LINE-1 methylation patterns of different loci in normal and cancerous cells

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ABSTRACT

This study evaluated methylation patterns of long interspersed nuclear element-1 (LINE-1) sequences from 17 loci in several cell types, including squamous cell cancer cell lines, normal oral epithelium (NOE), white blood cells and head and neck squamous cell cancers (HNSCC). Although sequences of each LINE-1 are homologous, LINE-1 methylation levels at each locus are different. Moreover, some loci demonstrate the different methylation levels between normal tissue types. Interestingly, in some chromosomal regions, wider ranges of LINE-1 methylation levels were observed. In cancerous cells, the methylation levels of most LINE-1 loci demonstrated a positive correlation with each other and with the genome-wide levels. Therefore, the loss of genome-wide methylation in cancerous cells occurs as a generalized process. However, different LINE-1 loci showed different incidences of HNSCC hypomethylation, which is a lower methylation level than NOE. Additionally, we report a closer direct association between two LINE-1s in different *EPHA3* introns. Finally, hypermethylation of some LINE-1s can be found sporadically in cancer. In conclusion, even though the global hypomethylation process that occurs in cancerous cells can generally deplete LINE-1 methylation levels, LINE-1 methylation can be influenced differentially depending on where the particular sequences are located in the genome.

INTRODUCTION

Long interspersed nuclear element-1 (LINE-1 or L1) sequences are highly repeated and widely interspersed human retrotransposon sequences (1). LINE-1s constitute about 17% of the human genome with up to 600 000 copies present (2). Approximately 3000–4000 copies remain in a full-length form and some may be retrotranspositionally active (3). By measuring the quantity of LINE-1 methylation, we found that LINE-1 methylation levels are varied. Whereas cancerous cells revealed a lower percentage of methylation, their normal tissue counterparts, normal tissues from different organs, showed tissue-specific levels of methylated LINE-1s (4). The levels of LINE-1 methylation were significantly different among different tissue types. Moreover, while the range of LINE-1 methylation levels in some tissues was narrow, some other tissues, such as the esophagus or thyroid, contained widely distributed LINE-1 methylation levels (4). The variation of methylation levels of LINE-1s in normal tissues and the hypomethylation observed in cancerous cells has been confirmed by several studies (5–18). These data indicate that the epigenetic event may be important for cellular functions, not only in cancer, but also in normal cells (4). Moreover, these findings led us to a question of whether methylation patterns of each LINE-1 are distinct despite the sequences being homologous.

In cancer, DNA methylation of the whole genome is generally depleted and this event is called 'global hypomethylation' (19–21). The causal mechanism of global hypomethylation is not known (22). Global hypomethylation may have several consequences on the multistep process of carcinogenesis. The most commonly recognized effects are to facilitate chromosomal instability (23–25) and to control gene expression (26). Interestingly, both consequences have been proposed to occur 'in cis', on nearby genomic sites of the hypomethylation (26–28). Therefore, if each LINE-1 locus possesses distinct methylation levels, LINE-1s may play different roles in cellular biology and cancer development, depending on their locations. This study evaluated the methylation status of different LINE-1s in normal cells and cancerous tissues.

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The finding may provide more information on the roles of the epigenetic modifications of repetitive sequences.

MATERIALS AND METHODS

Cells and tissue preparation

Eleven head and neck squamous cell cancer (HNSCC) cell lines (WSU-HN) (29), including WSU-HN 4, 6, 8, 12, 13, 17, 19, 22, 26, 30 and 31, were provided by Dr Silvio Gutkind (NIH, USA). HeLa (cervical cancer) and KB (oral cancer cell line, but with a HeLa contaminant) lines (ATCC, Manassas, VA, USA) were included. Cells were cultured in Dulbecco's modified Eagle's medium (Gibco BRL, Life Technologies, Pairly, UK), supplemented with 10% heat-inactivated fetal bovine serum (Sigma, St Louis, MO, USA). Cells were incubated at 37°C in 5% CO_2.

Normal oral epithelium (NOE) samples from 12 individuals were collected. Briefly, each subject used 20 ml of sterile 0.9% NaCl solution to rinse and gargle for 15 s then spit the solution into a sterile 50-ml closed container, which was kept at 4°C until the samples were processed for DNA collection (within 24 h of collection). Cells from the oral rinses were collected by centrifugation at 2500*g* for 15 min at 4°C. The supernatant was discarded and the pellets were washed with sterile PBS. Several samples of white blood cells (WBCs) from normal, healthy individuals were also collected. Several archival paraffin-embedded tissues, derived from HNSCCs, were obtained and prepared in 5 μm thickness sections on slides for microdissection as previously described (4).

