Published in final edited form as:
Dis Colon Rectum. 2014 August ; 57(8): 941–957. doi:10.1097/DCR.0000000000000160.

Epigenomic Characterization of Locally Advanced Anal Cancer: An RTOG 98-11 Specimen Study

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Abstract

Background—The Radiation Therapy Oncology Group 98-11 clinical trial demonstrated the superiority of standard 5FU/mitomycin-C over 5FU/cisplatin in combination with radiation in the treatment of anal squamous cell cancer. Tumor size (>5cm) and lymph node metastases are associated with disease progression. There may be key molecular differences (e.g. DNA methylation changes) in tumors at high-risk for progression.

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B. Drafting the article (EMS, SE, KW, BR, AB, DS) or revising it critically for important intellectual content (EMS, ES, KW, BR, AB, AA, JS, JM, JA, AM, AE, SH, DS).

C. Final approval of the version to be published (EMS, ES, KW, BR, AB, AA, JS, JM, JA, AM, AE, SH, DS).

Manuscript Category: Colorectal/Anal neoplasia

Conference Proceedings: Presented in part at the 2013 Meeting of the American Society of Colon and Rectal Surgeons, April 29th-May 1st, in Phoenix, AZ; Podium S27 and International Anal Neoplasia Society, November 22-24, 2013 in San Francisco, Abstract # 42

The authors have no financial disclosures.

Objectives—The objectives of this study were to determine if there are differences in DNA methylation at individual CpG sites and within genes among locally advanced anal cancers, with large tumor size and/or nodal involvement, compared to those that are less advanced.

Design—Case-case study among 121 patients defined as high-risk (tumor size>5cm and/or nodal involvement; n=59) or low-risk (≤5cm, node negative; n=62) within the mitomycin-C arm of RTOG98-11 trial. DNA methylation was measured using the Illumina HumanMethylation450 Array.

Settings—Tertiary care cancer center in collaboration with a national clinical trials cooperative group.

Patients—The patients consisted of 74 women and 47 men with a median age of 54 years (minmax 25-79).

Main Outcome Measures—DNA methylation differences at individual CpG sites and within genes between low and high-risk patients were compared using Mann-Whitney test (p-value<0.001).

Results—A total of 16 CpG loci were differentially methylated (14 increased and 2 decreased) in high vs. low-risk cases. Genes harboring differentially methylated CpG sites included known tumor suppressor genes and novel targets.

Limitations—This study only included patients in mitomycin-C arm with tumor tissue; however, this sample was representative of the trial.

Conclusions—This is the first study to apply genome-wide methylation analysis to anal cancer. Biologically relevant differences in methylated targets were found to discriminate locally advanced from early anal cancer. Epigenetic events likely play a significant role in the progression of anal cancer and may serve as potential biomarkers.

Keywords

anal cancer; methylation; genome-wide array; epigenetic; locally advanced anal cancer

INTRODUCTION

Anal cancer accounts for 4% of all lower gastrointestinal tract malignancies in the United States[1] and the incidence of anal cancer continues to rise by 2.6% per year.[2] In fact, the incidence rates of anal cancer have increased significantly in the past 30 years, jumping 160% in men and 78% in women. [3,4] The overall 5-year survival rate for anal cancer is 65.6%; however, this varies considerably by stage of diagnosis (80% for local disease; 60% for regional disease and 31% for distant disease at diagnosis).[5]

Radiation, 5-fluorouracil (5-FU), and mitomycin-C have remained the components of standard combined modality therapy over the last several decades despite the completion of several clinical trials.[6-9] Of these, the Radiation Therapy Oncology Group (RTOG 98-11) evaluated two chemoradiation regimens for the treatment of anal canal carcinoma (standard mitomycin-based regimen versus an experimental cisplatin-based regimen). This trial included 644 patients and reported a significantly better 5-year disease-free survival in the

mitomycin-C arm versus cisplatin arm (68% vs. 58%, p=0.006).[6,7] While this treatment is effective, there are significantly associated morbidities and alternative dosing or novel targeted treatments are needed to reduce morbidity and improve outcomes for some patients.

Tumor size and nodal status are strong clinical prognostic factors for anal cancer.[10] Within RTOG 98-11, patients with more advanced cancer at diagnosis, e.g. large tumors (>5cm diameter) and/or positive lymph nodes, had poorer disease-free survival outcomes than those with less advanced disease.[7] However, both early and locoregionally advanced tumors have heterogeneous outcomes. We hypothesized that there are underlying biological differences between larger size and/or node-positive tumors that put tumors at high risk of progressing and not responding to treatment. These differences may explain some of the variability in patient outcomes given the same treatment.

One such biological alteration important to carcinogenesis is DNA methylation, an epigenetic modification.[11,12] Epigenetic alterations encompass changes in chromatin structure, histone modification, and DNA methylation and play an important role in gene expression. Methylation of DNA occurs specifically at discrete sites termed CpG dinucleotides, where a cytosine (C) precedes a guanine (G). These CpG sites or loci generally reside in clusters known as CpG islands and are often associated with gene promoter regions.[12] Dense DNA methylation in CpG islands can result in the epigenetic silencing of tumor suppressor genes and are an important part of the process of carcinogenesis. [13] Epigenetic gene silencing has been demonstrated in HPV-associated cervical cancer and commonly occurred in pathways that important in the carcinogenesis.[13,14] For example, aberrant activity of the well described oncogenic Wnt/β-catenin pathway is prominent in numerous cancer types and genes encoding several key regulators of this pathway, such as *CDH1*, *APC* and *WIF1*, are frequently silenced via dense methylation of CpG islands in cervical cancer.[15-17]

Although likely important, the role of DNA methylation in the development of anal cancer remains very poorly studied. [18,19] Zhang *et al*. provided the first evidence of aberrant methylation in anal cancer among 11 candidate genes compared to normal tissue.[18] Subsequently using an array-based assay analyzing >1500 CpG sites, we observed differences in DNA methylation patterns in 20 genes in the progression from normal anal mucosa, carcinoma *in situ*, to invasive anal carcinoma in a small set of cases.[19] To date, a broad high-throughput characterization of methylation events in advanced anal cancer is clearly lacking. The objectives of this study were to determine if there are differences in DNA methylation at individual CpG sites and clusters within genes among locally advanced anal cancers, with large tumor size and/or nodal involvement, compared to those that are less advanced.

MATERIAL AND METHODS

RTOG 98-11 Trial

As reported,[6,7] this US Gastrointestinal Intergroup trial RTOG 98-11 evaluated combinations of external beam irradiation (XRT) plus chemotherapy in a 2-arm phase III randomized trial comparing XRT plus concurrent 5-FU and mitomycin-C with induction 5-

FU+cisplatin followed by concurrent XRT plus 5-FU+cisplatin.[6] Patients were excluded if their primary diagnosis was T1 or M1, severe comorbid conditions (including AIDS), or prior malignancy within the last 5 years.[6] Randomization was stratified by gender, clinical nodal status (positive or negative), and size of the primary tumor (2-5cm or >5cm). For anal cancer, clinical lymph node staging was conducted by physical examination and radiological imaging. All patients enrolled in RTOG 98-11 signed an IRB approved informed consent form. The use of de-identified tissues was IRB-approved by the University of South Florida IRB. This study includes patients from the Mitomycin-C arm of RTOG 98-11 that had archived tumor tissue available (N=186).

