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DNA methylation of imprinted gene control regions in the regression of low-grade cervical lesions

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Abstract

The role of host epigenetic mechanisms in the natural history of low-grade cervical intraepithelial neoplasia (CIN1) is not well characterized. We explored differential methylation of imprinted gene regulatory regions as predictors of the risk of CIN1 regression.

A total of 164 patients with CIN1 were recruited from 10 Duke University clinics for the CIN Cohort Study. Participants had colposcopies at enrollment and up to five follow-up visits over three years. DNA was extracted from exfoliated cervical cells for methylation quantitation at CpG (cytosine-phosphate-guanine) sites and human papillomavirus (HPV) genotyping. Hazard ratios (HR) and 95% confidence intervals (CI) were estimated using Cox regression to quantify the effect of methylation on CIN1 regression over two consecutive visits, compared to non-regression (persistent CIN1; progression to CIN2+; or CIN1 regression at a single time-point), adjusting for age, race, high-risk HPV (hrHPV), parity, oral contraceptive and smoking status.

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Median participant age was 26.6 years (range: 21–64.4 years), 39.0% were African-American, and 11% were current smokers. Most participants were hrHPV-positive at enrollment (80.5%). Over one-third of cases regressed (n=53, 35.1%). Median time-to-regression was 12.6 months (range: 4.5–24.0 months). Probability of CIN1 regression was negatively correlated to methylation at *IGF2AS* CpG 5 (HR=0.41; 95% CI=0.23–0.77) and *PEG10* DMR (HR=0.80; 95% CI=0.65–0.98).

Altered methylation of imprinted *IGF2AS* and *PEG10* DMRs may play a role in the natural history of CIN1. If confirmed in larger studies, further research on imprinted gene DMR methylation is warranted to determine its efficacy as a biomarker for cervical cancer screening.

Keywords

methylation; imprinted genes; cervical cancer; neoplasia; epigenetics; HPV

Introduction

As of 2014, an estimated 250,000 women were living with cancer of the cervix in the United States (US).[1] At current incidence and mortality rates, approximately 13,000 women will be diagnosed with cervical cancer in the US in 2017, resulting in over 4,000 subsequent deaths.[2] Though overall rates of cervical cancer in the US have decreased over time, the highest rates of cervical cancer incidence and mortality occur in the Southern states.[2]

Nearly all invasive cervical cancers are caused by the human papillomavirus (HPV), a sexually transmitted infection that affects over 79 million people in the US.[3–5] While most HPV infections clear spontaneously, oncogenic or high-risk HPV (hrHPV) types often lead to persistent HPV infection and subsequent high-grade cervical intraepithelial neoplasia (CIN2+), a risk factor for progression to invasive cervical cancer.[6]

Current cervical cancer prevention strategies include the use of cytology-based testing (Pap testing) as a primary screening tool, with the addition of HPV testing to increase screening sensitivity for the detection of CIN2+ among women 30 years and older, as well as primary hrHPV screening.[7] HrHPV testing is more sensitive, although less specific, than liquid-based cytology for the detection of high-grade (CIN2+).[8] A relatively small proportion of low-grade CIN cases progress to CIN2+, while most CIN1 cases regress to normal epithelia.[9] Follow-up of low-grade CIN is recommended in the US until regression to normal colposcopic impression or negative cytology, leading to a high burden of cost and decreased clinical visit adherence.[7, 10] Therefore, it would be advantageous to identify novel biomarkers that can differentiate CIN1 cases which progress from CIN1 cases which regress.

Epigenetic profiles have been hypothesized as potential diagnostic biomarkers for susceptibility to cervical cancer.[11, 12] Modifications of the epigenome include DNA methylation at cytosine-guanine dinucleotide sequences (CpG sites) which can affect the expression of genes involved in cancer tumorigenesis.[13] Genomic imprinting involves inheritance of parent-of-origin specific epigenetic modifications controlling allele-specific gene expression.[12, 13] Imprinted genes often exist in clusters and are regulated by imprinting

centers, which can include differentially methylated regions (DMRs) that are rich in CpG sites.[13]

Loss of imprinting (LOI) due to aberrant methylation at DMRs has been linked to various growth and developmental disorders,[14] including Beckwith-Wiedemann Syndrome (BWS).[15] In case-control studies, differential methylation of targeted imprinted genes has been associated with cancer outcomes, such as Wilms' Tumor of the kidney,[16] breast cancer,[17, 18] colorectal cancer,[11, 19, 20] and prostate cancer.[21] Preliminary analyses have also found dysregulated expression of imprinted genes involved in tumor suppression (e.g. *HYMAI, PEG3, PLAGL1, MEST, CDKN1C*) in cervical cancer specimens compared to normal cervical tissue.[22] Studies have examined the influence of methylation patterns on the expression of HPV E6/E7 oncogenic proteins which deactivate host cell tumor suppressor p53 and thus may promote cervical carcinogenesis.[23, 24] The influence of host aberrant methylation at imprinted gene control regions on the natural history of low-grade CIN has not been assessed.

It is important to establish molecular-based methods of differentiating CIN1 cases which progress versus regress to improve clinical management. The current study examines whether aberrant DNA methylation patterns of imprinted genes influence regression of low-grade CIN in the Cervical Intraepithelial Neoplasia Cohort Study (CINCS).

