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DNA Methylation Profile at the DNMT3L Promoter:

A Potential Biomarker for Cervical Cancer

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Abstract

Epigenetic events play a prominent role during cancer development. This is evident from the fact that almost all cancer types show aberrant DNA methylation. These abnormal DNA methylation levels are not restricted to just a few genes but affect the whole genome. Previous studies have shown genome-wide DNA hypomethylation and gene-specific hypermethylation to be a hallmark of most cancers. Molecules like DNA methyltransferase act as effectors of epigenetic reprogramming. In the present study we have examined the possibility that the reprogramming genes themselves undergo epigenetic modifications reflecting their changed transcriptional status during cancer development. Comparison of DNA methylation status between the normal and cervical cancer samples was carried out at the promoters of a few reprogramming molecules. Our study revealed statistically significant DNA methylation differences within the promoter of *DNMT3L*. A regulator of de novo DNA methyltransferases *DNMT3A* and *DNMT3B, DNMT3L* promoter was found to have lost DNA methylation to varying levels in 14 out of 15 cancer cervix samples analysed. The present study highlights the importance of DNA methylation profile at *DNMT3L* promoter not only as a promising biomarker for cervical cancer, which is the second most common cancer among women worldwide, but also provides insight into the possible role of *DNMT3L* in cancer development.

Keywords

DNMT3L; DNA methylation; cervical cancer; biomarker; nuclear reprogramming

INTRODUCTION

Correlation between epigenetic modifications and cancer development has been established firmly over the past few years.1 Most cancers exhibit genome-wide hypomethylation2 and gene-specific hypermethylation.3 Many examples of silencing of tumor suppressor genes by

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DNA methylation status are known.4 DNA repair genes have also been found to be silenced by aberrant DNA methylation in certain cancers.5 On the other hand, hypomethylation of a number of oncogenes has been observed.6,7 In addition, many studies have shown abnormal DNA methylation levels for genes involved in genomic imprinting, cell cycle regulation, metabolic regulators, etc.1

Reprogramming molecules like DNA and histone methyltransferase, histone acetylases and deactylases are the epigenetic effector molecules which can reprogram genetic information.8 These molecules are essential for normal development as any change to the epigenetic status of genetic loci in response to an environmental cue would have to be perpetuated through these reprogramming molecules. The possibility, therefore, exists that in the altered environment of cancer cells, epigenetic status of the genome undergoes changes due to deregulation of these genes. This deregulation could either be due to genetic mutations within these genes or due to changes in their epigenetic profile. We sought to explore the possibility that the reprogramming genes themselves undergo epigenetic modifications reflecting their changed transcriptional status during cancer development. To examine this possibility, we undertook a pilot study, wherein, we analysed DNA methylation status for the promoters of a few reprogramming genes in a small number of normal and cancer cervix samples collected from city based cancer hospital of Hyderabad, India.

Cervical neoplasia is one of the major causes of death among women in India, especially in rural areas.9-11 It is also the second most common cancer among women worldwide, with an estimated 493,000 new cases and 274,000 deaths in the year 2002.12 It is well known that infection with high risk HPV type is a major etiological risk factor for cancer cervix. The development of HPV vaccines and trials are promising to bring down the cancer cervix incidence.13,14 However the results of the efficacy of these vaccine trials in India are yet unknown. Implementation of rigorous cancer screening programmes are therefore urgently needed. While VIA, Pap-Test and HPV-typing have proved to be important in screening of cancer cervix,12 each of these tests have some limitations. Therefore, use of other adjunctive molecular biomarkers like DNA methylation will prove to be more beneficial for early detection and diagnosis. Recent evidence suggests that epigenetic modifications such as DNA methylation and histone modifications may play a major role in cancer development.15 For cancer cervix in particular, a few studies have examined changes in DNA methylation for a few genes related to apoptosis, cell cycle, DNA repair and tumor suppression.16

The main aim of our study was to identify loci, which showed unambiguous DNA methylation difference between the normal and cancer groups within the selected reprogramming genes. Two types of reprogramming molecules were examined in our study: direct effectors of epigenetic modifications like DNA and histone methyltransferases and regulators of these direct effectors. The regulators of effector molecules (like *EED* and *DNMT3L*) are not transcriptional regulators but interact directly with the effector proteins and modulate their function.17-19 A recent hypothesis proposes that cancer probably arises from stem cells1 suggesting the possibility that in a subset of cancer cells some of the markers might have stem cell like epigenetic signatures. Therefore, in addition to reprogramming molecules, we also examined some stem cell markers.