COBRA PCR

Combined bisulfite restriction analysis (COBRA) (30) for genome-wide LINE-1s (COBRALINE1) and COBRA for unique L1 sequences (CU-L1) were both performed as previously described, with some modifications (4). LINE-1s were selected by blat using L1.2 sequence to http://genome.ucsc.edu and full-length intronic LINE-1s with the representative COBRALINE-1 CpG dinucleotides were selected. All selected LINE-1s are listed in Supplementary Table S1. After extraction, all DNA samples were treated with sodium bisulfite as previously described (4,31). Briefly, genomic DNA was denatured in 0.22 M NaOH for 10 min at 37°C. Thirty microliters of 10 mM hydroquinone and 520 μl of 3 M sodium bisulfite were both added for 16–20 h at 50°C. The DNA was purified and incubated in 0.33 M NaOH for 5 min at 25°C, ethanol-precipitated, then washed with 70% ethanol and resuspended in 20 μl of TE buffer. Two microliters of bisulfited DNA were subjected to 35 cycles of PCR with two primers, as listed in Supplementary Table S2, at an annealing temperature of 53°C. The amplicons were digested in 30 μl reaction volumes with 2 U of TaqI or 8 U of TasI in $1 \times$ TaqI buffer (MBI Fermentas) overnight at 65°C and then electrophoresed on 8% non-denaturing polyacrylamide gels. The intensities of DNA fragments were measured on a PhosphoImager, using the ImageQuant software (Molecular Dynamics, GE Healthcare, Slough, UK). The TaqI positive methylated

amplicons yielded 80-bp DNA fragments, whereas the TasI positive unmethylated amplicons resulted in 98-bp fragments (previously reported as 97 bp). The LINE-1 methylation level was calculated as a percentage (the intensity of the TaqI-digested methylated LINE-1 fragment divided by the sum of the TasI-digested unmethylated LINE-1 and TaqI-positive amplicons). For CU-L1, several other methylated and unmethylated digested amplicons, as listed in Supplementary Table S2, were also measured. The methylation levels among several CpG dinucleotides were compared. All COBRA assays were performed in duplicate. The same set of DNAs was applied as positive controls in each set of the COBRA experiments. The PCR products of some of the CU-L1 were cloned into the pGEM-T easy vector (Promega, Santhan, UK) and sequenced.

Statistical analyses

Statistical significance was determined by applying an independent sample *t*-test and Pearson's correlation coefficient to the data, as specified.

RESULTS

COBRA for unique to LINE-1 sequence (CU-L1)

Previously, we investigated the methylation of genome-wide LINE-1s by investigating the methylation status of two CpG dinucleotides at the 5′-end of LINE-1s (4). This study compares the methylation status of LINE-1s across different loci. Because there are variations in both lengths and sequences of LINE-1s, we compared methylation levels of LINE-1s between locations by comparing methylation levels of the same representative CpG dinucleotides: TaqI positive 80-bp methylated DNA fragments and TasI positive 98-bp unmethylated DNA fragments (Figure 1A). We selected a set of 17 full-length LINE-1s located within introns. All LINE-1s possessed both representative CpG dinucleotides. Selected LINE-1s (Supplementary Table S1), a schematic representation of the assay (Figure 1A) and typical examples of PCR results (Figure 1B) are shown. We modified the COBRA protocol (30) to devise a PCR protocol to evaluate LINE-1 methylation status in the entire genome (COBRALINE-1) and at a specific locus (CU-L1) by replacing the LINE-1 forward primer with an oligonucleotide locating on unique sequence 5′ to LINE-1. Briefly, DNA is treated with bisulfite to distinguish between methylated and unmethylated sequences before PCR (32). Treatment with bisulfite converts unmethylated cytosines, but not methylated cytosines, to uracils and then thymines after PCR. This generates detectable methylation-dependent changes in the restriction pattern of PCR-amplified LINE-1 sequences (Figure 1A). The modified DNA was amplified by 5′-UTR LINE-1 bisulfited sequence primers and then digested with TaqI and TasI restriction enzymes, which recognize methylated and unmethylated sequences, respectively. The level of LINE-1 methylation in each sample was calculated by dividing the measured intensity of TaqI digestible amplicons by the sum of the TasI and TaqI products (4) (Figure 1A).