Materials and Methods

Study population

From June 2010 – April 2014, women attending ten Duke University hospitals and clinics in Durham, North Carolina were invited to participate in CINCS, as previously described.[25] Briefly, all clinics used Duke-affiliated pathology laboratories for cytology and histological evaluation. The CINCS cohort is comprised of 1,303 women who were referred for a colposcopy following an abnormal liquid-based cytology result. Participants were eligible if they provided written consent, were new visitors to the clinic, 21–79 years old, English or Spanish speakers, and able to give informed consent. We excluded women who had received previous treatment for cervical lesions—cold knife conization (CKC), electrosurgical excision procedure (LEEP), cryotherapy, or hysterectomy; had moved out of the study area; or did not intend to receive follow-up care at one of the 10 Duke clinics. Women who were diagnosed with CIN1 at enrollment and had at least one follow-up visit with HPV and methylation data were included in the present statistical analyses. Approval for this study was granted by the Institutional Review Boards at Duke University (Durham, NC, USA), North Carolina State University (Raleigh, NC, USA) and University of North Carolina (Chapel Hill, NC, USA).

Data collection and laboratory analyses

At enrollment, participants had a physician-directed cervical examination with a colposcopy-directed biopsy. Women diagnosed with CIN1 by colposcopic impression without biopsy (n=29) at enrollment were also included in the study, as prevalence of hrHPV (63%) was comparable to the hrHPV prevalence among those who underwent a biopsy

(59%). Study participants attended a clinical visit approximately every 6 months for the first two years, and every 12 months for the final third year. During each follow-up visit, all women underwent a liquid-based cytology (LBC) test. For participants with abnormal cytology results, clinic physicians performed colposcopy examination. Directed biopsies at follow-up colposcopy visits occurred only if clinically necessary, according to the physician's best judgement and per clinical guidelines for management of precancerous cervical lesions.[7] Study staff administered a questionnaire to ascertain information on any behavioral and clinical characteristics at enrollment and follow-up visits, including age, race/ethnicity, current smoking status, history of oral contraceptive use and parity.

Ascertainment of Cervical Cytology and Histology

To conduct a LBC test, the clinic physician utilized a spatula and cytobrush to obtain exfoliated cervical cells. Cervical exfoliated cell specimens were suspended in a vial containing ThinPrep® solution (Hologic®, Malborough, MA, USA) for cytological assessment. All study clinic pathologists evaluated LBC cytology according to Bethesda criteria.[26] The residual LBC cervical exfoliated cell specimens were stored at 4°C prior to HPV DNA testing.

Biopsy results were also reviewed and graded for severity by a pathologist at Duke-affiliated pathology laboratories. All histological biopsy specimens were tested for adequacy using the 2012 American Society for Colposcopy and Cervical Pathology (ASCCP) guidelines.[7] Information on cytology and histology were abstracted from patient medical records.

HPV Testing and Typology

HPV typology was assessed using cervical exfoliated cells from the enrollment pelvic exam. ThinPrep® specimens were collected during the same enrollment visit and sent to Johns Hopkins University and the University of Hawaii Cancer Center for laboratory testing, as previously described.[27, 28] Following DNA extraction, HPV status was determined by targeted amplification of a 450bp region of the HPV L1 genome using PGMY09/PGMY11 primers.[27, 28] Amplification of the human β-globin gene was included as an internal control for sample sufficiency. Specimens identified as HPV-positive were subsequently genotyped using the HPV Linear Array® (Roche Diagnostics, Branchburg, NJ, USA). This assay is designed up to 37 high-risk and low-risk genotypes.

Assessment of DNA Methylation in Imprinted Differentially Methylated Regions (DMRs)

Nucleic acid extraction—DNA was extracted from the LBC cell pellet using a protocol for simultaneous nucleic acid extraction provided by Teltest (Friendswood, TX) for DNA Stat60 reagents. Nucleic acids were aliquoted, barcoded, and stored at −80°C until required.

DNA methylation analysis—DNA methylation was measured using genomic DNA at differentially methylated regions (DMRs) regulating genomic imprinting of *IGF2/H19*, *IGF2AS* (*IGF2*-antisense), *MESTIT1/MEST*, *Kv*DMR, *MEG3*, *PLAGL1/HYMAI*, and *PEG3*, *PEG10* imprinted domains, using Sequenom (San Diego, CA) MassARRAY EpiTYPER assays. Bisulfite-treated DNA was processed using the EZ-96 DNA Methylation Kit (Zymo Research Corporation, Irvine, CA) to convert unmethylated DNA cytosine bases

to uracil bases, leaving methylated cytosines unchanged per manufacturer's protocol. We used Sequenom (San Diego, CA) EpiDesigner software to design primers complementary to bisulfite-converted DNA in regions without CpG nucleotides, adding a T7 promoter site to all forward primers. Polymerase chain reaction (PCR) assays were performed on the treated DNA samples using HotStarTaq DNA Polymerase (Qiagen, Hilden, Germany). PCR products were treated with Shrimp alkaline phosphatase (SAP, Sequenom, San Diego, CA) followed by transcription and T cleavage reactions according to the protocol. Cleanup and sequencing were performed according to the EpiTYPER user guide. Matrix-assisted laser desorption/ionization and time-of-flight mass (MALDI-TOF) spectrometry analysis was performed on resulting transcripts using the MassARRAY system (Sequenom). Fragments generated from the PCR assay differed by size and mass, allowing for quantification of methylated forms of each targeted fragment.

