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Gynecol Oncol. Author manuscript; available in PMC 2017 September 01.

Published in final edited form as:

Gynecol Oncol. 2016 September ; 142(3): 566–573. doi:10.1016/j.ygyno.2016.07.006.

NOVEL EPIGENETIC CHANGES IN *CDKN2A* ARE ASSOCIATED WITH PROGRESSION OF CERVICAL INTRAEPITHELIAL NEOPLASIA

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Abstract

Objective—To conduct a comprehensive mapping of the genomic DNA methylation in *CDKN2A*, which codes for the p16^{INK4A} and p14^{ARF} proteins, and 14 of the most promising DNA methylation marker candidates previously reported to be associated with progression of low-grade cervical intraepithelial neoplasia (CIN1) to cervical cancer.

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Conflicts of interest disclosures: The other authors declare that there are no conflicts of interest.

DATA AVAILABILITY

All sequencing data generated in this study are deposited at the Gene Expression Omnibus and are available in Series GSE76986. Interim link for reviewers: http://www.ncbi.nlm.nih.gov/geo/query/acc.cgi?token=izyrcmsafpoljgh&acc=GSE7698

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Methods—We analyzed DNA methylation in 68 HIV-seropositive and negative women with incident CIN1, CIN2, CIN3 and invasive cervical cancer, assaying 120 CpG dinucleotide sites spanning *APC, CDH1, CDH13, CDKN2A, CDKN2B, DAPK1, FHIT, GSTP1, HIC1, MGMT, MLH1, RARB, RASSF1, TERT* and *TIMP3* using the Illumina Infinium array. Validation was performed using high resolution mapping of the target genes with HELP-tagging for 286 CpGs, followed by fine mapping of candidate genes with targeted bisulfite sequencing. We assessed for statistical differences in DNA methylation levels for each CpG loci assayed using univariate and multivariate methods correcting for multiple comparisons.

Results—In our discovery sample set, we identified dose dependent differences in DNA methylation with grade of disease in *CDKN2A, APC, MGMT, MLH1* and *HIC1*, whereas single CpG locus differences between CIN2/3 and cancer groups were seen for *CDH13, DAPK1* and *TERT*. Only those CpGs in the gene body of *CDKN2A* showed a monotonic increase in methylation between persistent CIN1, CIN2, CIN3 and cancers.

Conclusion—Our data suggests a novel link between early cervical disease progression and DNA methylation in a region downstream of the *CDKN2A* transcription start site that may lead to increased p16^{INK4A}/p14^{ARF} expression prior to development of malignant disease.

INTRODUCTION

The vast majority of low grade cervical intraepithelial neoplasia (CIN1) will regress spontaneously without treatment [1, 2]. CIN1 that contains oncogenic human papillomavirus (HPV), particularly HPV 16 or 18, the two oncogenic HPV types that are associated with over 70% of cervical cancers, are more likely to persist and progress [3–5]. CIN1 is commonly over-treated and over-managed because of the inability to distinguish the few CIN1 likely to progress. Additionally, lesion persistence and progression to high-grade cervical intraepithelial neoplasia (CIN2/3) are more common in HIV-seropositive women, which were previously thought to be driven by global immune dysfunction. Understanding the acquired molecular events that determine which few early CIN lesions will progress to cancer is a major goal with clear clinical implications.

Transcription regulation of host genes through the methylation of CpG dinucleotide sites within DNA is increasingly accepted as playing a critical role in tumorigenesis [6–11]. However, because of the cross-sectional design of prior studies, it remains unclear whether the identified epigenetic changes preceded or followed the disease. Also, prior studies focused primarily on gene promoter regions where DNA methylation would be expected to silence genes, whereas the broad profile of aberrant DNA methylation events within genes associated with cervical tumorigenesis remains unknown [6, 12]. This is a major limitation, since differential methylation of intragenic sites (i.e., within exonic and intronic regions) have been associated with differential, and often increased, expression of tumor suppressor genes predictive of clinical progression in cervical cancer [13, 14]. In particular, a number of studies have found conflicting associations between aberrant methylation and expression of known tumor suppressor genes, including *CDKN2A*, which codes for the p16^{INK4A} and p14^{ARF} proteins, with cervical cancer [15, 16] and high-grade CIN [17–19]. To address these limitations, we prospectively recruited and followed women with CIN1, CIN2, CIN3 and cancer, and collected cervical tissue for in-depth DNA methylation analysis and

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sequencing of CpG loci spanning across 15 candidate tumor suppressor genes, including *APC, CDH1, CDH13, CDKN2A, CDKN2B, DAPK1, FHIT, GSTP1, HIC1, MGMT, MLH1, RARB, RASSF1, TERT* and *TIMP3*, that have been previously reported to be associated with progression of CIN1 to cervical cancer [6].

