Frequent changes in subtelomeric DNA methylation patterns and its relevance to telomere regulation during human hepatocarcinogenesis

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A telomere, which is a specialized structure composed of tandem repeats of DNA and associated proteins, is crucial for chromosome integrity. Dysfunctional telomeres result in chromosome end-to-end fusion, which leads to chromosomal instability.

Telomere length is maintained by at least two mechanisms: telomerase and homologous recombination. Telomerase adds telomeric repeat sequences to the 3' end of telomeres. Most cancer cells possess active telomerase, and thus, telomere length is maintained during successive proliferation. In telomerase-negative cancer cells, telomere is maintained by telomere recombination, which results in long telomeres with heterogeneity. This mechanism is known as alternative lengthening of telomeres (ALT).

Mapping and sequencing have been performed in detail on human subtelomeric sequence assemblies within a distance of 500 kb of the telomere. A total of 941 transcripts, including 214 single-copy genes, were found in subtelomeric sequence assemblies, and the transcript density is similar to that found genome wide. Similar to pericentromeric regions, subtelomeres have a high density of DNA repeats and CpG islands, which are susceptible to DNA methylation by DNA methyltransferases (DNMTs). Telomeres, however, do not contain CpG sequences.

Recent evidence indicates that epigenetic modification of subtelomeric and telomeric chromatin exerts influence on the regulation of telomere length. In DNMT-deficient cells, demethylation of subtelomeric regions induced telomere elongation, which was suggested to result from increased homologous recombination in telomeric sequences. In telomerase knockout mouse cells, telomere shortening was associated with...
features of open chromatin, revealing decreased DNA methylation and increased histone acetylation in subtelomeres. These data suggest that subtelomeric DNA methylation is implicated in telomere metabolism.

Hepatocellular carcinoma (HCC) is the seventh most common cancer worldwide, and more than 80% of HCC cases are caused by chronic hepatitis B virus (HBV) infection. Telomere length becomes shorter in the early stages of human hepatocarcinogenesis, and these short telomeres are thought to be one of the factors that can induce chromosomal instability, an important mechanism of HBV-related hepatocarcinogenesis. Epigenetic modification has been implicated as a critical event in carcinogenesis. Ablation of oncoproteins and tumor suppressor genes has frequently been found in human cancers. Epigenetic modifications of human subtelomeres, however, have not been characterized in tumors. Moreover, their impact on telomere length regulation and telomere function has not been examined during tumorigenesis, particularly during hepatocarcinogenesis.

In this study, we examined the subtelomeric methylation status at six Cpg islands in 32 pairs of HCCs and their adjacent noncancerous liver tissues (non-HCCs) using methylation-specific PCR (MSP), and at one Cpg island in 19 pairs of HCCs and their adjacent non-HCCs using bisulfite genomic sequencing (BGS). The subtelomeric methylation pattern was dynamically altered during hepatocarcinogenesis, and the methylation ratios of 18p and 21q correlated with telomere length in HCCs, and change in methylation of 7q and 21q correlated with telomere length changes during hepatocarcinogenesis.

Material and Methods

Tissues

Thirty-two HCCs and their adjacent non-HCCs were sampled from resected liver specimens, frozen directly in liquid nitrogen, and stored at -80°C. The study included 26 male and six female patients with an age range of 40 to 67 years (mean ± SD = 53 ± 7.98). Twenty-eight patients were positive for HBV, one had hepatitis C virus (HCV), one patient was an alcoholic and two had none of these conditions. Liver resection was performed due to metastatic colon cancers. The noncancerous liver tissues showed normal histology, except for mild steatosis. The specimens were supplied by the Liver Cancer Specimen Bank of the National Research Resource Bank Program of the Korea Science and Engineering Foundation (Ministry of Science & Technology, Republic of Korea).

Genomic DNA extraction

A 20- to 40-mg sample of frozen tissue was ground using a pestle in liquid nitrogen and treated with 700 μL of lysis buffer containing 20 μg/mL protease K (Cosmo Genetech, Seoul, Korea), 20 mM Tris-HCl (pH 8.0), 5 mM EDTA (pH 8.0), 400 mM NaCl and 1% SDS. Samples were incubated overnight at 42°C in a shaking water bath and then extracted three times with phenol or chloroform. After extraction, genomic DNA was isolated by isopropanol precipitation. The DNA pellet was washed with 70% ethanol, air-dried and then dissolved in 300 μL of water. DNA integrity and RNA contamination were checked by gel electrophoresis.