Statistical analyses

Methylation percentage was calculated at each CpG site of each imprinted gene DMR. Because imprinted gene DMRs are characterized by having one fully methylated allele and one unmethylated allele, the expected value of methylation for an imprinted gene in a diploid cell is approximately 50%. A total of 8 imprinted DMRs were considered *a priori* with 5 CpG sites for the *IGF2/H19* DMR; 10 CpGs for the *IGF2AS* DMR; 31 CpGs for the *MEST/MESTIT1* DMR; 27 CpG sites for the *Kv* DMR; 31 CpG sites for the *MEG3* DMR; 8 CpG sites for the *PLAGL1/HYMAI* DMR; 11 CpG sites for the *PEG10* DMR; and 12 CpG sites for the *PEG3* DMR. Genomic coordinates for each DMR have been previously published.[29] Four DMRs were excluded from the analyses due to extensive missing data at CpG sites. As a result, DMRs in the analysis included *IGF2AS*, *MEG3*, *PEG10* and the *Kv* DMR. Median percentages were calculated across DMRs to estimate methylation for *cis*-acting CpGs at a given region[12].

Regression of cervical lesions was defined as a diagnosis of negative/normal cytology (or histology if applicable) at two consecutive follow-up visits. Cytology results were utilized to determine regression status if the participants had missing histology data given no biopsy was performed, per conservative clinical practice. Women with a negative/normal screening cytology or histology at one follow-up time point only (e.g. regressed to negative/normal at the first follow-up visit, and had cervical abnormalities at the subsequent visit) were not considered to have regressed for the main study analyses. Cervical lesion persistence was defined as a diagnosis of low-grade histology (CIN1) at follow-up or low-grade lesions during cytology testing (e.g. low-grade squamous epithelial lesions (LSIL), or atypical squamous cells of undetermined significance (ASC-US). Progression was defined as a follow-up histological diagnosis of CIN2+, or as a cytological diagnosis of high-grade squamous epithelial lesions (HSIL), LSIL-H (LSIL, cannot exclude HSIL), or ASC-H (ASC, cannot exclude HSIL). For women who received treatment (LEEP, CKC, cryotherapy, or hysterectomy) at a follow-up visit, the histological diagnosis from the pre-treatment specimen was utilized.

A univariate analysis was performed to assess the distribution of methylation biomarkers and covariates. Kaplan-Meier product-limit method was used to estimate the cumulative

proportion of CIN1 regression, stratified by median methylation percentage at each DMR. The Log-rank test was used to assess differences between regression probabilities over time at methylation percentages below and above the median for each DMR (Figures 1a–1d). Cox proportional hazards regression models were employed to estimate unadjusted hazard ratios (HR) and 95% confidence intervals (95% CI) to examine associations between methylation at a specific CpG site, and CIN1 regression. Time-to-regression was measured from the date of enrollment to the date of the second consecutive negative/normal histological or cytological diagnosis. Participants contributed person-time to the longitudinal analyses up to the occurrence of regression or the date of the last attended clinical study visit. Participants who received treatment during the study were right censored at the date of procedure. Administrative censoring occurred at 3 years. Woman-months were calculated as the sum of person-time for all women at risk among each specific methylation exposure group.

We calculated a median methylation percentage to represent a summary measure of methylation across each candidate region (when applicable) to estimate HRs and 95% CIs in the univariate and multivariate Cox regression models. In the Cox models, methylation levels (treated as a continuous variable) were rescaled using the interquartile range (IQR) for each CpG site or the IQR for median methylation across the gene DMR. Confounders selected for the multivariable Cox regression model were determined *a priori* using conceptual models (directed acyclic graphs).[30] Covariates considered for the analyses included continuous age at enrollment, race/ethnicity, current smoking status at enrollment (current vs. non-current), history of oral contraceptive use (ever vs. never), parity (continuous), and hrHPV infection at enrollment. We considered a 2-level hrHPV variable (16,18, 31, 33, 35, 39, 45, 51, 52, 56, 58, 66, 68) infection vs. non-hrHPV infection, as well 3-level hrHPV variable (HPV-16/18 infection vs. non-HPV-16/18 hrHPV infection vs. lrHPV/no infection). Covariate modification was assessed using Akaike Information Criteria for model fit.

A sensitivity analysis was conducted to determine the change in estimate given a regression event at CIN1 regression at one follow-up visit. Further sensitivity analyses were also conducted to determine the impact of drop outs; exclusion of women who were hrHPV negative at enrollment; and the exclusion of women who had high-grade cytology (HSIL or higher) at their enrollment pap (preceding the enrollment colposcopy). All statistical analyses were conducted using SAS version 9.4 (SAS Institute, Cary, NC).

Of the 1,303 enrolled CINCS participants, 495 participant samples were tested for methylation (38%), of which 486 (98%) had HPV DNA laboratory results. Of these, 15 women with no CIN diagnosis, 230 women with a negative/normal histological diagnosis at enrollment, and 75 women with CIN2+ histological diagnosis were excluded. The remaining 164 CIN1 cases at enrollment were included in analyses.

