

DNA Methylation Profiling across the Spectrum of HPV-Associated Anal Squamous Neoplasia

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Abstract

Background: Changes in host tumor genome DNA methylation patterns are among the molecular alterations associated with HPV-related carcinogenesis. However, there is little known about the epigenetic changes associated specifically with the development of anal squamous cell cancer (SCC). We sought to characterize broad methylation profiles across the spectrum of anal squamous neoplasia.

Methodology/Principal Findings: Twenty-nine formalin-fixed paraffin embedded samples from 24 patients were evaluated and included adjacent histologically normal anal mucosa (NM; n = 3), SCC-*in situ* (SCC-IS; n = 11) and invasive SCC (n = 15). Thirteen women and 11 men with a median age of 44 years (range 26–81) were included in the study. Using the *SFP10 LIPA HPV-typing system*, HPV was detected in at least one tissue from all patients with 93% (27/29) being positive for high-risk HPV types and 14 (93%) of 15 invasive SCC tissues testing positive for HPV 16. Bisulfite-modified DNA was interrogated for methylation at 1,505 CpG loci representing 807 genes using the Illumina GoldenGate Methylation Array. When comparing the progression from normal anal mucosa and SCC-IS to invasive SCC, 22 CpG loci representing 20 genes demonstrated significant differential methylation (p < 0.01). The majority of differentially methylated gene targets occurred at or close to specific chromosomal locations such as previously described HPV methylation “hotspots” and viral integration sites.

Conclusions: We have identified a panel of differentially methylated CpG loci across the spectrum of HPV-associated squamous neoplasia of the anus. To our knowledge, this is the first reported application of large-scale high throughput methylation analysis for the study of anal neoplasia. Our findings support further investigations into the role of host-genome methylation in HPV-associated anal carcinogenesis with implications towards enhanced diagnosis and screening strategies.

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Introduction

Anal squamous cell cancers (SCC) account for 4% of all lower gastrointestinal tract malignancies in the United States with an estimated 6,230 new cases and 780 deaths expected in 2012 [1]. The incidence of anal cancer continues to rise steadily, with an average increase of 2.4% per year reported between 1992 and 2009 [2,3]. Infection with human papillomavirus (HPV) has been demonstrated to be the primary causative agent in the development of SCC of the anogenital tract, including cancers of the cervix, vulva, vagina and anus [4]. Although infection with HPV is a common event, the subsequent development of cancer is rare, suggesting that additional molecular events are required for malignant transformation [5].

Anal intraepithelial neoplasia (AIN) is the precursor lesion of invasive anal SCC. It is generally thought that high grade AIN (HGAIN) or carcinoma *in situ*, but not low grade lesions, are at risk of malignant progression to invasive anal SCC. It is estimated that the risk of malignant progression of HGAIN is approximately 10% but may be higher in immunocompromised individuals [6]. Consequently, the optimal method for screening and managing patients with HGAIN (e.g. prophylactic treatment vs. observation) remains somewhat controversial [7,8]. Molecular biomarkers of anal neoplasia have the potential to more accurately risk stratify HGAINs and also may improve the low sensitivity of HGAIN diagnosis found with anal PAP smears, thus reducing the need for costly invasive biopsies [9].

Table 1. Primers for Bisulfite Sequencing,

Gene	Forward Primer	Reverse Primer	Annealing Temp	MgCl ₂	Amplicon size (bp)
<i>HOXA5</i>	TTA TTA GGA TGT ATT AAT TGT TAG GT	CAA AAT TCA AAA CTA CTA ACA AAA C	48.2	2.5	192
<i>TGFB3</i>	GAT TGA GGT TTG GTA AGA AGG TGT A	ACT AAA AAT CAA AAC CCA ACA AAA C	56.5	2.5	167
<i>KRT1</i>	TAG AGT AGG AGA TAG ATA TTA G	TCC AAT ATA AAA CTT AAA TCA CC	48.2	2.75	176

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It is well recognized that genetic mutations occur in cancer cells and that these exert disease-associated changes in gene expression and/or function. Very little is known about the specific genetic events that drive anal carcinogenesis; although it has been reported that alterations in *TP53*, *DCC*, *APC* and *FHIT* may be contributing factors [10,11]. Cancer cells also exhibit aberrant epigenetic alterations which appear to play a prominent role in cancer development. DNA methylation is a key aberrant epigenetic event that has been documented in virtually every tumor type studied and is amongst the earliest disease-associated changes observed during tumorigenesis [12]. HPV may influence the host transcriptome via a number of epigenetic mechanisms [5,13] including HPV E7 oncoprotein-mediated alterations in the activity of DNA methyltransferases (DNMTs) [14,15], histone deacetylases (HDACs), and pCAF acetyltransferase [14,16].

Despite the likely importance of aberrant DNA methylation in the pathogenesis of anal SCC, there has only been one report investigating methylation in anal cancer. Zhang et al. evaluated the methylation status of 11 candidate genes identified from studies of other HPV-associated malignancies [9]. They reported higher methylation in HGAIN and anal cancer for two genes compared to normal mucosa or low-grade lesions suggesting a role for DNA methylation in anal carcinogenesis. Additional epigenetic targets unique to anal cancer may be uncovered by a more comprehensive high-throughput methylation array approach. However, due in part to the limited quantity and quality of anal cancer tissue specimens, broad scale genomic techniques have not been widely applied to this disease site. The successfully met objectives of this study were first, to demonstrate the feasibility of investigating DNA methylation in anal cancer utilizing methylation array technology and second, to identify CpG loci that were differentially methylated in invasive SCC compared to pre-invasive and/or normal mucosa.

