

Telomerase activity is associated with an increase in DNA methylation at the proximal subtelomere and a reduction in telomeric transcription

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ABSTRACT

Tumours and immortalized cells avoid telomere attrition by using either the ribonucleoprotein enzyme telomerase or a recombination-based alternative lengthening of telomeres (ALT) mechanism. Available evidence from mice suggests that the epigenetic state of the telomere may influence the mechanism of telomere maintenance, but this has not been directly tested in human cancer. Here we investigated cytosine methylation directly adjacent to the telomere as a marker of the telomere's epigenetic state in a panel of human cell lines. We find that while ALT cells show highly heterogeneous patterns of subtelomeric methylation, subtelomeric regions in telomerase-positive cells invariably show denser methylation than normal cells, being almost completely methylated. When compared to matched normal and ALT cells, telomerase-positive cells also exhibit reduced levels of the telomeric repeat-containing-RNA (TERRA), whose transcription originates in the subtelomere. Our results are consistent with the notion that TERRA may inhibit telomerase: the heavy cytosine methylation we observe in telomerase-positive cells may reflect selection for TERRA silencing in order to facilitate telomerase activity at the telomere. These data suggest that the epigenetic differences between telomerase-positive and ALT cells may underlie the mechanism of telomere maintenance in human tumorigenesis and highlight the broad reaching consequences of epigenetic dysregulation in cancer.

INTRODUCTION

Critical shortening of telomeric repeats is linked to the limited capacity for normal human cells to divide and the subsequent onset of replicative senescence (1,2). The ability to elongate and maintain telomeres is a key feature of germline cells, tumours and immortalized cells (3–5). In around 85% of tumours, telomere length maintenance is achieved by activation of telomerase (6); telomerase activity is not detectable or present at very low levels in most normal human somatic cells (3). About 10–15% of tumours exhibit telomere stabilization in the absence of telomerase activity, via the alternative lengthening of telomeres (ALT) mechanism (7). ALT is a recombination-based mechanism characterized by telomere length heterogeneity (8), and the presence of specific PML bodies known as ALT-associated PML bodies (APBs) (9) and extrachromosomal telomeric repeat DNA (10). It is not known why some tumours use telomerase and others use ALT to maintain telomere length.

Telomeres are normally maintained in a heterochromatin-like state, carrying epigenetic modifications characteristic of transcriptional repression (11). Telomeric repeats are associated with histone modifications typical of heterochromatin and proteins such as HP1 (12). Methylation of cytosines in CpG dinucleotides is a well-characterized marker of transcriptional silence; sequences maintained as heterochromatin, such as retrotransposons and pericentromeric regions, are usually heavily methylated (13). Although mammalian telomeric TTAGGG repeats lack CpG dinucleotides, the immediately adjacent subtelomeric regions are CpG-rich and in human somatic cells are methylated (14,15). Subtelomeric regions also carry repressive histone modifications

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The authors wish it to be known that, in their opinion, the first two authors should be regarded as joint First Authors.

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characteristic of heterochromatin (16). Thus, telomeric regions can be considered as epigenetic structures that are maintained as constitutive heterochromatin (11).

Recent studies in the mouse have suggested that there is a relationship between the epigenetic state of telomeres and telomere maintenance. Mice lacking the DNA methyltransferase DNMT1 that show dramatic losses of subtelomeric DNA methylation display heterogeneous telomere lengthening reminiscent of that seen in ALT tumours (17). Although the observed recombination may not have been telomere specific, but rather a global consequence of genome-wide methylation loss, and while the involvement of telomerase in the telomere lengthening cannot be excluded (17), these results raise the possibility that the epigenetic state of the telomere affects telomere length regulation. More recent evidence from human cancer cell lines suggests that reduced methylation at a large subtelomeric CpG-rich repeat is associated with longer telomeres (18).

We hypothesized that the epigenetic state of the telomere influences the telomere maintenance mechanism used in human cancer. By analysing subtelomeric methylation and telomeric transcription, we find that the epigenetic state of the telomere differs markedly between cells that use ALT and cells that use telomerase. Unexpectedly, our results support a model in which telomerase activity, rather than ALT, is governed by the epigenetic state of the telomere.