Results

Median age of CIN1 cases at enrollment (n=164) was 26.6 years (range: 21–64.4 years; Table 1). Nearly half of participants were non-Hispanic White (47%), while over one-third were Black (39%). Only 11% of participating women were current smokers, whereas most

had a history of oral contraceptive use (78%). Over 80% of participants had laboratory-confirmed infection with any hrHPV type at enrollment (81.3%). A total of 25 women had infection with either HPV16 or 18: 12% had HPV-16 infection, 5.3% with HPV-18 and 0.6% with both. The most prevalent hrHPV genotypes in the sample were HPV 66 (19.9%) and HPV 51 (13.3%; Table A1 in Appendix).

Median DMR methylation levels were 58% for *IGF2AS*; 29% for *Kv* DMR, 52% for *MEG3*, and 32% for *PEG10* (Table 2). Within the *Kv* and *PEG10* DMRs, there was ≤30% methylation at most CpG sites. There was little variation in *Kv* DMR methylation (DMR IQR = 0.07) and in *PEG10* methylation (DMR IQR = 0.07) among participating women with CIN1.

Median study follow-up time was 10.5 months (range: 0.9–30.8). Thirteen women (n=13; 8.5%) dropped out after enrollment. No differences were observed between those who dropped out (n=13) compared to those who had at least one follow-up visit (n=151; data not shown). Over the 3-year study duration, a total of 53 (35.1%) women regressed from CIN1, compared to 98 (64.9%) who did not regress (including 41 women with persistent CIN1, 20 women progressed to CIN2+, and 37 women who regressed at only one visit). Median time to CIN1 regression was 12.6 months (range: 4.5–24.0 months). Approximately 60% of follow-up diagnoses assessed for the longitudinal analysis were defined by pathology-confirmed histology (57%), as a large proportion of women (43%) with abnormal screening results had CIN1 colposcopic impression at their follow-up colposcopy visit and thus did not warrant a biopsy, per the clinician's best judgement.

The unadjusted estimated cumulative probability of CIN1 regression plots showed that women with methylation percentages above the median at *IGF2AS* CpG 5, and at the *PEG10* DMR had lower incidence of regression over a 3-year period as compared to women with methylation percentages below the median (Figures 1a & 1d). No notable differences were observed in CIN1 regression probability at the *Kv* and *MEG3* DMRs (Figures 1b–1c).

There was at least a 40% decrease in the probability of CIN1 regression for women with higher methylation versus lower methylation at *IGF2AS* CpG 5 (unadjusted HR, 0.57; 95% CI: 0.34, 0.79; Table 3). At the *PEG10* DMR, women with higher methylation had a 21% decrease in likelihood of CIN1 regression versus lower methylation (unadjusted HR, 0.79; 95% CI: 0.65–0.97). After adjusting for continuous age, hrHPV status, race, current smoking status, continuous parity and history of oral contraceptive use, estimates for CIN1 regression were similar to unadjusted estimates— the probability of CIN1 regression decreased by 59% for women with higher methylation at *IGF2AS* CpG 5 versus lower methylation (adjusted HR, 0.41; 95% CI, 0.23–0.76). Within the *PEG10* DMR, the likelihood of CIN1 regression decreased by 20% for women who had higher methylation (adjusted HR: 0.80, 95% CI, 0.65–0.98 versus lower methylation).

No differences in CIN regression rates were observed stratified by hrHPV status (Table A2 in Appendix). Similarly, there was no evidence of modification by race/ethnicity or by other covariates (data not shown). Data suggest potential modification by age, where women 25

years of age and older with higher methylation at MEG3 DMR had 50% decreased likelihood of regression (HR, 95% CI: 0.54, 0.32–0.92; Table 4).

A sensitivity analysis was conducted to consider time to CIN1 regression at a single follow-up visit, and increased methylation at both *PEG10* and *IGF2AS* DMRs remained associated with a decreased probability of CIN1 regression (refer to Appendix Table A1). An increase in methylation at *Kv* DMR resulted in a 20% decrease in the probability of first CIN1 regression (adjusted HR: 0.81; 95% CI, 0.63–1.01). Exclusion of either hrHPV-negative participants or participants with high grade cervical cytology at their enrollment Pap test did not significantly change adjusted HR estimates (data not shown).

Discussion

This longitudinal study of 164 CIN1 patients is among the first to prospectively examine aberrant methylation patterns of regulatory regions of imprinted genes and their association with low-grade CIN regression. Women with higher levels of methylation at the *IGF2AS* DMR CpG 5 and the *PEG10* DMR had a lower 3-year cumulative probability of CIN1 regression as compared to women with lower levels of methylation. A decrease in the probability of CIN1 regression due to increased methylation at *Kv* DMR was also observed over one follow-up visit. These findings may implicate these DMRs as potential epigenetic biomarkers of a lower regression potential in low-grade CIN cases, and thus higher risk of high-grade precancer or more severe disease.

IGF2AS is a paternally expressed component of a downstream imprinted center, IC1 (located on human chromosome 11p15.5) that promotes cell proliferation.[31] Abnormal methylation of *IGF2* may be associated with mechanisms involved in cervical tumorigenesis. Aberrant DNA methylation of the *IGF2* DMRs and other sequences regulating imprinted genes have been previously associated with higher risk of cervical dysplasia and invasive cancer in cross-sectional and case studies in Tanzania and France.[32, 33] In contrast with our findings, decreased methylation at the *IGF2* DMR was associated with an increased risk of invasive cervical carcinoma.[33] Though a notable association was found between aberrant methylation at *IGF2AS* CpG 5, we could not make conclusions regarding the entire regulatory *IGF2AS* region from these data. However, the presence of abnormal methylation patterns at *IGF2* warrants additional research.