MATERIALS AND METHODS

Collection of specimens

The present study was approved by the institutional bioethical committee and patients consent was taken for the sample collection. The collection of cervical biopsy specimen has been described earlier.20 Briefly, the biopsy specimen were collected from women,

(attending the cancer clinic at the MNJ cancer research hospital) diagnosed with invasive cancer. Cervical tissue specimens collected from women undergoing hysterectomy, whose pathological report confirmed absence of neoplasia constituted the control group. Cervical scrapes were also collected using Ayer's spatula from asymptomatic healthy women attending rural health camps. The cervical scrapes were collected in methanol based fixative and cells were further collected by centrifugation and frozen immediately. The DNA was extracted by phenol chloroform based method following proteinase K digestion.

HPV detection

DNA isolated from cervical tissues was tested for presence of HPV using a PCR based line blot assay as described earlier.21

DNA methylation analysis

DNA methylation for the selected genes was analysed by bisulfite sequencing with an aim to define the DNA methylation profile for the selected genetic loci and identifying the most informative CpG's within that region. Sodium bisulfite modification was done as described previously by Hajkova et al. (2002).22 Briefly, approximately 1 mg of *Eco*RI digested genomic DNA was denatured in a boiling water bath for 5 minutes, snap chilled and incubated with 0.3 M NaOH for 15 minutes at 50°C. The denatured DNA was mixed with equal volume of 2% LMP Agarose (Sea Plaque Agarose, BMA) and 10 ml aliquots were pipetted into the cold mineral oil to form beads. The beads were transferred into the bisulfite modification solution and incubated on ice for 30 minutes and then at 50°C for 3.5 hours. Subsequently, the beads were washed with TE (pH 8.0) treated with 0.2 N NaOH and again washed with 1X TE (pH 8.0). The beads were stored in a minimal volume of 1X TE and before PCR were washed with sterile water. PCR primers specific to the converted DNA were used to amplify specific CpG islands. The primers used in our study (designed using Methprimer23) are given in (Supplementary Table T1). The PCR products were electrophoresed on 1.5% agarose gels and the specific bands were eluted, ligated to T-tailed vector and transformed. To obtain methylation profile, about sequences from 8 or more clones for each sample were analysed. The efficiency of bisulfite conversion was analysed by calculating the percentage conversion of cytosines in non-CpG context. In our study, the non-CpG "C to T conversion rate" was 95.7 ± 0.64 (standard error of mean).

RNA isolation and Real-Time quantitative PCR

Total RNA was isolated from Hela and SiHa cells using the RNeasy-RNA isolation kit (Qiagen) as per the given instructions. The RNA was DNase I treated and quantified in a spectrophotometer. Total RNA (1mg) from both cell lines was reverse transcribed using oligo$(dT)_{21}$ primer and Superscript III (Invitrogen) according to manufacturer's instructions in a reaction volume of 20 ml. RT-PCR was performed in triplicates in a reaction volume of 10 ml containing 1ml of the cDNA (from the 20 ml reaction), 1X SYBR Green PCR Master Mix and 150 nM primers. The reactions were performed in a 7900HT Fast Real Time PCR system (ABI) starting with an incubation at 95°C for 5 minutes followed by 40 cycles of 15 seconds at 94°C, 30 seconds at 62°C and 30 seconds at 65°C with fluorescence detection after the extension step of each cycle. The specificity of the reaction was analyzed by performing a melting curve analysis at 95°C for 15 seconds after the amplification and confirmed by electrophoresing the products on a 1.5% agarose gel. Relative amounts of *DNMT3L* transcripts were normalized against the levels of b-*ACTIN* RNA in each cDNA sample.