A

B
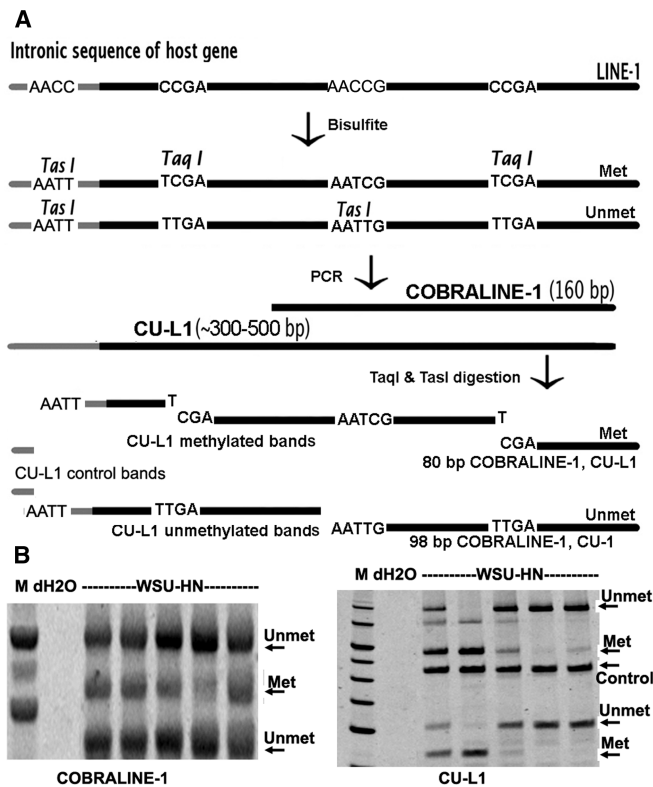
Figure 1. COBRA for unique L1 sequence (CU-L1) and COBRA for genome-wide LINE-1s (COBRALINE1). COBRA was performed as previously described (30), with some modifications (4). (**A**) Schematic representation of CU-L1 and COBRALINE-1, showing LINE-1 sequence in relation to the 5′ unique sequence. AACCG and CCGA are LINE-1 sequences, following bisulfite treatment and PCR, unmethylated AACCG sequences are converted to AATTG (TasI site) and methylated sequences are converted from CCGA to TCGA (TaqI site). The amplicon sizes of CU-L1 are between 300 and 500 bp and for COBRALINE-1 are 160 bp. After digestion, COBRALINE-1 yielded two TasI-digested unmethylated L1 sequence fragments of 62 and 98 bp and one 80 bp TaqI-digested methylated L1 sequence. The LINE-1 methylation level was calculated as a percentage (the intensity of methylated LINE-1 divided by the sum of the unmethylated LINE-1 and the TaqI-positive amplicons). CU-L1 usually has additional TaqI site(s) and AATT sequences; therefore, there are additional methylated, unmethylated and control bands. Met and unmet are methylated and unmethylated sequences, respectively. Control bands are digested DNA fragments without candidate CpG restriction sequences. (**B**) A typical example of results from COBRALINE-1 and CU-L1. The ranges of intensity between methylated and unmethylated bands of CU-L1 were wider than COBRALINE-1. The unmet, met and control arrows indicate unmethylated, methylated and control LINE1 sequences, respectively. M is a standard size marker. The –ve is dH$_2$O. Several samples from WSU-HN cells are shown.

We designed the CU-L1 PCR to constitute several digested fragments, including methylated, unmethylated and control, which are without candidate CpG restriction sequences, so that we could check for multiple CpG dinucleotides containing TaqI sites and an unmethylated CpG containing TasI site. Therefore, the intensities of each methylated and unmethylated band could be measured to compare the methylation status among several CpG nucleotides. Figure 2A and B provides examples of the positive linear correlation between methylated fragments from the CU-L1 locations. Direct correlations between

unmethylated fragments were also demonstrated (Figure 2C and D). Therefore, the methylation levels of the representative COBRALINE-1 TaqI and TasI sites represent methylation levels of each LINE-1 in a positive correlated fashion.

To evaluate the relationship among all of the 5′ LINE-1 CpGs, CU-L1 amplicons were cloned and sequenced. Figure 3 demonstrates examples of CU-L1 sequences. Direct correlation of methylation levels between CU-L1 and bisulfite sequencing can be observed. Both CU-L1 and bisulfite sequences demonstrated low L1-*LRP2* methylation levels in WSU-HN 6 and 22 cells and high methylation levels in WSU-HN 26 cells, compared with a sample of NOE. Therefore, CU-L1 methylation levels were applied to compare methylation levels between each DNA sample. The bisulfite sequences (Figure 3) demonstrated that even though they were directly correlated, the methylation statuses of the CpG dinucleotides from 5′ LINE-1 are not equally distributed. We found that the CpGs around TaqI and TasI COBRALINE-1 digested sites might be more prone to be demethylation than others (Figure 3).

Interestingly, even though a universal characteristic of cancer development is clonal in origin and selective clonal expansion, bisulfite sequencing indicated that LINE-1 methylation patterns of each DNA strand from the same locus are not identical (Figure 3). This indicates that LINE-1 methylation may be dynamic. Moreover, there are mixtures of CU-L1 alleles with distinguishably different methylation statuses. For example, the methylation status of L1-*LRP2* can divide the chromosomes of the cancer cell lines, WSU-HN 6 and 22, into two groups. The DNA from one group was almost completely unmethylated and that from the others was partially methylated (Figure 3). This result may suggest that LINE-1 hypomethylation in cancer may also be a dynamic process and either different chromosomes or cells may be unequally influenced by the global hypomethylation process.

