

RESEARCH COMMUNICATION

Line-1 Hypomethylation in Multistage Carcinogenesis of the Uterine Cervix

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Abstract

Objective: To evaluate characteristics of global hypomethylation in evolution of cervical cancer. **Materials and Methods:** Eight cases of squamous cell carcinoma (SCC) and seven cases of carcinoma in situ (CIS) were studied. Each of the SCC samples contained CIS, and all SCC and CIS samples contained normal ectocervical epithelium. Microdissection was performed to separate normal epithelium, CIS and SCC prior to DNA extraction. Hypomethylation levels of long interspersed nuclear elements (LINE-1 or L1) were measured with a combined bisulfite restriction analysis (COBRA) PCR (polymerase chain reaction) protocol. The percentage of L1 hypomethylation for SCC, CIS and normal epithelium was compared. **Results:** In the SCC cohort, the L1 hypomethylation level showed progressive increase comparing normal epithelium ($59.4 \pm 8.86\%$) to CIS ($64.37 \pm 7.32\%$) and SCC ($66.3 \pm 7.26\%$) (repeated measurement ANOVA, $P = 0.005$). A significantly greater L1 hypomethylation level was found in CIS ($62.06 \pm 3.44\%$) compared to normal epithelium ($60.03 \pm 3.69\%$) (paired t-Test, $P = 0.03$). No significant difference in L1 hypomethylation level was noted between CIS of the two sample groups (unpaired t-Test, $P = 0.2$). **Conclusions:** In our study, there was a significant correlation between the degree of hypomethylation and progression from normal ectocervical mucosa to CIS and invasive cancer. Laboratory assessment of biopsies for this molecular event may have clinical significance.

Keywords: Uterine cervical cancer - LINE-1 methylation - global hypomethylation - carcinogenesis

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Introduction

Carcinoma of the cervix uteri is an important cause of death of women worldwide, especially in developing countries (Cannistra and Niloff, 1996; Pornthanakasem et al., 2001). Invasive squamous carcinoma develops through a multistage process of carcinogenesis, and sexually transmitted human papillomavirus is the major etiological factor for cancer development (Cannistra and Niloff, 1996; Pornthanakasem et al., 2001; Tavassoli and Devilee, 2003). Viral oncoproteins E6 and E7 have been shown to interact with tumor suppressor gene products, induce chromosomal abnormalities, and change cellular phenotypes (Pornthanakasem et al., 2001; Tavassoli and Devilee, 2003; Duensing and Munger, 2004). Identification of genetic events during cancer progression is important, not only because it provides a basic knowledge of the disease, but may also have clinical implications given that some of the genetic alterations precede morphological changes in biopsies.

Genome-wide losses of DNA methylation have been demonstrated in several human cancers in which there is downregulation of methylated CpG dinucleotides, which are dispersed throughout the genome both in genes and

noncoding repetitive sequences (Sugimura and Ushijima, 2000; Kaneda et al., 2004; Chalitchagorn et al., 2004). Although it remains controversial as to whether the epigenetic change occurs at the initial step or during progression of carcinogenesis (Chalitchagorn et al., 2004; Feinberg et al., 1988), Kim et al (1994) has demonstrated that global hypomethylation increased progressively during the evolution of cervical cancer. Assessment of this epigenomic alteration may have clinical significance, but technical difficulty with conventional methods, such as Southern blotting, has made the evaluation impractical for routine usage.

Using combined bisulfite restriction analysis (COBRA) polymerase chain reaction (PCR), we have recently developed a quantitative method to analyze the methylation status of long interspersed nuclear elements (LINE-1 or L1), called COBRALINE-1 (Chalitchagorn et al., 2004). Our modified PCR protocol targets short amplicon sizes of the widely distributed L1, and is thus highly suitable for formalin-fixed paraffin-embedded material. The purpose of this study was to evaluate L1 hypomethylation status during cervical carcinogenesis using this technique, and to assess the degree of hypomethylation in relation to the morphological changes.

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Materials and Methods

Samples

Two groups of samples were used in this study. The first group included eight cases of invasive squamous carcinoma (SCC) stage I of the cervix uteri obtained from radical hysterectomy. The second group included seven cases of carcinoma in situ (CIS) with free resection margins, obtained from conization or total abdominal hysterectomy. All SCC specimens contained CIS and normal ectocervical epithelium; all CIS samples contained normal ectocervical epithelium.

Laboratory Investigations

Formalin-fixed paraffin-embedded unstained tissue sections were manually microdissected under an inverted light microscope to separate normal epithelium, CIS and SCC prior to DNA extraction. COBRALINE1 analysis was performed as previously described (Chalitchagorn et al., 2004). Briefly, unmethylated cytosines in DNA samples were changed to uracil by treatment with sodium bisulfite. The modified DNA was, then, amplified by 5'UTR L1 bisulfited sequence primers as follows: 5'-CCGTAAGGGGTTAGGGAGTTTTT-3' and 5'-RTAAAACCTCCRAACCAAATATAAA-3' and digested by TaqI and TasI restriction enzymes, which recognize methylated and unmethylated sequences, respectively. TaqI or TasI-positive amplicons had been previously cloned and sequenced, revealing complete methylation and unmethylation of all linked CpG dinucleotides, respectively (Chalitchagorn et al., 2004). Reproducibility of the test was confirmed by our previous and other subsequent studies (Chalitchagorn et al., 2004; Matsuzaki et al., 2005). In this study, the level of L1 hypomethylation in each sample was expressed as a percentage, dividing the measured intensity (PhosphorImager) of TasI digestible amplicons by the sum of TasI and TaqI products.