Paternally-expressed *PEG10* also appears to have a role in increased cell proliferation.[34] Decreased methylation was associated with overexpression of *PEG10* in hepatocellular carcinoma samples.[35] In contrast, our current findings demonstrated that an increase in methylation of *PEG10* DMR may be associated with a higher risk of cervical precancer, as evidenced by a lower risk of CIN1 regression. It is possible that regulatory mechanisms of *PEG10* may differ in CIN from its involvement in hepatocellular carcinogenesis. Further investigation on the relationship between *PEG10* methylation and CIN development in a larger cohort may provide insight on methylation patterns that are indicative of the risk of CIN progression.

Interestingly, age appeared to modify regression rates for *DLK1/MEG3* DMR. Relatively older women (≥ 25 years of age) were less likely to regress over time given increased methylation relative to younger women. The *MEG3* DMR is maternally expressed and is reciprocally imprinted with paternally-expressed *DLK1* on chromosome 14q32[36]. Though little research describes aberrant methylation of *MEG3*, hypermethylation has been implicated as a potential biomarker in cervical cancer.[37] When estimating time-to-regression at a single follow-up visit, increased methylation at the *Kv* DMR also decreased the probability of regression relative to women who had lower levels of methylation. The *Kv* DMR is maternally methylated, comprised of the imprint control region IC2 located at Chr11p15.5, which regulates at least 11 imprinted genes.[38] Imprinting at this region controls transcription of the long non-coding RNA *KCNQ1OT1*, which regulates the expression of *cyclin-dependent kinase inhibitor 1C* gene (*CDKN1C*), an inhibitor protein involved in cell proliferation and growth regulation.[39] While studies on *Kv* DMR in the context of cervical dysplasia and ICC are limited, changes in methylation at *Kv* DMR/IC2 have been positively associated with colorectal cancer[20] and breast cancer.[17]

Infection with hrHPV, the primary cause of invasive cervical cancer, may interact with regulation of imprinted genes among women with cervical dysplasia. Hypermethylation of imprinted genes *PEG3* and *PEG1/MEST* were positively correlated with hrHPV infection, suggesting it may serve as an intermediate in CIN development.[33, 40] Imprinted tumor suppressor *CDKN1C*, controlled by IC2/*Kv* DMR, was upregulated during E2 (HPV viral regulatory protein)-mediated HeLa cell senescence and concomitant repression of E6/E7 HPV viral oncogenes.[41] These findings implicate downregulation of *CDKN1C*, leading to upregulated cervical cell proliferation and subsequent cervical tumor development.[41] Inhibition of cell apoptosis due to loss of E2 expression in cervical carcinogenesis may be mediated in part by aberrant methylation and subsequent deregulation of pivotal genes, some of which are imprinted and implicated in cervical cancer development pathways.[33, 41]

A major advantage of this study was the ability to prospectively assess the association between methylation markers and CIN. Accounting for time in estimating the probability of CIN1 regression improved the strength of the association. The findings here, comparable to previous studies, further support the consideration of imprinted gene biomarkers as a screening tool for LSIL/CIN1 cases.[33, 40]

Among potential limitations, this study did not assess HPV infection at study follow-up, which would have allowed for a more stringent definition of cervical lesion regression, including HPV negative status at follow-up. However, the decision to define the cervical regression as two consecutive negative screening results, rather than one, strengthened the robustness of the outcome. We also had limited power to assess the association of methylation on CIN regression stratified by individual HPV type, although no evidence of modification was found. Future work should include capturing type-specific HPV infection status at all follow-up visits with a larger cohort to determine the extent to which persistent hrHPV infection plays a role in the association between methylation patterns and the natural history of cervical dysplasia. The current study was also limited by the possibility that women with CIN1 at enrollment may have been misclassified. To address this, a sensitivity analysis was conducted by excluding women with high-grade cervical cytology at

enrollment, producing similar results. The 2012 ASCCP guideline update,[7] which shifted follow-up algorithms for CIN management, likely affected the variability in the number of visits and duration between follow-up visits for each participant. Obtaining data on changes in smoking habits and other time-dependent behavioral/lifestyle factors also would broaden future research in order to investigate their influence on methylation patterns among women with CIN.[42] The incorporation of RNA/gene expression data would further characterize the influence of aberrant methylation among women with and without disease.

It is critical to understand risk factors that determine the natural course of CIN in order to improve the effectiveness of current cervical cancer screening methods. These study findings indicate further investigation into *IGF2* and *PEG10* DMRs as diagnostic biomarkers in women with low-grade CIN is warranted. Characterization of potential cervical tumorigenesis pathways related to the dysregulation of imprinted gene networks would help to establish novel epigenetic biomarkers in CIN management to reduce cervical cancer incidence, while avoiding the unnecessary follow-up of patients at relatively lower risk of progression to high-grade precancerous lesions or more severe.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Novelty and Impact

Few studies have assessed DNA methylation of imprinted genes as a potential biomarker for cervical dysplasia. Successful characterization of the natural history of low-grade cervical lesions may improve current screening approaches.