RTOG Tissues and DNA extraction

Archived formalin fixed paraffin embedded (FFPE) tumors were macrodissected and genomic DNA was isolated using the QIAamp DNA FFPE Tissue Kit (QIAGEN, Valencia, CA). DNA quality was evaluated using the qPCR Illumina FFPE QC kit (Illumina, San Diego, CA) in triplicate. Samples with sufficient DNA (≥250ng) that met criteria for inclusion by the QC assay (Illumina, San Diego, CA) were included.

Bisulfite Modification and Infinium Methylation Analysis

DNA methylation cannot be measured directly and requires treatment with sodium bisulfite which converts all unmethylated cytosines to uracil, while methylated cytosines are not altered. Thus, genomic DNA is first sodium bisulfite-modified using the EZ DNA Methylation kit (Zymo Research, Orange, CA). Bisulfite-modified DNA was prepared using the Infinium HD FFPE DNA Restore kit (Illumina, San Diego, CA). Methylation was interrogated using the Infinium HumanMethylation450K BeadChip following manufactures specifications throughout the whole-genome amplification, fragmentation, hybridization, base extension, counterstaining and scanning). A Tecan Liquid Handling robot with the Te-Flow apparatus was used for the single base extension and staining, and the chips were scanned on a single HiScanSQ System (Illumina Inc.). The Infinium HumanMethylation450K BeadChip incorporates both Infinium I (methylated and unmethylated beads per CpG locus) and Infinium II assays (one bead type with the methylated state determined at the single base extension step after hybridization) to evaluate DNA methylation status at 485,512 CpG loci, which covers 99% of annotated genes and 96% of defined CpG islands.[20,21]

Statistical Analysis

Patients were classified as low-risk with tumor diameter of less than 5 cm and node negative and advanced high-risk with tumor diameter of more than 5 cm and/or node positive). Patients were also grouped individually by tumor size (≤5cm *vs.* >5cm) or nodal status (negative *vs.* positive). Differences in patient characteristics were determined by chi-square, Fisher's exact, or t-test. Overall survival was defined as the time from randomization to death due to any cause. Disease-free survival was defined as the time from randomization to local, regional, or distant failure, second primary tumor, or death due to any cause.[6] Differences in survival by clinical risk group were estimated univariately with the Kaplan-Meier method and the clinical risk groups were compared using the log-rank test.

Methylated CpG loci were defined on the Infinium array as a β-value, which was calculated for the 485,512 CpG-loci from unmethylated (U) and methylated (M) signal [M / (U+M +100)] and assigned a range between 0 and 1 (unmethylated to 100% methylated). β-values with a corresponding detection *p-value*>0.05 were set as missing. Methylation data were pre-processed using the R statistical software package and Bioconductor packages methylumi and wateRmelon.[22] β-values were normalized using the normalizeViaControls function in the methylumi package. Chip-wide controls and Multi-Dimensional Scaling plots were used to visualize data quality. β-values were analyzed as continuous variables. To quantitate differential methylation between high and low-risk groups, we calculated a delta β = $\beta\text{-value}_{high} - \beta\text{-value}_{low}$. A threshold of delta β>0.1 was required to identify potentially meaningful biologic changes in methylation. Differential methylation between groups was analyzed using Mann-Whitney test and students t-test at level of significance of p-value<0.001. Differential methylation across regions of DNA required ≥5 CpG loci that were significantly different per annotated gene at a p-value<0.05 by Mann-Whitney or T-test. Statistical analysis was performed using the R statistical software package, and MATLAB packages.

RESULTS

Patient Characteristics

We identified 186 patients from RTOG 98-11 randomized to the Mitomycin-C arm with archived tumor tissue available for this study. A total of 121 cases had sufficient DNA for methylation analysis. There were no differences in tumor characteristics and outcomes between patients eligible for the methylation analysis to those in the Mitomycin-C arm that were not included, therefore the sample of 121 anal cancer patients was representative of patients randomized to the Mitomycin-C arm of RTOG 98-11 (data not shown). The median age of the 121 patients was 54 (range 25-79; **Table 1**). A majority of the patients were women (61%), Caucasian (87%), and highly functional (95% had Karnofsky Performance Status ≥ 80). Sixty-two patients (51%) were classified as low-risk and 59 (49%) as high-risk. There were 88 (73%) and 33 (27%) patients with tumor sizes of ≤ 5cm and >5cm, respectively. Nodal status was determined as node negative (N0) in 85 (70%) patients and node positive (N+) in 36 (30%) patients. There were no statistically significant differences in distribution of gender or age across clinical risk groups (**Table 1**).

Risk groups associated with patient outcomes

The classification of risk groups as low- and high-risk was confirmed by examining the relationship between clinical risk group and outcome **(Figure 1)**. The 5-year disease-free survival rates were 89% for low-risk and 49% for the high-risk group (p-value<0.0001, **Figure 1A**). The 5-year overall survival rates were 92% vs. 64% for low- and high-risk groups, respectively (p-value=0.0003, **Figure 1B**). Similar differences in survival were observed when considering tumor size or nodal status individually (data not shown). These data confirm that within the 121 cases, the risk groups were accurately classified and are representative of the entire Mitomycin-C arm of RTOG 98-11.

Differentially methylated CpG sites by risk group

At a p-value<0.001, 16 CpG loci were differentially methylated between low- and high-risk groups (**Table 2**). Of these, 14 loci had increased methylation in high-risk tumors. These 16 CpG sites were located in 7 defined genes and one uncharacterized genomic region (See **Online Table 1** for gene details). The individual contribution of tumor size and nodal status on methylation differences was also examined. A total of 68 CpG loci from 59 genes were differentially methylated in large tumors (**Table 3**). There were 61 sites with increased methylation levels within large tumors and 7 sites with decreased methylation. When considering nodal status, 8 CpG loci (5 increased and 3 decreased methylation) from 6 unique genomic regions were differentially methylated in tumors with nodal involvement (data not shown). There was no overlap in individual differentially methylated CpG sites between tumor size and nodal status. Only 2 genes (3.5%) identified in the tumor size analysis were also significantly different by clinical risk group (**Table 2** and **3**) and 2 genes (33%) from the nodal analysis overlapped with risk group findings (data not shown).

Differential methylation within genomic regions

An individual methylated CpG site itself can serve as a detectable biomarker regardless of downstream impact on gene expression. However, prognostic biomarkers with functional biological relevance are of great interest. Given that functionally significant methylation is often associated with methylation across clusters of CpG sites, we next examined whether there were methylation differences in clusters of CpG sites within genomic regions between the high and low risk groups. At a p-value<0.05, we identified 6 genes with clusters of CpG sites differentially methylated in high-risk tumors (**Table 4**). **Figure 2** plots a 361 bp region of the genome encoding the Paraoxonase 3 (PON3) gene that includes 13 CpG sites examined within the CpG island. Box plots represent the median and interquartile range (25^{th} and 75^{th} percentiles) of methylation levels in high (aqua blue boxes) and low-risk (maroon red boxes) tumors. The median methylation for each of the 9 CpG sites (32% of CpG sites examined in the PON3 gene) was significantly lower in high-risk tumors than low-risk tumors. **Figure 3** plots a ~2kb region of the SAL-Like 3 gene (SALL3) that includes 10 CpG sites within the CpG island. Overall, 8 out of 27 total sites examined had significantly higher methylation in the high-risk tumors. The genomic region with the most differentially methylated sites (LOC728392) does not have a defined function, but does have predicted gene coding regions and an identified CpG island. **Figure 4** plots 8 out of 27 CpG sites examined across 776 bps of a CpG island that all have higher methylation in high-risk tumors. When examining clusters of differentially methylated sites by tumor size, we identified 20 regions (**Table 4**), of which 14 genes had exclusively higher methylation in large tumors and 3 had exclusively lower methylation. Only three genomic regions had clusters of significantly different CpG methylation by nodal status, all of which were increased in node positive cases (**Table 4)**.