Bisulfite modification of genomic DNA

Bisulfite modification of genomic DNA was performed using the EZ DNA methylation kit (Zymo Research Corp., Orange, CA) following the manufacturer’s instructions. Specifically, 1 μg of genomic DNA was mixed with 5 μL of M-dilution buffer in a total volume of 50 μL, and the sample was incubated at 37°C for 15 min. Cytosine-to-thymidine conversion was followed by adding 100 μL of CT conversion reagent, which was then incubated at 50°C for 15 hr. Modified DNA was purified via Zymo-Spin 1 Columns, dissolved in 30 μL of purified water and used immediately or stored at -20°C until use.

Methylation-specific PCR

MSP primers were designed with MethPrimer (http://www.urogene.org/methprimer/). The primer sequences for both methylated and unmethylated regions are provided in Table 1. CpGenome™ Universal Methylated DNA (Chemicon, Temecula, CA) was used as a control. The PCR mixture contained 1 μL of bisulfite-modified genomic DNA, 1.5 μL of 10 μM forward primer, 1.5 μL of 10 μM reverse primer, 2.5 μL of 10× PCR buffer, 2 μL of 25 mM MgCl2, 2 μL of 2.5 mM dNTP and 0.2 μL of AmpliTaq Gold (Applied Biosystems, Foster City, CA) in a total volume of 25 μL. PCR reactions began at 95°C for 10 min and were followed by 40 cycles of amplification [denaturation at 95°C for 30 sec, annealing for 30 sec (temperatures are given in Table 1), and extension at 72°C for 30 sec]. A final extension at 72°C for 5 min concluded the reactions. PCR products were separated by electrophoresis on 2% agarose gels.

Bisulfite sequencing analysis

Primers for BGS were designed using MethPrimer. The primer sequences were 5’-GGA GTT TAG GAT TTA GAT TTG GTT TTA G-3’ (sense) and 5’-AAC AAA TTA AAA AAT CCC CTC TAC C-3’ (antisense), resulting in a 298-bp product from chromosome 10q. These primers were specific for modified DNA but did not contain any CpG sites in their sequence, enabling both methylated and unmethylated DNA to be amplified by the same primer set. PCR was performed under the same conditions as MSP with the following changes: 5 μL of bisulfite-modified genomic DNA, 1 μL of forward primer, 1 μL of reverse primer, denaturation at 94°C and annealing at 55°C. The PCR product was subjected to T-vector cloning (Invitrogen, Carlsbad, CA). Cloned DNA was column-purified (Qiagen, Hilden, Germany), and two to nine clones were randomly chosen for automated sequencing (Cosmo Genetech, Seoul, Korea).
Telomere terminal restriction fragment length analysis

Telomere length was measured using Southern hybridization,19,26 and the mean telomere length was calculated as described by Kruk et al. 27 Briefly, 10 µg of genomic DNA was digested with Hinf I overnight, purified by phenol extraction and isolated by ethanol precipitation. DNA concentration was determined by spectrometry, and 2

Next, we compared the methylation ratios of individual subtelomeric regions. High levels of methylation were detected on chromosomes 7q (mean ± SD = 78 ± 19.7), 18p (67 ± 14.1) and XpYp (74 ± 11.8), whereas low levels of methylation were found on 8q (28 ± 8.4), 17q (38 ± 21.8) and 21q (26 ± 16.6) in both HCCs and non-HCCs (Table 2 and Fig. 3a). Chromosome 7q revealed a high incidence of high methylation ratio (>70% methylation ratio) in both HCCs (29/32) and non-HCCs (27/31) (Table 2). Mean-

Statistical analysis

All statistical analyses were performed using SPSS 13 (SPSS, Chicago, IL). Data were analyzed with two-sided p values from a 2 × 2 contingency table determined by a χ2 test and by Levene’s test for equality of variances. Significance was set at a p value <0.05.