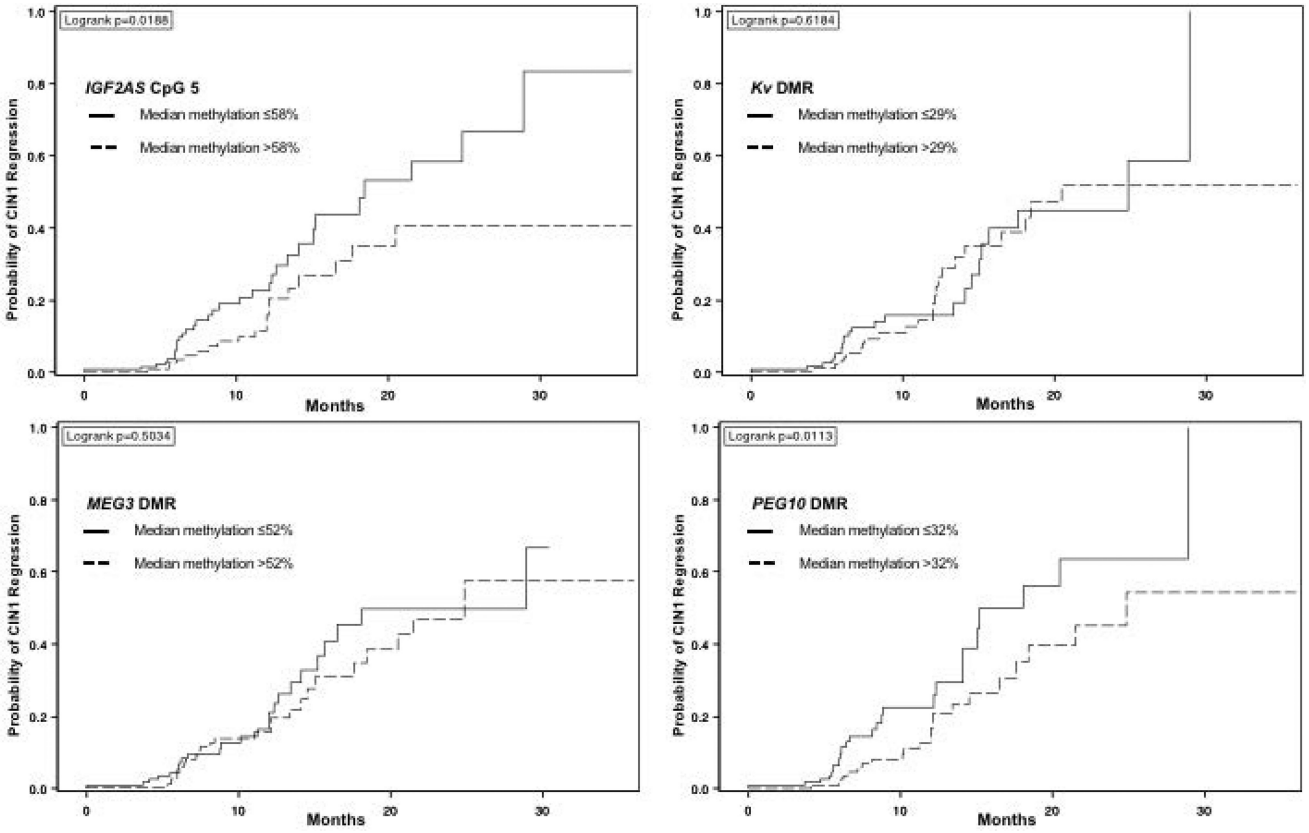

Figure 1.

a. Time to CIN1 regression for *IGF2AS* (at CpG 5), stratified at median methylation percentage*

*CIN = cervical intraepithelial neoplasia

b. Time to CIN1 regression for *Kv* DMR, stratified at median methylation percentage*

* CIN = cervical intraepithelial neoplasia; DMR = differentially methylated region (median methylation of all CpG sites)

c. Time to CIN1 regression, stratified at median *MEG3* DMR methylation percentage*

* CIN = cervical intraepithelial neoplasia; DMR = differentially methylated region (median methylation of all CpG sites)

d. Time to CIN1 regression, stratified at median *PEG10* DMR methylation percentage*

* CIN = cervical intraepithelial neoplasia; DMR = differentially methylated region (median methylation of all CpG sites)

