18p11.31	LOC388458	5′ end
2C35	4	chr10:23502265-23502665	65	1.11	10p12.2	LOC729385	5′ end
2D74	4	chr3:26639210-26639610	70	0.94	3p24.2	LRP15	5′ end
3C02	4	chr5:3649107-3649507	77	1.03	5p15.33	IRX1	5′ end
3D41	4	chr12:100127408-100127808	63	0.85	12q23.2	SLC5A8	5′ end
1E11	3	chr19:34709075-34709475	62	0.93	19q12	AK094793	5′ end
1F22	3	chr9:125817263-125817663	66	0.98	9q33.3	LHX2	Body
2C39	3	chr16:12903167-12903567	79	1.04	16p13.12	CR627300	5′ end
2D48	3	chr7:27231338-27231738	58	0.64	7p15.2	—	Intergenic
2E09	3	chr2:233058586-233058986	70	0.96	2q37.1	ECEL1	Body
2E30	3	chr18:43027591-43027991	79	0.90	18q21.1	FUSSEL18	5′ end
2E64	3	chr12:113370724-113371124	65	0.77	12q24.21	—	Intergenic
2F77	3	chr13:101850696-101851096	64	0.66	13q33.1	FGF14	Body
3B46	3	chr2:71952217-71952617	low	low	2p13.2	—	Intergenic
3F02	3	chr6:28818575-28818975	low	low	6p22.1	—	Intergenic
3F81	3	chr5:66335590-66335990	71	0.72	5q12.3	MAST4	5′ end
4C16	3	chr16:88565887-88566287	64	0.73	16q24.3	AFG3L1	5′ end
4D08	3	chr17:72952782-72953182	low	low	17q25.3	SEPT9	Body

*Cloned RLGS spots lost three or more times. An additional 79 cloned spots were lost at least once.
† March 2006 freeze of the human genome.
‡ NotI sites are either in CpG islands or within 200 bp of a CpG island.
§ Annotated gene, mRNA, or spliced EST within 5 kb of the CpG island.
‖ Within 5 kb of transcriptional start site and/or including exon 1.

blood lymphocyte DNA to identify CpG dinucleotides that best distinguished normal DNA from tumor DNA. Figure 1*B* and *C* show the bisulfite sequencing for 2C24 and 2C12, respectively, in normal PBL and the two corresponding tumors (patient 2 and 5). Consistent with the RLGS profile, bisulfite sequencing from patient 5 shows only ~30% methylation for 2C24 (Fig. 1*B*) and nearly no methylation for 2C12 (Fig. 1*C*). In patient 2, however, where RLGS spot loss of 2C24 is complete and loss of 2C12 is partial, bisulfite sequencing showed ~90% (Fig. 1*B*) and 50% (Fig. 1*C*) methylation, respectively. Taken together, these data show the high level of correspondence between the spot loss shown by RLGS and the degree of methylation detected by bisulfite sequencing, thus further supporting that RLGS spot loss represents DNA methylation.

Figure 2*A* similarly shows the RLGS profile for fragment 2C58 (CpG island in the promoter region of the *NOL4* gene) and spot loss that is visually apparent in the cervical tumor of patient 7 and no apparent loss in the cervical tumor of patient 20 (patient with no RLGS spots loss). We present Southern blot as another method of confirming and estimating the degree of methylation, in Fig. 2*B*. Southern hybridization was performed on tumor and paired normal DNA digested with *Not*I plus *Eco*RV and hybridized with a portion of the 2C58 *Not*I/*Eco*RV restriction fragment. Lane 1 shows a control normal bone marrow DNA digested with *Eco*RV only to show the size of the *Eco*RV fragment. All remaining lanes show the double digest to see if *Not*I can cut the *Eco*RV fragment. Figure 2*B* is a representative example of the Southern analysis. In all cases, the normal DNAs have reduced or no hybridization to the larger *Eco*RV fragment and stronger hybridization of the smaller *Not*I/*Eco*RV fragment, indicative of digestion and, therefore, lack of methylation of the *Not*I sites. In the tumor DNAs, however, the opposite is true. Nearly all the hybridization signal is at the larger *Eco*RV fragment, indicating a lack of digestion and, therefore, hypermethylation of the *Not*I sites. These data serve as further evidence that the RLGS spot loss of 2C58 is indicative of hypermethylation of the *Not*I site found in the promoter region of the *NOL4* gene.