METHODS

Study sample

Subjects included 68 women with histologically-confirmed CIN1 (N=27), CIN2 (N=8), CIN3 (N=10), and invasive cervical carcinoma (N=23) treated at the affiliated teaching hospitals for Albert Einstein College of Medicine – Montefiore Medical Center and North Bronx Hospital Center. HIV-seropositive and negative patients who had incident, histologically-confirmed CIN1 were enrolled and followed prospectively every 3 to 6 months for a maximum of 2 years (4 visits) with repeat biopsies done at each visit. Liquid-based cytology samples were also collected for HPV genotyping by polymerase chain reaction (PCR) protocol described below [20]. CD4+T-cell counts and HIV viral load levels in the HIV-seropositive patients assessed within six-months of their CIN/cervical cancer diagnosis were abstracted from the electronic medical records. Written informed consent was obtained from all subjects prior to participating prior in the study under IRB approved protocols. Patient characteristics are described in Table 1.

Each subject had a biopsy of the colposcopically identified lesion for standard pathologic evaluation by two pathologists [KW, MA] and scored following lower anogenital squamous terminology (LAST) criteria [21], plus an adjacent biopsy for methylation analysis of their genomic DNA, and a biopsy of a colposcopically ‘normal’ area that was confirmed normal by H&E stain of slide during tissue processing. All biopsies were snap-frozen in liquid nitrogen within two minutes of tissue procurement. Women that had CIN1 at initial diagnosis but had no CIN at follow-up based on a pathologically normal biopsy samples collected 6 to 12 months later were defined as ‘regressed CIN1’, and those that had CIN1 on consecutive visits were defined as ‘persistent CIN1’. Our biopsy approach allowed us to assess the association between DNA methylation in a newly detected CIN1 lesion with disease regression prospectively, as well as changes in methylation in lesions over time. Women with CIN2/3 and cervical cancer were recruited with similar fresh tissue collection as for CIN1 cases, but without prospective follow-up, as these subjects required immediate treatment.

DNA methylation analyses

As is typical for genomic studies, we employed multiple assays and independent platforms to confirm our findings. Out of the subjects that had numerous time-points of tissue collection, a subset of cervical biopsies were processed and analyzed, chosen based on the amount of available tissue from the biopsy and total nucleic acid content, with two partially overlapping sample sets selected serially, first for initial analysis using the Illumina Infinium platform (discovery cohort, N=29), followed by HELP-tagging and Targeted Bisulfite Sequencing (validation cohort, N=54). Where possible, subject samples were tested in batches balanced by disease grade, HIV status and age at diagnosis (±10 years).

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Illumina Infinium assay—Changes in DNA methylation associated with CIN progression to cervical cancer were assessed using the Illumina Infinium assay with the HumanMethylation27 DNA Analysis BeadChip (Illumina Inc., CA). DNA methylation levels at individual CpG loci spanning 15 candidate genes represented on the Illumina beadchip (2–25 CpG loci per gene site) were determined by measuring the fraction of methylated signal over the total signal (unmethylated + methylated fractions) in each genomic DNA sample. Bisulfite conversion of cervical tissue genomic DNA (500ng) was carried out using the EZ DNA Gold methylation kit (Zymo Research Inc., CA). Normalized M values were generated using the R package HumMeth27KQCReport function, including the X chromosome data and using an average probe p-value of 0.03 as the cutoff for sample inclusion [22]. Individual beadchip controls (DNA sample-dependent and sample-independent) confirmed efficient bisulfite conversion of DNA, hybridization specificity, base extension and target removal for all genomic DNA samples. A complete description of these controls is available from the manufacturer. Chromosome (chr) locations, RefSeq and Genebank accession numbers were based on National Center for Biotechnology Information (NCBI) build 36 (http://www.ncbi.nlm.nih.gov/mapview/stats/BuildStats.cgi?taxid=9606&build=36&ver=1).

HELP-tagging assay—To further map the CpG rich regions within candidate genes that showed differential methylation with grade of cervical disease on the Illumina array, we employed a massively-parallel sequencing protocol (HELP-tagging) developed at Einstein [23]. HELP-tagging is a more comprehensive and quantitative assay than array-based methods like the Illumina beadchip platform [24]. DNA from the cervical samples was digested with HpaII, ligated to a customized Illumina adapter that contains an EcoP15I site allowing isolation of the adjacent 27 base pair (bp) sequence, and treated with EcoP15I to generate a "tag" adjacent to the HpaII site (CCGG). We then ligated the complementary Illumina adapter to the other end of the tag, and generated a 120bp library by PCR. Because HpaII cannot cleave methylated DNA, the number of reads generated for a locus was inversely proportional to the methylation at that site, a quantitative outcome that was enhanced by using a pooled MspI representation for normalization. Results were analyzed using the Wasp cyberecosystem [25] and linked to a local mirror of the University of California, Santa Cruz (UCSC) Genome Browser for visualization.

Targeted Bisulfite Sequencing (TBS)—Validation of candidate regions identified using HumanMethylation27 and HELP-tagging was performed using an orthogonal bisulphite sequencing approach for nucleotide-resolution CpG mapping of specific regions. We designed bisulfite-conversion-based PCR primers within target regions using the University of California San Francisco (UCSF) MethPrimer tool [26]. We reduced the possibility of off-target amplicons using BiSearch to interrogate to human genome [27]. Primer sets were further optimized for PCR using random primer pools controlling for secondary structures using Life Technologies' Multiple Primer Analyzer tool. We bisulfite converted 500ng of purified genomic DNA using a high throughput 96 well plate. After bisulfite conversion, all samples were pre-amplified using an equimolar primer mix. We then added custom dual indexed adapters and amplified the libraries using a Fluidigm Access Array (Fluidigm Inc., CA). We sequenced the resulting amplicon library using the Illuminia MiSeq platform and

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150bp paired end sequencing. After spatially filtering out read clusters by removing reads that were too close to produce high confidence index reads, we aligned reads to the human genome and calculated methylation ratios using BSMAP 2.7.3. Only high confidence CpGs containing coverage of at least 50 effective reads were considered, and methylation ratios were averaged in 50bp intervals across the assay regions.

