Genome-Wide Hypomethylation in Head and Neck Cancer Is More Pronounced in HPV-Negative Tumors and Is Associated with Genomic Instability

Kristy L. Richards1,4*, Baili Zhang1, Keith A. Baggerly2,5, Stefano Colella1,6*, James C. Lang7, David E. Schuller7, Ralf Krahe1,3,5,6*

1 Department of Genetics, University of Texas M. D. Anderson Cancer Center, Houston, Texas, United States of America, 2 Department of Bioinformatics and Computational Biology, University of Texas M. D. Anderson Cancer Center, Houston, Texas, United States of America, 3 Department of Thoracic/Head and Neck Medical Oncology, University of Texas M. D. Anderson Cancer Center, Houston, Texas, United States of America, 4 Division of Cancer Medicine, University of Texas M. D. Anderson Cancer Center, Houston, Texas, United States of America, 5 Graduate Program in Human and Molecular Genetics, University of Texas at Houston Graduate School in Biomedical Sciences, Houston, Texas, United States of America, 6 Graduate Program in Genes and Development, University of Texas at Houston Graduate School in Biomedical Sciences, Houston, Texas, United States of America, 7 Molecular Biology and Cancer Genetics Programs, Ohio State University, Comprehensive Cancer Center, Columbus, Ohio, United States of America

Abstract

Loss of genome-wide methylation is a common feature of cancer, and the degree of hypomethylation has been correlated with genomic instability. Global methylation of repetitive elements possibly arose as a defense mechanism against parasitic DNA elements, including retrotransposons and viral pathogens. Given the alterations of global methylation in both viral infection and cancer, we examined genome-wide methylation levels in head and neck squamous cell carcinoma (HNSCC), a cancer causally associated with human papilloma virus (HPV). We assayed global hypomethylation levels in 26 HNSCC samples, compared with their matched normal adjacent tissue, using Pyrosequencing-based methylation assays for LINE repeats. In addition, we examined cell lines derived from a variety of solid tumors for LINE and SINE (Alu) repeats. The degree of LINE and Alu hypomethylation varied among different cancer cell lines. There was only moderate correlation between LINE and Alu methylation levels, with the range of variation in methylation levels being greater for the LINE elements. LINE hypomethylation was more pronounced in HPV-negative than in HPV-positive tumors. Moreover, genomic instability, as measured by genome-wide loss-of-heterozygosity (LOH) single nucleotide polymorphism (SNP) analysis, was greater in HNSCC samples with more pronounced LINE hypomethylation. Global hypomethylation was variable in HNSCC. Its correlation with both HPV status and degree of LOH as a surrogate for genomic instability may reflect alternative oncogenic pathways in HPV-positive versus HPV-negative tumors.

Introduction

DNA methylation is an epigenetic DNA modification that occurs via the action of DNA methyltransferases on CpG dinucleotides. Methylated regions of DNA are associated with chromatin remodeling, generally occurring in areas of more condensed chromatin and decreased transcriptional activity [1,2]. Renewed interest in this process arose after it was recognized that two types of aberrant methylation patterns are present in cancer cells [1,3]. The first is gene-specific hyper-methylation, where CpG islands in the promoter regions of genes acquire increased methylation, generally leading to reduced expression of the downstream gene. The second is genome-wide hypo-methylation, a large percentage of which occurs in repetitive DNA elements. In malignancy, global methylation is often aberrantly reduced, whereas gene-specific methylation is often aberrantly increased. While the effects of gene-specific hypermethylation (e.g., reduced expression of a gene that is important for growth control) are easily appreciated, the effects of reduced global methylation are more vague [1,3].

It has been hypothesized that DNA methylation initially evolved as a defense mechanism against viral and other DNA pathogens as a way to silence foreign DNA sequences [4–6]. This is consistent with the observation that LINE and SINE (Alu) elements, originating from transposable elements, are heavily methylated in normal cells. Methylation of the HPV viral genome upon integration into the host genome has been reported, and changes in methylation of HPV DNA have been associated with tumorigenesis [7,8].
Global methylation is also clinically relevant, as demonstrated by associations between clinical outcome and global methylation levels in a number of cancer types [9–11]. From a mechanistic standpoint, global methylation appears to be related to cancer progression, since loss of global methylation tends to become more pronounced as precancerous lesions progress [12,13]. Furthermore, in colon cancer cells, loss of LINE methylation is inversely correlated with microsatellite instability and is directly correlated with chromosome instability [14,15].