Methylight assay development and validation. Neither RLGS analysis nor bisulfite sequencing is appropriate for large scale, highly sensitive detection of DNA methylation in clinical samples. Accordingly, we developed Methylight assays using both methylation-specific primers and methylation-specific probes as a robust method to accurately and sensitively quantify DNA methylation in large sample sets. However, to know specifically which CpG dinucleotides should be used in these primers and probe to best distinguish between normal and tumor DNA alleles, bisulfite sequencing is needed.

We performed bisulfite sequencing on five tumor DNAs demonstrating methylation by RLGS for 2C58 and 2C24, as well

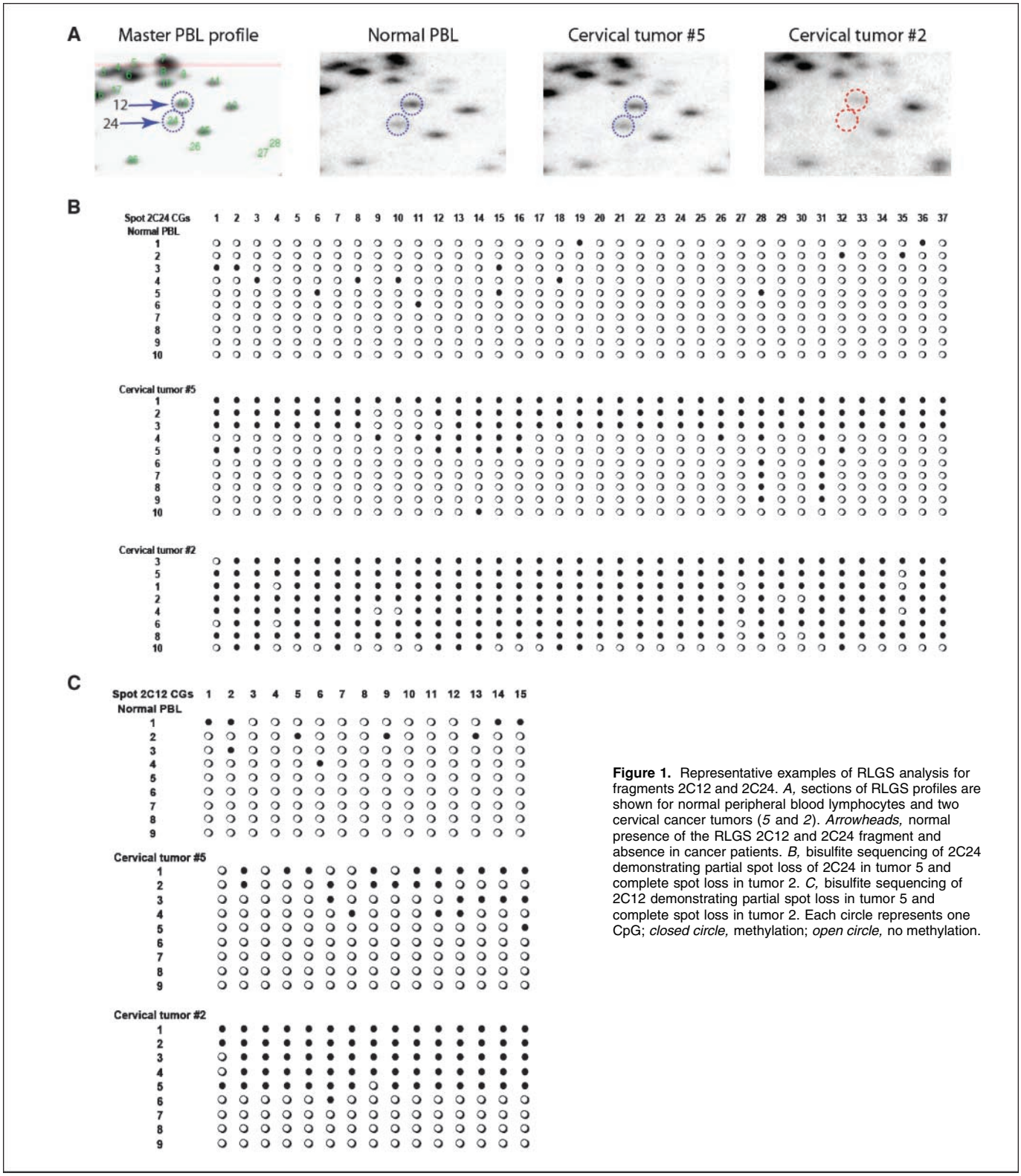

Figure 1. Representative examples of RLGS analysis for fragments 2C12 and 2C24. *A,* sections of RLGS profiles are shown for normal peripheral blood lymphocytes and two cervical cancer tumors (*5* and *2*). *Arrowheads,* normal presence of the RLGS 2C12 and 2C24 fragment and absence in cancer patients. *B,* bisulfite sequencing of 2C24 demonstrating partial spot loss of 2C24 in tumor 5 and complete spot loss in tumor 2. *C,* bisulfite sequencing of 2C12 demonstrating partial spot loss in tumor 5 and complete spot loss in tumor 2. Each circle represents one CpG; *closed circle,* methylation; *open circle,* no methylation.

as on five normal PBL DNAs. Figure 2*C* shows the results of the bisulfite sequencing for 2C58 (data from two normal PBL DNAs is not shown). These data show the variability of methylation at each CpG dinucleotide in both normal and tumor DNA. Regions where primers for Methylight assays were designed are highlighted in yellow. These regions were selected because most tumor alleles showed methylation, whereas most normal alleles were unmethylated. The area in green indicates where the methylation-specific probe was designed, again corresponding to sites at which tumor DNA was frequently methylated and normal DNA was

unmethylated. Finally, regions at which there was considerable methylation of normal DNA and were therefore unsuitable for including in primers and probes are shown in orange. These data further confirm the RLGS analysis and support the use of bisulfite sequencing data in designing Methylight assays for detecting tumor-specific alleles. A Methylight assay for 2C24 was similarly designed (data not shown).