HPV DNA genotyping

DNA extracted from liquid-based cytology smears was digested with proteinase-K/ Laureth-12, precipitated and purified in ethanol, and amplified by PCR with Gold-Taq using a well-described the MY09/MY11 protocol [28, 29], followed by Southern blot hybridization with generic probes for HPV and an oligonucleotide for human β-globin DNA (as a control). PCR products positive by Southern blot were analyzed using biotinylated type-specific oligonucleotide probes for >40 different HPV types, including high-risk types (16,18,31,33,35,39,45,51,52,56,58 & 59)[30]. Samples that tested positive by the generic probe mix but negative by all type-specific probes were considered to represent low-risk HPV types.

Statistical analyses

We ranked the most significant predictors of disease progression comparing average CpG methylation levels in lesion samples assessing cross-sectionally by disease grade from CIN1 to cancer, and prospectively, comparing regressing vs. persistent CIN1 lesions. We tested for statistical differences in DNA methylation levels for each CpG loci assayed using univariate parametric and non-parametric tests, where appropriate. Significant CpG sites (corrected $p<0.05$) were further restricted on a magnitude of difference in DNA methylation (e.g., of at least ±0.2 difference in beta for the Illumina protocol) considered sufficient to result in a 'biologically' significant difference on expression [31]. This approach was chosen to reduce the potential for redundant predictors and over-selection that may arise. Disease group methylation values were illustrated using box plots showing the median, interquartile range (25th and 75th percentile), and upper and lower adjacent Tukey values [32].

Using the HELP-tagging assay, we assessed for differences in DNA methylation at all HpaII sites proximal to the Illumina probes within candidate genes using parametric and non-parametric tests. We estimated the relative odds of methylation at each HpaII site for CIN2/3 and cervical cancer compared to persistent CIN1 using polytomous multivariate logistic regression models implemented in the *nnet* package in R. We used a change in point estimate selection method to assess for confounding (e.g., by detection of high-risk HPV DNA or HIV serostatus) comparing covariate adjusted OR controlling *a priori* for sample batch and age [33]. In addition, we performed non-parametric tests for trend using the Jonckheere-Terpstra test implemented using the *clinfun* R package on a covariate-adjusted data generated using the *ComBat* R package. A similar approach was used to test for significant differences in average DNA methylation measured across the multiple 50bp regions assessed by targeted bisulfite sequencing. We employed a strict method to control for multiple testing by setting a Bonferroni threshold of significance using an alpha=0.05 divided by either the number of assayed HpaII sites in a specific region for Help-tagging or the number of bins in each amplicon for TBS. All statistical tests were two-tailed.

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RESULTS

We analyzed DNA methylation using the Illumina Infinium (HumanMethylation27) platform in a discovery sample set of HIV-negative women with incident CIN1 followed prospectively, including 6 with CIN1 that regressed and 11 with persistent CIN1, plus a convenience sample of 3 CIN2, 2 CIN3 and 7 cervical cancer patients. A total of 120 CpG loci spanning 15 candidate genes, including tumor suppressor genes *APC, CDH1, CDH13, CDKN2A, CDKN2B, DAPK1, FHIT, GSTP1, HIC1, MGMT, MLH1, RARB, RASSF1, TERT* and *TIMP3*. Nine CpG loci (8%) did not pass initial quality control (average control probe p-values <0.03 for all samples) and were excluded. We identified multiple CpG loci that showed significant dose-dependent increases in methylation with grade of disease, including several within the *CDKN2A* gene (Table S1). With respect to the other genes that had significant differences in methylation with grade of disease, *CDKN2A, APC, MGMT, RASSF1* and *HIC1* showed dose-dependent increases, whereas *MLH1, CDH13, RARB, CDKN2B, TERT* and *DAPK1* showed larger differences either between persistent CIN1 and CIN2/3, or between CIN2/3 and cancer (Figure S1).

Interestingly, further assessment of the *CDKN2A* gene revealed monotonic increases in methylation between persistent CIN1, CIN2, CIN3 and cancers for CpG located downstream of the transcription start site (chr9:21958106–21958899 NCBI build 36), whereas no differences, or even decreases, in methylation were seen for loci within or near the $p14^{ARF}$ promoter region (chr9:21983444–21986348; Figure 1). Subgroup analyses excluding CIN2, and CIN1 cases that regressed, remained significant for most of the CpG loci identified in the larger sample, including within the downstream regions of *CDKN2A, APC, MGMT, HIC1* and *MLH1* (Table S1).

