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Subtelomeric DNA methylation and telomere length in human cancer cells

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ABSTRACT

Subtelomeric epigenetic modifications are known to be associated with telomere length. We examined subtelomeric DNA methylation at seven sites for five chromosomes by methylation-specific PCR (MSP) and two sites for two chromosomes by bisulfite genomic sequencing (BGS) in 20 human cancer cell lines and subsequently analyzed their association with telomere length. Full-methylation (55/140) was more frequently found compared to un-methylation (35/140) ($p = 0.01$). Subtelomeric-methylation patterns varied from region to region; full-methylation and un-methylation were dominant at one of 9q sites (20/20) and 9p (18/20), respectively. MSP and BGS data exhibited no apparent correlation between methylation status and telomere length. In addition, Hep3B subclones that possessed different telomere lengths exhibited no changes in methylation status according to telomeres. In summary, subtelomeres might form distinct chromatin structures from region to region and effect of subtelomeric DNA methylation on telomere regulation might be little.

1. Introduction

Located adjacent to gene-poor subtelomeric regions made up of TTAGGG tandem repeat sequences [1,2], telomeres are bound by a specialized protein complex known as 'shelterin,'which plays fundamental roles in the regulation of telomere length along with protection of the chromosome from degradation and from recognition as a target for non-homologous end joining [3–6]. Telomeres are thought to be maintained by at least two mechanisms: telomerase activity and recombination. Telomerase synthesizes telomeres at the chromosome ends and thus regulates the length of telomeric repeats [7–9]. Telomere length can, in some cases, be maintained by an alternative mechanism, such as a telomerase-independent telomere length mechanism (alternative lengthening of telomeres, or ALT) based on homologous recombination-mediated DNA replication among telomeric sequences [10–12].

Recent evidence indicates that epigenetic modification of telomeric chromatin has an influence on the regulation of telomere length [13]. DNA methylation is one epigenetic chromatin modification, and it plays important roles in gene expression and imprinting, as well as in heterochromatin assembly [14,15]. DNA methylation is mediated by DNA methyltransferases (DNMTs) known as DNMT1, DNMT3a, and DNMT3b, and three DNMTs have been identified in both humans and mice [16–19]. These DNMTs play a role in maintaining DNA methylation patterns at the pericentric major satellites of heterochromatin, ensuring proper centromeric function [20–22].

Mammalian telomeres and subtelomeres have features similar to pericentromeric regions. In sequence

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composition, the telomere, subtelomere, and pericentromere have a high density of DNA repeats. Telomeres do not contain genes, whereas subtelomeres are gene-poor [23]. Telomeres also do not contain the CpG sequence that is susceptible to methylation by DNMTs, while subtelomeres have a high density of CpG sequences [24,25]. A role for DNA methylation at the subtelomere has recently been reported in mice, with the discovery of hypermethylated mouse subtelomeric DNA [26,27]. De-methylation of subtelomeric regions in DNMT-deficient cells results in telomere lengthening caused by increased homologous recombination in telomeric sequences [27]. Reintroduction of DNMTs 3a and 3b into DNMT-deficient cells restores methylation at the subtelomere and results in less telomeric homologous recombination [26,27]. In telomerase knock-out mouse cells, decreased DNA methylation is a consequence of telomere shortening [26]. DNA methylation at the subtelomere is therefore implicated as an important regulator of telomeres, raising the possibility that DNA methylation levels might have a close association with telomere length. However, epigenetic modifications of human subtelomeres and their impact on telomere length regulation and telomere function remain uncharacterized.

In this study, we examined the subtelomeric DNA methylation status at seven sites in five chromosomes by methylation-specific PCR (MSP) and two sites in two chromosomes by bisulfite genomic sequencing (BGS) and analyzed their possible association with telomere length in human cancer cell lines.

2. Methods and materials

2.1. Cell Cultures and establishment of stable cell lines

Cell lines used in this study were obtained from the American Type Culture Collection (ATCC, MA, USA) and the Korean Cell Line Bank (KCLB, Seoul, Korea). YCC cell lines have been established at the Yonsei Cancer Center at Yonsei University College of Medicine (Seoul, Korea). Cells were cultured at 37 °C under 5% CO_2 in Minimum Essential Medium, RPMI 1640, or Dulbecco's modified Eagle's MEM supplemented with 10% fetal bovine serum (GIBCO, NY, USA), 100 U/mL penicillin, and 0.1 mg/mL streptomycin (GIBCO).