Statistical analysis

To determine whether the differences in DNA methylation levels that we observe between cancer and normal cervical samples are statistically significant, we performed t-test for each CpG at all the loci examined. The t-test was done using two-tailed distribution and taking into account unequal variance in the two data sets.

RESULTS

Comparison of DNA methylation profiles between normal and cancer cervix samples

In the present study promoter methylation for a few genes was examined in normal and neoplastic cervical samples. The control group constituted histopathologically normal cervical epithelium obtained either as surgically removed tissue sample or endocervical scrapes. Recently it was shown that the exfoliated scraped cells have similar gene expression profile as the cervical tissue and hence these are considered as a good sample material for molecular biomarker studies.24 A PCR based line-blot approach was used for HPV typing. Almost all the cervical cancer specimens used in the study were classified as high-grade squamous cell carcinoma and the major HPV type detected was HPV-16 (Fig. 2).

Several studies previously have shown that the levels of DNA methylation are altered within the promoter of *RASSF1A*, a tumor suppressor gene.16,25-27 As a control for our studies we therefore examined the promoter DNA methylation for *RASSF1A*. As can be seen from (Fig. 1), the difference between normal and cancer samples (hypermethylation) was restricted to only one sample (CC3). Amongst the reprogramming molecules, we analysed the two de novo DNA methyltransferase *DNMT3A* and *DNMT3B*.28 *EZH2*, a histone H3Lys27 methyltransferase,18 was examined from amongst the histone methyltransferases. *EED*, a part of the histone methylation complex PRC218 and *DNMT3L*, which regulates the *de novo* DNA methylation activity of *DNMT3A* and *DNMT3B*,17,19 were analysed as they are known to regulate the functioning of histone H3Lys27 and DNA methyltransferases respectively. The stem cell markers *OCT4* and *NANOG29,30* and *BLIMP1*, a critical determinant of germ line31 were also analyzed.

The de novo methyltransferases *DNMT3A* and *DNMT3B*, the polycomb complex proteins *EED* and *EZH2* and germ line determinant *BLIMP1* were found to be mostly unmethylated both in normal as well as cancer cervix samples (Fig. 2 for summary and supplementary Figs. S1-5 respectively). Hypomethylation of these genes correlated with their expression levels as these genes are normally expressed in cervical tissues (from the data available on the UniGene database at the NCBI website). *OCT4* gene is normally transcriptionally silent, concomitantly its promoter is methylated in differentiated cells.32 As expected, the promoter of *OCT4* was found to be predominantly methylated in both the groups (Fig. 2 and supplementary Fig. S6). *NANOG* was found to be partially methylated (methylated at some CpG's and unmethylated at other CpG's, Supplementary Fig. S7) in both the groups. The difference between the percentage of clones showing methylated or unmethylated CpG residues was not statistically significant.

DNMT3L is normally expressed at very low levels and moreover, its expression is observed only in testis, ovaries and thymus in humans.33 In mice, its maximum expression is found in ES cells where its promoter region is unmethylated.34 In humans, *DNMT3L* is not expressed in cervical tissue (based on the data available on the UniGene database available at the NCBI website). We found most of the CpG's (except 4th) within the *DNMT3L* promoter to be methylated in normal cervix samples (Fig. 3). The cancer cervix samples on the other hand showed interesting differences in comparison to the normal samples. Three types of DNA methylation profiles were observed for the cancer samples. CC2 showed a profile very similar to normal samples with all the CpG's methylated. CC3 was found to

have negligible methylation at all the CpG within the promoter of *DNMT3L* (Fig. 3). As shown above, CC3 also showed extensive changes in DNA methylation for the promoter of *RASSF1A* (Fig. 1). CC1, CC4, CC5, CC6 and CC8 were found to have methylation only at CpG no. 3, 6 and 11 and have lost methylation at all the other CpG's (Fig. 3). To substantiate our finding, we further analysed 8 more cancer cervix samples and 3 more normal cervix samples for DNA methylation at the *DNMT3L* promoter. As tabulated in Figure 2, all the cancer samples (CC9 to CC11 and CC14 to CC18) showed changes in DNA methylation levels at the *DNMT3L* promoter as compared to normal. CC15 and CC17 showed complete loss of DNA methylation at all the CpG sites analysed. For CC9, 10, 11, 14, 16 and 18 while some CpG's had lost methylation completely, most of the CpG's showed decrease in the percentage of methylation. Comparsion of the two groups for DNA methylation using t-test showed that the difference at eight of the 11 CpG's was significant ($p < 0.05$, denoted by + below each CpG for *DNMT3L*, (Fig. 2 and Supplementary Fig. S8). For five out of these eight CpG's this difference was highly significant ($p < 0.001$, denoted by * in Fig. 2 and supplementary Fig. S8).