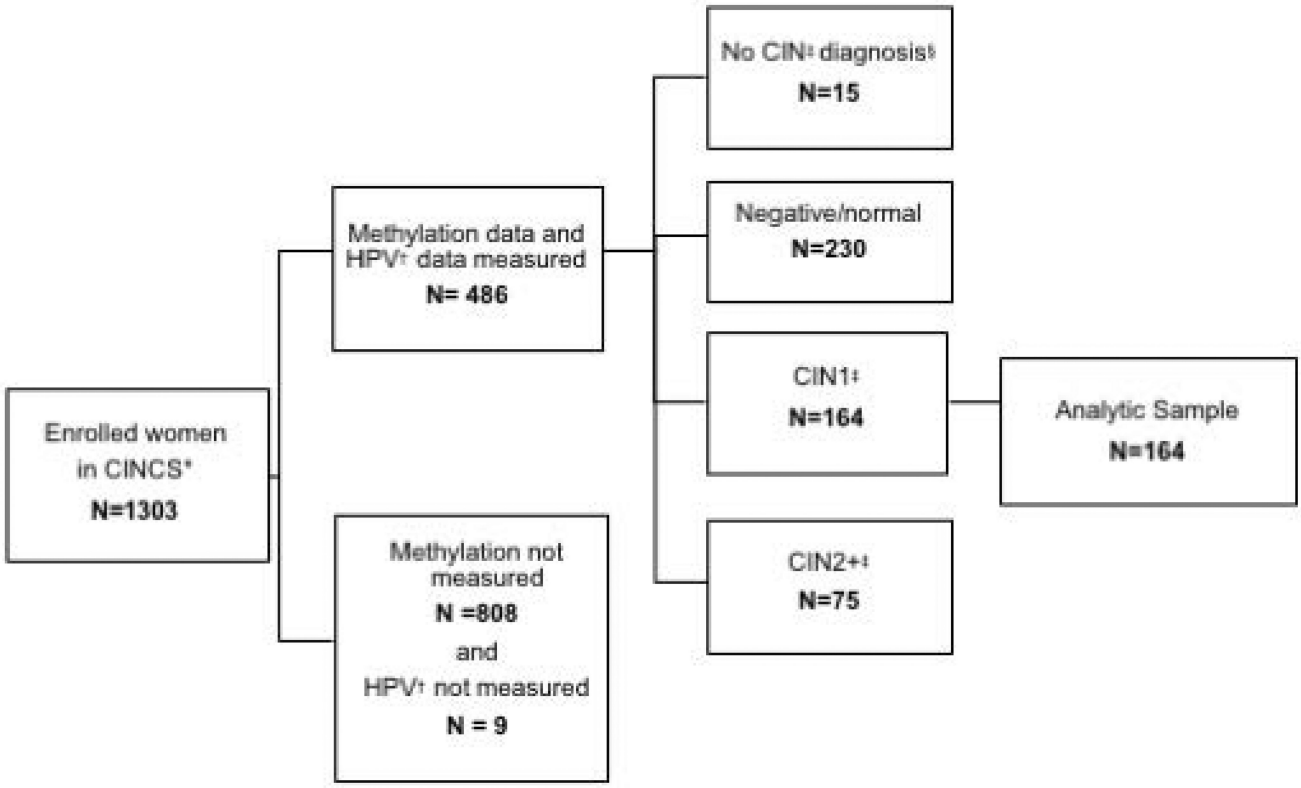

Figure 2.

CINCS* study population flowchart

* CINCS = Cervical Intraepithelial Neoplasia Cohort Study

† HPV = Human papillomavirus

‡ CIN= Cervical intraepithelial neoplasia

§ No colposcopy/unable to grade

Table 1

Characteristics of 164 women with CIN1 at enrollment in the CINCS Study [*]

Enrollment characteristic	N	(Range)%
Age (years)		
Median	26.6	(21.0–64.4)
18–24	65	39.6
25–29	56	34.2
30–34	13	7.9
35+	30	18.3
High-Risk HPV [†]		
Negative	32	19.5
Non-16/18 Positive[‡]	106	64.6
16/18 Positive	26	15.9
Race		
Non-Hispanic White	77	47.0
Black/African-American	64	39.0
Other[§]	23	14.0
Current Smoker		
No	146	89.0
Yes	18	11.0
Ever Use of Oral Contraceptives[//]		
No	33	21.7
Yes	119	78.3
Parity[//]		
Nulliparous	93	57.4
Primiparous (1)	29	17.9
Multiparous (2+)	40	24.7

[*] CIN = Cervical Intraepithelial Neoplasia; CINCS = Cervical Intraepithelial Neoplasia Cohort Study

[†] HPV=human papillomavirus

[‡] Includes high-risk HPV types 31, 33, 35, 39, 45, 51, 52, 56, 58, 59 and 68

[§] Other includes Hispanic/Asian/Pacific Islander/Native American/Multiracial

[//] Numbers do not add up to the total sample size due to missing data

Table 2

Imprinted gene DMR[§] methylation distributions in 164 CIN1 patients at Enrollment[*]

Gene Name	CpG site	N[†]	Mean	Median	SD[‡]	Interquartile Range	Range
IGF2AS	5	157	0.47	0.58	0.32	0.57	(0.00, 0.96)
Kv							
	DMR[§]	144	0.30	0.29	0.05	0.07	(0.16, 0.42)
	1	143	0.25	0.26	0.06	0.07	(0.12, 0.39)
	6	144	0.25	0.26	0.06	0.08	(0.08, 0.41)
	8,9	144	0.27	0.27	0.05	0.07	(0.03, 0.40)
	10,11,12	142	0.23	0.23	0.06	0.07	(0.03, 0.38)
	15	144	0.30	0.31	0.06	0.08	(0.14, 0.43)
	17,18	144	0.30	0.31	0.05	0.07	(0.14, 0.49)
	20	144	0.31	0.31	0.06	0.06	(0.10, 0.47)
	21	144	0.30	0.30	0.06	0.05	(0.12, 0.47)
	22	144	0.24	0.24	0.04	0.04	(0.12, 0.43)
	24	144	0.29	0.30	0.08	0.10	(0.11, 0.44)
	26,27	144	0.55	0.55	0.06	0.07	(0.35, 0.70)
MEG3[//]							
	DMR[§]	152	0.49	0.52	0.11	0.19	(0.26, 0.82)
	3	152	0.57	0.59	0.16	0.17	(0.11, 1.00)
	6	150	0.54	0.54	0.09	0.28	(0.31, 0.96)
	15	152	0.40	0.39	0.10	0.14	(0.00, 0.80)
	16	150	0.38	0.38	0.10	0.15	(0.00, 0.82)
	20,21	150	0.43	0.42	0.09	0.14	(0.05, 0.75)
	22	150	0.54	0.54	0.09	0.17	(0.31, 0.96)
	23	152	0.46	0.46	0.10	0.14	(0.21, 0.95)
	26,27,28	152	0.55	0.55	0.11	0.21	(0.03, 0.89)
	29,30,31	152	0.57	0.56	0.12	0.19	(0.12, 1.00)