Table 1. MSP primer information

| Chromosome | Distance to telomere (kb) | Forward primer sequence | Reverse primer sequence | Product size (bp) | Annealing Tm (°C) |
|------------|--------------------------|-------------------------|-------------------------|------------------|-----------------|
| 7q         | 104                      | TTTGGTTTTTTATATGTT      | GACCTAAACCACTCAAACCG    | 200              | 60              |
|            | U                        | TTTGGTTTTTTATATGTT      | CAACCTAAACCACTCAAACCG   | 201              | 60              |
| 8q         | 27                       | TTTTTATTTTTTTTTTTTCC    | GAAGCCTAATGCTAATCCG     | 168              | 58              |
|            | U                        | TTTTTATTTTTTTTTTTTCC    | CAAACACTAATCATACCCCAAC  | 167              | 58              |
| 17q        | 4                        | TATTTAGAAGGGGATGTC      | ATATTAACCCCGATCTCTCCGT  | 121              | 60              |
|            | U                        | TATTTAGAAGGGGATGTC      | AATATTAACCTCAAATCTACATT | 122              | 60              |
| 18p        | 2                        | GATTTTTTTCGGTAGATTAAA   | TAAAAATTTAACCCTATCTGA   | 168              | 59              |
|            | U                        | GATTTTTTTCGGTAGATTAAA   | CTAAAAATTTAACCCTATCTCA  | 169              | 59              |
| 21q        | 31                       | CGTAAGTGGTTAGTAAACGCGG  | TTTTTATAACTCGAAAAACACAGAA | 135             | 58              |
|            | U                        | GTTTGTAAGTGGATTATAATGTTG | TTTTTATAACTCAAAAAACACAAA | 139             | 58              |
| XpYp       | 40                       | TATTGAGTAGATGATGGTGATTC | AATCTACAAAAAAGCAAAAGCG  | 112              | 61              |
|            | U                        | TATTGAGTAGATGATGGTGATTC | ATCTACAAAAAAGCAAAAGCG   | 107              | 61              |

Abbreviations: M: methylated sequence; U: unmethylated sequence.

Results

 Primer design for MSP and BGS

For the design of MSP and BGS primers, we selected CpG islands at chromosomes that are known to have no sequence gaps and no telomere-like repeat sequences at subtelomeric regions.10,11 MSP was performed on chromosomes 7q, 8q, 17q, 18p and 21q and XpYp at locations 104, 27, 4, 2, 31 and 40 kb from telomeres, respectively. Figure 1 shows a representative MSP result for chromosome 7q with a map of MSP primers. BGS, which provides a quantitative methylation ratio, was performed on chromosome 10q at a location 26 kb upstream of the telomere, and a map of BGS primers and results are shown in Figure 2.

Subtelomeric methylation in HCCs and non-HCCs

MSP was determined at six chromosomal regions in 32 pairs of HCC tissues (192 sites total) and adjacent non-HCC samples (192 sites total). Methylation was quantified by measuring the intensity of the DNA bands. The methylation ratio (%) of each sample is summarized in Table 2.

First, we compared the methylation ratios of individual subtelomeric regions. High levels of methylation were detected on chromosomes 7q (mean ± SD = 78 ± 19.7), 18p (67 ± 14.1) and XpYp (74 ± 11.8), whereas low levels of methylation were found on 8q (28 ± 8.4), 17q (38 ± 21.8) and 21q (26 ± 16.6) in both HCCs and non-HCCs (Table 2 and Fig. 3a). Chromosome 7q revealed a high incidence of high methylation ratio (>70% methylation ratio) in both HCCs (29/32) and non-HCCs (27/31) (Table 2). Meanwhile, none of the samples exhibited a methylation ratio higher than 70% on 8q and 21q, where the methylation ratio was within the range from 0 to 39% and from 0 to 68%, respectively (Table 2).

Next, we compared the methylation ratio between HCCs and non-HCCs in individual regions (Fig. 3a). Methylation
Figure 1. A representative result of methylation-specific PCR (MSP) in the subtelomeric region. (a) Location of the MSP target region on chromosome 7q. Chromosomes, location of CpG islands (including distances to telomeres and adjacent genes) and CpG dinucleotide sites are shown. MSP target primers are indicated. (b) Results of MSP in the subtelomeric region on chromosome 7q. M and U indicate amplification from methylated and unmethylated sequence-specific primers, respectively. Hepatocellular carcinomas (HCCs) and adjacent non-HCCs are indicated by T and NT, respectively. Methylated DNA provided by the manufacturer (Chemicon, Temecula, CA) was used as a control (indicated by C). Patients are identified by numbers. [Color figure can be viewed in the online issue, which is available at wileyonlinelibrary.com.]
ratio of 21q \( (p = 0.007) \) and 18p \( (p = 0.056) \) in HCCs were higher than those in non-HCCs (Fig. 3a). Meanwhile, 7q, 8q, 17q and XpYp showed little difference in methylation ratio between HCCs and non-HCCs. Notably, 7q and 21q in HCCs exhibited a wider distribution of methylation ratio compared to non-HCCs \( (p = 0.011, p = 0.015, \) respectively) (Fig. 3a).