Correlation of DNA methylation at DNMT3L promoter with its expression

Bisulfite sequencing analysis for the *DNMT3L* promoter was performed for the cervical cancer cell lines, HeLa and SiHa. Low levels of DNA methylation was observed in HeLa cells whereas in SiHa most of the CpG's (except 7th CpG) within the *DNMT3L* promoter were found to be methylated (Fig. 4A). To examine the level of *DNMT3L* expression in these cell lines we carried out Real-Time quantitative PCR using primers spanning 1st/2nd and 3rd/4th exon of *DNMT3L*. Relative amounts of *DNMT3L* transcripts were normalized against the levels of b-*ACTIN*. As can be seen from Figure 4B, there is a 8-fold difference in the expression level of *DNMT3L*.

DISCUSSION

In the present study we have examined the possibility that the reprogramming genes themselves undergo epigenetic modifications reflecting their changed transcriptional status during cancer development. A few stem cell markers were also analysed in our study to probe the hypothesis that cancer probably arises from stem cells.1 In addition, we have also explored the possibility of using DNA methylation as adjunct biomarker along with the use of HPV and Pap smear test in the screening of cervical cancer. While HPV is the known etiological risk factor for cancer cervix, recent studies have established a correlation of epigenetic changes, especially those in DNA methylation, with neoplastic development.15 DNA methylation profiles for genetic loci are good cancer biomarkers, not only because DNA is a much more stable bio-molecule in comparison to RNA and can survive routine processing for histopathology but also because DNA methylation changes have been observed very early during cancer development.1 Moreover, the changes in DNA methylation pattern can be correlated with the status of RNA expression.35

Since this was a pilot study we restricted ourselves to only a few samples (15 cancer and 6 normal) and focussed on statistically significant DNA methylation changes. As the amount of DNA available from these samples was limiting, DNA methylation analysis of only nine genes was undertaken. In this investigation, we were able to corroborate previous studies which have demonstrated changes in the level of DNA methylation at the *RASSF1A* gene. 25,26,27 We surprisingly observed only one cancer patient showing hypermethylation at the *RASSF1A* promoter. More importantly, we observed interesting differences in the DNA methylation profile at the *DNMT3L*. A nuclear reprogramming related gene, *DNMT3L* regulates the DNA methylation function of de novo methyltansferases, *DNMT3A* and *DNMT3B*.17,19 Whereas CC3, CC15 and CC17 showed complete loss of methylation for the *DNMT3L* promoter, we found that 11 out of the other 12 cancer samples also had a

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methylation profile different from normal cervix samples. The differences observed were statistically significant ($p < 0.05$) for eight of the 11 CpG's analysed for this region. No significant DNA methylation difference was observed for *DNMT3A* and *DNMT3B*, the de novo methyltransferases; *EZH2* and *EED*, which are involved in histone methylation (at H3 lys27) and the stem cell markers.

The difference in the DNA methylation at the promoter of *DNMT3L* is significant as *DNMT3L* is a regulator of de novo methyltransferases and expression of this molecule in tissues where it is normally not expressed could in turn activate *DNMT3A*. This could cause aberrant DNA methylation (changes in the level of methylation as well as DNA methylation at sites which are normally not methylated). We were unable to analyse the expression level of *DNMT3L* in the cancer and normal samples due to the limitation of specimen available. However, we found correlation of DNA methylation within *DNMT3L* promoter with its expression in the cervical cancer cell lines that we analysed. HeLa cell line, which had lost methylation at the *DNMT3L* promoter, showed 8 fold more expression than SiHa cell line (promoter is predominantly methylated) *DNMT3L* was not expressed. Furthermore, in mice, DNA methylation has been shown to control the expression level of *DNMT3L*.34 It is also important to note that a regulator of epigenetic effector molecule (*DNMT3L*) showed changed methylation pattern upon cancer development whereas the effector molecules (*DNMT3A* and *DNMT3B*) did not show any change.