A general concern with the COBRA method is the misinterpretation of cases of a mutation or polymorphism at a restriction site. For COBRALINE-1, this type of error is irrelevant because of the vast number of targeted LINE-1 sequences in the genome (4). For CU-L1, we were able to check for the possibility of a mutation error. If there is a mutation at the TaqI restriction site, for example TCGA to TTGA, then the homozygous or heterozygous polymorphic sample will result in a false positive as completely unmethylated for two or one alleles, respectively. Therefore, CU-L1 methylation levels should dramatically deviate from the linear correlation of the linked CpGs. However, striking deviation was rare when methylation levels between CpGs were compared (Figure 2A). Nevertheless, because CU-L1 contains several methylated and unmethylated fragments, alternative sites could be selected if the density of the representative sites deviated strikingly from the group (Figure 2A). Moreover, in normal cells, particularly WBCs, CU-L1s of most loci usually demonstrated methylation levels in a limited range without a striking deviation (Figure 4A). In conclusion, mutations or polymorphisms of these CU-L1
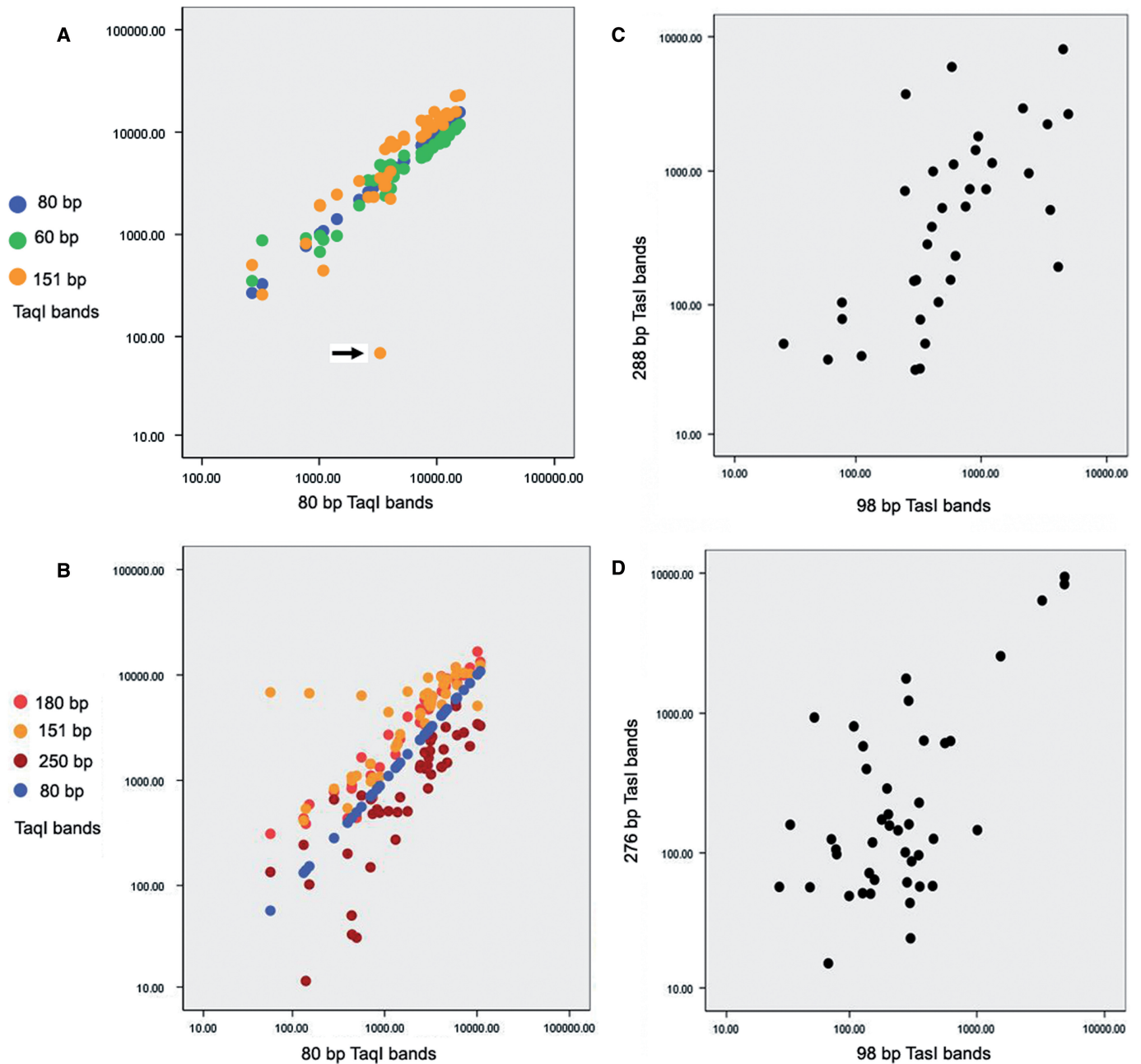
Figure 2. Correlation between the bands of CU-L1. Pixel counts of CU-L1 fluorescent intensities from 19 cell lines, 12 WBCs and 12 NOEs are shown. Examples of two LINE-1 loci are shown. (**A** and **B**) Comparison between methylated bands. (**C** and **D**) Comparison between unmethylated bands. (A and C) The L1-*EPHA3*-IVS5 locus. (B and D) The L1-*MGC4217* locus. Each band was named according to the restriction enzymes and sizes of the digested fragment. Most samples demonstrate a linear correlation. The arrow indicates a striking deviation of the 151-bp TaqI band from the same sample.

restriction sites are rare and several informative amplicons help prevent interpretation errors.