We hypothesized that global methylation levels in cancer, represented by levels of LINE and SINE (Alu) methylation, might be correlated with viral infection. We, therefore, examined LINE methylation in relation to HPV status in head and neck cancers. In contrast to cervical cancers, which are nearly all associated with HPV infection, HNSCC is virally mediated in only a subset of cases (25–30%) [16]. To determine the impact of viral infection on global methylation levels in HNSCC, we developed Pyrosequencing-based methylation assays for repetitive DNA elements and compared HPV-positive with HPV-negative cancers. Previous publications have suggested that LINE methylation is variable in HNSCC, but did not find an association between HPV DNA status and LINE methylation levels [17,18]. Here we show that global hypomethylation is variable in HNSCC and correlates with both HPV status and genomic instability.

Results
LINE/SINE (Alu) assays are precise and reproducible, but show only moderate correlation between LINE-1 and Alu methylation levels

To assay global methylation levels, we adapted our Pyrosequencing-based Methylation Analysis (PMA) assay [19] to assess methylation of repetitive LINE and SINE (Alu) elements, similar to genome-wide methylation assays reported previously [20]. To validate the assays, we performed a series of tests: (1) mixing experiments with known amounts of methylated/unmethylated DNA; (2) methylation studies on a variety of cancer cell lines to compare LINE to SINE methylation and to survey global methylation across a broad range of malignancies; and (3) methylation of samples from different ages and genders to eliminate these factors as possible confounders of global methylation measurements.

Stepwise increments of methylated DNA were prepared by mixing universally unmethylated (U2ML) DNA with universally methylated (UM.L) DNA in various proportions. The mixed samples were then bisulfite treated and subjected to our PMA LINE-1 (LINE) and Alu (SINE) assays. Linear regression analysis showed that LINE-1 and Alu methylation levels were very closely associated with levels predicted by input fraction of methylated DNA ($r = 0.995, p$-value $< 0.0005$ for LINE-1, and $r = 0.980, p$-value $< 0.0005$ for Alu; Figure S1). It should be noted that even when input DNA was completely methylated, the maximum global methylation percentage as measured by the PMA assay is just over 50%, not 100%. This is because individual repetitive elements have diverged in sequence over time; and CpG dinucleotides, if mutated toTpG dinucleotides, are indistinguishable from unmethylated CpG after the bisulfite treatment. This level of background noise is taken into account by normalizing each result to the universally methylated control (i.e., reporting the percent methylated reference, or PMR).

Four pools of normal DNA samples (from peripheral blood leukocytes) were generated to assess the influence of age and gender on LINE-1 and Alu methylation levels. Each pool (females ≤40 years old, males ≤40 years old, females >40 years old, males >40 years old) contained DNA from at least five individuals. There were no sex- or age-dependent differences between the pools in LINE-1 or Alu methylation levels (data not shown).

Each LINE-1 and Alu methylation assay was tested on a panel of 23 cancer cell lines. Different CpG sites were compared by means of three distinct LINE-1 assays and three distinct Alu assays, each derived from different regions of the LINE-1 and Alu consensus sequences, respectively (Table 1). As a control, we also tested seven normal lymphoblastoid cell lines, which showed normal levels of methylation (all >80% in our LINE-1 assays). In contrast, as expected from previous reports [12–14,21], many of the tumor cell lines showed global hypomethylation, which was most pronounced in the LINE-1 assays (Figure 1). To check the consistency between LINE and SINE methylation levels (represented by our LINE-1 and Alu assays, respectively), we compared the degree of correlation between results from the three LINE-1 assays, between