We could not draw any correlation between grade of cancer cervix examined and change observed in DNA methylation for any of the genes analysed. CC3 was classified as grade IV cancer cervix sample and showed the most striking DNA methylation difference at the promoter of both *RASSF1A* and *DNMT3L* but CC6, was also graded IV and did not show DNA methylation difference for the *RASSF1A* promoter. CC6 did show DNA methylation differences at the *DNMT3L* loci but similar changes were also observed in other cancer samples (except CC2) which were not typed as grade IV cancers. In addition, the number of samples for each grade that we analysed were too less to statistically make any correlation between DNA methylation changes and grade of the cancer.

In conclusion, our pilot study has identified a strong correlation of DNA methylation changes at the promoter of *DNMT3L* with cancer cervix. Since more than 90% of the cancer samples analysed were found to show a change in DNA methylation profile for the *DNMT3L* promoter the possibility therefore exists for *DNMT3L* promoter methylation to be a potential biomarker for cervical cancer. Studies are ongoing to test our findings not only on a larger cohort of cancer cervix patients but also to examine whether similar differences exist in other cancer type.

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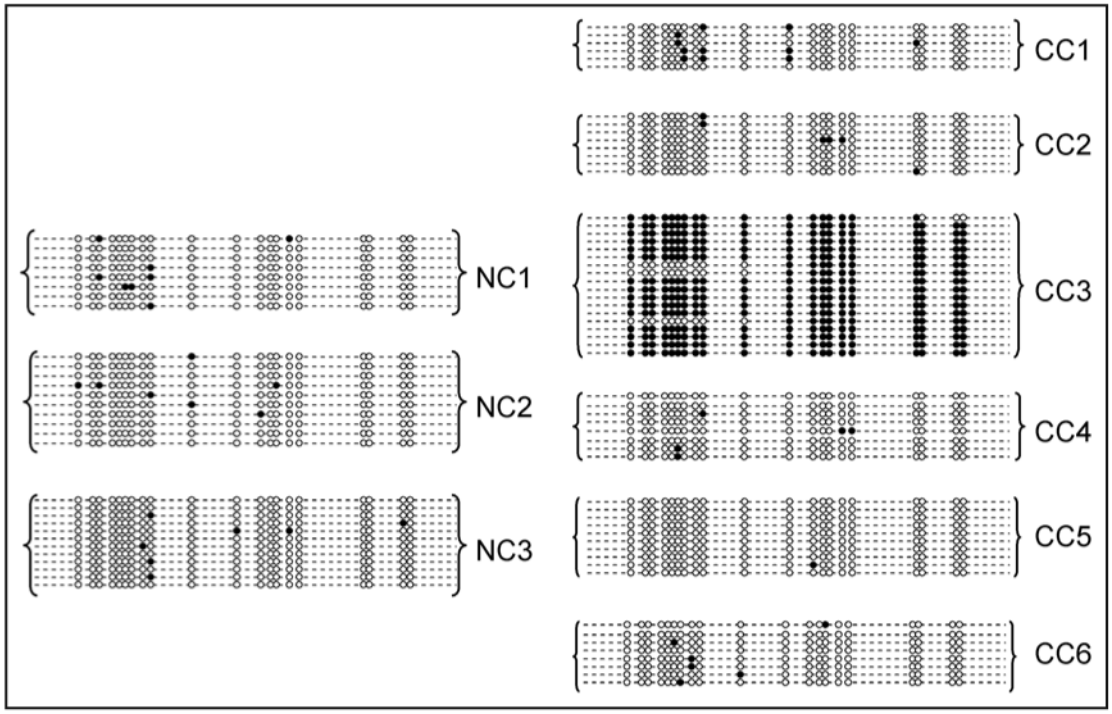

Figure 1.
Comparative analysis of DNA methylation at the *RASSF1A* promoter. Bisulfite sequencing analysis was performed on DNA isolated from normal and cancer cervix patients. Each horizontal line indicates a single clone from the respective PCR products after bisulfite treatment. Circles denote CpG dinucleotides present within the sequence. The positions are not drawn to scale. Open circles indicate no methylation. Filled circles represent methylated cytosine. Each bracketed profile represents individual sample. Normal cancer cervix samples are prefixed with NC and cancer samples have CC as a prefix.