MATERIALS AND METHODS

Human cells, SV40-transformed cells and tumour cell lines

Peripheral blood mononuclear cells (PBMC) were obtained from adult donors (aged 69, 74, 78 and 81 years) or umbilical cord blood, with informed consent and the approval of the St. Vincent's Hospital Human Research Ethics Committee. All cell lines were grown in DMEM with 10% fetal bovine serum and penicillin/streptomycin. Tumour-derived cell lines were derived from primary human osteosarcomas (143BTK-, SAOS-2, G292, U2OS, SJSA-1 and MG63) or lung carcinomas (SK-LU-1 and A2182), and were obtained from the American Type Culture Collection. SV40-transformed cells were derived by transfecting fibroblasts from a single individual with SV40 early region gene constructs, isolating individual foci of morphologically transformed cells, and culturing them continuously; cells that became immortalized were categorized as telomerase-positive based on a telomerase activity assay (3) or as ALT-positive based on the absence of telomerase activity and on the presence of telomere length heterogeneity (19) and APBs (9) (Bonnetin, P., Englezou, A. and Reddel, R., unpublished data). Genomic DNA was isolated from peripheral blood and cultured cells by standard phenol-chloroform extraction.

In silico analysis of subtelomeric CpG islands

The endmost 2 kb of both p and q arms of human chromosomes, as counted from the proximal end of the telomeric repeats, was screened for CpG islands

(<http://www.urogene.org/methprimer/>). Each CpG island was rated for potential methylation analysis based upon the availability and uniqueness of sequence, and proximity to the end of the chromosome. Three regions, on the p arms of chromosomes 2, 4 and 18, were chosen for analysis (see Table S1).

Bisulphite allelic sequencing

About 2 µg of genomic DNA was subjected to bisulphite conversion (Epitect, Qiagen). PCR was performed on 100 ng bisulphite converted DNA using primers and parameters as detailed in Table S1. Resultant PCR amplicons were cloned into a plasmid vector and sequenced. Sequences were excluded from further analysis if they contained more than one unconverted non-CpG cytosine. The percentage methylation at each allele was calculated as the number of methylated CpGs across all sequenced alleles divided by the total number of CpGs.

Genomic 5-methylcytosine content

The total 5-methylcytosine content of tumour-derived cell lines was determined by high performance liquid chromatography (HPLC). Genomic DNA (2 µg) was digested with nuclease P1 (Sigma) and calf intestinal phosphatase (New England Biolabs). HPLC was performed on a Waters Alliance 2695 with an isocratic elution (2.5–16% methanol gradient in 50 mM phosphate buffer, pH 4.5) as previously described (20).

Quantitation of telomeric transcript

The telomeric repeat-containing transcript TERRA was quantitated in normal fibroblasts as well as ALT and telomerase-positive cell lines by slot-blot. A total of 2 µg DNase-treated total RNA was transferred to a nylon membrane and probed with oligonucleotides end-labelled with ³²P-γ-ATP by T4 polynucleotide kinase (New England Biolabs) or DNA probes body-labelled with ³²P-α-dCTP by random priming (PrimeAGene kit, Promega). Hybridizations were performed in UltraHyb or UltraHyb Oligo (Ambion); the membranes were subjected to low-stringency (oligonucleotide probes) or high-stringency (DNA probes) washes.

Quantitation of telomeric DNA

Telomeric DNA was quantitated by dot blot. Sheared genomic DNA (500 ng) was transferred to a nylon membrane and probed with a telomeric repeat plasmid body labelled with ³²P-α-dCTP (DECAprime II probe labelling, Ambion). Hybridization was performed overnight in Church buffer and the membrane subjected to high-stringency washes. After detection, the membrane was stripped and re-probed with an Alu repeat plasmid, as above.

Statistical analysis

Data were analysed using MedCalc statistical software version 9.3.8.0 (<http://www.medcalc.be/>). Student's *t*-test or one-way analysis of variance (ANOVA) with a Newman-Keuls *post hoc* test was used to compare average percent methylation and 5-methylcytosine content

between sample groups. The relationship between global 5-methylcytosine and subtelomeric methylation was analysed by Spearman's rank correlation. Average values are expressed \pm SD. Statistical significance was assigned where $P < 0.05$.

RESULTS AND DISCUSSION

Subtelomeric methylation in normal human cells

We first sought to determine the normal levels and patterns of subtelomeric cytosine methylation in human somatic cells. Subtelomeric methylation has been shown to be a reliable surrogate marker for the epigenetic state of the telomere (17); although it is known that human cells are normally enriched for cytosine methylation at the subtelomere (14,15), the extent and patterns of methylation are not known. We analysed subtelomeric methylation by choosing CpG islands very close to the telomere (within 2 kb), in order to estimate as closely as possible the epigenetic state of the telomere itself. We identified subtelomeric CpG islands on chromosomes 2p, 4p and 18p (Figure 1A and Table S1) and analysed their methylation in peripheral blood mononuclear cells (PBMC) from four infants (cord blood) and four older adults (69–89 years). We found them to carry relatively dense cytosine methylation (Figure 1B and Table S2). We observed little inter-individual variation, with the average percentage methylation across all loci in all individuals being $81 \pm 3\%$. Every allele examined showed methylation of most CpGs, with occasional unmethylated CpGs. There was

no difference between levels of subtelomeric methylation in young and old individuals ($P = 0.729$). This finding suggests that the heterochromatic state of human telomeres is maintained despite the known telomere attrition that occurs with age, especially in the first few years of life (21). Thus, there may be an important role for telomeric heterochromatin in the maintenance of telomere function and integrity, independent of telomere length.