LINE-1 methylation levels vary among locations and cell types

Our previous study showed that genome-wide LINE-1 methylation levels can be significantly different among normal tissue types (4). Moreover, some tissues, such as the thyroid and esophagus, demonstrated wider ranges in the methylation level. Here, we compared LINE-1 methylation levels of the 17 loci between NOE and

WBC (Figure 4A). Means of the LINE-1 methylation levels varied from highly methylated, which was the case for the L1-*PKP4*, L1-*EPHA3*-IVS5, L1-*EPHA3*-IVS15, L1-*ANTXR2* and L1-*COL24A1* fragments, to 24–41%, which was the case for the L1-*PPP2R2B* and L1-*PKG1* fragments. To support the premise that LINE-1 is completely methylated, the partial methylation levels of genome-wide LINE-1s would have to not only be reported by COBRALINE-1 (4), but also by several other techniques (9,18,33). The lower methylation levels of some of the
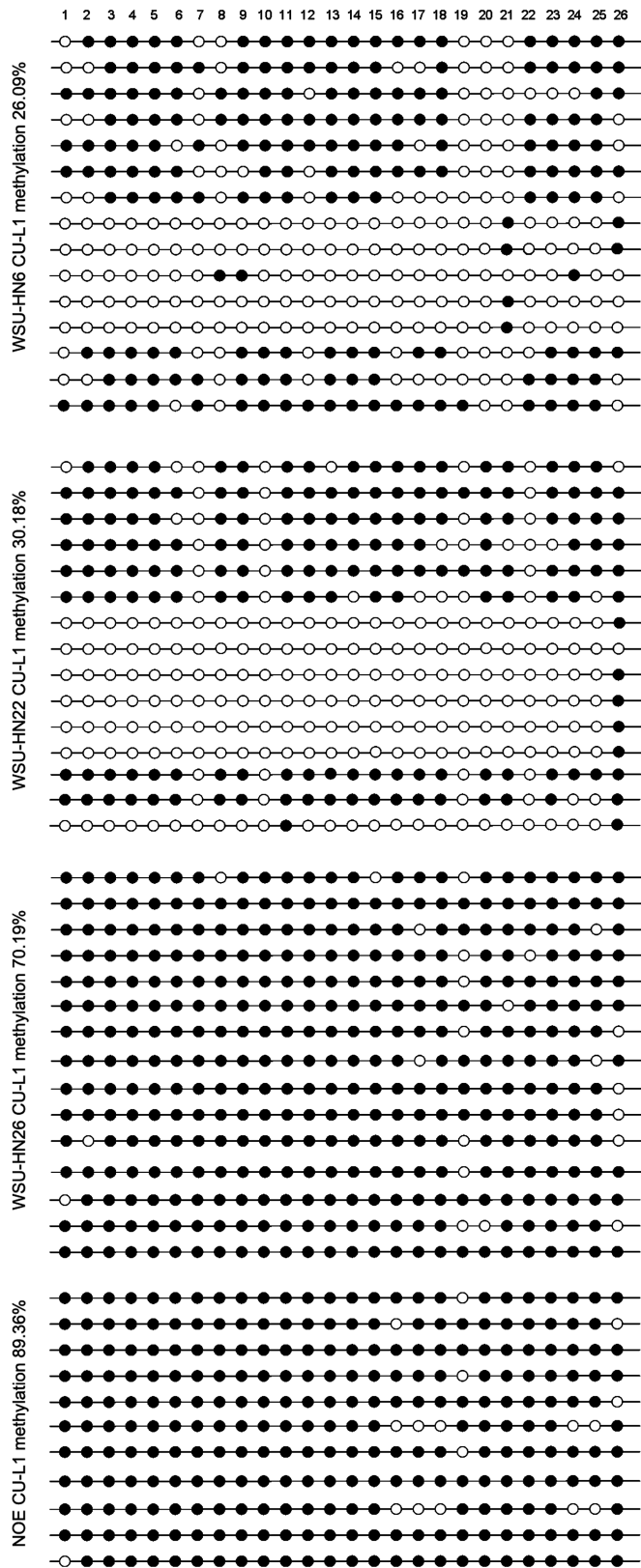
Figure 3. CU-L1s bisulfite sequences. CU-L1s bisulfite sequenced methylated CpG dinucleotides and non-methylated CpGs of each clone are illustrated with closed and open circles, respectively. The 80-bp (methylated) and 98-bp (unmethylated) TasI-digested fragments are the 20th and 19th CpGs, respectively. Sample names and CU-L1 methylation levels are shown on the left.

CU-L1s proved that not all LINE-1s are completely methylated in normal cells.