We note that three additional spots identified by RLGS as methylated in ≤13 cancers were also pursued for further analysis. Briefly, 2D45 and 4C46 showed little or no methylation by bisulfite sequencing, and Q-MSP assay development was therefore not further pursued. Although methylation of 2C12 in cancers was evident by bisulfite sequencing, methylation was also evident albeit at a lower level in normal specimens. Q-MSP assay for 2C12 was therefore not satisfactorily developed. In summary, Q-MSP assays for two RLGS-identified spots, 2C24 and 2C58, were successfully developed and subsequently evaluated for confirmation in an independent set of 15 cervical cancer cases.

We sought to confirm their methylation in an independent set of cervical cancers and to determine whether methylation of both genes could be detected in cytologic specimens diagnosed as CIN3. Our evaluation of the Methylight assays for 2C24 and 2C58 showed that the majority of the 15 independent tumors were again methylated for 2C24 (67%) or 2C58 (52%; Fig. 3) and that 73% of cancers were methylated for either marker. Of CIN3 cytology specimens evaluated, lower levels of methylation were found for 2C24 (25%) and 2C58 (38%), with 38% of CIN3 methylated for either 2C24 or 2C58.

Finally, we also evaluated an additional seven candidate genes (*DAPK1, CDH13, CDKN2A, TIMP3, SOCS2, hTERT,* and *CDH13*) to determine the added value of measuring methylation of other candidate genes in conjunction with the two novel markers identified in our investigation. These candidate markers were selected based on multiple reports that have shown that these markers are often methylated in cervical cancers (2, 3, 8, 27). The most frequently methylated genes were *DAPK*, methylated in 67% of cancers, and *SOCS2*, methylated in 53% of tumors. DAPK and *SOCS2* were methylated in 33% and 0% of CIN3 cytology specimens, respectively. Taken together, we found that 93% of (the 15 tumors in the validation set) tumors showed methylation for at least one of four genes, *DAPK1, SOCS2, 2C24,* or *2C58*, and that 43% of liquid-based cytology specimens of CIN3 were methylated at one or more of these genes. All cancers were methylated for at least one or more of the nine total markers evaluated and 13 of the 15 tumors had more than one locus methylated (Fig. 3).

Discussion

Identification of a marker panel composed of targets frequently methylated in cervical cancer may permit the development of a clinical assay for distinguishing HPV infections that are likely to progress to cancer from the majority, which are destined to regress.