To assess whether changes in DNA methylation predict persistence of low-grade cervical lesions, a clinically intervenable endpoint in-of-itself, CIN1 cases (n=15) with two or more follow-up visits and biopsy samples with sufficient DNA for bisulfite conversion were also assayed using the Illumina Infinium platform. We assessed for the changes in methylation comparing paired biopsies collected at diagnosis and 6–12 months later from women with persistent vs. regressing CIN1 lesions. No significant increases in *CDKN2A* methylation were detected for any of the measured CpG loci. Genes that showed changes (increases or decreases) in methylation associated with CIN1 persistence vs. regression included *CDH1, GSTP1, MGMT, MLH1, RASSF1* and *TIMP3*, but these did not remain significant after Bonferroni correction.

To further explore the apparent associations between DNA methylation and grade of disease, and to broaden coverage to include gene promoter regions not assayed by the Illumina Infinium platform, we assessed the methylation levels across 286 HpaII sites proximal to the HumanMethylation27 probes using HELP-tagging on a partially overlapping sample of 59 biopsies from HIV-seropositive and negative persistent CIN1 (n=20), CIN2 (n=8), CIN3 (n=8) and (n=23) cervical cancer cases. Figure 2a illustrates the distribution of percent DNA methylation (shown as box plots) across the four lesion grade groups at CpG loci within *CDKN2A*. A significant trend in increasing methylation with grade of disease was detected at 5 HpaII sites in the CpG island and shore approximately 1kb to 2.5kb upstream of the

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HumanMethlation27 probes. The trends were similar to those seen for neighboring CpG sites probed by the Illumina array, and after Bonferroni correction, the two closest loci within the CpG island shore remained significant. Although there was one CpG significantly differentially methylated in the *CDKN2A* p16^{INK4a} promoter (not assayed by the HumanMethylation27 beadchip), this result did not remain significant after Bonferroni correction. The *DAPK1* gene showed significant increases in DNA methylation at HpaII sites within the gene promoter, and decreasing methylation within the gene body, although only the gene body differences remained significant after Bonferroni correction (Figure 2b). In contrast, the *TERT* gene only showed a significant decrease in methylation with disease grade within its promoter after Bonferroni correction (Figure 2c). Other genes that showed significant trends (increases or decreases) in methylation with grade of disease after correction for multiple comparisons were *HIC1* and *RARB*, as well as for the *CDKN2B* gene region proximal to the *CDKN2A* promoter (Figure S2). A few CpG loci differences in methylation between persistent CIN1, CIN2, CIN3 and cancer lesions were seen in promoter regions of other genes like *APC, CDH13, CDH1, FHIT, MGMT, GSTP1* and *MLH1*, and in the gene body of *MGMT*, although these were not significant after Bonferroni correction.

We used multivariable polytomous logistic regression to assess the strength of association between increasing DNA methylation percentage and disease grade for the *CDKN2A, TERT, RARB, HIC1, DAPK1* and *CDKN2B* gene regions identified above, while accounting for potential confounding by sequencing batch and patient age. To optimize power, adjusted odds ratios (ORs) and 95% confidence intervals (CI) were generated comparing CIN1 to CIN2/3 and cervical cancer, respectively. Significant differences in DNA methylation between CIN1 and cancers persisted after adjustment for two of the CpG sites implicated in the gene body regions of *CDKN2A* (OR=1.09, 95%CI:1.02–1.18 and OR=1.08 (95%CI:1.01–1.15), while the ORs for the CIN2/3 and CIN1 comparison were somewhat attenuated and not significant (OR=1.05, 95%CI:0.98–1.12 and OR=1.01, 95%CI:0.95–1.07). Other gene regions that showed consistent results after covariate adjustment included the promoter regions of *TERT, RARB* and *HIC1*, and the gene body regions of *DAPK1* and *CDKN2B.*

Further adjustment for detection of high-risk and low-risk HPV types in the cervical samples and for HIV seropositivity yielded similar results, but generated unstable estimates due to over-stratification. To address this, we restricted the analyses to samples positive for high-risk HPV DNA (n=39), and saw consistent associations between DNA methylation within the downstream region of *CDKN2A* and disease grade for the cancer vs. CIN1 (OR=1.09, 95%CI:0.98–1.22 and OR=1.13, 95%CI:1.00–1.27) and CIN2/3 vs. CIN1 comparisons (OR=1.07, 95%CI:0.93–1.24 and OR=0.97, 95%CI:0.83–1.14). Similarly, for the HIV-negative group (n=44), the ORs for DNA methylation at the two *CDKN2A* CpG sites were similar for the cancer vs. CIN1 (OR=1.08, 95%CI:1.00–1.17 and OR=1.15, 95%CI:1.00–1.34) and CIN2/3 vs. CIN1 comparisons (OR=1.01, 95%CI:0.94–1.09 and OR=0.91, 95%CI:0.79–1.04), and even increased somewhat for HIV-seropositive subjects (n=15), for whom we could compare the CIN1 and the pooled CIN2/3 and cancer groups (OR=1.21, 95%CI:0.98–1.51 and OR=1.08, 95%CI:0.99–1.19), although the differences were not significant.