Statistical analysis

The L1 hypomethylation level among the three different tissues (normal, CIS and SCC) in the carcinoma group was assessed by repeated measurement ANOVA. To evaluate the differences in L1 hypomethylation level between CIS and normal epithelium in the CIS group, and between CIS in the two groups, paired and unpaired T-tests were used, respectively.

Results

Results are summarized in Figure 1. Of the eight invasive cancers, the degree of L1 hypomethylation level progressively increased from normal epithelium ($59.4 \pm 8.86\%$) to CIS ($64.37 \pm 7.32\%$) and SCC ($66.3 \pm 7.26\%$) (repeated measurement ANOVA, $P = 0.005$). In the CIS group, the CIS portion showed a significantly greater L1 hypomethylation level ($62.06 \pm 3.44\%$) compared to normal epithelium ($60.03 \pm 3.69\%$) (paired t-Test, $P = 0.03$). No significant difference in L1 hypomethylation level was noted between CIS of the two sample groups (unpaired t-Test, $P = 0.2$).

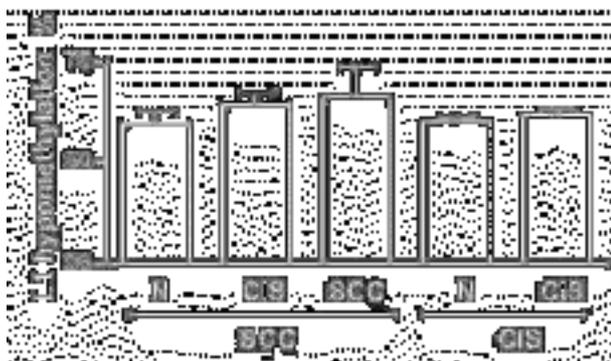


Figure 1. L1 Hypomethylation in Multistage Cervical Carcinogenesis. In samples showing invasive squamous cell carcinoma (SCC), the degree of L1 hypomethylation level progressively increases from normal epithelium (N) ($59.4 \pm 8.86\%$) to CIS ($64.37 \pm 7.32\%$) to SCC ($66.3 \pm 7.26\%$) (repeated measurement ANOVA, $P = 0.005$). In samples with carcinoma in situ (CIS) without nearby invasive cancer, significantly greater L1 hypomethylation was found in the CIS portion ($62.06 \pm 3.44\%$) compared to normal epithelium ($60.03 \pm 3.69\%$) (paired t-Test, $P = 0.03$). No significant difference in L1 hypomethylation status was noted in CIS with or without adjacent invasive carcinoma (unpaired t-Test, $P = 0.2$).

Discussion

LINE-1 (L1), a highly repeated interspersed human retrotransposon, is ubiquitous and constitutes approximately 17% of the human genome. Its methylation status, therefore, reflects the genome-wide methylation level (Chalitchagorn et al., 2004; Gilbert et al., 2004). Although the role of this epigenetic loss in cancer development has not been conclusive, hypothetical mechanisms include increasing genomic instability and interfering with the expression of linked genes (Chalitchagorn et al., 2004; Gilbert et al., 2004; Florl et al., 1999). With COBRALINE-1, we have shown previously that most human carcinomas possessed significantly greater L1 hypomethylation levels, when compared with their normal counterparts (Chalitchagorn et al., 2004). While hypermethylation takes place at specific genes, most cancers are globally hypomethylated, and L1 has been shown to be responsible for the overall losses of DNA methylation (Chalitchagorn et al., 2004; Florl et al., 2004; Feng et al., 2005).

In the present study, we first evaluated how global hypomethylation evolves during the multistep process of carcinogenesis of the cervix uteri. SCC specimens containing CIS and normal epithelium were microdissected and separately analyzed with COBRALINE1. In parallel with the previous investigation (Kim et al., 1994), which utilized a different technique, the degree of global hypomethylation increased progressively during the progression from normal cervical epithelium to CIS and then invasive cancer. Although differences in the hypomethylation level between CIS ($64.37 \pm 7.32\%$) and SCC ($66.3 \pm 7.26\%$) in the SCC group and between normal cervical epithelium ($60.03 \pm 3.69\%$) and CIS ($62.06 \pm 3.44\%$) in the other appears to be minimal, considering the fact that L1 segments constitute a significant component of the genome (17%), such magnitude of change would be critical to cellular functions.

Next, we determined whether the precancerous lesion

had different global hypomethylation level depending on whether there was nearby invasive carcinoma or not. To address this issue, CIS specimens with free resection margins were examined. Significantly greater L1 hypomethylation level was observed in CIS compared to normal epithelium. Taken together, the results from our study support the hypothesis that the genesis and progression of cervical carcinoma positively correlates with an increasing degree of global hypomethylation. Since routine formalin-fixed paraffin-embedded tissues are suitable for the COBRALINE1 analysis, identification of this molecular event might be a useful adjunct to the standard pathological examination of cervical specimens.

In conclusion, the results of present investigation indicate that global hypomethylation is an epigenetic change associated with the development and progression of uterine cervical cancer. An increasing degree of this genetic alteration significantly correlated with the morphological changes at biopsy examination. Hence, laboratory assessment of this molecular epigenetic event may be a useful part of the examination of cervical biopsies and could have clinical implications.

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