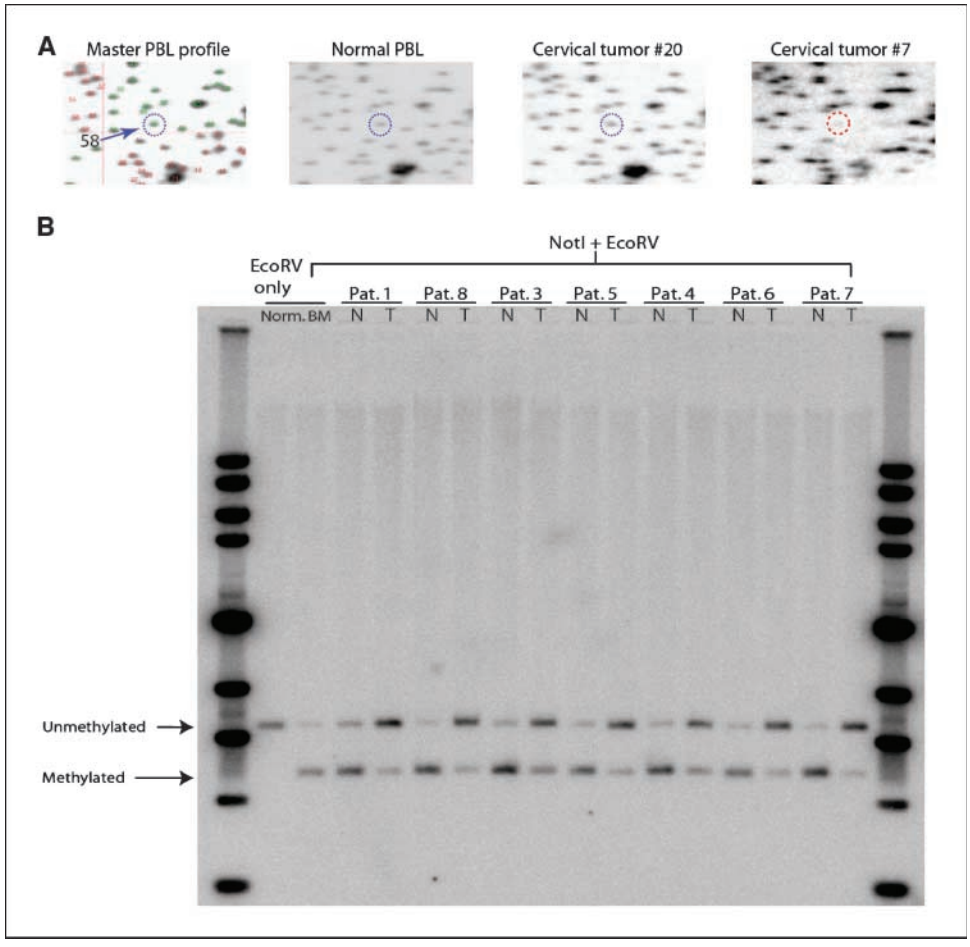

Figure 2. Representative examples of RLGS analysis for fragment 2C58. *A,* sections of RLGS profiles are shown from normal PBLs and two cervical cancer tumors. *Arrowheads,* normal presence of the RLGS 2C58 fragment, absence in tumor 7, and presence in tumor 20. *B,* Southern blot showing methylation of 2C58 in tumor 7. *Lanes 1* and *18,* 1-kb ladder; *lane 2,* normal bone marrow (*BM*) DNA, control sample (*Eco*RV digestion only); *lane 3,* normal bone marrow DNA, control sample (*Not*I and *Eco*RV digestion); *lanes 4* to *17,* cervix tumor (*Not*I and *Eco*RV digestion). *N*, normal; *T*, tumor.