Materials and Methods

Ethics Statement

Our study was approved by the Institutional Review Board at the University of South Florida as exempt and not requiring informed consent from study subjects. Data were collected and appropriately de-identified prior to analysis.

Case Identification and Tissue Collection

The records of all patients treated at the H. Lee Moffitt Cancer Center and Research Institute from 2000–2008 with the diagnosis of anal SCC or SCC *in situ* (SCC-IS) were reviewed. Patients with a pathological diagnosis of SCC or SCC-IS of the anus and sufficient formalin-fixed paraffin-embedded (FFPE) tissue for analysis were identified. Pertinent clinical data were collected retrospectively utilizing our institutional electronic medical record system. We identified 24 patients treated at the Moffitt Cancer Center that met our inclusion criteria. Median age for the 24 patients (13 females and 11 males) in the study population was 44

years (range 26–81). Five patients were immunocompromised secondary to HIV (n = 3) or immunosuppressive medications given for organ transplantation (n = 2).

To ensure the accuracy of diagnosis, tissue samples were re-reviewed and regions of histologically normal mucosa, SCC-IS, and SCC were marked by a dedicated gastrointestinal pathologist (DC). FFPE tissues were subsequently cut (15 µm thick) and meticulously macrodissected to reduce cross contamination. Prior to the macrodissection of each case, gloves and instruments were changed and the workspace was disinfected. Of note, for the purposes of this study, SCC-IS is considered equivalent to AIN III and HGAIN.

HPV Genotyping

DNA was extracted from FFPE tissues using QIAamp DNA FFPE Tissue Kit (Qiagen Inc, Valencia, CA). HPV genotyping was performed using the INNO-LiPA HPV Genotyping *Extra kit* (Innogenetics, Belgium). In brief, 100 ng of DNA was utilized for PCR amplification of a short fragment (65-bp) of the HPV L1 region with biotinylated primers (SPF₁₀) using the MJ PTC-200 DNA engine thermocycler. PCR products were hybridized to the AutoBlot 3000H 20 Strip, a probe-specific nitrocellulose test strip, placed on an adhesive LiPA-Scan Reading template and analyzed using the LiRAS for LiPA HPV v2.01 software (Innogenetics, Belgium). All assays included the amplification of a 270 bp fragment of HLA-DPB1 as a positive control for human DNA. All PCR runs met quality control standards, with all samples positive for internal positive controls and negative controls negative for each run. This system detects 28 HPV types (HPV 6, 11, 16, 18, 26, 31, 33, 35, 39, 40, 43, 44, 45, 51, 52, 53, 54, 56, 58, 59, 66, 68, 70, 73, and 82). High-risk HPV types were defined as the 12 high-risk types classified as group 1 carcinogens (16, 18, 31, 33, 35, 39, 45, 51, 52, 56, 58, and 59) [17].

Methylation Array

Genomic DNA (500 ng) was sodium bisulfite-modified using the EZ DNA Methylation kit (Zymo Research, Orange, CA) following the manufacturer's instructions. DNA methylation was measured using the Illumina GoldenGate methylation assay with the Cancer Panel 1 probes (Illumina, San Diego, CA) following standard protocols [18]. This bead array platform interrogated 1505 CpG loci that represent 807 cancer-related genes. In brief, following methylation-specific hybridization, allele specific oligonucleotides were extended and ligated to a locus-specific oligonucleotide (LSO), which served as the template for fluorescently labeled universal primers that amplify either unmethylated (U) or methylated (M) templates. Labeled DNA contained a unique IllumiCode address for hybridization to its complement bead type (~30 replicates per CpG site) on a Sentrix Array Matrix (SAM) plate. At each CpG site, U and M fluorescent intensities were measured, averaged across replicate beads, and compared to a panel of negative controls using the Illumina's BeadStudio