DISCUSSION

This study investigated whether DNA methylation differed between locally advanced (high-risk) or locoregionally-confined (low-risk) anal cancers. To address this question, we conducted the first genome-wide investigation of DNA methylation in anal cancer using a

well annotated set of tumors archived within the RTOG 98-11 trial. We identified individual CpG sites and clusters of CpG sites that were differentially methylated in locally advanced tumors. Furthermore, we identified a large number of CpG sites with differential methylation in larger (>5cm) tumors, regardless of nodal status. These differentially methylated regions provide clues for future studies that can examine whether this dense DNA methylation leads to transcriptional silencing of genes within large or advanced tumors. While the biological impact of methylation at an individual CpG site is unknown, the differentially methylated or unmethylated CpG sites identified in advanced or large tumors may be developed as clinically-applicable biomarkers

The findings of this study are in line with the growing appreciation that aberrant epigenetic events are critical in the process of cancer growth and progression. For example, differential methylation patterns have been reported in the progression of several malignancies, including prostate[93,94] bladder,[95,96] renal cell,[47] esophageal,[97] HPV-positive and HPV-negative head and neck,[98,99] and cervical cancers.[100,101] In general, these papers reported that increased DNA methylation within CpG islands was associated with increasing tumor aggressiveness[94,101] and some epigenetic events appear to be early markers of progression.[100] In cervical cancer, methylation of RASSF2 was associated with increased tumor vascular invasion and shorter survival time, independent of tumor stage.[100] Such differences in methylation provide biological insight into the mechanisms of carcinogenesis.

DNA methylation differences were observed in locally advanced anal cancers compared to early, less advanced tumors. Tumor suppressor genes are often targets of DNA methylation-mediated inactivation which in turn, contributes to cancer progression. A loss of methylation can also be associated with re-expression of suppressed oncogenic elements which can then also drive neoplastic growth. We identified 2 tumor suppressor genes with differentially methylated individual CpG sites or clusters of methylated sites in locally advanced anal cancers. SAL-like 3 (SALL3) has been reported to be methylated in hepatocellular cancer.[25] Secreted frizzled-related protein 2 (SFRP2) has been reported to be frequently methylated in several cancers, including cervical[34], HPV-positive and negative head and neck[27], and prostate[28] (**Online-Table 1**). Paraoxonase 3 (PON 3) has been reported as an imprinted gene and observed here to have reduction methylation in advanced anal cancer. Furthermore, there were a large number of differentially methylated CpG loci in large tumors, suggesting an accumulation of methylation events with progressive growth of a tumor. Of these, 7 genes are tumor suppressor genes previously reported as methylated in other cancers (**Online-Table 2**). Specifically, Early B-cell factor 3 (EBF3)[37] and Neuronal pentraxin I (NPTX1)[66,67] have been reported as epigenetically altered in HPV-associated oropharyngeal and cervical cancers, respectively. Increased methylation of EBF3 was highly correlated with HPV-16 infection in head and neck SCC.[46] In addition, several genes found to be methylated in locally advanced or large anal tumors encode for proteins that interact with HPV oncogenes. For example, cyclin-dependent kinase 6 (CDK6) regulates the activity of tumor suppressor pRb, which is a target of HPV oncoprotein E7. In HPV-positive cervical cancer cell lines, the inactivation of CDK6 was critical for HPV-associated carcinogenesis.[38] Notably, a large number of differentially methylated loci identified in this study occurred within genes that are not well characterized and may represent novel

methylation targets. Overall, this genome-wide methylation analysis identified several biologically-relevant methylated genes that have been consistently found to be methylated in other cancers (including those associated with HPV). This epigenetic silencing that may promote anal tumor growth and progression to advanced disease.

A striking number of epigenetic alterations in CpG loci were identified within components of the WNT/β-catenin pathway[102] in anal tumors. The clustering of epigenetic alterations in the WNT/β-catenin pathway among larger or high-risk anal cancers is similar to what has been reported in HPV-associated cervical cancer.[17,26,103-105] The WNT signaling pathway appears to be a target of HPV, with both epigenetic[106] and gene expression-related alterations.[107] It is well established that WNT/β-catenin signaling is a critical component of cancer progression and epigenetic alterations of both activators and inhibitors may promote aberrant cellular proliferation and carcinogenesis. These epigenetic changes within the WNT pathway warrant further exploration.

There were relatively few significant differentially methylated regions identified by nodal status or clinical risk groups. There are several possible explanations for the relatively few methylation differences by nodal status. First, epigenetic alterations may not play a role in nodal invasion and thus our limited number of aberrantly methylated loci represents the only underlying changes. Second, due to clinical lymph node staging (e.g. physical exam and/or radiological imaging) in anal cancer, there is a possibility that clinically node-negative patients had occult, undetected micrometastatic disease. This would not only dilute the molecular comparison but result in minimizing differences in outcomes between node-positive and node-negative patients. The observation of significantly better outcomes among node-negative patients provides evidence that misclassification of nodal status does not entirely explain these findings. Finally, the cross-sectional design of this study limited our ability to identify the sequence of epigenetic alterations in anal cancer progression and to distinguish alterations which may be drivers or bystanders of neoplastic progression. Future studies that determine the extent of epigenetic differences by nodal involvement and identify epigenomic signatures of patient outcomes are warranted.

This study represents the largest comprehensive genome-wide molecular analysis (in this case, DNA methylation) of anal cancers; a rare malignancy with low tissue availability. The RTOG 98-11 specimen archive is one of the largest fully annotated pre-treatment anal SCC tissue repositories available. Cases in this trial may not represent the general population with anal SCC, especially high-risk populations such as HIV or immunocompromised patients. HIV status of cancer patients was unknown. This study is limited to patients in the mitomycin-C arm with available tumor tissue; however, this sample is representative of the mitomycin-C arm when comparing the distribution of demographic, pathological factors and outcomes (**Figure 2**). Due to the limited availability of anal cancer tissues (especially fresh frozen), this study was unable to determine whether differences in methylation resulted in decreased mRNA transcription. However, differential methylation events at specific loci may still represent potential biomarkers of HPV-associated carcinogenesis. Furthermore, the characterization of clusters of differentially methylated CpG loci within CpG islands allows for the identification of biologically relevant targets for which expression is likely modulated by methylation and can be further investigated using *in-vitro* and *in-vivo*

laboratory models. The molecular biology of anal cancers is not well characterized and based on this study alone; we cannot determine the most important epigenetic alterations among those identified. However, this study provides important candidate targets for future validation in other patient populations and using different methods, such as identifying loss of mRNA or protein expression.