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As the Illumina Infinium HumanMethylation27 array and the HELP-tagging assay implicated similar regions within the target genes tested, but did not necessarily assay overlapping CpGs, we further verified these findings through TBS. We conducted fine epigenetic mapping of the all CpG loci within specific regions of the candidate genes, including *CDKN2A, CDKN2B, DAPK1, MGMT, RASSF1* and *TERT* in a subset of 53 biopsies (persistent CIN1 (n=20), CIN2, (n=7), CIN3 (n=7) and cervical cancer (n=19) cases). TBS confirmed there were significant increases in DNA methylation with grade of disease for a large number of CpG loci within the downstream and distal gene body regions of *CDKN2A* that overlapped with the HumanMethylation27 probes, whereas no significant differences were detected after Bonferroni correction for CpG loci located within the p16^{INK4a} and p14^{ARF} promoter regions, or in the CpG island shore identified upstream by the HELP-tagging assay (Figure 3a). The one other gene region that showed consistent increases in DNA methylation by TBS included the promoter of *DAPK1* (Figure 3b), although only the differences observed for *CDKN2A* remained significant after adjustment for patient age when modeled using multivariable polytomous logistic regression. Further adjustment for HPV DNA type or restriction on HIV serostatus did not substantively change the observed associations between DNA methylation and disease grade.

DISCUSSION

In this prospective study of epigenetic profiles in HIV-seropositive and negative women with different grades of CIN, including regressed and persistent CIN1, there were clear differences in the quantity and profile of methylated genes across grades of CIN and cervical cancer. Other cross-sectional studies in CIN have revealed patterns of aberrant DNA methylation in specific genes, including for tumor suppressor genes *CDKN2A* [34–37], *MGMT* [19, 38], *HIC1, APC, CADM1, MAL*, and *RARβ* [6, 9, 12]. However, as these prior studies were cross-sectional, the predictive nature and timing of the gene methylation has not yet been elucidated. One of the strengths of our study is the prospective collection at different time points and using subjects as their own controls, allowing us to more precisely investigate the predictive value of methylation of these specific genes. By studying these previously identified targets and many other genes along similar pathways as well as novel pathways, we had the ability to confirm previous results for validity, as well as identify other patterns of methylation yet unstudied.

Among the candidate genes identified, *MGMT* (represented by two CpG sites on the array) has been suggested as an intermediate to late event in cervical cancer [19, 38]. However, no differences were observed in *MGMT* for our patient samples of CIN after accounting for multiple testing. Moreover, when we conducted TBS of a separate but defined region of *MGMT*, we observed a markedly lower level of DNA methylation. In contrast, we now show that methylation of *CDKN2A*, which expresses the p16^{INK4A} and p14^{ARF} proteins, clearly begins to occur between low-grade and high-grade CIN. Immunohistochemistry staining for the p16^{INK4A} protein has been shown to be important in defining clinically-relevant, but histologically equivocal CIN [39, 40]. In advanced cervical cancers, the majority of cells had methylated *CDKN2A*, lack the p16^{INK4A} protein, but no longer express the HPV E7 oncoprotein. Thus p16^{INK4A} inactivation may be a mechanism of blocking the cyclin D-Rb

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pathway in invasive cervical cancer [41], whereas p14^{ARF} expression may be increased in HPV-positive precursor lesions [42–45].

Previous studies have also identified the intriguing result that *CDKN2A*, which is normally repressed in cycling cells by *EZH2* via H3K27me3, and frequently undergoes DNA hypermethylation in cancer, is often overexpressed in HPV-positive carcinoma [14]. Our data suggests a novel potential link between early cervical disease progression and DNA methylation of CpGs located within the 700bp downstream region of the transcriptional start site of *CDKN2A* that may lead to increased p16^{INK4A}/p14^{ARF} expression prior to development of malignant disease. Consistent with this epigenetically driven association, we have previously shown a positive correlation between downstream CDKN2A methylation and p16^{INK4A}/p14^{ARF} expression in HPV-associated head and neck cancer [46].

The mechanism by which hypermethylation occurs at the downstream CpG island region from the *CDKN2A* locus is not clear. Differential methylation events, including some associated with *CDKN2A*, have also been previously observed in cancer cell lines [47]. Cervical cell line data using methylation-specific PCR suggests DNA methylation of *CDKN2A* occurs only occasionally within early cervical cancer cells and is not always correlated with HPV E7 expression [41]. However, our study is unique in that it used biopsied human cervical tissue, thereby overcoming the limitation of high levels of DNA methylation observed in many cell lines when compared to corresponding patient samples [48].

Current treatment strategies for the management of CIN1 are not uniform. There is always a concern amongst women's health providers when a patient has persistent CIN1, which will regress spontaneously in the majority of cases but will progress in a significant minority. Other sensitive molecular markers such as HPV DNA testing has limited utility for identifying the lesions that might progress over time,[5, 49, 50] so providers are forced to closely observe these patients until they have a regressing lesion or they progress. This often adds stress to the patient, as well as the provider, and may lead to more aggressive management (e.g. cryotherapy, loop electrosurgical procedure, or cone biopsy) and over-treatment on the part of the provider [51], with associated long-term risks to future pregnancies [52]. New predictive point-of-care testing markers, such as establishing specific DNA methylation panels that can specifically identify clinically-relevant CIN that have a high risk of progressing have clear clinical implications.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

The study was supported in part by an American Cancer Society Research Scholar Grant (RSG 08-002-01-CCE, MHE), an Einstein Cancer Center Supplement (NIH CA013330, NFS and MHE), the MSTP program at Albert Einstein College of Medicine (NIH T32 GM007288, NAW), and Einstein's Center for Epigenomics.