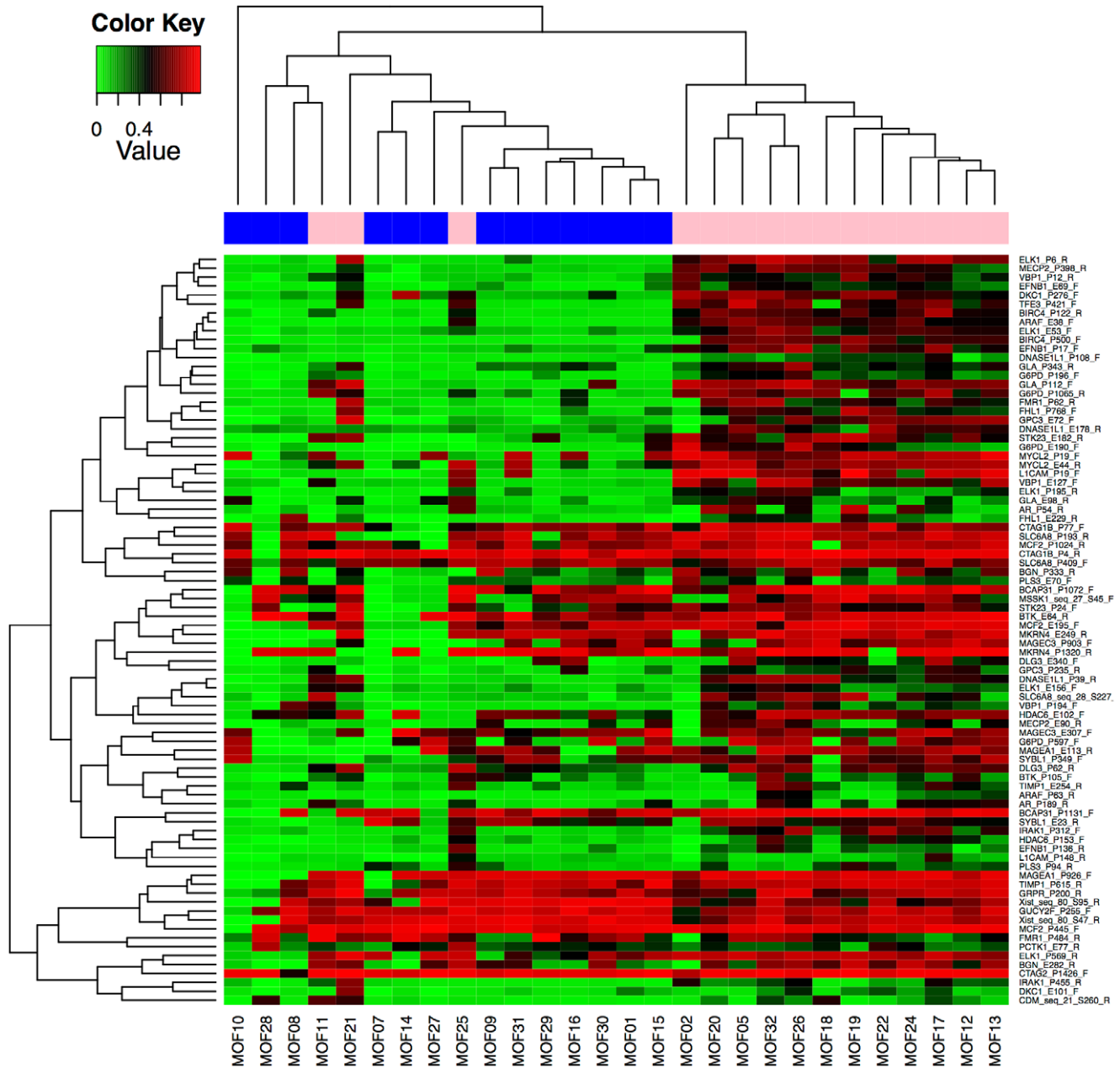


Figure 1. Gender Heatmap of X-linked CpG Loci. DNA methylation is involved in the transcriptional activation of genes on one of the two X chromosomes in female somatic cells. In general, male and female cases clustered together with methylation levels of the majority of X-linked genes correlating well with the gender of the tissue source (i.e. little-to-no methylation in male samples and hemi-methylation in female samples). Sample gender is represented above the heatmap (pink = female, blue = male). Methylation is represented by the beta value, or percent of total signal with green representing low methylation and red representing hemi-methylation. 51 of 84 loci within X-linked genes were differentially methylated ($p < 0.05$) between males and females.
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Methylation Module v3.2. Probes that were significantly different from negative controls were included in the analysis.

Bisulfite Sequencing

Primer design and amplification of target CpG site. Primer sets targeting *HOXA5*, *TGF β 3*, and *KRT1* genes were designed with MethPrimer software [19] or derived from the published literature (**Table 1**). The primers specifically amplified the CpG sites measured in the Illumina GoldenGate assay. Using 2 μ l of bisulfite-converted DNA as PCR template, a 50 μ l reaction

containing 0.02 μ M of each primer, 0.2 mM dNTP, 1 unit of HotStarTaq Plus polymerase and varying amounts of magnesium was performed. Initial denaturation was at 95°C for 15 min, followed by 40 cycles of 94°C for 1 min, 48.2°C–56.5°C annealing for 1 min and 72°C for 1 min; and a final extension cycle of 72°C for 10 min. PCR products were viewed with a 1.5% agarose gel stained with ethidium bromide. Target amplicons were gel-extracted and purified using the QIAquick Gel Extraction Kit (Qiagen Inc., Valencia, CA) according to the manufacturer's instructions.

Table 2. HPV Genotyping of Anal Tissues: Overall Prevalence and Across the Spectrum of Anal Neoplasia.

HPV Type	Overall HPV Prevalence* (n = 24 Patients)	Normal Mucosa (n = 3 Tissues)	SCC-in Situ (n = 11 Tissues)	SCC (n = 15 Tissues)
High Risk				
HPV-16	22 (92%)	3 (100%)	6 (54%)	13 (87%)
HPV-18	1 (4%)	–	1 (9%)	–
HPV-31	1 (4%)	–	1 (9%)	–
HPV-39	2 (8%)	–	1 (9%)	1 (7%)
HPV-52	6 (25%)	–	4 (36%)	2 (13%)
HPV-54	1 (4%)	–	1 (9%)	–
Low Risk				
HPV-6	3 (13%)	–	3 (27%)	–
HPV-11	2 (8%)	–	–	2 (13%)

*A total of 38 HPV infections were detected in 29 tissues, due to multiple infections.
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Cloning, transformation, and sequencing. Gel-purified PCR products were ligated into a TA cloning vector, pCR® 4-TOPO®, using Invitrogen's TOPO cloning kit (Life Technologies, Carlsbad, CA), transformed into *Escherichia coli* competent cells, and plated on 100 mg/ml LB-ampicillin agar plates. Ligation and transformation was confirmed by PCR (12.50 µl of Platinum Supermix, 0.5 µl each of 10 µM gene-specific forward primer, M13 Universal Reverse primer and colony template) using the following thermocycler conditions: 1 cycle at 95°C for 6 min; 40 cycles of 94°C for 30 secs, appropriate annealing temperature (Table 1) for 30 secs and 72°C for 1 min, and 1 cycle at 72°C for 10 min. PCR confirmed positive colonies were inoculated into LB-Amp broth and incubated overnight at 37°C. Plasmid DNA was extracted using the QIAGEN Miniprep Kit (Qiagen Inc., Valencia, CA) according to the manufacturer's instructions. Plasmid DNA was sequenced using M13 universal primers on the ABI 3600 sequencer (Applied Biosystems, Foster City, CA).