Hep3B cells were co-transfected with pBK-Hygro and pCMV-myc or pCMV-myc-TRF1 using WelFect Ex™ PLUS transfection reagent (WelGENE, Seoul, Korea) according to the manufacturer's instructions. Selection followed under treatment of hygromycin at 60 μg/mL for 2 weeks. Positive clones were screened by Western blot analysis with anti-myc antibody (9E10, Santa Cruz Biotechnology, CA, USA). Cells grown in 10 cm dishes to 80–90% confluency were split to 1–10, which was denoted as a passage.

2.2. Preparation of genomic DNA

High-molecular weight genomic DNA was isolated by a classical method using proteinase K. Briefly, cells were lysed in TNE buffer (20 mM Tris–HCl, pH 8.0, 400 mM NaCl, 5 mM EDTA, pH 8.0), 1% SDS, 20 mg/mL proteinase K (Invitrogen, CA, USA), and 10 mg/mL RNase A (Sigma, MO, USA) and incubated at 55 °C for 17 h. Phenol/chloroform extraction was repeated two times and DNA was isolated by isopropanol precipitation. DNA concentration was measured by Nanodrop (Nanodrop technologies, NY, USA).

2.3. Analysis of telomere length

Telomere length of cell lines used in this study was reported in a previous study [28] in which 3′ overhang and telomere were determined by in-gel hybridization. Briefly, genomic DNA (20 μg) digested with 30 units of *Hinf* I (Promega, WI, USA) for 16 h was fractionated on 0.7% agarose gels in 0.5× TBE buffer at 23 V for 17 h. The DNA was then stained with ethidium bromide. Gels were immersed in 2× SSC at room temperature for 30 min and dried in a gel dryer (Bio-Rad, CA, USA) at room temperature for 28–30 min. Prehybridization was then performed with the dried gel at 37 °C for 1 h in hybridization buffer (5× SSC, 5× Denhardt's reagent, and 10 mM sodium phosphate, pH 7.2) and 3′ overhang detection was followed [28]. After detection of overhang, gels were treated twice with denaturing solution (0.15 M NaCl, 0.5 M NaOH) at room temperature for 30 min and then treated with neutralizing solution (0.15 M NaCl, 0.5 M Tris, pH 7.4) and hybridized with the $d(CCCTAA)_4$ probe at 37 °C for 16–18 h. Hybridized gels were washed three times in 0.2X SSC at 37 °C for 1 h, and then exposed to X-ray film at −70 °C for 16–18 h. The intensity of each lane was quantified using a phosphorimager and Image Gauge Version 3.12 (Fujifilm, Tokyo, Japan) [28]. Oligonucleotide, $d(CCCTAA)_4$, was labeled at the 5′-end with [γ-^{32}P]ATP and T4 polynucleotide kinase (Takara, Tokyo, Japan) and purified using MicroSpin™G-50 Columns (Amersham, Buckinghamshire, England).

2.4. Bisulfite modification

Genomic DNA isolated from cell lines was modified by an EZ DNA Methylation kit (Zymo Research, CA, USA). Briefly, 1 μg of DNA in a volume of 50 μL was denatured by 5 μL M-dilution buffer for 15 min at 37 °C. Bisulfite-containing CT-Conversion Reagent (100 μL) was added and mixed, and samples were incubated at 50 °C for 16 h. Modified DNA was purified by Zymo-Spin 1 Columns and used immediately or stored at −20 °C.

2.5. Methylation-specific PCR (MS-PCR)

For methylation-specific PCR, two pairs of primers were employed. All primer sets were designed using the MethPrimer program (http://www.urogene.org/methprimer/index.html). Primer sequences and PCR annealing temperatures are shown in Table 1. CpGenome™ Universal Methylated DNA (Chemicon, CA, USA) was used as control DNA. PCR reactions were performed in a total volume of 25 μL containing 1× Taq PCR buffer, 2 mM $MgCl_2$, 0.6 μM each primer, 0.2 mM dNTPs, 0.6 U Taq-gold polymerase (Roche, Mannheim, Germany), and 1 μg bisulfite-treated DNA. PCR amplification was performed as follows: 95 °C for 10 min; followed by 40 cycles of 94 °C for 30 s, 58–62 °C for 30 s, and 72 °C for 30 s; and ending with an