| Assays | GenBank Number | F Primer Seq (5′-3′) | R Primer Seq (5′-3′) | Sequencing Primer (5′-3′) | Amplicon Size (bp) | No. CpG Sites |
|--------|---------------|----------------------|----------------------|--------------------------|-------------------|--------------|
| L1-1   | X52235        | ttattaggstattatatggg | GACGGGACACCGCTGATCGTTTA | tgggyttaggttagggtg (F) | 117               | 6            |
|        |               |                       |                       | ctttccaaataaaacactacc    |                   |              |
| L1-2   | X52235        | GACGGGACACCGCTGATCGTTTA | ggttagtttagttggagaa   |                         |                   |              |
|        |               |                       |                       | ccttccaaacacaaataaaat    |                   |              |
| L1-3   | M80343        | attagattagttatagttggg | GACGGGACACCGCTGATCGTTTA | gyttagtattgtgtggtg (F) | 123               | 5            |
|        |               |                       |                       | cccttacaccccaaat         |                   |              |
| Alu-1  | [26]          | GACGGGACACCGCTGATCGTTTA | ccaacaaaataaaat       |                         |                   |              |
|        |               |                       |                       | aaaccaaaaaaataaat       |                   |              |
| Alu-2  | [26]          | GACGGGACACCGCTGATCGTTTA | tcaacccrrraaaaataaa    |                         |                   |              |
|        |               |                       |                       | aataacaaaaatcaacaaac    |                   |              |
| Alu-3  | J00085        | gqqqagqagqgtttagttagga | GACGGGACACCGCTGATCGTTTA | tgaatattagqgtgggfa (G) | 104               | 4            |
|        |               |                       |                       | cactatcaacccaaataaaaatca |

The sequence for the biotin-labeled universal primer (5′-3′) for the 3-primer PCR is underlined. Biotin-GGGACACCGCTGATCGTTTA. F, sequencing primer extends in the forward direction; R, sequencing primer extends in the reverse direction. r, purine (A or G); y, pyrimidine (C or T). doi:10.1371/journal.pone.0004941.t001
results from the three Alu assays, and between the various LINE-1 and Alu assays. Linear regression analysis showed that the individual LINE-1 assay results were highly correlated with each other, for example \( r = 0.93 \) for the correlation between the L1-2 and L1-3 assay results (Figure S2A). The same high degree of correlation was present between results from the Alu assays, for example \( r = 0.82 \) for the correlation between the Alu-1 and Alu-3 assay results (Figure S2B). However, LINE-1 assay results were only moderately correlated with Alu assay results, for example \( r = 0.33 \) comparing L1-1 and Alu-1; Figure S2C). A similar level of moderate correlation between LINE-1 and Alu assays was reported previously in neuroendocrine tumors [22].

LINE-1 hypomethylation of HNSCC patient samples

Matching normal, primary tumor, and where available, lymph node metastases of head and neck patient samples (Table S1) were tested using our PMA LINE-1 assays (Figure 2). Because of the greater dynamic range of the LINE-1 assay and limited sample amounts, only LINE-1 assays were performed for the remainder of this study. In general, HNSCC primary tumors and metastatic lymph nodes were hypomethylated compared to their matching normal adjacent tissues. With the LINE1-1 assay, the mean primary tumor PMR was 65.5%, while the mean normal PMR was 90.0% (p-value = 6.7 \times 10^{-20} using a paired t-test). However, there was quite a bit of variability in the primary tumors, ranging from 31.2% (severe hypomethylation) to 90.8% (normal). The mean PMR in lymph node metastases (73.8%; range 31.8%–93.8%) was also highly variable, even with respect to the corresponding primary tumor, sometimes lower and sometimes higher than the PMR of the primary tumor with which it is associated.