Methylation Array Bioinformatics and Statistical Analyses

Methylation data were pre-processed by setting non-detected probes as N/A (Not Applicable) for samples in which the detection p value for the probe was >0.05. Chip-wide controls and Multi-Dimensional Scaling plots were used to visualize data quality. Methylation data were analyzed using the R statistical software package and Bioconductor packages. Internal functional validation of the assay was performed by unsupervised clustering to confirm separation by gender due to the presence of X-linked CpG sites on the array (Figure 1) [18]. Analysis of X-linked genes with respect to gender was performed using the Mann-Whitney non-parametric test with $p < 0.05$ as the threshold for significance. Clustering was performed using non-centered correlation as the similarity metric within R. X-linked methylation probes were then discarded prior to further analysis.

The number of paired, matched tissues was small; therefore, we examined the three groups of tissues in a pooled fashion to identify targets with differential methylation in pre-invasive (normal and SCC-IS) vs. invasive cancer samples. Due to non-normal distribution of beta ratios, we applied a Mann-Whitney test for differences in groups to a total of 1,421 CpG loci (1,505–84 X-linked loci) and selected significant loci using a conservative threshold to partially correct for multiple testing problems ($p < 0.01$). Boxplots were used to visualize aggregate methylation data, representing the median methylation of each group.

Additional analysis was performed using a Kruskal-Wallis non-parametric test to accommodate variables with three levels (Normal, SCC-IS and Invasive).

Results

HPV Genotyping

HPV genotyping was performed on adjacent histologically normal anal mucosa (n = 3), SCC-IS (n = 11), and SCC (n = 15) tissues. HPV viral DNA was detected in all tissues, with 38 total infections detected (33 high-risk and 5 low-risk types, Table 2). High-risk HPV types were detected in 93% (27/29) of tissues; 3 patients with SCC-IS were positive for low-risk HPV 6. HPV 16 was the most common infection, being detected in 76% (22/29) tissue samples overall and in 87% of SCCs. It was most often detected as a single infection (59%, 17/29 tissue sections). Two SCCs were positive for HPV 52; whereas HPV 18 was only detected in one SCC-IS. Infection with multiple HPV types was detected in 7 (29%) of 24 patients, of which two were HIV positive. The detection of low-risk and high-risk types occurred concurrently in 3 out of the 5 low-risk infections detected; specifically, two SCC tissues had co-infection with HPV 11 and 16. Of the SCC-IS tissues where HPV 6 was detected, only one had a concurrent high-risk type detected (HPV 52). No correlation between multiple infections and histological diagnosis (normal mucosa, SCC-IS, SCC) or immunocompetency was identified.

Quality Control Analysis of GoldenGate DNA Methylation Data

The GoldenGate array contains standard control probes that assess several parameters including 1) Allele specific extension, 2) Bisulfite Conversion, 3) Extension Gap, 4) First hybridization, 5) Gender, 6) Negative Control, 7) PCR Contamination, and 8) Second Hybridization [18]. All cases used for this study were reviewed and passed these quality control measures. Figure 1 presents the quality control analysis of the methylation probes by gender to confirm separation due to the presence of X-linked CpG sites on the array [18]. In an unsupervised cluster analysis of X-linked CpG sites, a distinct separation of patients by gender was observed with 51 of 84 (61%) X-linked genes found to be differentially methylated ($p < 0.05$) between specimens derived from male and female patients. These analyses support the internal validity of our methylation data.

Table 3. Genes with Differentially Methylated CpG Loci in the Progression of Anal Neoplasia.

Symbol	Product	Annotation	CpG Number*	P-value	
				MW [†]	KW [‡]
Growth Regulation and Cell Cycle Control					
<i>TGFβ3</i>	Transforming growth factor, beta 3	Controls proliferation and differentiation	cg17928876	0.0012	0.0063
<i>FRK</i>	Fyn-related kinase	SRC kinase family with epithelial tissue-specific expression	cg26557270	0.0054	0.0247
<i>PADI4</i>	Peptidyl arginine deiminase, type IV	Post-translational modification (arginine methylation and citrullination) of histones	cg19159961	0.0030	0.0155
<i>ID1</i>	Inhibitor of DNA binding 1 isoform a	Helix-loop-helix protein	cg09569033	0.0071	0.0228
Differentiation					
<i>S100A2</i>	S100 calcium binding protein A2	Small, acidic Ca(2+)-binding proteins in nucleus	cg09232826; cg21074565	0.0015; 0.0083	0.0040; 0.0325
<i>KRT1</i>	Keratin 1	Differentiation-dependent keratin	cg06030058	0.0015	0.0071
<i>KRT5</i>	Keratin 5	Primary keratin	cg04254916	0.0033	0.0154
<i>PRSS8</i>	Prostasin pre-protein	Trypsinogen; serine proteases	cg27436259	0.0022	0.0116
Angiogenesis					
<i>FLT1</i>	Fms-related tyrosine kinase 1	VEGF receptor tyrosine kinase (also VEGFR1)	cg21787743	0.0020	0.0041
<i>KDR</i>	Kinase insert domain receptor	VEGF Type III receptor tyrosine kinase (also VEGFR2)	cg04695981	0.0044	0.0197
Apoptosis					
<i>DAPK1</i>	Death-associated protein kinase 1	Calmodulin-dependent serine-threonine kinase	cg01984172	0.0021	0.0106
<i>HOXA5</i>	Homeobox A5	DNA-binding transcription factor	cg27409178	0.0037	0.0133
<i>TNFRSF10B</i>	Tumor necrosis factor receptor superfamily, 10b	Death domain associated receptor	cg07508317	0.0026	0.0112
<i>BCL2A1</i>	BCL2-related protein A1	Reduces pro-apoptotic cytochrome C release; blocks caspase activation.	cg27177709	0.0061	0.0262
<i>SEMA3B</i>	Semaphorin 3B isoform 1	Extracellular secreted protein important in axonal guidance; induces apoptosis	cg12999941	0.0083	0.0283
Other Processes					
<i>CCL3</i>	Chemokine (C-C motif) ligand 3	Macrophage inflammatory protein-1	cg05481196	0.0096	0.0374
<i>P2RX7</i>	Purinergic receptor P2X7 isoform b	Cell surface ATP receptor; ligand-gated ion channel	cg08688169	0.0096	0.0156
<i>CD9</i>	CD9 antigen	Cell surface tetraspanin (TM4SF) glycoprotein	cg19415774	0.0071	0.0228
<i>DIO3</i>	Deiodinase, iodothyronine, III	Selenoenzyme	cg18191511	0.0074	0.0133
<i>GABRA5</i>	Gamma-aminobutyric acid A receptor, alpha 5	heteromeric pentameric ligand-gated ion channels	cg02225257; cg20051555	0.0030; 0.0044	0.0112; 0.0044