Dr. Schlecht has served in the past as an advisory board member for *Merck, Glaxo-Smith Klein* and *PDS Biotechnology*. Dr. Einstein has advised, but does not receive an honorarium from any companies. In specific cases in the past 12 months, his employer during the conduct of this study, Montefiore Medical Center has received

payment for time spent for advice related to clinical trials from *Roche, Photocure, Papivax, Natera, Inovio*, and *PDS Biotechnology*. If travel is required for meetings with any industry, the company pays for Dr. Einstein's travel-related expenses. Also, Montefiore has received grant funding for research related costs of clinical trials that Dr. Einstein has been the overall PI or Montefiore PI from *Roche, Photocure, Fujiboro, Eli Lilly, PDS Biotechnology*, and *Becton-Dickinson*.

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RESEARCH HIGHLIGHTS

- There is inconsistent evidence for host gene DNA methylation in early cervical neoplasia.
- We conducted comprehensive mapping of DNA methylation loci in 15 candidate genes.
- Increased methylation of the downstream region of CDKN2A was associated with progression to cervical cancer.

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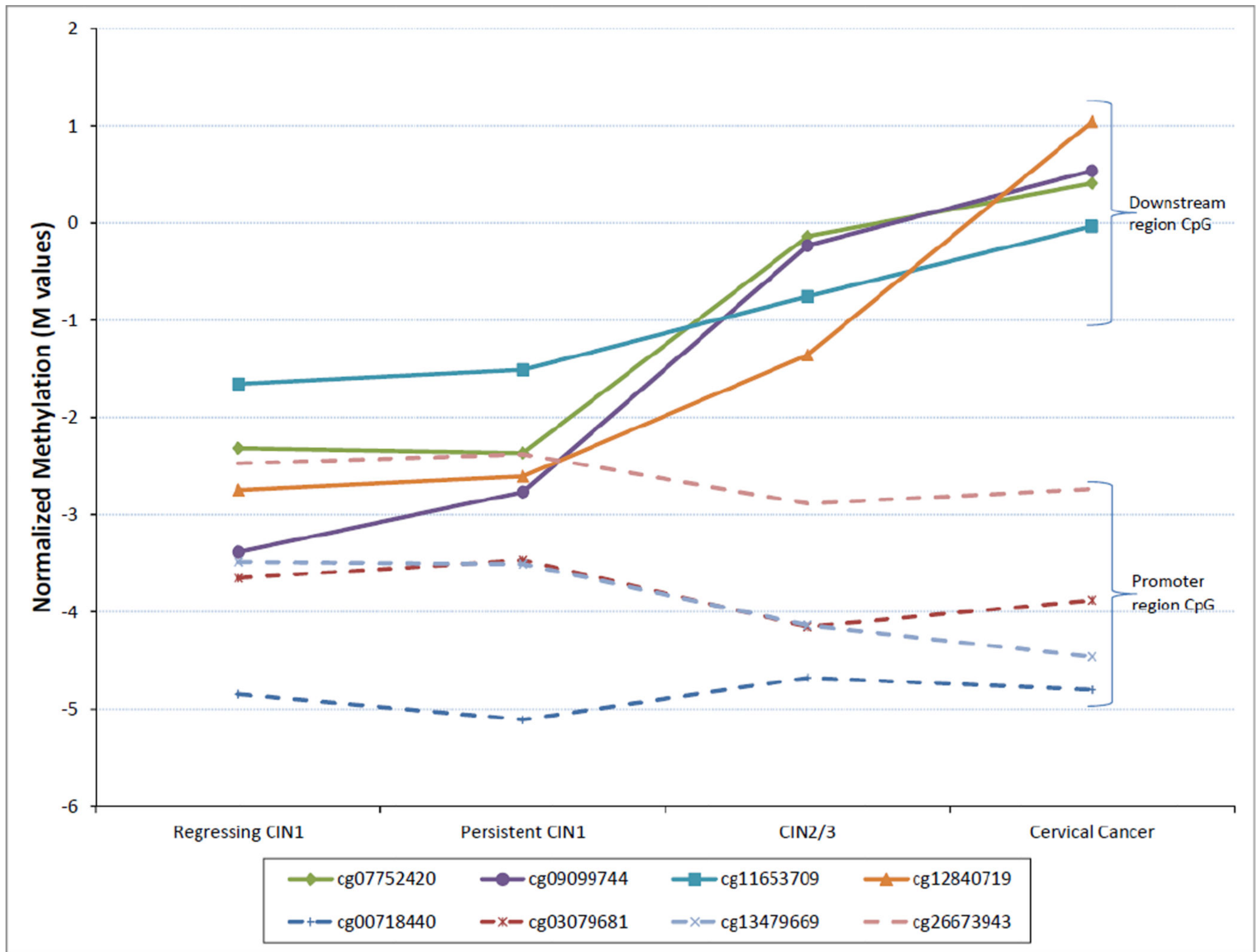

Figure 1.