HPV-positive tumors retain more LINE-1 methylation than HPV-negative tumors

Because there was so much variability in the HNSCC tumor PMR, we hypothesized that the level of global hypomethylation might vary in different subgroups of HNSCC. To explore the relationship between loss of LINE-1 hypermethylation and HPV status, we compared the mean LINE-1 methylation level of three
groups that differed by their HPV status. The first group (n = 8) was HPV negative (−/−); the second group (n = 8) contained HPV DNA, yet were transcriptionally silent with respect to the E6 viral mRNA; (+/−), HPV-positive but transcriptionally silent; and −/+, HPV negative.

doi:10.1371/journal.pone.0004941.g003

Levels of LINE-1 hypomethylation and LOH in HNSCC tumors are correlated

Colon cancer cell lines with more pronounced LINE hypomethylation were previously reported to have a higher degree of LOH [14,15]. To examine whether this correlation held true in our HNSCC patient samples, we performed a global LOH analysis using 10K SNPChip data. Genotypes were compared between normal adjacent tissue and matched tumor samples; informative loci were those that were heterozygous in the normal tissue. Figure 4 shows a plot of the percentage of informative loci for each primary tumor with LOH versus the degree of LINE-1 methylation in the same specimen. We fit a linear trend to the data, which showed a Pearson correlation of −0.494, with a p-value = 0.011 for the trend (Figure 3).

We also compared global methylation levels in matched normal adjacent tissues from the same three groups. In contrast to the results for the primary tumors, there were no differences in global methylation levels in the matching normal tissues and therefore no correlation with HPV status (Figure 3). Based on a previously reported association between LINE-1 methylation and T-N-M stage [18], we looked for such an association in our sample set, but found none (p-value = 0.31).

Discussion

We developed several LINE (LINE-1) and SINE (Alu) methylation assays with PCR primers in conserved regions of these repetitive elements. These assays utilize multiple CpG sites (3–6) to determine methylation levels and enable the simultaneous amplification of many individual LINE and SINE elements throughout the human genome as representative genomic landmarks for global methylation analysis. As expected, results from individual LINE-1 assays were very well correlated with results from other LINE-1 assays, despite measuring methylation in different regions of the LINE-1 sequence. The same is true for Alu assays, with high correlation between results from three different assays. However, when LINE-1 and Alu methylation levels were compared with each other, the correlation was more modest, only about 40%. The reasons for this lower correlation may be related simply to differences in assay sensitivity: LINE-1 assays ranged from 0–50% methylated in mixing studies, while Alu assays had less amplitude, ranging from 0–30% methylated, possibly secondary to higher inter-individual background noise due to sequence variation between individual Alu elements (Figure S1). Another possibility is that there is a functional or biological difference between the two types of repetitive DNA in their methylation maintenance. LINE repeats are more frequent in gene-poor regions of the genome, while Alu elements are more common in gene-rich regions [23]. Since global methylation can be measured by a variety of methods, these inter-assay differences should be considered when comparing results using different types of assays and studies.

Our results show decreases in global methylation in cell lines from a variety of cancer cell types (Figure 1), similar to previously published results for a variety of tumor types [12–14,21]. It is worth noting that the cancer cell lines in general had lower levels of methylation than the HNSCC samples, perhaps reflecting a longer time to accumulate methylation loss or the clonal nature of these cell lines. However, some cell lines (e.g., KKO) had little if
any loss of methylation. This implies, as for the primary HNSCC tumors, that there is variability in the underlying biology. Further research into what causes this variability could provide important information about pathways of and the role of epigenetic alterations in cancer progression.

Our results demonstrate a positive correlation between maintenance of normal LINE methylation and HPV-positivity. In addition, maintenance of normal LINE methylation was also correlated with less LOH or genome instability. If LOH is viewed as a surrogate for chromosome instability (CIN), this result is consistent with the previously reported result that colon cancers also have an association between CIN and loss of LINE hypermethylation [14,15]. Thus, it is tempting to speculate that LINE methylation and CIN are causally related, perhaps via methylation’s known association with more densely chromatin packaging, which may translate into more fidelity during chromosome segregation and therefore less LOH. However, our results and previously reported results are purely correlative; functional studies will be necessary before this causal relationship can be established.