*CpG locus label within the GoldenGate methylation array.

[†]Mann-Whitney test for methylation differences between two groups (pre-invasive vs. invasive SCC) using a conservative threshold to partially correct for multiple testing problems ($p < 0.01$).

[‡]Kruskal-Wallis non-parametric test performed to test three levels (Normal, SCC-IS and Invasive).

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Differential Methylation in Invasive SCC vs. Pre-invasive Anal Tissue

To optimize the numbers of cases per comparison group, methylation profiles were compared between pre-invasive anal tissue (normal and SCC-IS) and invasive SCC specimens using the Mann-Whitney test. A total of 22 CpG loci corresponding to 20 genes was noted to have significant ($p < 0.01$) differential methylation between the two groups (**Table 3**). Of these, all but 2 CpG loci demonstrated increased methylation in invasive SCC compared to pre-invasive tissues (**Figure 2** and **Figure S1**). Methylation levels of these 2 CpG sites both within the *GABRA5* gene were significantly lower in invasive SCC compared to pre-

invasive tissues. (**Table S1** provides detailed annotation for genes containing differentially methylated CpG loci).

There were three distinct patterns of methylation observed in the differentially methylated CpG panel (**Figure 2**): 1) Loci with low methylation levels (median beta value < 0.3) in all tissue types (e.g. *ID1*, *KDR*, *TNFRSF10B*, and *SEMA3B*) but with a statistically significant difference between non-invasive and invasive tissues; 2) loci with high methylation levels (median beta ≥ 0.3) in all tissue types (e.g. *GABRA5*, *BCL2A1*, *CCL3*, *DIO3*, *FRK*, *KRT1*, *KRT5*, *P2RX7*, *PRSS8*, and *TGFB3*) but with a statistically significant difference between non-invasive and invasive tissues; and 3) loci with little to no methylation in non-invasive tissues (beta values < 0.3) with a significant increase above the threshold into