BGS performed on chromosome 10q in 19 pairs of HCCs and their adjacent non-HCCs showed that methylation frequency ranged from 44 to 76% in non-HCCs and from 7 to 93% in HCCs (Fig. 2b). HCCs showed greater variance in methylation frequency \( (p = 0.003; \) Fig. 2c) although the mean value of methylation frequency \( (58 \pm 21.9) \) was similar.
to that found in non-HCCs (62 ± 8.2). Ten HCCs were found to have higher methylation ratio than their adjacent non-HCCs, and nine HCCs had lower ratios than the adjacent non-HCCs (Fig. 2b).

We evaluated differences in methylation ratio of individual chromosomes between HCCs and their adjacent non-HCCs (Fig. 3b). The difference in methylation ratio was determined as follows: methylation ratio of HCC - methylation ratio of adjacent non-HCC. The difference in methylation ratio of 17q and 21q widely dispersed, and 8q, 18p and XpYp showed a narrow range of the difference (Fig. 3b).

### Frequent subtelomeric DNA methylation pattern changes during hepatocarcinogenesis

We evaluated changes in methylation pattern at each site and categorized them into three groups: hypermethylation, hypomethylation and no change. For instance, hypermethylation changes

| Patient No. | 7q | 8q | 17q | 18p | 21q | XpYp |
|-------------|----|----|-----|-----|-----|------|
| NT | T  | NT | T   | NT  | T   | NT  | T   | NT  | T   | NT  | T   | NT  | T   | NT  | T   |
| 1  | 87 | 94 | 23  | 27  | 49  | 44  | 63  | 68  | 29  | 48  | 85  | 83  |
| 2  | 85 | 89 | 29  | 25  | 39  | 55  | 49  | 59  | 37  | 66  | 74  | 73  |
| 3  | 81 | 71 | 32  | 39  | 1   | 32  | 59  | 65  | 47  | 26  | 82  | 79  |
| 4  | 79 | 48 | ND  | 0   | 42  | 21  | 59  | 56  | 25  | 61  | 61  | 65  |
| 5  | 76 | 73 | 31  | 34  | 37  | 49  | 72  | 71  | 5   | 17  | 63  | 42  |
| 6  | 59 | 20 | 22  | 17  | 69  | 23  | 66  | 72  | 18  | 49  | 83  | 82  |
| 7  | 78 | 87 | 31  | 34  | 45  | 22  | 48  | 64  | 8   | 27  | 82  | 79  |
| 8  | 79 | 72 | 35  | 37  | 29  | 39  | 33  | ND  | 24  | 24  | 99  | 87  |
| 9  | 73 | 14 | 35  | 31  | 13  | 44  | 32  | 34  | 11  | 47  | 95  | 93  |
| 10 | 67 | 62 | 19  | 22  | 58  | 27  | 51  | 43  | 17  | 53  | 84  | 86  |
| 11 | 88 | 87 | 32  | 18  | 32  | 7   | 71  | 75  | 15  | 15  | 73  | 76  |
| 12 | 89 | 73 | 17  | 39  | 100 | 80  | 70  | 75  | 45  | 48  | 61  | 71  |
| 13 | 79 | 89 | 34  | 38  | 31  | 23  | 73  | 83  | ND  | 8   | 57  | 57  |
| 14 | 85 | 86 | 29  | 38  | 20  | 33  | 84  | 80  | 13  | 68  | 71  | 80  |
| 15 | 90 | 77 | 22  | 19  | 22  | 44  | 60  | ND  | 22  | 31  | 72  | ND  |
| 16 | 77 | 73 | 37  | 35  | 27  | 26  | 60  | 58  | 19  | 24  | 68  | 73  |
| 17 | 74 | 92 | 39  | 35  | 34  | 85  | 67  | 77  | 17  | 14  | 82  | 72  |
| 18 | 74 | 86 | 18  | 22  | 42  | 53  | 43  | 37  | 39  | 43  | 73  | 82  |
| 19 | 73 | 71 | 23  | 25  | 31  | 56  | 75  | 85  | 48  | 27  | 67  | 75  |
| 20 | 70 | 72 | 36  | 13  | 47  | 33  | 74  | 81  | 2   | 1   | 93  | 87  |
| 21 | 84 | ND | 14  | 17  | 31  | 10  | 69  | 83  | 5   | 48  | 79  | 68  |
| 22 | 90 | 95 | 27  | 27  | 25  | 18  | 70  | 83  | 21  | 19  | 80  | 70  |
| 23 | 75 | 80 | 28  | 31  | 92  | 92  | 70  | 73  | 11  | 25  | 74  | 75  |
| 24 | 80 | 76 | 36  | 30  | 40  | 28  | 74  | 66  | 18  | 7   | 58  | 55  |
| 25 | 85 | 96 | 36  | 30  | 48  | 2   | 49  | 72  | 22  | 34  | 52  | 57  |
| 26 | 89 | 95 | 15  | 19  | 0   | 24  | 67  | 72  | 19  | 44  | 82  | 82  |
| 27 | 83 | 86 | 27  | 36  | 33  | 40  | 60  | 79  | 7   | 20  | 57  | 60  |
| 28 | 76 | 99 | 19  | 16  | 29  | 50  | 48  | 64  | 13  | 21  | 80  | 80  |
| 29 | 74 | 85 | 21  | 21  | 25  | 15  | 79  | 79  | 40  | 45  | 78  | 85  |
| 30 | 67 | ND | 36  | 38  | 37  | 54  | 85  | 83  | 13  | 18  | 79  | 68  |
| 31 | 85 | 73 | 24  | 23  | 87  | 48  | 79  | 75  | 16  | 0   | 57  | 50  |
| 32 | 87 | 76 | 33  | 39  | 27  | 38  | 88  | 87  | 14  | 34  | 77  | 79  |