The novel finding in our results is that HPV-positivity is correlated with maintenance of LINE methylation. A recent epidemiologic study identifying risk factors for LINE-1 hypermethylation reported no significant association between HPV DNA status and LINE-1 methylation levels [17]. However, there were methodological differences between that and our study, including the use of both HPV E6 DNA and RNA status in the assignment of HPV status, the use of a different methylation assay, and the use of methylation level as a continuous variable in the analysis. Although Furniss and colleagues did not show a direct association, they showed that outcomes varied by LINE-1 methylation levels for HPV-negative tumors [23]. Further studies are needed to clarify the association between HPV status and LINE methylation. One possible hypothesis is that in an attempt to silence the HPV virus, infected cells induce a more exuberant methylation response, harkening back to the origins of methylation as a viral defense mechanism. Although this will again require mechanistic studies or perhaps studies in additional types of virally mediated cancers (e.g., Hepatitis B and C mediated HCC compared with non-virally induced HCC, or EBV+ lymphoma vs. EBV- lymphoma) to establish causality, our results along with those of Furniss and colleagues [17] certainly provide more support for the hypothesis that HPV-positive HNSCC and HPV-negative HNSCC represent distinct biologic entities that arise via separate oncogenic pathways.

In summary, we developed several new LINE (LINE-1) and SINE (Alu) whole-genome methylation assays, which we applied to determine the methylation status of a variety of cancer cell lines and HNSCC primary tumor samples. We find that cancer cell lines in general have decreased methylation levels of repetitive elements. Even more variability in LINE-1 methylation was noted within HNSCC samples. Importantly, we discovered a correlation between HPV-negativity, increased genome instability, and loss of genome methylation. This correlation reinforces the concept that HPV-positive and HPV-negative cancers are biologically distinct and provides a basis for future studies to further define the biologic mechanism underlying these findings.

**Materials and Methods**

**Patient samples and cell lines**

Matched tumor/normal adjacent tissue samples, and when available cervical lymph node metastases from HNSCC patients were collected at Ohio State University as described previously (Table S1) [24]. Genomic DNA was extracted from the DNA-protein phase of TriZol-extracted tissues according to the manufacturer’s suggestions (Invitrogen). DNA was extracted using the PureGene kit (Gentra) on cell pellets from four HNSCC cell lines (H1395, H520, H2170, SK-MES-1 and SW-900), one breast cancer cell line (MCF7), one cervical cancer cell line (HeLa), three brain cancer cell lines (U251, SK-N-AS and M059K), one uterine cancer cell line (AN3CA), one sarcoma cell line (HT1080), one kidney cancer cell line (HEK293), and six colon cancer cell lines (LoVo, SW48, HCT-15, LDL-1, COLO 502DM and RKO) according to the manufacturer’s suggestions. In addition, DNA from the lymphoblastoid cell line BL1395 was used as a matching control to H1395. All cell lines are available from ATCC (Manassas, VA).

**Normal pools and methylated controls**

Four pools of normal samples were generated representing different genders and ages. DNA samples were obtained from anonymous blood donors and were a gift of Dr. Michael J. Siciliano (University of Texas M. D. Anderson Cancer Center). Three of the pools (females older than 40 years of age, females age 40 or under, and males age 40 or under) were each comprised of five individuals per pool. The fourth pool (males older than 40 years of age) was comprised of six individuals. Commercially prepared universally methylated and universally unmethylated DNA (U.M.C and U2M.C, respectively) were obtained from Chemicon. U.M.L (universally methylated DNA) was generated as a positive control by treating normal peripheral blood leukocyte (PBL) DNA with the CpG methylase M.SssI (New England Biolabs) [25]. U2M.L (universally unmethylated DNA) was generated as a negative control by amplifying the same DNA used to generate the positive control DNA using the GenomiPhi kit (GE Healthcare) as described by the manufacturer.