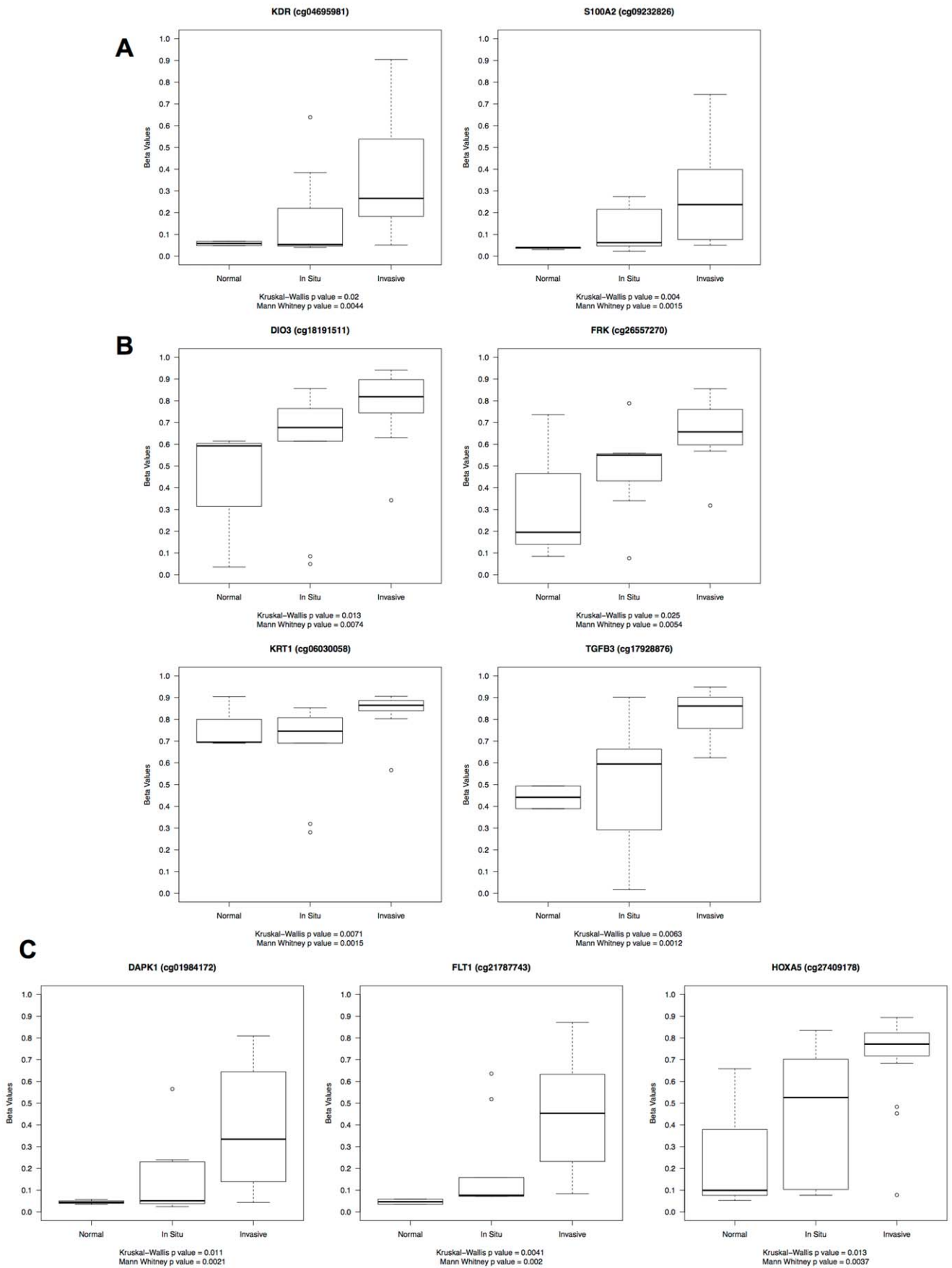


Figure 2. Boxplot Representations of Selected Differentially Methylated CpG Loci. Boxplots illustrating methylation levels of selected significant differentially methylated CpG sites (Mann-Whitney $p < 0.01$) across histologic subtypes (normal and SCC-IS vs. invasive CC). Three patterns are identified: A: Loci with low methylation levels (median beta value < 0.3) across all tissues. B: Loci with high methylation levels (median beta value ≥ 0.3) across all tissues. C: Loci demonstrating little to no methylation in non-invasive tissues with significant levels of methylation in invasive SCC. doi:10.1371/journal.pone.0050533.g002

high methylation levels (beta ≥ 0.3) in invasive SCC (e.g. *CD9*, *DAPK1*, *FLT1*, *HOXA5*, and *PADI4*). For *S100A2*, one CpG site demonstrated low methylation levels in all tissues (median beta value < 0.3) while the other was consistent with pattern 3; however, the general trend was suggestive of a low to high methylation pattern across both sites within this gene.

Although the number of samples in each group was relatively small, and particularly, in the normal tissue group, we performed an exploratory analysis to identify targets with differential methylation across all three groups (normal, SCC-IS and SCC). Using a Kruskal-Wallis non-parametric test and p -value < 0.01 , 7 probes had differential methylation across the 3 groups, four of which (*S100A2*, *FLT1*, *TGF β 3* and *KRT1*) were identified in our binary analysis (Table 3 and Figure 2) and three were not (*BMP4*, *EV12A*, and *IL1RN*). In fact, the top 20 significant and near significant ($p < 0.02$) differentially methylated loci from the Kruskal-Wallis analysis and the top 20 significant ($p < 0.01$) loci from the Mann-Whitney analysis were highly similar with 13 CpG sites in common.

Measurement of Methylated Targets by Bisulfite Sequencing

Given the scarcity of these specimens, a confirmatory measurement of methylation using conventional bisulfite sequencing was performed in a total of 9 anal tissues (8 SCC and 1 SCC-IS) for which there was sufficient remaining tissue and/or genomic DNA. We selected 3 genes for the determination of methylation status by

bisulfite sequencing: *TGF β 3* and *KRT1* had high methylation in all tissue types while *HOXA5* demonstrated a progression of methylation from low to high levels across tissues. Within this study, the median beta values for CpG sites within *TGF β 3*, *KRT1* and *HOXA5* genes were 0.86, 0.86 and 0.77, respectively. Within the set of 9 patients, the average methylation levels at the CpG site of interest, as determined by bisulfite sequencing, for *TGF β 3*, *KRT1* and *HOXA5* were very similar to the corresponding average array beta values (63% vs. 69%; 68% vs. 76% and 63% vs. 63%, respectively). On an individual tissue specimen level, we demonstrated evidence of methylation (median beta value of ≥ 0.3) at the CpG site of interest in $\geq 30\%$ of corresponding clones by bisulfite sequencing at concordance rates of 88% (8 of 9 clones) for *TGF β 3*, 75% (6/8) for *HOXA5* and 100% (9/9) for *KRT1*. In all cases of non-concordance, a low array beta value was associated with a higher percentage of methylated clones by sequencing. Our findings confirm previous reports of high concordance between GoldenGate methylation array technology and conventional methylation assays [20,21].

Discussion

The molecular events involved in HPV-associated anal carcinogenesis, including alterations in host genome methylation, remain poorly studied. With respect to HPV characterization, all patients in our study were found to be infected with one or more HPV genotypes. HPV 16 was the predominant oncogenic subtype

Table 4. Chromosomal Mapping of Genes with Differentially Methylated CpG Loci.