Gene Name	CpG site	N[†]	Mean	Median	SD[‡]	Interquartile Range	Range
PEG10							
	DMR[§]	140	0.30	0.32	0.08	0.07	(0.00, 0.45)
	5	137	0.31	0.32	0.10	0.07	(0.02, 0.83)
	6	138	0.26	0.27	0.12	0.14	(0.00, 0.47)
	7	139	0.32	0.34	0.10	0.07	(0.00, 0.85)

*CIN = Cervical Intraepithelial Neoplasia; CINCS = Cervical Intraepithelial Neoplasia Cohort Study

†Numbers do not add up to the total sample size due to missing data

‡SD = Standard Deviation

§DMR = Differentially methylated region; Median across all CpG loci for candidate gene

‖*MEG3* intronic differentially methylated region

Table 3

Analysis of CIN Regression, stratified by Imprinted Gene DMR/CpG Site CIN1 cases in North Carolina *

Gene Name	CpG Site	Woman-Months	Regression Events (N) †	Unadjusted HR ‡ (95% CI)	Adjusted HR (95% CI) ‡§
IGF2AS	5	2742	53	0.57 (0.34, 0.94)	0.41 (0.23, 0.76)
Kv					
	DMR//	2514	49	0.81 (0.55, 1.19)	0.83 (0.55, 1.26)
	1	2485	48	0.78 (0.54, 1.14)	
	6	2514	49	0.75 (0.50, 1.12)	
	8,9	2514	49	1.06 (0.74, 1.50)	
	10,11,12	2473	48	0.92 (0.64, 1.30)	
	15	2514	49	0.73 (0.51, 1.05)	
	17,18	2514	49	0.87 (0.59, 1.27)	
	20	2514	49	0.89 (0.67, 1.17)	
	21	2514	49	0.89 (0.66, 1.19)	
	22	2514	49	1.02 (0.76, 1.37)	
	24	2514	49	0.70 (0.45, 0.99)	
	26,27	2514	49	1.10 (0.80, 1.53)	
MEG3 ¶					
	DMR//	2762	53	0.80 (0.52, 1.21)	0.89 (0.57, 1.38)
	3	2762	53	0.88 (0.58, 1.33)	
	6	2720	53	0.90 (0.61, 1.35)	
	15	2762	53	0.67 (0.49, 0.91)	
	16	2741	52	0.76 (0.54, 1.06)	
	20,21	2725	52	0.75 (0.54, 1.03)	
	22	2720	53	0.90 (0.60, 1.35)	
	23	2762	53	0.92 (0.68, 1.25)	
	26,27,28	2762	53	0.78 (0.53, 1.15)	
	29,30,31	2762	53	0.85 (0.57, 1.28)	
PEG10					

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Gene Name	CpG Site	Woman-Months	Regression Events (N)[†]	Unadjusted HR[‡] (95% CI)	Adjusted HR (95% CI) ‡§
	DMR[//]	2517	50	0.79 (0.65, 0.97)	0.80 (0.65, 0.98)
	5	2461	49	0.57 (0.42, 0.78)	
	6	2475	50	0.66 (0.47, 0.92)	
	7	2510	50	0.85 (0.69, 1.05)	

[*] CIN = cervical intraepithelial neoplasia; 151 cases with at least one follow-up visit

[†] Regression event defined as histologically or cytologically negative diagnosis over two consecutive follow-up visits

[‡] HR= Hazard ratio; 95% CI= 95% confidence intervals; continuous methylation levels rescaled using interquartile range for each CpG site

[§] Adjusted for continuous age, high risk-HPV, race, smoking, continuous parity and history of oral contraceptive use

[//] DMR = differentially methylated region (median methylation of all CpG sites)

[¶] *MEG3* intronic differentially methylated region

Table 4

Analysis of CIN Regression Imprinted Gene DMRs among CIN1 cases in North Carolina: Stratified by Age at Enrollment [*]

Gene	CpG Site	Overall	Age <25 years N=56	Age ≥25 years N=78
			HR (95% CI)[†‡]	
IGFAS	5	0.41 (0.23, 0.76)	0.41 (0.17, 0.97)	0.65 (0.34, 1.25)
Kv	DMR[§]	0.83 (0.55, 1.26)	0.73 (0.40, 1.34)	0.86 (0.52, 1.41)
MEG3	DMR[§]	0.89 (0.57, 1.38)	1.53 (0.80, 2.94)	0.54 (0.32, 0.92)
PEG10	DMR[§]	0.80 (0.65, 0.98)	0.60 (0.43, 0.85)	0.88 (0.69, 1.12)

[*] CIN = cervical intraepithelial neoplasia; 151 cases with at least one follow-up visit

[†] HR=Hazard ratio; 95% CI= 95% confidence intervals; continuous methylation levels rescaled using interquartile range for each CpG site

[‡] Adjusted for race, current smoking, continuous parity and history of oral contraceptive use

[§] DMR = differentially methylated region (median of all CpG sites

[‖] *MEG3* intronic differentially methylated region