**Primers and PCR conditions**

PCR primers and sequencing primers were designed by using PSQ Assay Design software (Biotage) [26]. Three assays for SINE (Alu) elements and three assays for LINE-1 elements were designed (Table 1). PCR was performed in a 25 µl reaction containing Qiagen HotStart Taq master mix (Qiagen) using 1 µl bisulfite treated DNA, Pyrosequencing was carried out using the PSQ96HS system (Biotage) according to the manufacturer’s suggestions. In addition, DNA amplification using bisulfite treated DNA was stored at –20°C until use. PMA is a Pyrosequencing-based technology that can analyze CpG methylation at multiple sites in a single assay. After a PCR amplification using bisulfite treated DNA, Pyrosequencing was carried out using the PSQ96HS system (Biotage) according to the manufacturer’s protocol including single strand binding protein (PyroGold reagents). The results were analyzed using Q-CpG Software.
software (Biotage), which calculates the methylation percentage \(\frac{mC}{(mC+cC)}\) for each CpG site, allowing quantitative comparisons. The methylation index (called MI) was calculated as the average value of \(\frac{mC}{(mC+cC)}\) for all examined CpG sites in the assay. In general, there was very good agreement in methylation levels among individual CpG sites in the same assay. Bisulfite-treated UML.L was used as the universally methylated reference. PMA data for these global methylation assays are reported as a percentage of methylated reference (PMR) value, normalizing the MI of each sample to the MI of the universally methylated reference (UML.L) DNA [25].

Commercial universally methylated DNA (UML.C) and universally unmethylated DNA (U2M.C) (Chemicon) were also used to perform this analysis, and the result was linear \(r=0.983, p\text{-value}<0.0005\) for LINE-1; Figure S1). However, commercially available U2M was not completely unmethylated, since a pure U2M.C sample had residual methylation of approximately 26\%, different from U2M.L prepared in our lab, which had expected 0\% methylation. We therefore used our own universally unmethylated DNA (U2M.L) for all further studies.

Detection of HPV16 E6 DNA and E6 RNA to determine HPV status

Quantitative real-time PCR was performed to detect either HPV16 E6 DNA or E6 cDNA using the same primer/probe set for both. The primers were designed using the HPV16 serotype, which accounts for \(\geq 90\%\) of all HPV-positive HNSCC cases [27]. To control for possible genomic DNA contamination in the cDNA, amplifications of cDNA from DNase-treated RNA that had been prepared using the SuperScript First Strand Synthesis System (Invitrogen) both with and without the addition of reverse transcriptase were compared. PCR was performed in a 25 µl reaction containing IQ supermix master mix (Biorad) using 25 ng of genomic DNA or 5 µl of cDNA. Forward primer \(5'\text{-CTGCAATGTGTTTAGAGCCCA-3'}\) and reverse primer \(5'\text{-TCATGTATAGTTGTTTGCAGCTCTGT-3'}\) were added to a final concentration of 200 nM each. Texas Red labeled Fluorescein was added to each reaction at a final concentration of 320 nM. DOC) amplification was carried out at the following conditions: denaturation at 95°C for 15 sec, 60°C for 1 min and annealing in real time using an iCycler PCR machine and software (Biorad). Samples that did not amplify were scored as negative, and all samples were grouped into 3 categories based on these results: (+/+), positive for both E6 DNA and E6 RNA; (+/−), positive for E6 DNA, but transcriptionally silent; and (−/−), negative for E6 DNA and RNA. HPV status was successfully determined for 24 of the 26 HNSCC samples, and these were used for subsequent analyses utilizing HPV status.

10K SNP Chip LOH analysis

HNSCC genomic DNAs were extracted using the standard TriZol-extraction protocol; they were further purified by ethanol precipitation before and after whole genome amplification using the GenomiPhi kit (GE Healthcare). The Affymetrix 10K XbaI131 array contains approximately 11,500 SNPs with an average spacing of 210 kb. Standard Affymetrix protocols were followed for these assays. Briefly, about 250 ng genomic DNA was digested with XbaI and then ligated to adaptors. Next, one-primer amplification was carried on by using the GeneAmp PCR System 9700 (Applied Biosystems). After purification with Qiagen MinElute 96 UF, a total of about 20 µg of PCR product was fragmented and labeled with biotin. Hybridization was performed in the Affymetrix GeneChip Hybridization Oven at 48°C for 16–18 hours. Arrays were washed and stained with the Affymetrix GeneChip Fluidics Station 400 and were scanned with the Affymetrix GeneArray 2500 Scanner. Image processing was performed with GCOS 1.0 software and genotypes were generated with GTYPE 2.0 or higher software.