Median DNA methylation (M) in CIN1 (by disease progression), CIN2/3 and cervical cancer samples for CpG loci within *CDKN2A*. The Illumina CG codes for each CpG tested on the Illumina Infinium (Human Methylation 27) array are listed in the figure legend with the corresponding line markers. CpGs located downstream or near the gene promoter region are indicated in the right margin.

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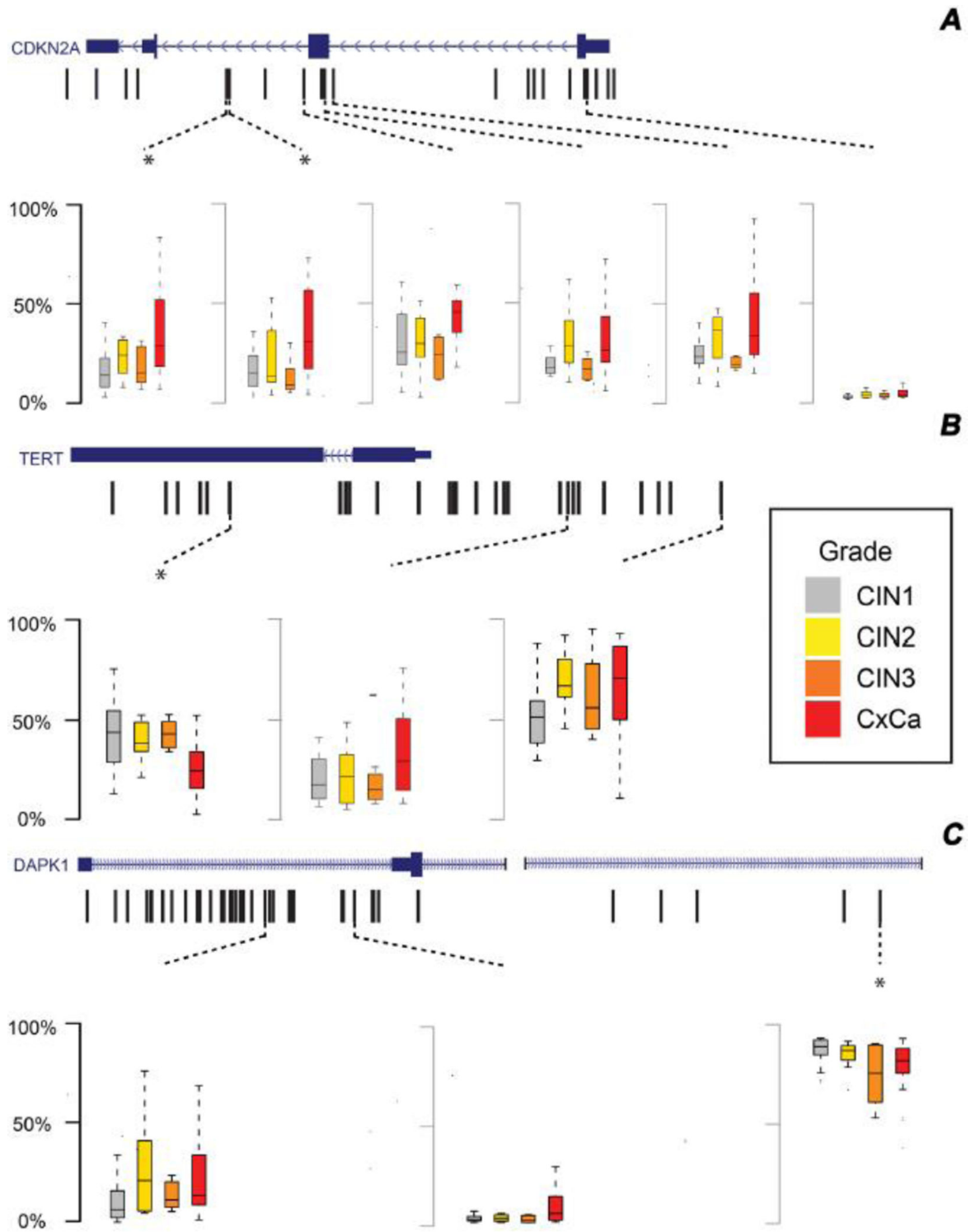

Figure 2.

Gene maps showing CpG loci with significant methylation differences between CIN1, CIN2, CIN3 and cervical cancer samples for (A) *CDKN2A*, (B) *TERT*, and (C) *DAPK1*. Refseq maps for each gene are shown on top, with the CpGs assayed by HELP-tagging indicated by black hash marks. Box plots summarizing median, interquartile range and adjacent percent (%) methylation values are shown below each map for CpGs with significant statistical differences in methylation across lesion groups (uncorrected $p<0.05$), with comparisons that remained significant after Bonferroni correction marked by an asterisk.