LINE-1 methylation patterns are distinguishable between NOE and normal WBCs. Even though there was limited range, when COBRALINE-1s were compared between NOE and WBCs, they were significantly different, $P < 0.01$. Similarly, methylation levels between WBCs and NOE of some CU-L1 loci were also different, such as in the case of the L1-*PPP2R2B* $(P < 10^{-9})$ and L1-*CNTNAP5* $(P < 10^{-10})$ fragments. Interestingly, in normal cells, most loci possessed limited ranges, within 4–6% of CU-L1s, while some loci, such as L1-*SPOCK3*, demonstrated a wider range of up to 45.46%. The wider ranges could most frequently be found in NOE rather than WBCs, such as at the L1-*FAM49A*, L1-*LOC286094* and L1-*LRP2* loci. This result is similar to our previous COBRALINE-1 finding that the ranges of genome-wide LINE-1 methylation levels differ across tissues (4).

Characteristics of LINE-1 hypomethylation in cancer

Genome wide LINE-1 methylation levels in cancerous tissue are lower in variable degrees than in the representative normal cells (4). To evaluate the cancer-specific epigenetic process in detail, we reported CU-L1s from 17 loci in 11 WSU-HN, HeLa and KB cell lines and compared our findings with those from a number of NOE (Figure 4B). LINE-1 hypomethylation, which is a methylation level lower than that in NOE, can be observed in all loci. However, each locus had a different number of samples with hypomethylation. Three to six samples out of 13 were found to have hypomethylation at the L1-*PKP4*, L1-*SPOCK3* and L1-*MGC42174* loci. On the contrary, the L1-*FAM49A*, L1-*LOC286094*, L1-*LRP2*, L1-*CDH8* and L1-*PRKG1* loci demonstrated hypomethylation in 12–13 samples out of 13 samples. Moreover, the level of hypomethylation varied when comparisons were made between loci. For example, WSU-HN19 cells had completely unmethylated L1-*COL24A1* and L1-*ADAMTS20* loci, but were barely hypomethylated at the L1-*EPHA3-IVS5* and L1-*EPHA3-IVS15* loci (Figure 4B). Different CU-L1 levels were observed even if the cells were from the same origin. WSU-HN30 and WSU-HN31 were two cell lines derived from the same patient, but from different tissue origins. While most CU-L1 levels were similar, different levels were found at some loci, such as at L1-*EPHA3-IVS15* (Figure 4B). Similar findings were found in HeLa and KB cells, which also had a HeLa contaminant. The hypomethylation level at the L1-*ANTXR2* locus was higher in KB cells than it was in HeLa. On the contrary, the L1-*COL24A1* locus in HeLa was more hypomethylated than in KB cells (Figure 4B). The differences in the CU-L1 methylation pattern between cell lines were similar to a previous report where a distinctive pattern of LINE-1 methylation was reported in different cell lines (6). This result may suggest that the global hypomethylation pattern of cancer cells can be variable. It is important to note that hypermethylation of CU-L1 could be observed sporadically, for example, the methylation level in WSU-HN8 at the L1-*CNTNAP5* locus (Figure 4B). Therefore, each LINE-1 locus may have
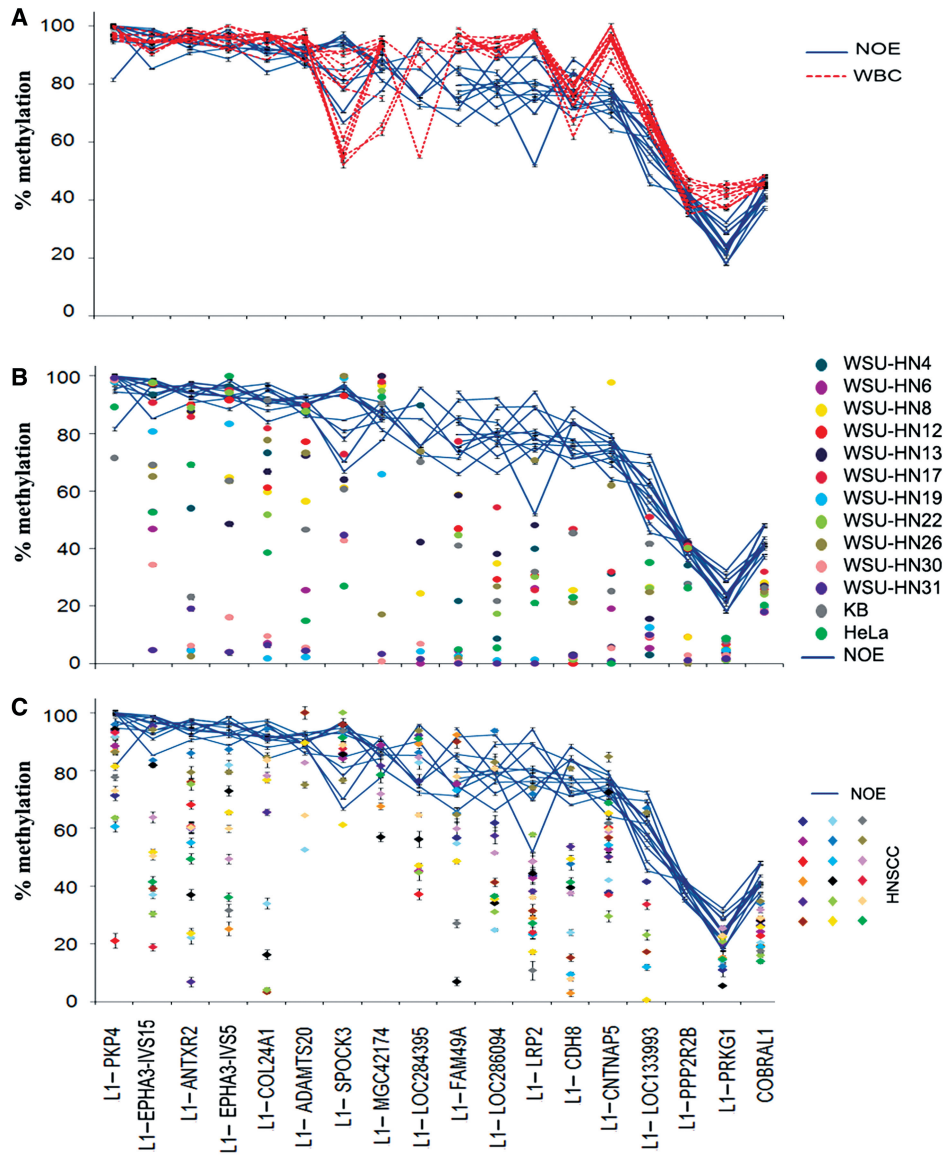
Figure 4. CU-L1 methylation levels. (**A**) NOE and WBC, (**B**) NOE and epithelial cell lines, (**C**) NOE and HNSCC. Each dot represents means ± SD each CU-L1 methylation level. Dashed and solid lines connect the measurements from the same samples.