Gene Symbol	Chromosomal Location	Relation to HPV or hotspots
<i>TGFβ3</i>	14q24.3	HPV integration site
<i>GABRA5</i>	15q12	HPV integration site
<i>FRK</i>	6q22.1	HPV integration site
<i>CCL3</i>	17q12	HPV integration site
<i>KDR</i>	4q12	HPV integration site
<i>BCL2A1</i>	15q25.1	Close to 15q25.3 HPV integration site
<i>HOXA5</i>	7p15.2	HPV16 and 18 hypermethylation hotspot
<i>DAPK1</i>	9q21.33	Close to 9q21.31 HPV18 hypermethylation hotspot
<i>DIO3</i>	14q32.31	Close to 14q32.33 HPV 16 and 18 hypermethylation hotspot
<i>P2RX7</i>	12q24.31	Close to 12q24.33 HPV 16 and 18 hypermethylation hotspot
<i>S100A2</i>	1q21.3	Close to 1q21.1 HPV16 and 18 hypomethylation hotspot
<i>KRT1</i>	12q13.3	Potential novel hypermethylation hotspot with KRT5 and CD9
<i>KRT5</i>	12q13.3	Potential novel hypermethylation hotspot with KRT1 and CD9
<i>CD9</i>	12q13.3	Potential novel hypermethylation hotspot with KRT1 and KRT5
<i>PRSS8</i>	16p11.2	Telomeric region
<i>ID1</i>	20q11.2	Telomeric region
<i>FLT1</i>	13q12.2–12.3	Telomeric region
<i>TNFRSF10B</i>	8p21.3	No associations
<i>SEMA3B</i>	3p21.31	No associations
<i>PADI4</i>	1p36.13	No associations

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identified; however, HPV 18, 32, 39 and 52 were also observed. The HPV prevalence and type distribution are similar to previous reports in anal cancer [22–26]. In the current investigation, we utilized an FFPE-tissue compatible methylation array to evaluate the methylation status of 1,505 CpG loci representing 807 genes. We identified a panel of 20 CpG loci representing 19 genes with increased methylation in the progression from pre-invasive normal and SCC-IS tissues to invasive anal SCC. Of these CpG loci, 6 had low methylation levels in normal and pre-invasive tissues but were hypermethylated in invasive SCC. From a biological standpoint, this panel of CpG sites may represent biomarkers of anal neoplastic progression from non-invasive to invasive anal neoplasia and may reflect carcinogenesis-related epigenetic alterations with a transition from an unmethylated to methylated state.

We identified a panel of CpG loci that were differentially methylated in HPV-associated anal SCC (**Table 3** and **Table S1**). These epigenetic events occurred in genes encoding proteins that play a role in growth regulation/cell cycle control (e.g. TGF- β 3[27–29], FRK[30–32], PADI4[33–36], and ID[37–41]) and critical regulation of apoptosis (e.g. TNFRSF10B[42–47], DAPK1[9,48–50], HOXA5 [51–54], BCL2A1[55–59], and SEMA3B[60–62]). CpG loci that were shown to have high methylation levels across the spectrum of anal tissues but with a significant difference between non-invasive tissues and invasive SCC may be markers of early epigenetic alterations in HPV-induced carcinogenesis. For example, methylation of CpG loci in two keratin genes, *KRT1* and *KRT5*, the products of which contribute to the differentiation of squamous epithelium, may be early biomarkers of HPV-associated carcinogenesis[63–65]. The HPV oncogenes E6 and E7 primarily target p53 and Rb, respectively, for degradation [66]. Interestingly, methylation levels of several CpG loci occurred within genes that either interact with or are part of the p53 or Rb pathways, including *PADI4* [33,35], *HOXA5* [67], *FRK* [32], and *DAPK1* [50] (**Table S1**). Several differentially methylated loci identified in this study appear within genes that may be potential novel targets in the setting of cancer, such as *FRK*, *BCL2A1*, *GABRA5*, *DIO3*, *P2RX7*, *CCL3* and *ID*. Overall, there appears to be a clustering of methylation in CpG sites across genes with similar functions within anal SCC and these epigenetic differences may have potential utility as biomarkers of HPV-associated carcinogenesis (**Table S1**) [68–93].

Zhang et al. evaluated the methylation status of 11 candidate genes (*DAPK1*, *IGSF4*, *MLH1*, *HIC1*, *RARB*, *p14*, *TP73*, *MGMT*, *RASSF1*, *APC*, and *CDKN2A*) in a set of 172 anal biopsies, which is the only published analysis of DNA methylation in anal cancer [9]. They reported an increased frequency of *DAPK1* and *IGSF4* methylation during the progression from normal mucosa to SCC-IS to invasive SCC. In our study, we have indeed confirmed that CpG loci within *DAPK1* were among the most significantly methylated sites with increasing methylation across the spectrum of anal carcinogenesis. However, neither of the *IGSF4* probes demonstrated significant differential methylation in our study ($p=0.029$ and 0.052 respectively by Mann-Whitney, data not shown). While data are limited in anal cancer, there is evidence for aberrant DNA methylation in HPV-associated cervical cancer [5,9,94]. Of the significant differentially methylated sites identified in our anal cancer study, *DAPK1* is the only methylation target that has been similarly shown to be methylated in invasive cervical SCC (55%) and high-grade pre-invasive lesions (52%), with reduced methylation in normal and low-grade lesions [48,49,95–98]. These data support a possible role for the epigenetic alteration of *DAPK1* in HPV-associated carcinogenesis.