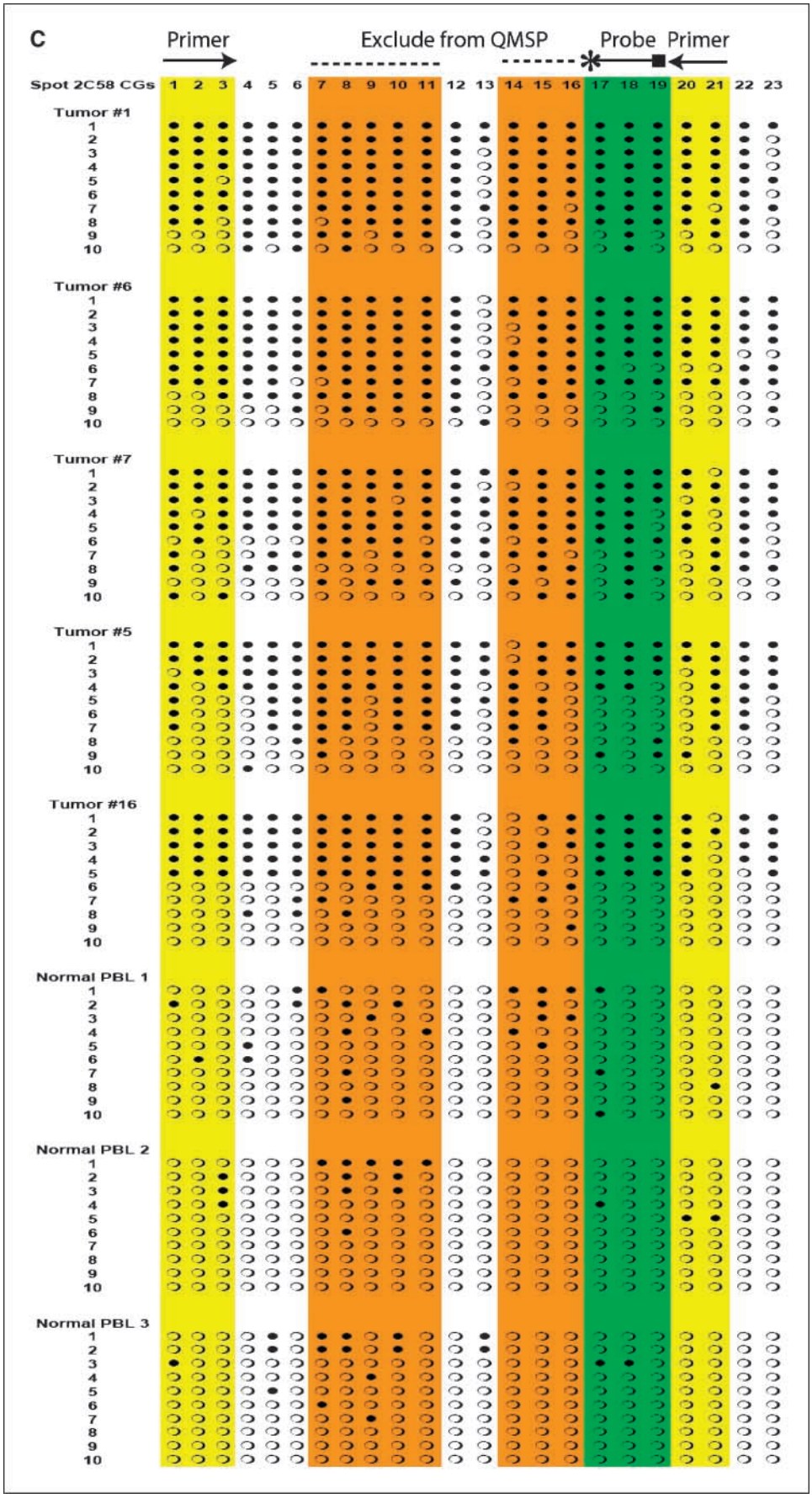

Figure 2 *Continued. C,* bisulfite sequencing of RLGS spot 2C58 on five tumors that had RLGS spot loss and three normal PBL DNAs. Methylight primer design (*yellow region*) shows the regions where primers were designed. Regions were thus selected where most tumor alleles are methylated. *Green region,* where the methylation-specific probe is designed, specifically to regions where almost all tumor alleles but no normal alleles are methylated. *Orange regions,* where primer and probe design are avoided because of an unacceptable level of methylation in normal alleles. Each circle represents one CpG; *closed circle,* methylation; *open circle,* no methylation.