Methylation ratio = [(DNA band intensity amplified from M primers)/(DNA band intensity amplified from M primers + DNA band intensity amplified from U primers)] × 100.

Abbreviations: ND: no detection.
were defined as a ≥10% increase in the methylation ratio of HCC compared to that of adjacent non-HCC, and hypomethylation changes were defined as a ≤−10% decrease in the methylation ratio of HCC (Fig. 4a). When the difference in the methylation ratio was between +10% and −10%, the band patterns in HCC and the adjacent non-HCC appeared to be nearly identical, leading us to define methylation changes in these ranges as no changes. Results are summarized in Figure 4a.

Changes in the methylation pattern between HCCs and their adjacent non-HCCs were found at a frequency of 42% (78/185) (Fig. 4a). Hypermethylation changes were found at 47 sites (25%) and hypomethylation changes at 31 sites (17%). No change was found at the remaining 107 sites (58%) (Fig. 4b). Hypermethylation changes were more frequent than hypomethylation changes with marginal significance ($p = 0.070$, $\chi^2$ test; Fig. 4b). Chromosomes 7q, 17q and 21q exhibited frequent changes in methylation patterns with a frequency of 14 (14/30, 47%), 26 (26/32, 81%) and 18 (18/31, 58%), respectively. Pattern changes were relatively rare for chromosomes 8q (3/31, 10%), 18p (10/30, 33%) and XpYp (7/31, 23%) (Fig. 4c). Notably, hypermethylation changes were prevalent at 18p (10/10, 100%) and 21q (15/18, 83%), and hypomethylation changes were more frequent at XpYp (6/7, 86%).

The sites with methylation change were evaluated in each patient. Changes in methylation pattern occurred at more than two out of six sites in most HCC patients (29/32, 91%). Eleven patients were found with changes in methylation at three sites, and four patients at four sites, and one patient showed no changes at all tested sites (Figs. 4a and 4d).

**Subtelomeric DNA methylation ratio and telomere length in HCCs and their adjacent non-HCCs**

The telomere length of 32 pairs of HCCs and non-HCCs was measured by Southern blot analysis (Fig. 5a), and the results...
Figure 4. Changes in subtelomeric methylation patterns during hepatocarcinogenesis. (a) Methylation pattern changes between hepatocellular carcinomas (HCCs) and their adjacent non-HCCs. Hypermethylation (≥10% increase in methylation ratio in HCC compared to its adjacent non-HCC), hypomethylation (≤−10% decrease in methylation ratio in HCC), and no changes are indicated for six subtelomeric regions by black, gray and white, respectively. Nd indicates not determined. (b) Incidence of subtelomeric hypermethylation, hypomethylation, and no change during hepatocarcinogenesis. (c) Subtelomeric methylation pattern changes in individual chromosomal regions. Hypomethylation and hypermethylation changes are indicated by gray and black bars, respectively. (d) Frequent subtelomeric methylation pattern changes in individual HCC patients. Sites with a change in methylation were counted in each patient. Out of six sites, 14 patients showed two sites with change in methylation, 11 patients three sites and four patients four sites.
were summarized in Figure 4a. The telomere length in HCCs ranged from 4.5 to 12.7 kb, with the mean of 8.2 ± 2.39 kb, and non-HCCs had a telomere length from 5.6 to 11.7 kb, with the mean of 8.7 ± 1.20 kb (Fig. 4a).