Statistical analysis

In order to assess the association between levels of methylation and levels of HPV load (a similar analysis was also used to assess the association between levels of methylation and T-N-M stage), we proceeded as follows. All sample methylation values were converted to ranks. One missing value for the LINE1-1 assay, for sample P2, was imputed using the P2 value from the LINE1-3 assay (correlation between the LINE1-1 and LINE1-3 assays is 0.98). These ranks were then scaled (weighted) by HPV load, with weights of 0, 1 and 2 for −/−, +/−, and +/+ respectively. The final association “score” was the sum of these weighted ranks. The null distribution for this score was assessed by simulations in which we repeatedly allocated samples to HPV groups at random. We ran one million simulations, and defined our \(p\)-value as two times the proportion of cases in which the simulation score was as large or larger than the one we actually saw. Our simulated \(p\)-value was 0.011.

Supporting Information

Table S1 Clinical and molecular features of HNSCC samples. Found at: doi:10.1371/journal.pone.0004941.s001 (0.12 MB DOC)

Figure S1 Dynamic range of PMA LINE-1 and Alu Assays. Mixing experiments were performed to determine PMA methylation levels measured with varying proportions of universally methylated and unmethylated DNA. UML.C and U2M.C represent commercially available (Chemicon) universally methylated and unmethylated DNAs, respectively. UML.L and U2M.L were generated from the same DNA by in vitro modification in our laboratory. Found at: doi:10.1371/journal.pone.0004941.s002 (0.10 MB TIF)

Figure S2 Correlation analysis of SINE and LINE global methylation assays. A variety of tumor and normal cell line DNAs were assayed with all six of our LINE and SINE assays (three LINE1-1 assays and three Alu assays). A. LINE-1 assays were highly correlated with each other. B. Alu assays were highly correlated with each other. C. However, LINE-1 and Alu assays were only moderately correlated. Two representative plots are shown for each series of comparisons. Found at: doi:10.1371/journal.pone.0004941.s003 (0.19 MB TIF)

Acknowledgments

We thank Tamer Ahmed for expert technical assistance and Linda L. Bachinski for critical reading of and comments on the manuscript.

Author Contributions

Conceived and designed the experiments: KLR BZ SC RK. Performed the experiments: BZ SC. Analyzed the data: KLR BZ KAB SC RK. Contributed reagents/materials/analysis tools: KAB JCL DES RK. Wrote the paper: KLR BZ KAB RK.
References