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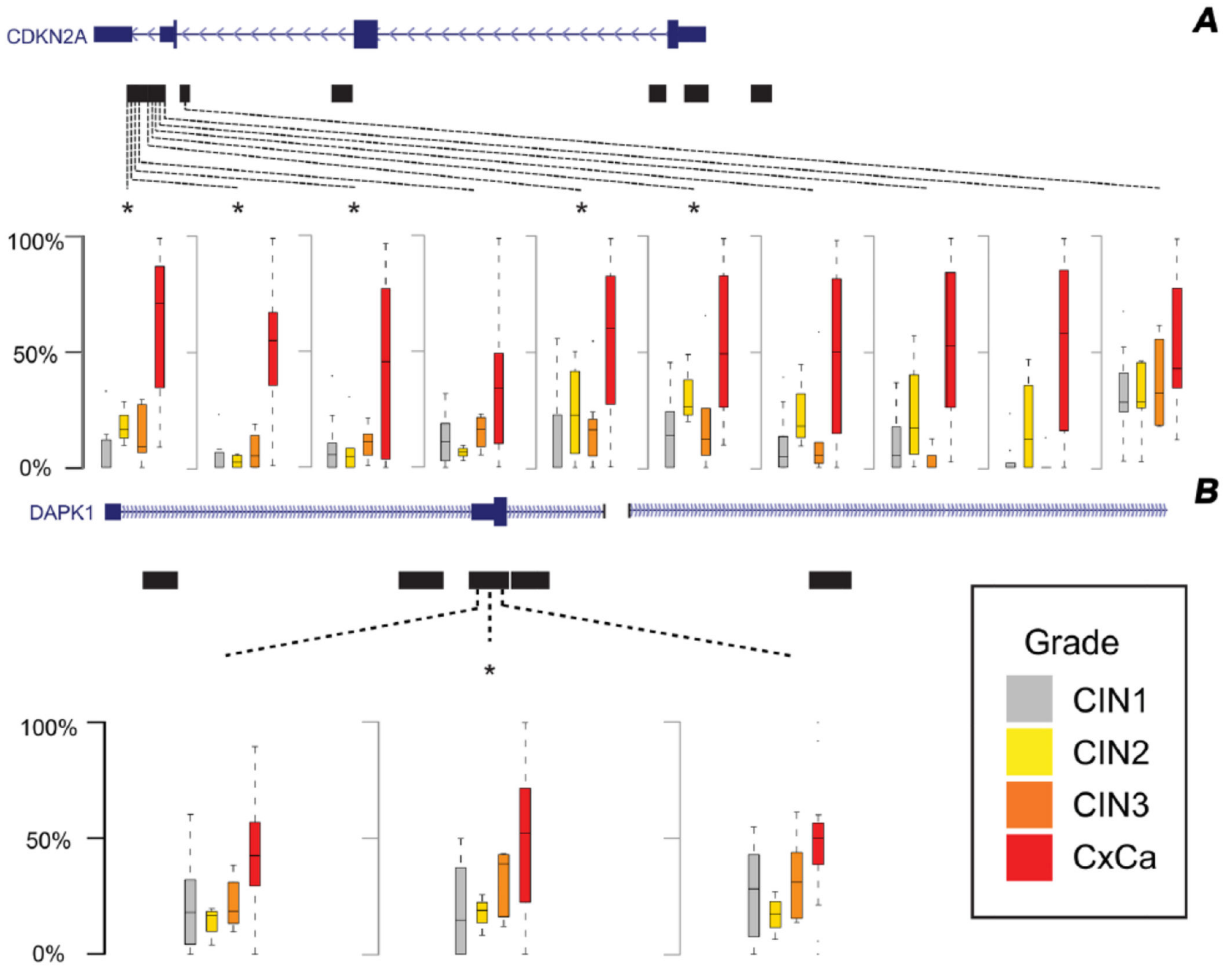

Figure 3.

Gene maps showing CpG regions with significant differences in percent (%) methylation between CIN1, CIN2, CIN3 and cervical cancer samples for (A) *CDKN2A* and (B) *DAPK1* genes as measured by bisulfite sequencing. The mapped regions are shown by black bars. Box plots are shown for CpGs with significant statistical differences in methylation across lesion groups (uncorrected $p<0.05$), with comparisons that remained significant after Bonferroni correction marked by an asterisk.

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Table 1

Distribution of study subject characteristics by disease grade

	CIN1 (N=27)		CIN2 (N=8)		CIN3 (N=10)		Cancer (N=23)	
Age (range)*	37.5	(24–56)	35	(21–52)	37	(29–50)	54.3	(24–75)
Race								
African-American	11	41%	6	75%	4	40.0%	12	52%
Caucasian	10	37%	2	25%	6	60.0%	9	39%
Asian/Other	0	0%	0	0%	0	0.0%	1	4%
Unknown	6	22%	0	0%	0	0.0%	1	4%
Ethnicity								
Hispanic	15	56%	5	62.5%	6	60.0%	5	22%
Non-Hispanic	12	44%	3	37.5%	4	40.0%	18	78%
HIV serostatus								
HIV negative	20	74%	3	37.5%	9	90.0%	22	96%
HIV positive	7	26%	5	62.5%	1	10.0%	1	4%
HPV DNA status								
HPV negative	5	19%	1	12.5%	2	20.0%	0	0%
HPV DNA positive	22	81%	7	87.5%	8	80.0%	23	100%
High-risk HPV positive†	15	56%	5	62.5%	8	80.0%	23	100%

*Mean age (range).

†High-risk HPV included types 16, 18, 31, 33, 35, 39, 45, 51, 52, 56, 58 & 59.