Using a genome-wide methylation analysis, this study has demonstrated that significant epigenetic alterations occur in the progression from early to later stage locally advanced anal cancer. The overall differences in methylation may lend clues to understanding the molecular alterations that occur with the malignant progression of anal cancer. Effective methylation-related biomarkers may ultimately guide modification of treatment for high risk patients (~50% of RTOG 98-11 patients), including radiation dose intensification, closer monitoring of dose completions and/or gaps in treatment and even possibly the development of novel targeted, radiosensitizing agents. An emerging option for dose modification for anal cancer patients is intensity modulated radiation therapy, which has been associated with less acute toxicity as reported in the RTOG-0529 trial[108] and theoretically less potential for accelerated tumor repopulation due to treatment breaks. Furthermore, these findings are also concordant with observations that HPV infection may be associated with extensive epigenetic modifications in the host genome that may impact on tumor development and behavior. Similar to other malignancies, these data suggest that the WNT pathway may play an important role in the progression of anal cancer. Further exploration of the potential roles of methylation-related biomarkers including the development of refined and validated epigenetic signatures of prognosis will be useful in optimizing outcomes in patients with anal cancer.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

The authors thank Sean Yoder and Chaomei Zhang in the Moffitt Molecular Genomics Core for running the Infinium HumanMethylation 450K arrays.

Funding: This was funded by a grant from American Society of Colon and Rectal Surgeons Norman Nigro LPG-09 grant (Shibata/Siegel PI); Miles for Moffitt Milestone Awards (Siegel and Shibata), and LeSportsac Cancer Research Fund (Shibata/Hoffe). Radiation Therapy Oncology Group (RTOG) conducted the RTOG98-11 trial that was supported by RTOG grant U10 CA21661 and CCOP grant U10 CA37422 from the National Cancer Institute (NCI). This manuscript's contents are solely the responsibility of the authors and do not necessarily represent the official views of the National Cancer Institute.

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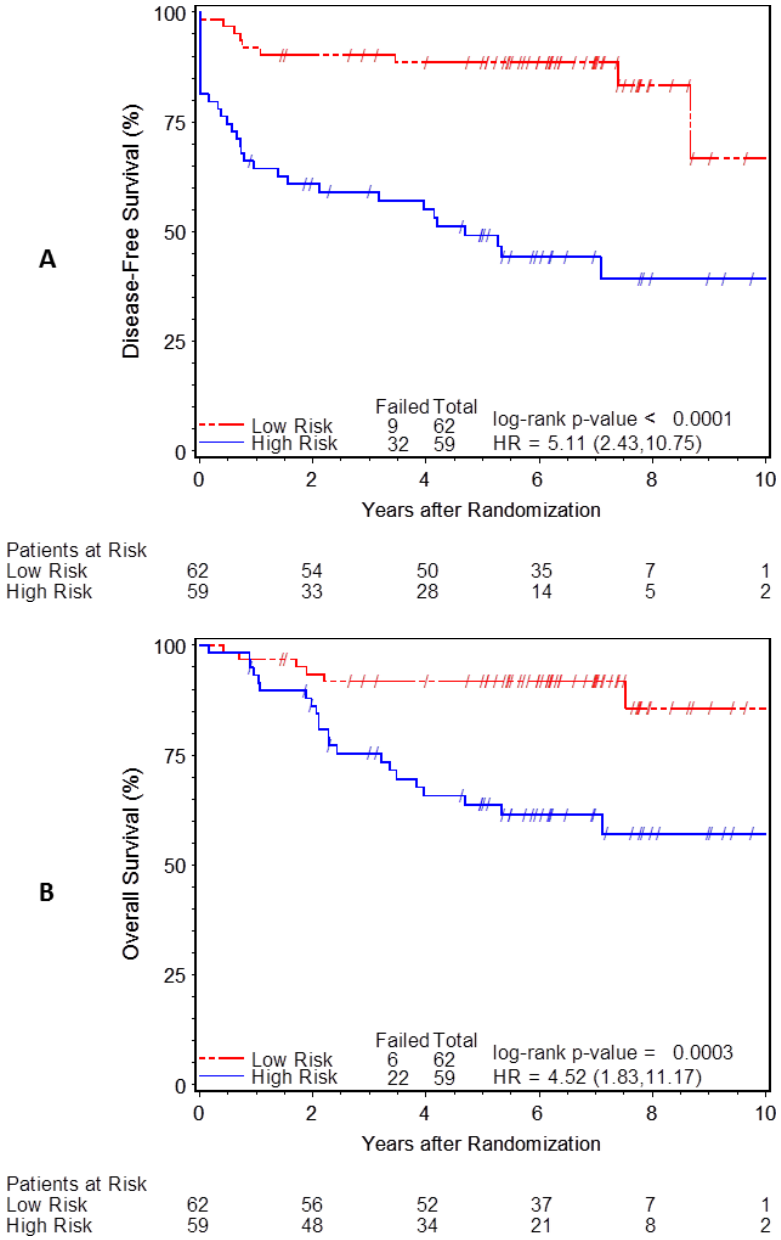

Figure 1.

Kaplan-Meier estimates of disease-free (A) and overall (B) survival by clinically low (N=62) and high (N=59) risk group. Differences in probability of disease-free survival (DFS) or overall survival (OS) by low (red) and high-risk (blue) groups tested using the log-rank test and hazard ratio (HR) estimated using cox-proportional hazards models.

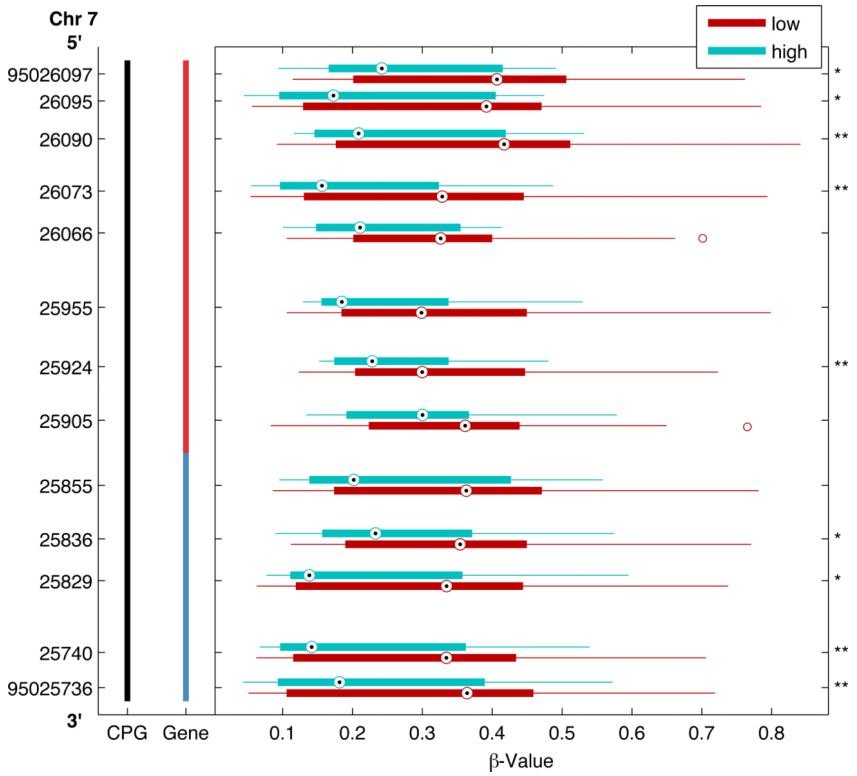

Figure 2.