Although methylated DNA is stable and can be sensitively detected using semiquantitative assays, efforts to develop an optimal panel based on candidate markers have yet to come to fruition. Accordingly, we performed RLGS profiling to identify novel targets suitable for assay development that could be combined with previously reported targets to constitute a new panel.

Our RLGS analysis of 20 unselected cervical squamous cancers showed methylation at one or more loci in 19 tumors with a median number of alterations of 9, suggesting that identification of a sensitive methylation panel for detecting invasive cancer is feasible. However, given that even the most frequently detected marker, NOL4, was not detected in 15% of the discovery tumor set, it seems probable that an effective assay would require a panel including several markers. Although some candidate genes have been evaluated repeatedly in cervical cancer, consistency between studies has been limited, probably reflecting differences in specific sequences targeted, assay methods, and specimen type, among and other factors (2, 3, 28). Nonetheless, methylation of at least one candidate gene evaluated in any given panel has been shown in the majority of cervical cancers. Based on this candidate gene approach, a small fraction of CpG islands in the genome (29, 30) has been interrogated. Only one study to date has taken a comprehensive approach to evaluating methylation; Sova and colleagues (13) used cell lines to first identify novel sites before demonstrating their methylation in human samples. Here, we take a complementary approach by first evaluating the human genome for cervix-specific methylation genes and further confirming their methylation in another independent set of human cervix tumors.

We report sequences from the RLGS fragments lost in three or more tumors as potential genes methylated in cervical cancer. Of these 25 RLGS loci, all but three had sequence characteristics consistent with CpG-island DNA. Chromosomal regions previously showed as regions involved in loss of heterozygosity in cervix cancers and also demonstrating hypermethylation activity include chromosome 3p (31). Recurrent losses have further been detected in chromosome region 18q12-22. Because these chromosomal losses suggest potential loss of candidate tumor suppressor genes, the overlap between our RLGS fragment loss in the same chromosomal regions [3p25.3 for *LHFPL4* (2C24) and 18q12.1 for *NOL4* (2C58)] provide further evidence for the inactivation of a tumor suppressor gene at 3p and potentially 18q.

Most notably, we identified the nucleolar-localized protein (NOL4) as a gene frequently methylated in cervical cancer. Although NOL4 has been identified to possess functional nuclear and nucleolar localization signals (32), it has not previously been associated with cervical cancer. Increasingly, however, there is evidence demonstrating that such nucleolar proteins, once considered to function solely for ribosomal transcription and subunit assembly, also possess important roles in cell cycle and apoptosis (33, 34). Recently, the nucleolar protein NOL7 has been linked as a tumor suppressor gene for cervical cancer (35). Specifically, Hasina et al. (35) provided both *in vitro* and *in vivo* evidence that NOL7 plays a critical role in the apoptotic index of cervical cancer cells specific to the angiogenic switch, whereby vascular endothelial growth factor production is decreased and angiogenesis inhibitor thrombospondin-1 production is increased. Additional nucleolar proteins relevant in cancer biology also include the nucleolar protein NOL8, identified as an oncogene for diffuse-type gastric cancers (36). Finally, tumor suppressor genes, such as p14, whose protein products localize in the nucleolus, have also been identified (37). We therefore believe that determining the biological role that NOL4 plays in cervical cancer is of significant interest and confirmation of its role as a potential tumor suppressor gene is warranted. Although immunohistochemistry staining of NOL4 antibodies would yield important clues regarding expression of the gene in cervix tissues of different progressive disease stages (e.g., low-grade disease, precancer, and cancer), at present, no human antibody is available for NOL4. We therefore encourage the development of such antibodies so that the biological relevance of both genes in cancer etiology can be fully pursued.