1. Jones PA, Baylin SB (2002) The fundamental role of epigenetic events in cancer. Nat Rev Genet 3: 415–428.
2. Robertson KD (2005) DNA methylation and human disease. Nat Rev Genet 6: 597–610.
3. Jones PA, Baylin SB (2007) The epigenomics of cancer. Cell 128: 683–692.
4. Bird AP (1993) Functions for DNA methylation in vertebrates. Cold Spring Harb Symp Quant Biol 58: 261–265.
5. Liu WM, Maca MJ, Ruben CM, Schmid CW (1994) Alu transcripts: cytoplasmic localisation and regulation by DNA methylation. Nucleic Acids Res 22: 1087–1095.
6. Yoder JA, Walsh CP, Bestor TH (1997) Cytosine methylation and the ecology of intragenomic parasites. Trends Genet 13: 335–340.
7. Rosi F, Arab A, Klévenz B, zur Hausen H (1993) The effect of DNA methylation on gene regulation of human papillomaviruses. J Gen Virol 74 ( Pt 5): 791–801.
8. Turan T, Kalantari M, Calleja-Macias IE, Cubir HA, Caschieri K, et al. (2006) Methylation of the human papillomavirus-18 L1 gene: a biomarker of neoplastic progression? Virology 349: 175–183.
9. Hsiung DT, Marsit CJ, Houseman EA, Eddy K, Furniss CS, et al. (2007) Global DNA methylation level in whole blood as a biomarker in head and neck squamous cell carcinoma. Cancer Epidemiol Biomarkers Prev 16: 108–114.
10. Pattamadilok J, Huapai N, Rattanatanyong P, Vasurattana A, Triratanachat S, et al. (2007) LINE-1 hypomethylation level as a potential prognostic factor for epithelial ovarian cancer. Int J Gynecol Cancer.
11. Tangkijvanich P, Hourpai N, Rattanatanyong P, Wisedopas N, Mahachai V, et al. (2007) Serum LINE-1 hypomethylation as a potential prognostic marker for hepatocellular carcinoma. Clin Chim Acta 379: 127–133.
12. Chalitchagorn K, Shuangshoti S, Hourpai N, Kongruttanachok N, Tangkijvanich P, et al. (2004) Distinctive pattern of LINE-1 methylation level in normal tissues and the association with carcinogenesis. Oncogene 23: 8841–8846.
13. Hoffmann MJ, Schulz WA (2005) Causes and consequences of DNA hypomethylation in human cancer. Biochem Cell Biol 83: 296–321.
14. Matsuzaki K, Deng G, Tanaka H, Kakur S, Murza S, et al. (2005) The relationship between global methylation level, loss of heterozygosity, and microsatellite instability in sporadic colorectal cancer. Clin Cancer Res 11: 43564–43569.
15. Ogino S, Kawasaki T, Nosho K, Ohnishi M, Sweeney N, et al. (2008) LINE-1 hypomethylation is inversely associated with microsatellite instability and CpG island methylator phenotype in colorectal cancer. Int J Cancer 122: 2767–2773.
16. Fakhr C, Gillieson ML (2006) Clinical implications of human papillomavirus in head and neck cancers. J Clin Oncol 24: 2606–2611.
17. Furniss CS, Marsit CJ, Houseman EA, Eddy K, Kelsey KT (2008) LINE region hypomethylation is associated with lifestyle and differs by human papillomavirus status in head and neck squamous cell carcinomas. Cancer Epidemiol Biomarkers Prev 17: 966–971.
18. Smith JM, Myllarz WK, Mithani SK, Califano JA (2007) DNA global hypomethylation in squamous cell head and neck cancer associated with smoking, alcohol consumption and stage. Int J Cancer 121: 1724–1728.
19. Colella S, Shen L, Baggyer KA, Isa J, Krahe R (2003) Sensitive and quantitative universal Pyrosequencing methylation analysis of CpG sites. Biotechniques 35: 146–150.
20. Yang AS, Estacio MR, Doshi K, Kondo Y, Tajara EH, et al. (2004) A simple method for estimating global DNA methylation using bisulfite-PCR of repetitive DNA elements. Nucleic Acids Res 32: e38.
21. Estacio MR, Gharibyan V, Shen L, Ibrahim AE, Doshi K, et al. (2007) LINE-1 hypomethylation in cancer is highly variable and inversely correlated with microsatellite instability. PLoS ONE 2: e399.
22. Choi IS, Estacio MR, Nagano Y, Kim do H, White JA, et al. (2007) Hypomethylation of LINE-1 and Alu in well-differentiated neuroendocrine tumors (pancreatic endocrine tumors and carcinoid tumors). Mod Pathol 20: 802–810.
23. Lander ES, Linton LM, Birren B, Nusbaum C, Zody MC, et al. (2001) Initial sequencing and analysis of the human genome. Nature 409: 860–921.
24. Colella S, Richards KL, Baggyer KA, Tazavachis S, Clarke JW, et al. (2008) Molecular signatures of metastasis in head and neck cancer. Head&Neck in press.
25. Weisenberger DJ, Campan M, Long TI, Kim M, Woods C, et al. (2005) Analysis of repetitive element DNA methylation by MethyLight. Nucleic Acids Res 33: 6823–6836.
26. Price AL, Eskin E, Pevzner PA (2004) Whole-genome analysis of Alu repeat elements reveals complex evolutionary history. Genome Res 14: 2254–2262.
27. Gillison ML, Koch WM, Capone RB, Spafford M, Westra WH, et al. (2000) Evidence for a causal association between human papillomavirus and a subset of head and neck cancers. J Natl Cancer Inst 92: 709–720.