We analyzed the correlation between the methylation ratio and telomere length in HCCs and non-HCCs (Fig. 5b). The methylation ratio of 18p negatively correlated with telomere length in both HCCs (p = 0.037) and non-HCCs (p = 0.084), although significance was marginal in non-HCCs (Fig. 5b). A similar result was seen in 7q in HCCs with marginal significance (p = 0.077) (Fig. 5b). Meanwhile, chromosome 21q in HCCs showed a positive correlation with telomere length (p = 0.025). Chromosomes 8q, 17q, YpYp and 10q, however, showed no significant correlation.

Next, we examined whether the difference in methylation ratio between HCCs and non-HCCs was related to telomere length changes during hepatocarcinogenesis (Fig. 6). Methylation changes negatively correlated with telomere length changes in 7q (p = 0.013); methylation changes proceeded further toward hypomethylation as telomere lengthened from non-HCCs to HCCs. Conversely, there was a tendency toward hypermethylation changes in 21q as telomere length
elongated from non-HCCs to HCCs ($p = 0.057$). Meanwhile, chromosomes 8q, 17q, 18p, XpYp and 10q showed no significant correlations.

**Discussion**

Subtelomeres and telomeres are generally heterochromatic.\textsuperscript{15,28,29} This study showed that subtelomeric methylation status appeared to vary from region to region on six chromosomes in HCCs and their adjacent non-HCCs. Most samples exhibited high levels of methylation in 7q, 18p and XpYp, and relatively low levels in 8q, 17q and 21q. This suggests that chromatin structure varies among subtelomeric domains, and that both compacted and relaxed chromatin structures coexist at subtelomeres.

Overall subtelomeric methylation status was similar in HCCs and their adjacent non-HCCs; however, at individual regions, HCCs in 18p and 21q had higher methylation ratio, and HCCs in 7q, 21q and 10q showed a wider distribution of methylation ratio compared to non-HCCs. Moreover, methylation patterns appeared to have been dynamically altered from non-HCCs to HCCs. In fact, methylation patterns were different between HCCs and their adjacent non-HCCs at a frequency of 42% (78/185 sites). Fifteen patients (15/32, 47%) appeared to have methylation pattern changes at three to four sites among the six chromosomal sites. These results indicated frequent methylation changes in subtelomeric regions during hepatocarcinogenesis. The MSP and BGS sites examined in this study are located far from the genes; thus, alteration of subtelomeric DNA methylation is assumed to exert little effect on the expression of adjacent genes. Instead, frequent methylation changes in subtelomeric regions might affect local chromatin structures. Telomeric repeat-containing RNA (TERRA) is known to have a function in orchestrating chromatin remodeling,\textsuperscript{30} and TERRA transcription is regulated by CpG methylation at their promoters,\textsuperscript{31} suggesting that there might be putative connection between subtelomeric DNA methylation and telomere function.

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Figure 6. Relationship between subtelomeric methylation changes and telomere length changes during hepatocarcinogenesis. Methylation changes are evaluated as follows: $\Delta$methylation = methylation ratio of HCC – methylation ratio of adjacent non-HCC. Similarly, telomere length changes ($\Delta$telomere) were obtained as follows: telomere length of HCC – telomere length of adjacent non-HCC. Correlation between changes in methylation and those in telomere length was analyzed. $p$ value < 0.05 was marked with *, and a marginal significance (0.1 < $p$ value < 0.05) was marked with **.
CpG methylation and TERRA expression. Detection of TERRA expression in hepatocarcinogenesis and the association of TERRA expression with subtelomeric CpG methylation would provide better understanding of the role of subtelomeric chromatin in hepatocarcinogenesis. Further studies with tumor samples from various tissues (e.g., the breast, colon and gastric tissues) and systematic experimental tests for more subtelomeric regions might clarify the association between subtelomeric methylation and tumorigenesis.