different potential for developing carcinogenic-related hypomethylation. This may indicate that LINE-1 hypomethylation is dynamic and influenced in *cis* by factors located on the same chromosome.

Similar patterns of LINE-1 methylation can be observed *in vivo*. Figure 4C shows the CU-L1 results from 17 microdissected HNSCC paraffin-embedded sections. Because the quality of DNA derived from these sample types was poor, not all CU-L1s yield positive amplicons. Nevertheless, these samples demonstrated LINE-1 methylation patterns that were similar to those in the HNSCC cell lines. The specific characteristics included a variable degree of frequent LINE-1 hypomethylation and sporadic events of L1-*CNTNAP5* hypermethylation (Figure 4C).

We evaluated the directions of relationships between methylation levels of two that were between genome-wide

and a specific locus or between two loci. Correlations are reported as Pearson correlation coefficients (*r*). The *r*-values between −0.5 and 0.5 were considered not significant. The multiple white spots in Figure 5 represent *r*-values between the COBRAs, listed on the *x*-axis, and the other *r*-values. We observed positive correlations between LINE-1 methylation levels between genome-wide and a specific locus or between two loci more frequently in WSU-HN than in NOE cells. This is demonstrated by the fact that most *r*-values between the LINE-1s of WSU-HN cells, but not of NOE cells, are frequently closer to one (Figure 5A and B). Hence, methylation levels of LINE-1s in cancer cells may result from a generalized process of global demethylation. However, methylation of certain loci, such as L1-*SPOCK3* and L1-*PKP4*, did not have a positive correlation with their cancer genome or other loci (Figure 5A).
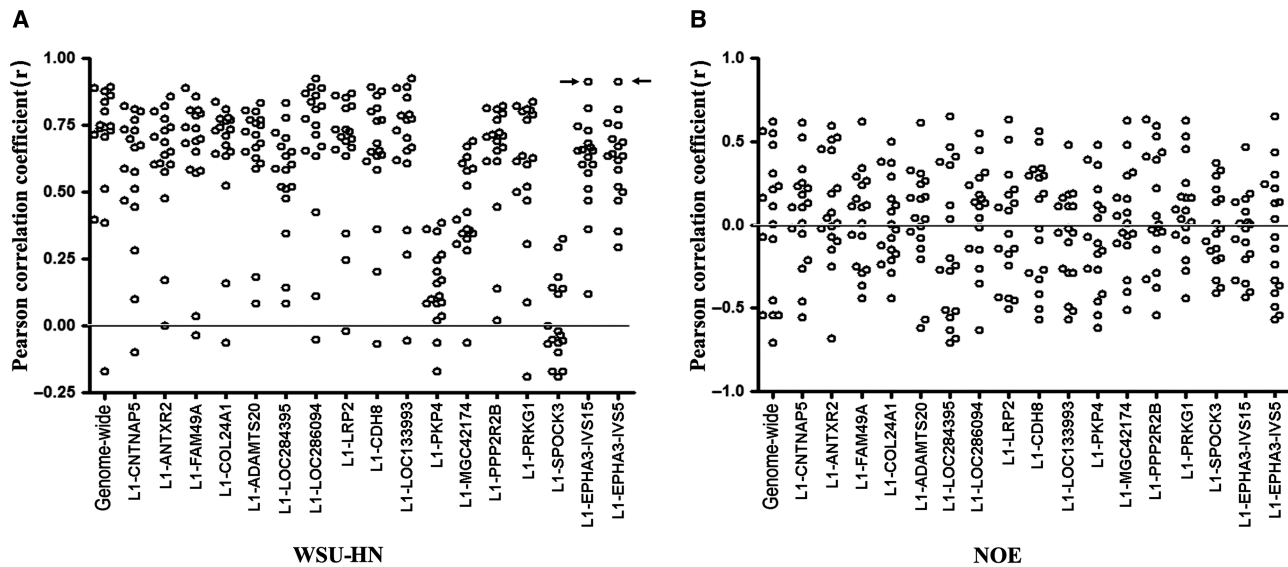
Figure 5. COBRALINE-1 and CU-Ls correlation. Pearson correlation coefficient values between LINE-1 and genome-wide loci of (**A**) WSU-HN cells and (**B**) NOE cells. Arrows indicate an association between loci L1-*EPHA3*-IVS5 and L1-*EPHA3*-IVS15.