Differentially methylated CpG Loci by clinical risk group across genomic regions of the Paraoxonase 3 (PON3) gene. A representative region of the genome from chromosome 7 (Chr 7) encoding the Paraoxonase 3 (PON3) gene is presented spanning 361 base pairs (bp) from 5' (top) to 3' (bottom) with genomic coordinates on vertical axis. This region includes 13 CpG loci within a CpG island located 1500 bp (blue) and 200 bp (red) from the gene transcriptional start site (vertical bar). For each CpG loci, boxplots illustrate the median (dot) and interquartile range [25th (low boundary of box) and 75th (upper boundary of box) percentiles] of β-values in high (aqua blue boxes) and low-risk (maroon red boxes) tumors. Significantly different median methylation at each CpG loci is noted: * $p<0.05$; ** $p<0.01$.

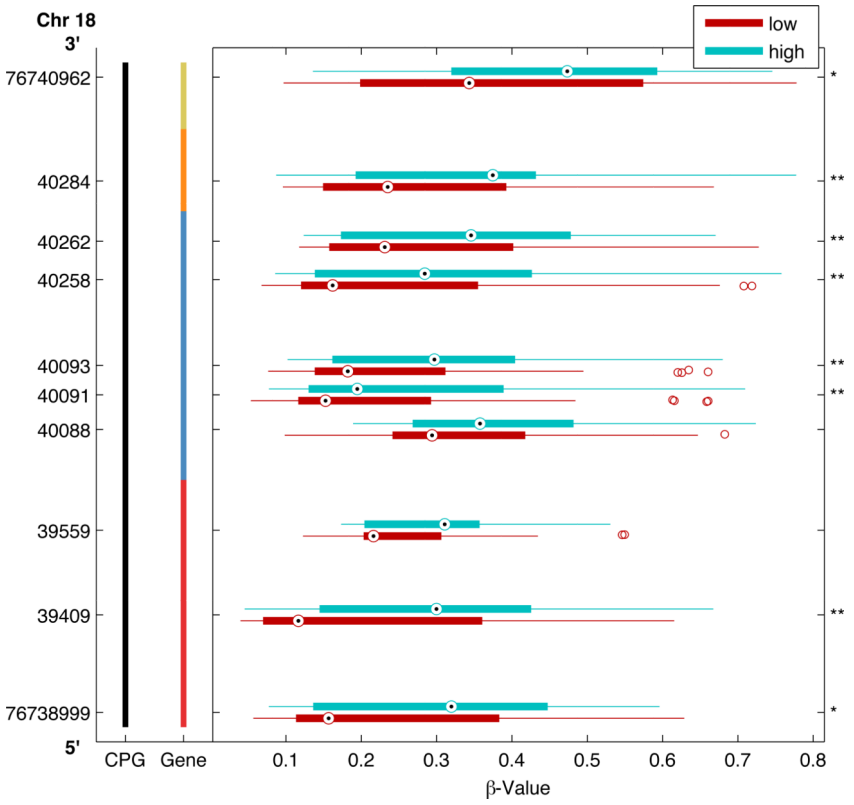

Figure 3.

Differentially methylated CpG Loci by clinical risk group across genomic regions of the SAL-Like 3 (SALL3) gene. A representative region of the genome from chromosome 18 (Chr 18) encoding the SALL3 gene is presented spanning 2Kb from 3' (top) to 5' (bottom) with genomic coordinates on vertical axis. This region includes 10 CpG loci within a CpG island (black bar) located 200 bp (red) and 1500 bp (blue vertical bar) from the gene transcriptional start site or within the first exon (orange) or body (mustard) of the gene. For each CpG loci, boxplots illustrate the median (dot) and interquartile range [25th (low boundary of box) and 75th (upper boundary of box) percentiles] of β-values in high (aqua blue boxes) and low-risk (maroon red boxes) tumors. Significantly different median methylation at each CpG loci is noted: * $p<0.05$; ** $p<0.01$.

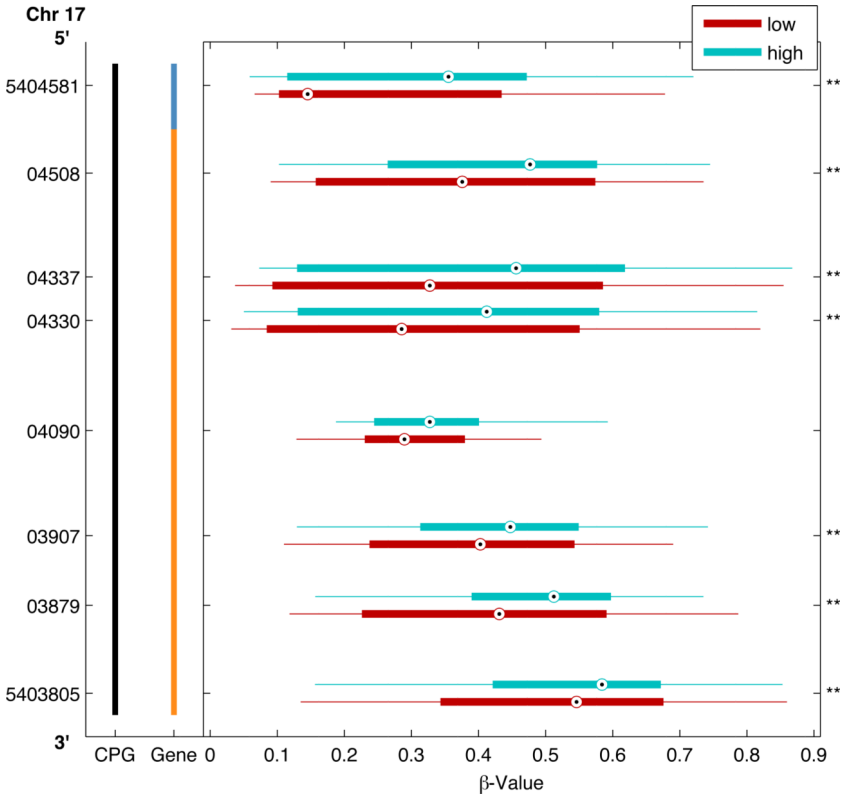

Figure 4.

Differentially methylated CpG Loci by clinical risk group across the LOC728392 genomic region. A representative region of the genome from chromosome 17 (Chr 17) encoding the uncharacterized transcript LOC728392 spanning from 5' (top) to 3' (bottom) with genomic coordinates on vertical axis. This region includes 8 CpG loci within a CpG island located 1500 bp (blue vertical bar) from the gene transcriptional start site or within the first exon (orange vertical bar). For each CpG loci, boxplots illustrate the median (dot) and interquartile range [25th (low boundary of box) and 75th (upper boundary of box) percentiles] of β-values in high (aqua blue boxes) and low-risk (maroon red boxes) tumors. Significantly different median methylation at each CpG loci is noted: * $p<0.05$; ** $p<0.01$.