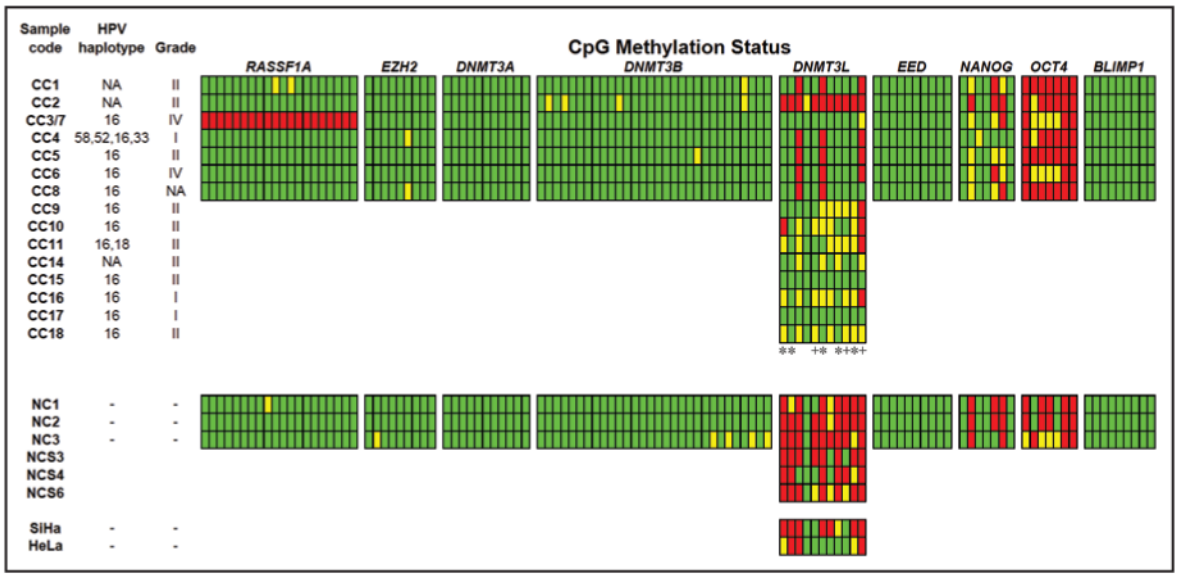

Figure 2.

Summary of DNA methylation results on the 9 genes analysed. Each colored box represents one CpG dinucleotide. Each gene is represented by colored boxes equal to the number of CpG analysed. Respective color denotes the percentage of clones showing methylation at individual CpG dinucleotide. Green: 0-34%, Yellow: 34-66%, Red: 66-100%. Note the remarkable difference in the methylation levels for *DNMT3L* between normal and cancer cervix samples (except CC2). Symbols below each CpG box for *DNMT3L* denotes statistical significant difference (calculated using t-test) in methylation for the respective CpG between normal and cancer samples (+ - $p<0.05$, * - $p<0.001$). No correlation was found between the grade of tumor and changes in DNA methylation levels. The sequences of the primers designed for bisulfite PCR using Methprimer are provided in (Supplementary Table T1).