Recently, a negative correlation between subtelomeric methylation frequency and telomere length was reported for several human cancer cell lines. In these studies, the methylation frequency and telomere length was reported for between subtelomeric methylation and tumorigenesis. More subtelomeric regions might clarify the association between subtelomeric methylation and tumorigenesis.

In our study, correlations between telomere length and methylation ratio were found in certain regions of subtelomeres. Methylation at 7q gradually decreased with telomere shortening of HCCs, and a similar relationship was found at 18p in both HCCs and non-HCCs, indicating that hypomethylation in these regions might be related to long telomeres. These results are consistent with previous studies, reporting that loss of subtelomeric heterochromatin is concomitant with substantial telomele elongation. Interestingly, hypomethylation changes on 7q increased when telomere lengthened from non-HCC to HCC, suggesting that structural change of 7q to relaxed chromatin might be related to telomere lengthening from non-HCC to HCC. Recent studies have indicated that a loss of DNMT3 results in increased telomeric recombination, which is a typical feature of ALT cells. It is, thus, interesting to determine whether ALT features such as APBs, ALT-associated PML bodies, increased in telomere elongated HCCs. Conversely, hypomethylation of 21q was frequent in short telomeres of HCCs, and the extent of hypomethylation changes at 21q appeared to be increased when telomere shortened from non-HCCs to HCCs, suggesting that a loss of subtelomeric heterochromatin at 21q might be associated with telomere shortening. A loss of subtelomeric and telomeric heterochromatic features were found to be related to telomere lengthening in DNMT deficient cells and telomere shortening in telomerase-negative and TRF2 overexpressed cells as well, suggesting a diverse role of telomeric chromatin in telomere regulation. It is not clear how subtelomeric methylation in different regions can have positive, negative or no correlation with telomere length in hepatocarcinogenesis. Subtelomeric chromatin in specific regions might differently involve in telomere regulation. For instance, the demethylation of certain subtelomeric regions might induce telomere regulators, e.g., TERRA, that in turn is involved positively or negatively in telomere regulation during hepatocarcinogenesis.

It was noted that 21q in HCCs had higher and wider methylation ratio compared to non-HCCs, and 21q showed frequent alteration on the methylation status during hepatocarcinogenesis. Similarly, 7q exhibited frequent methylation changes and a hypervariable methylation ratio in HCCs. Meanwhile, regions on 8q, 18p and XpYp, which showed no great difference in methylation ratio between non-HCCs and HCCs and less-frequent methylation changes, exhibited little correlation with telomere length changes. These results suggest that the subtelomeric regions revealing frequent and dynamic methylation pattern changes might be potential regions whose chromatin structures affect telomere length regulation.

In summary, the subtelomeric methylation patterns dynamically changed during hepatocarcinogenesis. Subtelomeric regions that exhibited diverse methylation ratios in HCCs and frequent methylation change from non-HCCs to HCCs tended to be associated with telomere length regulation in hepatocarcinogenesis. This study demonstrated a potential association between subtelomeric epigenetic modifications and telomere regulation during human hepatocarcinogenesis.

References

1. de Lange T. T-loops and the origin of telomeres. Nat Rev Mol Cell Biol 2004;5: 323–9.
2. Artandi SE, Chang S, Lee SL, Alson S, Gottlieb GI, Chin L, DePinho RA. Telomere dysfunction promotes non-reciprocal translocations and epithelial cancers in mice. Nature 2000;406:641–5.
3. Blasco MA, Lee HW, Hande MP, Samper E, Lansdorp PM, DePinho RA, Greider CW. Telomere shortening and tumor formation by mouse cells lacking telomerase RNA. Cell 1997;91:25–34.
4. Maser RS, DePinho RA. Connecting chromosomes, crisis, and cancer. Science 2002;297:565–9.
5. Rudolph KL, Chang S, Lee HW, Blasco M, Gottlieb GI, Greider C, DePinho RA. Longevity, stress response, and cancer in aging telomerase-deficient mice. Cell 1999;96:701–12.
6. Autexier C, Lue NF. The structure and function of telomerase reverse transcriptase. Annu Rev Biochem 2006;75: 493–517.
7. Kim NW, Piatyszek MA, Prowse KR, Harley CB, West MD, Ho PL, Coviello GM, Wright WE, Weinrich SL, Shay JW. Specific association of human telomerase activity with immortal cells and cancer. Science 1994;266:2011–5.
8. Shay JW, Bacchetti S. A survey of telomerase activity in human cancer. Eur J Cancer 1997;33:787–91.
9. Bryan TM, Englezou A, Dalla-Pozza L, Dunham MA, Reddel RR. Evidence for an alternative mechanism for maintaining telomere length in human tumors and