Table 1

Characteristics of low- and high-risk clinical groups with anal cancer

	Low Risk[a] (n=62)		High Risk (n=59)		Total (n=121)		p-value[b]
	n	%	n	%	n	%	
Median Age (min-max)	54 (37-78)		53 (25-79)		54 (25-79)		
Mean age (SD)	55.5 (9.7)		54.4 (10.4)		55.0 (10.0)		0.54
Gender							
Male	25	40%	22	37%	47	39%	0.73
Female	37	60%	37	63%	74	61%	
Race							
White	52	84%	53	90%	105	87%	0.33
African American/Other	10	16%	6	10%	16	13%	
KPS							
60-70	3	5%	3	5%	6	5%	1.00
80-100	59	95%	56	95%	115	95%	
Mean tumor size (SD)	3.5 (0.9)		5.6 (2.3)		4.5 (2.0)		--[c]
T-Stage							
T2	60	97%	24	41%	84	69%	--[c]
T3/T4	2	3%	35	59%	37	31%	
N-Stage							
N0	62	100%	23	39%	85	70%	--[c]
N1	0	0%	11	19%	11	9%	
N2	0	0%	14	24%	14	12%	
N3	0	0%	5	8%	5	4%	
Nx	0	0%	6	10%	6	5%	

Abbreviations: min, minimum; max, maximum; SD, standard deviation, KPS, Karnofsky Performance Status

[a] Low-risk = tumors ≤5 cm and N0; High-risk= tumors >5 cm and/or N+

[b] Statistical differences in patient characteristics between the two groups were determined by Chi-square test, Fisher's exact test, or t-test.

[c] Testing not applicable for tumor size and N-stage, as those variables define the risk groups. Similarly for T-stage, which is highly associated with tumor size by AJCC 1997 version definition.

Table 2

Differentially Methylated CpG Loci in Clinically defined Low and High-risk anal cancers

Symbol	Product	Association with Disease	CpG Number[a]	Median Beta value			p-value[c]	
				Low Risk	High Risk	Dif[b]	MW	T-Test
ADAT3[d]	Adenosine deaminase, tRNA-specific 3	Not reported as target of methylation or involved in cancer	cg20243900	0.64	0.75	0.11	5.48E-04	2.01E-04
GSG1L	Germ Cell Associated 1 (GSG1)-Like	Possible susceptibility locus for colon cancer in AOM mouse models; Not reported as target of methylation.	cg09528825	0.07	0.18	0.11	3.12E-02	8.15E-04
KIAA0319	KIAA0319	Dyslexia-associated gene, Not reported as target of methylation or involved in cancer	cg18428180	0.24	0.09	−0.15	1.29E-03	4.87E-04
LOC-728392	LOC728392	Not reported as target of methylation or involved in cancer	cg18671949	0.11	0.40	0.29	4.96E-05	9.84E-06
			cg06462347	0.24	0.53	0.28	1.43E-04	5.17E-05
			cg27230784	0.14	0.51	0.37	4.44E-05	4.58E-05
			cg22298430	0.15	0.47	0.32	1.49E-04	7.13E-05
			cg01433610	0.38	0.52	0.15	1.66E-03	8.04E-04
PARD3	Partitioning defective 3 homolog	Lost in prostate cancer[23], involved in cell motility; Associated with WNT/β-catenin pathway[24]; Not reported as a target of methylation.	cg26795540	0.48	0.58	0.10	7.57E-04	1.60E-03
SALL3	SAL-Like 3	Methylated in HCC[25]; interacts with DNMT3A to reduce methylation	cg13794993	0.22	0.38	0.17	6.89E-04	4.15E-04
			cg14007067	0.14	0.31	0.17	1.27E-03	8.36E-04
			cg06954658	0.16	0.27	0.11	1.47E-03	4.78E-04
			cg16433156	0.14	0.25	0.11	1.34E-03	5.46E-04
			cg05080154	0.11	0.29	0.18	2.36E-03	9.22E-04
SFRP2	secreted frizzled-related protein 2	Methylated in several cancers, including cervical[26]; head and neck[27], and prostate[28]	cg22178613	0.16	0.38	0.22	1.10E-03	8.78E-04
ST6GAL2	ST6 beta-galactosamide alpha-2,6-sialyltranferase 2	Upregulated in breast cancer[29]. Not reported as target of methylation.	cg13168617	0.72	0.57	−0.15	1.75E-03	8.25E-04
SCAMP4[d]	Secretory Carrier Membrane Protein 4	Not reported as target of methylation or involved in cancer	cg20243900	0.64	0.75	0.11	5.48E-04	2.01E-04

[a] CpG number is the unique identifier for the loci provided by Illumina; multiple loci within a differentially methylated region listed in descending order according to genomic position.

[b] Difference in median β-value between high and low risk tumors

[c] Statistical differences in β-values between the two groups were determined by Mann-Whitney or student's t-test

[d] CpG loci located in a genomic sequence that overlaps for ADAT3 and SCAMP4 genes.

Table 3

Annotation of genes with differentially methylated CpG Loci by diameter of primary tumor (≤ 5 cm vs. >5 cm)

Symbol	Product	Association with Disease	CpG Number[a]	Beta value			p-value[c]	
				≤5 cm	>5 cm	Dif.[b]	MW	T-Test
ADAT3[d]	Adenosine deaminase, tRNA-specific 3	Not reported as target of methylation or involved in cancer	cg01397065	0.57	0.74	0.16	2.75E-03	2.30E-04
AGFG2	ArfGAP with FG repeats 2	Associated with HIV infection; Not reported as target of methylation or involved in cancer	cg03431524	0.43	0.55	0.12	6.56E-04	1.06E-03
ALDH1L2	Aldehyde dehydrogenase 1 family, member L2	Methylated in alcoholism[30]. Not reported as involved in cancer	cg16527105	0.67	0.83	0.16	9.98E-04	9.81E-03
ASPSCR1	Alveolar soft part sarcoma (ASPS) chromosome region, 1	Translocated in ASPS and renal cell carcinoma; Not reported as target of methylation.	cg11511084	0.58	0.71	0.13	4.85E-04	5.96E-04
BDNFOS	Brain derived neurotrophic factor (BDNF) antisense RNA	Not reported as target of methylation or involved in cancer	cg23330212	0.59	0.42	−0.16	8.81E-04	4.95E-04
BLVRA	Biliverdin reductase A	Altered expression in HCC[31], renal[32] and vaginal[33] cancer. Upregulated after p53 loss[34] and chemotherapy[35]. Not reported as a target for methylation.	cg14579118	0.62	0.74	0.11	1.87E-03	6.39E-04
BRE	Brain and reproductive organ-expressed	TNFRSF1A modulator[36]; Up-regulated in HCC and esophageal cancer; Not reported as target of methylation	cg13861527	0.60	0.77	0.18	3.98E-04	2.05E-04
C2orf74	Chromosome 2 open reading frame 74	Not reported as target of methylation or involved in cancer	cg01648237	0.32	0.16	−0.16	9.18E-04	1.82E-03
C6orf136	Chromosome 6 open reading frame 136	Not reported as target of methylation or involved in cancer	cg13016528	0.53	0.64	0.11	5.29E-04	3.60E-04
CCDC63	Coiled-coil domain containing 63	Not reported as target of methylation or involved in cancer	cg02855409	0.24	0.37	0.13	4.31E-03	8.76E-04
			cg10995082	0.32	0.50	0.18	8.03E-03	4.22E-04
			cg19006003	0.33	0.48	0.15	2.86E-03	3.25E-05
CDK6	Cyclin-dependent kinase 6	Upregulated in lymphoma, leukemia, and melanoma[37]; inactivation in HPV+ cervical cells critical[38]; Not reported as a target for methylation.	cg23628117	0.71	0.83	0.12	7.60E-04	1.51E-03
COX8A	Cytochrome c oxidase subunit VIIIA (ubiquitous)	Related to tumor progression and chemo-resistance in glioma[39], breast[40] and esophageal[41] cancers; loss of COX subunits increases chemo-sensitivity[41]; HIV inhibits COX activity[42]; Not reported as a target of methylation.	cg17292384	0.60	0.72	0.12	3.56E-04	1.09E-04
CYP27C1	Cytochrome P450, family 27, subfamily C, 1	Uncharacterized; Not reported as target of methylation or involved in cancer.	cg08022717	0.63	0.76	0.13	1.38E-03	5.10E-04
DMRT3	Doublesex and MAB-3 related transcription factor 3	Silenced or lost in lung SCC[43]. Not reported as target of methylation.	cg14176274	0.27	0.39	0.12	2.60E-04	4.45E-04