Until recently, the mechanisms responsible for the association between HPV infection and epigenetic alterations have been somewhat speculative with limited *in vitro* data. HPV has been shown to upregulate and augment DNMT [14,15,99,100] and HDAC activity [14,16]; thus it is biologically plausible that epigenetic alterations may play a role in HPV-induced carcinogenesis. Leonard et al. [15] have demonstrated that the transfection of the episomal forms of both HPV16 and 18 result in the induction of DNMT1 and DNMT3B expression and subsequent alterations in methylation status of numerous host genes across the genome. Of interest, they observed that the majority of HPV-induced methylation targets appeared to be non-random and were associated with cis-acting events (e.g. increased CpG dinucleotide density, CpG sites near telomeres and known HPV integration sites) and clustered across genes within specific chromosomal locations (e.g. HPV methylation hotspots) [15]. We determined that 19 of the 22 differentially methylated CpG loci identified in our study occurred in genes that fit the criteria for these “methylation-prone” areas (**Table 4**). *TGFB3*, *GABRA5*, *KDR*, *FRK* and *CCL3* all localize directly to known HPV integration sites while *BCL2A1* is immediately adjacent to one such site [15,101–105]. *HOXA5* is located at an exact HPV 16 and 18 hypermethylation hotspot while *DAPK1*, *DIO3*, and *P23X7* are all immediately adjacent to a described hotspot [15]. *FLT1*, *PRSS8* and *ID1* are located in the telomeric regions of their respective chromosomes. Finally, we have noted what appears to be a novel hotspot at 12q13.3 with *KRT1*, *KRT5* and *CD9* all mapping directly to this locus. Our findings lend further support to the notion that HPV-associated methylation events appear to occur in a non-random fashion and suggest that there may be similarities in epigenetic alterations across HPV-associated cancers. A critical area for future research will be to distinguish between epigenetic alterations that are oncogenic drivers versus those that may simply be bystander effects of HPV infection.

To our knowledge, this study is the first to demonstrate the feasibility of a large-scale evaluation of DNA methylation in FFPE tissues from anal SCC, which in addition includes corresponding HPV genotyping. We acknowledge the fact that the GoldenGate array interrogates a number of selected known methylation targets and as such, does not represent a full evaluation of genome-wide events. In addition, with this array, we were unable to determine whether differences in methylation at specific CpG loci had biological consequences, such as gene silencing. However, differential methylation events at specific loci may still represent potential biomarkers of HPV-associated carcinogenesis. This study is certainly limited by its small sample size, which unfortunately is inherent with the relative paucity of anal cancer tissues treated at one center. Normal anal mucosa was identified by histologic review; however, these tissues were HPV-positive and adjacent to SCC-IS or SCC, thus, do not represent purely unaffected anal mucosa. Non-neoplastic anal tissues infected by HPV may have altered epigenetic profiles but nonetheless are relevant as the earliest end of the spectrum of HPV-associated anal carcinogenesis. In this study, due to a small sample size, we were unable to fully analyze differences in methylation by HPV genotype. However, in an exploratory analysis, we identified a panel of 27 CpG loci that were differentially methylated ($p<0.05$ by Mann-Whitney) between the HPV16+ and HPV16- tissues (data not shown). Larger studies are needed to fully evaluate the impact of HPV-related factors, such as genotype or number of infections, on epigenetic profiles and associated biologic pathways.

Our data provide preliminary evidence that the progression from normal anal mucosa and anal SCC-IS to invasive SCC is associated with distinct alterations in host genome methylation

which in turn may be a consequence of high risk HPV infection. With the application of broad high-throughput methylation profiling, we have generated a candidate list of progressively methylated CpG loci across the spectrum of anal squamous neoplasia, including sites within genes previously unassociated with anal SCC. Despite the small numbers of patients evaluated in this study, our findings are in agreement with what is known about the impact of HPV on epigenetic profiles including increased methylation at or close to specific chromosomal locations including previously described HPV methylation “hotspots” and viral integration sites. Our work represents an important initial step in understanding the epigenetic events associated with anal carcinogenesis. A critical translational step would be to apply broad methylation assays to identify a relevant panel of methylation biomarkers that could not only refine the diagnosis of HGAIN or SCC-IS but also serve as an indicator for increased risk of subsequent malignant transformation to invasive SCC. Molecular guidance ultimately may reduce the burden of potentially unnecessary screening, biopsy and treatment procedures. Further evaluation of methylation events in anal squamous neoplasia is clearly warranted in larger patient populations and may contribute not only to a better understanding of anal carcinogenesis, but also to the development of novel biomarkers that may ultimately impact on enhanced diagnosis, screening and prevention.

Supporting Information

Figure S1 Boxplot Representations of All Differentially Methylated Loci. Boxplots illustrating methylation levels of 22

significant differentially methylated CpG sites representing 20 genes (Mann-Whitney $p < 0.01$) across histologic subtypes (normal and SCC-IS vs. invasive CC). Three patterns are identified: A: Loci with low methylation levels (median beta value < 0.3) across all tissues. B: Loci with high methylation levels (median beta value ≥ 0.3) across all tissues. The two CpG sites within the *GABRA5* gene demonstrated reduced methylation with neoplastic progression C: Loci demonstrating little to no methylation in non-invasive tissues with significant levels of methylation in invasive SCC. (TIF)

Table S1 Genes with Differentially Methylated CpG Loci in the Progression of Anal Neoplasia with Full Annotation.

(DOC)

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Author Contributions

Conceived and designed the experiments: EMS DS. Performed the experiments: JMH BR AE AA DC. Analyzed the data: EMS SE XQ AB DS. Contributed reagents/materials/analysis tools: EMS DC WMG ARG DS. Wrote the paper: JMH EMS DS.

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