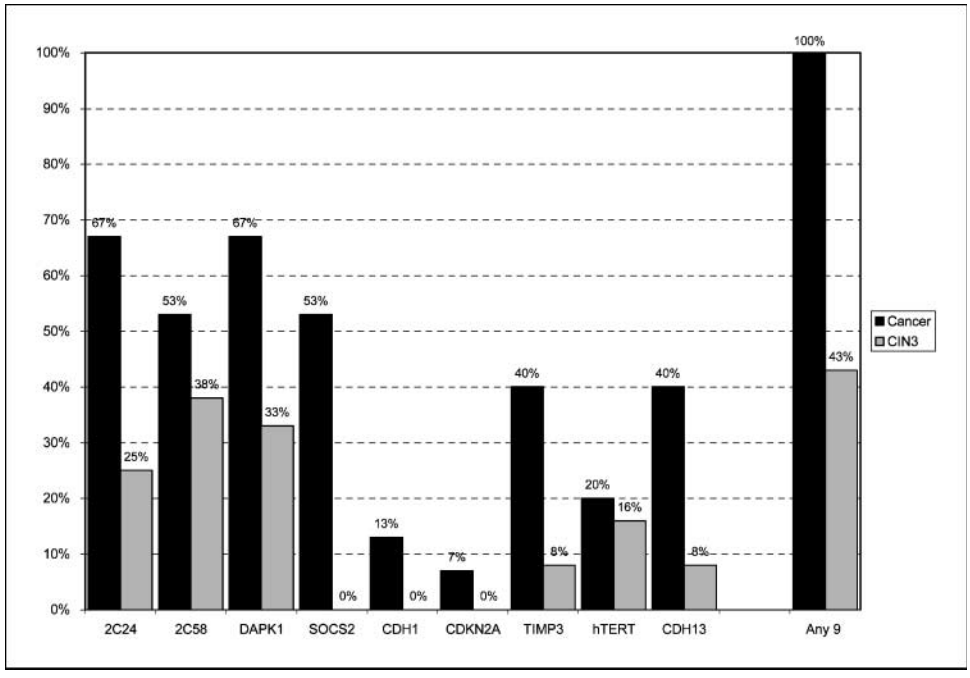

Figure 3. Detection of hypermethylation of two novel genes and seven previously published candidate genes in an independent set of 15 cervical cancer tissues and 12 CIN3 cytology specimens.

Appendix A. Gene symbol, name, chromosomal location, function, primer sequences, and annealing temperature (Ta) for Methylight assays

Gene symbol	Gene name (Entrez Gene)	Chromosomal location	Gene function	Forward primer sequence (5′-3′)
DAPK	Death-associated protein kinase 1	9q34.1	Apoptosis	TCGTCGTCGTTTCGGTTAGTT
CDH1	Cadherin 1, type 1, E-cadherin (epithelial)	16q22.1	Adhesion	AATTTTAGGTTAGAGGGTTATCGCGT
CDKN2A	Cyclin-dependent kinase inhibitor 2A (melanoma, p16, inhibits CDK4)	9p21	Cell cycle control	TGGAGTTTTCGGTTGATTGGTT
TIMP3	TIMP metallopeptidase inhibitor 3	22q12.1-q13.2	Metalloendopepdidase inhibitor	GCGTCGGAGGTTAAGGTTGTT
SOCS2	Suppressor of cytokine signaling 2	12q	Negative regulator of growth hormone/insulin-like growth factor signaling pathway	TCCCTTCCCCGCCATT
hTERT	Telomerase reverse transcriptase	5p15.33	Elongates telomeres	GGATTCGCGGGTATAGACGTT
CDH13	Cadherin 13, H-cadherin (heart)	16q24.2-q24.3	Adhesion	AATTTCGTTCGTTTTGTGCGT
2C24	LHFPL4	3p25.3	Lipoma HMGIC fusion partner-like 4; transmembrane protein	GGATTCGGGATTGGTACG
NOL4 (2C58)	Nucleolar protein 4	18q12	RNA binding	TTTTAGACGTTTCGTTTTCG
ACTB	β actin		Cell motility	TGGTGATGGAGGAGGTTTAGTAAGT

NOTE: For *DAPK, CDH1, TIMP3, SOCS2, hTERT,* and *CDH13,* we replaced the BHQ-1 for TAMRA at the 3′ end.

We demonstrate that RLGS is a powerful genomic scanning tool, effective for identifying candidate genes that are targets of hypermethylation in cervical cancer. An important strength of the current study is that we used human tissues; data suggest that use of cell lines for marker discovery may be limited by overestimation of the frequency of hypermethylation (21). Our use of *Not*I as the landmark enzyme is also advantageous, because it favors detection of methylation in CpG islands within promoters, suggesting that candidate markers detected with this method are more likely to be linked to functionally relevant biological events (38). Consistent with our results, previous studies have found that tumors vary greatly in the frequency of methylation at candidate regions, but sites of methylation are found in nearly every tumor that is tested at many loci. A limitation of our study is that we were unable to assess HPV type and other pathologic factors. However, RLGS profiles of tumor DNA were compared with normal DNA from the same individuals in most instances, which may have minimized the effect of this limitation. Another limitation of our approach is that although powerful, RLGS covers ~1,200 CpG islands (4%) of the 29,000 in the human genome. Thus, follow-up studies that use complementary approaches such as array-based approaches to identify methylation are encouraged for more complete coverage in discovery efforts.