Symbol	Product	Association with Disease	CpG Number[a]	Beta value			p-value[c]	
				≤5 cm	>5 cm	Dif.[b]	MW	T-Test
EBF3	Early B-cell factor 3	Methylated in pancreatic[44], gastric[45]; and HPV+ head and neck SCC[46]	cg04804618	0.16	0.34	0.18	8.81E-04	1.59E-03
			cg14737286	0.28	0.43	0.14	1.41E-03	8.06E-04
FAM150A	Family With Sequence Similarity 150, Member A	Increased methylation by Infinium in more aggressive renal cell cancers[47]	cg07076175	0.45	0.60	0.15	1.53E-04	1.14E-04
			cg10094616	0.17	0.42	0.24	2.91E-04	1.43E-04
			cg22862746	0.18	0.36	0.18	6.56E-04	9.57E-04
			cg09442654	0.36	0.61	0.25	6.70E-04	1.62E-04
FOS	FBJ murine osteosarcoma viral oncogene homolog	Oncogene; AP-1 activates transcription and methylation of TSG and HPV[48]; Induces EMT	cg23404711	0.52	0.63	0.11	5.40E-04	5.38E-04
FZD10	Frizzled family receptor 10	Member of WNT/β-catenin signaling pathway; Not reported as target of methylation	cg13859208	0.49	0.61	0.12	4.92E-05	2.07E-06
GET4	Golgi to ER traffic protein 4 homolog (S. cerevisiae)	Associated with ubiquitination and ER; Not reported as target of methylation or involved in cancer	cg24580076	0.46	0.66	0.20	1.46E-04	9.10E-05
GSG1L	Germ Cell Associated 1 (GSG1)-Like	Possible susceptibility locus for colon cancer in AOM mouse models; Not reported as a target of methylation.	cg03921753	0.15	0.30	0.15	5.76E-04	8.10E-04
			cg09528825	0.07	0.37	0.29	1.38E-03	3.35E-04
			cg03394150	0.08	0.29	0.21	2.31E-03	8.97E-04
GULP1	Engulfment adaptor PTB domain 1	Possible TSG; inhibits TGF-B induced growth	cg19202813	0.72	0.57	−0.15	5.52E-04	1.02E-03
HDAC2	histone deacetylase 2	Overexpressed in several cancers, including prostate[49] and ovarian[50] cancer; regulator of DNA methylation. Not reported as a target of methylation.	cg15069235	0.55	0.69	0.15	6.28E-04	5.73E-04
HORMAD 2	HORMA domain containing 2	Novel cancer/testis gene; expressed in lung cancer (Chinese)[51]. Not reported as a target of methylation.	cg24211826	0.59	0.71	0.12	5.40E-04	7.70E-04
HOXA6	homeobox A6	Methylated in meningioma[52] and lymphoid malignancies[53].	cg23129930	0.45	0.60	0.15	5.89E-04	9.02E-04
HS6ST1	heparan sulfate 6-O-sulfotransferase 1	HPV binds heparan sulfate protoglycans for cell entry[54]; activity reported in ovarian cells[55]; Not reported as target for methylation.	cg08472795	0.44	0.58	0.14	5.64E-04	5.79E-04
KIR3DX1	killer cell immunoglobulin-like receptor, three domains, X1	Regulated by DNA methylation[56]. KIR3DX1 not reported to be a target for methylation or involved in cancer.	cg10731960	0.64	0.77	0.13	1.02E-03	4.58E-04
KLHL29	Kelch-like family member 29	Not reported as target of methylation or involved in cancer	cg00537210	0.44	0.58	0.14	7.45E-04	1.27E-03
MECOM	MDS1 and EVI1 complex locus	Amplification early event in several cancers, including ovary, head and neck, and cervical cancers. Not reported as a target of methylation	cg20528780	0.51	0.40	−0.12	2.85E-04	7.94E-04
MFI2	Melanotransferrin	Expressed in melanoma[57] and other cancers; Not reported as target of methylation	cg00477017	0.38	0.48	0.10	4.54E-04	8.00E-04

Symbol	Product	Association with Disease	CpG Number[a]	Beta value			p-value[c]	
				≤5 cm	>5 cm	Dif.[b]	MW	T-Test
MIR200B	MicroRNA 200b	Regulated by DNA methylation; epigenetic silencing promotes EMT[58]; Altered in oral[59] and head and neck[60].	cg14161399	0.77	0.91	0.14	1.87E-03	1.19E-05
MIR200A[d]	MicroRNA 200a		cg02825344	0.60	0.72	0.12	2.75E-03	7.00E-04
MMP9	Matrix metallopeptidase 9	Tumor-associated tissue remodeling and metastasis. Not reported as target of methylation.	cg17664577	0.26	0.46	0.19	7.77E-04	8.27E-04
MRPS22	Mitochondrial ribosomal protein S22	Overexpressed in breast cancer[61]; Not reported as a target of methylation.	cg11277156	0.66	0.76	0.10	2.36E-05	1.09E-06
MSI2	Musashi RNA-binding protein 2	Overexpressed in leukemia[62] and medulloblastoma[63]; downstream regulator of WNT-related modulation of p21[64]. Not reported as target of methylation	cg04486382	0.64	0.84	0.20	6.99E-04	1.54E-03
			cg19810715	0.64	0.82	0.18	4.44E-04	3.26E-03
NPTX1	Neuronal pentraxin I	Methylated in cancer, including colon[65] and cervical cancer[66],[67].	cg20853771	0.68	0.79	0.11	2.18E-03	2.43E-04
OR2C3	Olfactory receptor, family 2, subfamily C, 3	Not reported as target of methylation or involved in cancer	cg20320823	0.56	0.69	0.12	6.99E-04	4.33E-04
PAK1	p21 protein-activated kinase 1	Upregulated in several cancers[68],[69]; involved in WNT/β-catenin and RAS pathways; Increased cell motility; interaction with HPV[70]	cg24536703	0.62	0.72	0.11	3.98E-04	6.91E-05
PDE3B[d]	Phosphodiesterase 3B,	Pro-apoptotic in CML[71]; associated with cisplatin resistance in head and neck SCC[72]	cg21901307	0.24	0.35	0.11	2.72E-04	1.36E-03
PLA2G2A	Phospholipase A2, group IIA	Regulated by WNT/β-catenin pathway; expression associated with improved survival in gastric[73] and esophageal[74] cancers	cg13211559	0.72	0.83	0.12	3.98E-04	1.88E-03
PLEKHG1	Pleckstrin homology domain containing, family G member 1	Oncogene; activation leads to aberrant Rho GTPase targets; Not reported as a target for methylation.	cg26852242	0.66	0.80	0.14	8.27E-04	4.47E-04
PLEKHG3	Pleckstrin homology domain containing, family G member 3	Oncogene; activation leads to aberrant Rho GTPase targets; Not reported as a target for methylation.	cg11802553	0.66	0.77	0.11	2.43E-04	3.02E-04
PON3	Paraoxonase 3	Imprinted in mouse genome; Over-expressed in several cancers[75]; anti-apoptotic	cg04080282	0.48	0.37	−0.10	4.39E-03	7.39E-04
			cg08898155	0.41	0.24	−0.16	3.20E-03	5.54E-04
			cg11435506	0.33	0.16	−0.17	2.91E-03	2.10E-04
PPP1R9A	Protein phosphatase 1, regulatory subunit 9	Imprinted gene; Not reported to be involved in cancer.	cg09724492	0.38	0.52	0.14	4.47E-03	5.00E-04
RERE	Arginine-glutamic acid dipeptide (RE) repeats	Expression reduced in neuroblastoma and leukemia[76]. Not reported to be a target of methylation.	cg19679865	0.62	0.75	0.13	3.64E-04	2.97E-04
PSMA1[d]	Proteasome subunit, alpha type, 1	Mediates bortezomib resistance in AML[77]; may promote tumor growth; loss radio-sensitized NSCLCs. Not reported as a target for methylation.	cg21901307	0.24	0.35	0.11	2.72E-04	1.36E-03