Interestingly, both of the loci had less hypomethylation in many samples (Figure 4B). Therefore, they may be less influenced by the global hypomethylation process. The highest correlation value observed in cancerous cells, was between the two LINE-1s inserted in the same gene, L1-*EPHA3*-IVS5 and L1-*EPHA3*-IVS15 ($P < 10^{-16}$; Pearson $r = 0.913$) (Figure 5A). These characteristics of CU-L1 methylation levels suggest that loss of LINE-1 methylation in cancers, in addition to being a result of genome-wide hypomethylation, may also be influenced by locus-specific pressure.

DISCUSSION

Methylation patterns of LINE-1s in different normal tissue types and hypomethylation characteristics in many types of epithelial cancers have been previously reported (4–18). To our knowledge, this is the first report to demonstrate methylation patterns of LINE-1s at different genomic locations. It is surprising that even though LINE-1s are repetitive sequences, methylation levels of LINE-1s among different loci can be different. In normal cells, some loci possess high methylation levels, whereas many loci are only partially methylated. This suggests that *cis*-elements, or factors on the same chromosome near each LINE-1, may influence the epigenetic modification. LINE-1 methylation levels of some loci represent a tissue-specific pattern, as shown by the different levels between NOE and WBC. Interestingly, Eller and colleagues (34) demonstrated that LINE-1 elements are frequently excluded from housekeeping regions. Moreover, hyper-methylation of LINE-1s has been reported during abnormal overgrowth and differentiation of human placenta (11). It is therefore possible that LINE-1 methylation is associated with tissue-specific phenotypes of the cells in relation to the genome function in *cis*. Finally, in some regions (e.g. L1-*SPOCK3*), this epigenetic control may be

a dynamic process. Wider ranges of methylation levels could be observed with a greater number of LINE-1 loci in NOE than in WBC. Interestingly, we previously found intermediate ranges of genome-wide LINE-1 methylation levels in head and neck squamous cells and wider ranges in esophageal and thyroid tissues (4).

LINE-1 hypomethylation is a common epigenetic process in cancer that may progressively evolve during the multistage carcinogenesis process (4,5,7,12). The underlying mechanism of this process is not known. Because the methylation levels of most LINE-1 loci in cancerous cells are positively correlated with each other and on a genome-wide level, we concluded that the mechanism leading to global hypomethylation is a generalized process that simultaneously influences dispersed loci. Our experiments, however, have demonstrated that the locus-specific epigenetic modifications of some loci may also be related to carcinogenesis. Evidence that supports this hypothesis includes the data that show different hypomethylation incidences from different LINE-1 loci, the direct association between the two LINE-1s in *EPHA3* introns and hypermethylation of some loci, such as L1-*CNTNAP5*, in some HNSCCs. In conclusion, even though global hypomethylation processes can generally and progressively deplete LINE-1 methylation levels, locus-specific methylation of LINE-1s can differentially be influenced by carcinogenic processes depending on where the particular LINE-1s are located in the genome.

The level of LINE-1 hypomethylation is positively associated with how the tumor progresses (4,5,7,12). Consequently, the potential roles of a PCR-based LINE-1 methylation analysis for cancer diagnosis has been evaluated by several studies (8–10,12,13). Differential methylation levels of normal tissues, however, limit the utility of COBRALINE-1. For example, the application of COBRALINE-1 to analyze circulating free cell DNA should be limited because serum DNA contains a mixture

of DNA released from normal and cancerous cells (35). The cancer-induced global hypomethylation differentially influences each LINE-1 locus depending on their locations. Moreover, variations of methylation levels in normal cells occur specifically at some loci. Consequently, since there are a vast number of LINE-1s, researchers can test and select for LINE-1 loci that yield specific information related to cancer development. The CU-L1 methylation levels may therefore be an important tool for cancer diagnosis in the future.

SUPPLEMENTARY DATA

Supplementary Data are available at NAR Online.

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