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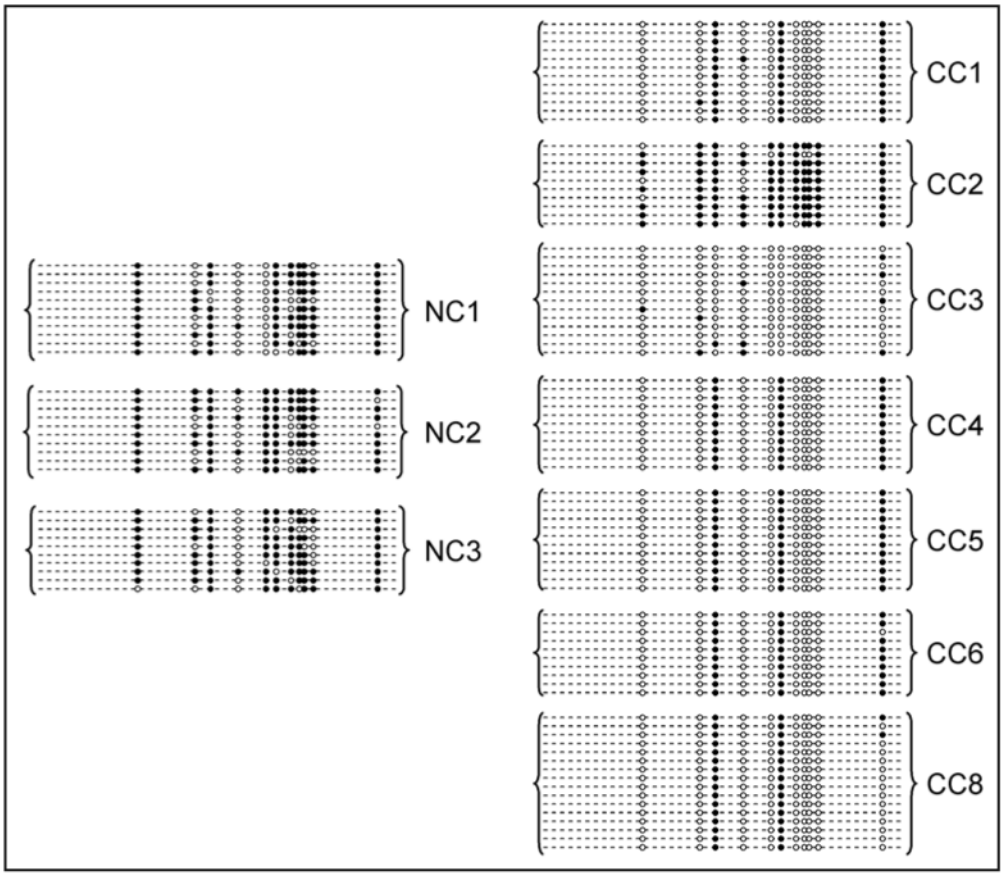

Figure 3.
Comparative analysis of DNA methylation at the *DNMT3L* promoter. Bisulfite sequencing analysis was performed on DNA isolated from normal and cancer cervix patients as described in materials and methods. See (Fig. 1) legend for explanation of the figure.

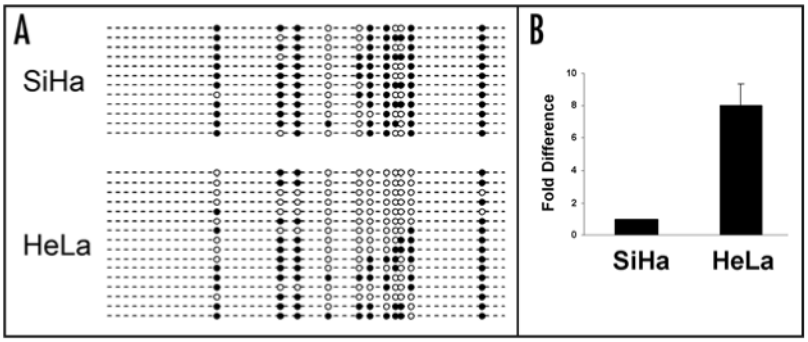

Figure 4.

DNMT3L promoter methylation is correlated to its expression. (A) Bisulfite analysis of *DNMT3L* promoter for HeLa, and SiHa cell lines. (B) Real-time QRT-PCR analysis for *DNMT3L* mRNA extracted from SiHa and Hela cervical cancer cell lines. Results are shown as fold difference in the *DNMT3L* expression levels. Each experiment was done in triplicate and was repeated thrice and normalized against the amount of b-*ACTIN* mRNA in each sample.