Symbol	Product	Association with Disease	CpG Number[a]	Beta value			p-value[c]	
				≤5 cm	>5 cm	Dif.[b]	MW	T-Test
RNASEN	Ribonuclease type III, nuclear	Regulates cell proliferation; increased copy number in cervical cancer[78]	cg04590036	0.68	0.82	0.14	4.44E-04	5.74E-04
RREB1	Ras responsive element binding protein 1	Tumor marker in melanoma; downregulated in pancreatic cancer[79]; regulated by miRNA[80]	cg03137792	0.62	0.83	0.21	1.28E-03	7.00E-04
SCAMP4[d]	Secretory Carrier Membrane Protein 4	Not reported as target of methylation or involved in cancer	cg01397065	0.57	0.74	0.16	2.75E-03	2.30E-04
SEPP1	Selenoprotein P 1	SNPs in prostate[81] and esophageal risk[82]; mercury induced hypomethylation[83].	cg08626131	0.51	0.66	0.15	2.79E-04	2.32E-04
SLC9A3	Solute carrier family 9, subfamily A, member 3	Downregulated in Ulcerative Colitis[84]; Not a reported target for methylation.	cg06058576	0.68	0.91	0.23	1.98E-04	3.64E-04
SNTB1	Syntrophin, beta 1, basic component 1	Expression associated with lung cancer survival[85]; Interacts with the HTLV-1 TAX protein[86]; Not reported as a target of methylation.	cg04318006	0.12	0.23	0.10	8.10E-04	1.68E-03
STMN2	Stathmin-like 2	Involved in WNT/β-catenin pathway; novel β-catenin transcriptional target in HCC[87]; age-related methylation in germ cells[88].	cg00398130	0.45	0.57	0.12	3.08E-03	7.09E-04
SURF6	Surfeit 6	Not reported as target of methylation or involved in cancer.	cg01832218	0.57	0.72	0.14	1.93E-04	5.13E-05
TMEM196	Transmembrane protein 196	Not reported as target of methylation or involved in cancer.	cg18505401	0.33	0.49	0.16	8.45E-04	5.94E-04
TRA2B	Transformer 2 beta homolog	Oncogenic; amplified in lung, ovary, cervix and head/neck[89]; downregulation-improved survival.	cg12825509	0.52	0.62	0.10	5.40E-04	1.33E-04
TTC9	Tetratricopeptide Repeat Domain-Containing 9	Upregulation leads to increased cell motility in breast cancer; Not reported as target of methylation.	cg01634544	0.35	0.52	0.17	3.19E-04	4.04E-04
VWDE	von Willebrand factor D and EGF domains	Not reported as target of methylation or involved in cancer.	cg02935154	0.21	0.42	0.21	7.60E-04	6.10E-04
			cg16278512	0.15	0.37	0.21	2.72E-04	8.68E-04
WNT9A	wingless-type MMTV integration site family, member 9A	Bi-directional promoter for WNT9A/CD558500 silenced by methylation[90]; mutated in colon cancer[91]	cg26452081	0.42	0.53	0.12	1.89E-04	1.97E-04
ZHX2	ZHX2 zinc fingers and homeoboxes 2	TSG; methylated in HCC[92]	cg01801603	0.29	0.41	0.12	7.45E-04	1.29E-03

[a] CpG number is the unique identifier for the loci provided by Illumina; multiple loci within a differentially methylated region listed in descending order according to genomic position.

[b] Difference in median β-value between large and small tumors

[c] Statistical differences in β-values between the two groups were determined by Mann-Whitney or student's t-test

[d] CpG loci located in a genomic sequence that overlaps for ADAT3/SCAMP4, MIR200A/MIR200B; and PDE3B/PSMA1 genes.

Table 4

Differentially methylated CpG loci across genomic regions[a] by risk group, tumor size and nodal status

Gene	Number of Methylated Loci / Total CpG sites (%)	Loci with decreased methylation[b]	Loci with Increased methylation
Risk Group			
LOC728392	7 / 15 (46.7)	0	7
PON3	9 / 28 (32.1)	9	0
VWC2	8 / 26 (30.8)	0	8
PREX2	6 / 20 (30.0)	0	6
SALL3	8 / 27 (29.6)	0	8
SLITRK3	7 / 25 (28.0)	0	7
Tumor Size			
SVIP	10 / 12 (83.3)	0	10
C2orf43	8 / 14 (57.1)	8	0
ENPP5	7 / 13 (53.8)	0	7
PON3	14 / 28 (50.0)	14	0
C2orf74	7 / 16 (43.8)	7	0
DPY19L2P4	7 / 16 (43.8)	0	7
FOXI2	9 / 22 (40.9)	0	9
TMEM178	6 / 15 (40.0)	0	6
STK32A	7 / 18 (38.9)	1	6
VWC2	10 / 26 (38.5)	0	10
STK33	8 / 22 (36.4)	0	8
PREX2	7 / 20 (35.0)	0	7
RGS17	6 / 20 (30.0)	0	6
NHSL1	8 / 44 (18.2)	0	8
NKAIN3	6 / 38 (15.8)	0	6
GRID2	6 / 47 (12.8)	1	5
SGK1	6 / 51 (11.8)	0	6
HOXB3	6 / 54 (11.1)	0	6
CDK6	7 / 66 (10.6)	0	7
HOXC4	8 / 126 (6.3)	4	4
Nodal Status			
LOC728392	7 / 15 (46.7)	0	7
SALL3	6 / 27 (22.2)	0	6
SLITRK3	6 / 25 (24.0)	1	5

[a] Gene regions defined by Illumina.

[b] Number of CpG Loci that had significantly decreased methylation (lower β-value in high-risk, >5cm, or N+ groups) and increased methylation (higher β-value in high-risk, >5cm, or N+ groups)