Our results are of particular interest from a diagnostic point of view. In the limited set of cytologic CIN3 samples tested, we found that methylation occurred at a lower frequency than in cancer. This lower rate of detection of methylation in cytologic CIN3 than cancer may reflect the fact that cytologic specimens contain many ostensibly normal cells and are also restricted to superficial epithelial layers. Alternatively, methylation events that are associated with progression of CIN3 to cancer may not be evident yet in some examples of CIN3. Notably, most women diagnosed with CIN3 are 15 to 20 years younger than those diagnosed with cancer, and data suggest that invasion typically occurs after many years of CIN3 persistence. Accordingly, many examples of CIN3 detected by routine screening are not incipient cancer precursors. If the absence of methylation events shows good negative predictive value for cancer, it may be a useful marker for safely lengthening the screening interval in some cases.

In summary, using a genomic scanning method, we report the first discovery effort with validation for identifying a novel panel of candidate genes methylated in cervical neoplasia. Methylight assays developed for two genes, *NOL4* and *LHFPL4*, showed a high rate of methylation in an independent set of 15 cervical neoplasias and a slightly lower rate of methylation in CIN3 specimens. To confirm whether these genes are specific to cervical cancer or play a broader role in cancer etiology, we believe evaluating methylation of related (e.g., other HPV-associated tumors) or unrelated tumors should be pursued. For early diagnosis of cervical cancer, we believe that in building a methylation panel, these two markers can serve as a foundation upon which other potential candidate markers should be investigated, including those recently identified as promising for cervical cancer: *TSLC1, DcR1, TWIST1, MT1G, NMES1, RRAD, SFRP1, SPARC*, and *TFP12* (10, 13, 39, 40, 41). In addition, comprehensive detection of methylation markers in precursor stages should be pursued using optimized sensitive assays. Evaluation of all promising markers to date is needed in a large population-based epidemiologic study to help define which genes comprise a methylation profile that could be translated as biomarkers of risk for progressive cervical neoplasia.

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Appendix A. Gene symbol, name, chromosomal location, function, primer sequences, and annealing temperature (Ta) for Methylight assays (Cont'd)

Reverse primer sequence (5′-3′)	Probe sequence (5′-3′)	Ta	Reference
TCCCTCCGAAACGCTATCG	6FAM5′-CGACCATAAACGCCAACGCCG-3′TAMRA	60°C	(11)
TCCCCAAAACGAAACTAACGAC	6FAM5′-CGCCCACCCGACCTCGCAT-3′TAMRA	60°C	(11)
AACAACGCCCGCACCTCCT	6FAM5′-ACCCGACCCCGAACCGCG-3′TAMRA	60°C	(23)
CTCTCCAAAATTACCGTACGCG	6FAM5′-AACTCGCTCGCCCGCCGAA-3′TAMRA	60°C	(11)
TTGTTTTTGTCGCGGTGATTT	6FAM5′-CCGAAAAACTCAAAACACCGCAAAATCAT-3′TAMRA	60°C	(11)
CGAAATCCGCGCGAAA	6FAM5′-CCCAATCCCTCCGCCACGTAAAA-3′TAMRA	60°C	(11)
CTACCCGTACCGAACGATCC	6FAM5′-AACGCAAAACGCGCCCGACA-3′TAMRA	60°C	(11)
CGCCGATACACGA	6FAM5′-CGGTTCGGCGTTTTTAGT-3′TAMRA	65°C	
AAAAATCCGAAACCCGTAT	6FAM5′-TCGTTCGTTTAGCGTTTTTG-3′TAMRA	60°C	
AACCAATAAAACCTACTCCTCCCTTAA	6FAM-ACCACCACCCAACACACAATAACAAACACA-3′TAMRA	60°C	(23)

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