Int. J. Cancer: 128, 857–868 (2011) © 2010 UICC
tumor-derived cell lines. Nat Med 1997;3:1271–4.
10. Riethman H, Ambrosini A, Castaneda C, Finklestein J, Hu XL, Madunuri U, Paul S, Wei J. Mapping and initial analysis of human subtelomeric sequence assemblies. Genome Res 2004;14:18–28.
11. Ambrosini A, Paul S, Hu S, Riethman H. Human subtelomeric duplication structure and organization. Genome Biol 2007;8:R151.
12. Brock GJ, Charlton J, Bird A. Densely methylated sequences that are preferentially localized at telomere-proximal regions of human chromosomes. Genet 1999;240:269–77.
13. Steinert S, Shay JW, Wright WE. Telomere instability and telomerase reactivation in dysplastic nodules of human hepatocarcinogenesis. J Hepatol 2003;39:786–92.
14. Blasco MA. Carcinogenesis Young Investigator Award. Telomere epigenetics: a higher-order control of telomere length in mammalian cells. Carcinogenesis 2004;25:1083–7.
15. Gonzalez S, Jiao I, Fraga MF, Chen T, Li E, Esteller M, Blasco MA. DNA methyltransferases control telomere length and telomere recombination in mammalian cells. Nat Cell Biol 2006;8:416–24.
16. Benetti R, Garcia-Cao M, Blasco MA. Telomere length regulates the epigenetic status of mammalian telomeres and subtelomeres. Nat Genet 2007;39:243–50.
17. Zhai Y, Zhou G, Deng G, Xie W, Dong X, Zhi L, Yao Z, et al. Estrogen receptor alpha polymorphisms associated with susceptibility to hepatocellular carcinoma in hepatitis B virus carriers. Gastroenterology 2006;130:2001–9.
18. Oh BK, Jo Chae K, Park C, Kim K, Jung Lee W, Han KH, Nyun Park Y. Telomere shortening and telomerase reactivation in dysplastic nodules of human hepatocarcinogenesis. J Hepatol 2003;39:786–92.
19. Oh BK, Kim YJ, Park C, Park YN. Up-regulation of telomere-binding proteins. TRF1, TRF2, and, TIN2 is related to telomere shortening during human multistep hepatocarcinogenesis. Am J Pathol 2005;166:73–80.
20. Laurent-Puig P, Legoix P, Bluteau O, Belghiti J, Franco D, Binot F, Monges G, Thomas G, Bioulac-Sage P, Zucman-Rossi J. Genetic alterations associated with hepatocellular carcinomas define distinct pathways of hepatocarcinogenesis. Gastroenterology 2001;120:1763–73.
21. Plenz R, Caselitz M, Bleck JS, Gebel M, Finklestein J, Hu XL, Mudunuri U, Paul S, Azzalin CM, Reichenbach P, Khoriauli L, Giulotto E, Lingner J. Telomeric repeat containing RNA and RNA surveillance factors at mammalian chromosome ends. Science 2007;318:798–801.
22. Nergadze SG, Farnung BO, Wischniewski H, Khoriauli L, Vitelli V, Chawla R, Giulotto E, Azzalin CM. CpG-island promoters drive transcription of human telomeres. RNA 2009;15:2186–94.
23. Vera E, Canela A, Fraga MF, Esteller M, Blasco MA. Epigenetic regulation of telomeres in human cancer. Oncogene 2008;27:6817–33.
24. Benetti R, Gonzalez S, Jiao I, Schotta G, Klatt P, Jenuwein T, Blasco MA. Suv4-20h1 deficiency results in telomere elongation and derepression of telomere recombination. J Cell Biol 2007;178:925–36.
25. Baird DM, Rowson J, Wynford-Thomas D. Telomere length regulation of subtelomeric chromatin. Proc Natl Acad Sci USA 2005;102:4634–8.
26. Oh BK, Kim YJ, Park C, Park YN. Telomere shortening and telomerase reactivation in dysplastic nodules of human hepatocarcinogenesis. J Hepatol 2003;39:786–92.