Subtelomeric methylation is heterogeneous in ALT cells but universally increased in telomerase-positive cells

Once we had established the patterns of subtelomeric methylation in healthy human cells, we compared subtelomeric methylation in human tumour-derived cell lines that use either ALT ($n = 4$) or telomerase ($n = 4$) as the telomere maintenance mechanism. The ALT tumour cell lines displayed highly heterogeneous patterns of subtelomeric methylation (Figure 2 and Table S2). Individual subtelomeres showed highly variable methylation, with some loci displaying dramatically less methylation than normal PBMC, and others showing more; there was variation both between loci and between ALT cell lines. One of the four ALT cell lines (SK-LU-1) did not show a loss of methylation at any locus examined. In contrast to ALT cells, telomerase-positive tumour cell lines invariably showed dense methylation at all subtelomeric loci examined ($97 \pm 1\%$); nearly all alleles were completely methylated (Figure 2 and Table S2). Telomerase-positive cells had significantly more subtelomeric methylation than both ALT cells ($P = 0.013$) and normal PBMC ($P < 0.001$).

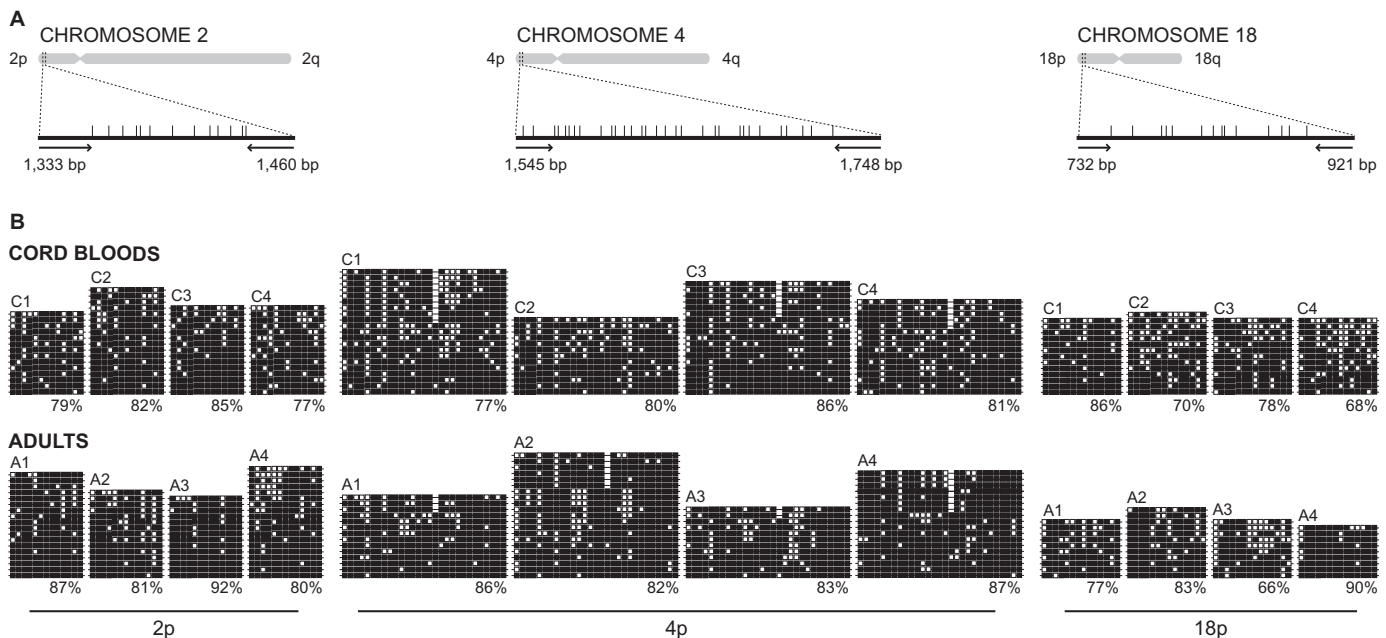


Figure 1. Allelic methylation patterns at human subtelomeric regions in normal cells. (A) Subtelomeric regions of chromosomes 2p and 18p analysed by bisulphite sequencing. CpGs are represented by small vertical lines and PCR primers by arrows. (B) Bisulphite sequencing maps from normal human PBMC are shown below the corresponding region in (A). Within each map, horizontal lines show the methylation pattern of individual alleles: black squares represent methylated CpGs and white squares unmethylated CpGs. The average percent methylation for each individual is shown at the bottom right of each map. The gap in some alleles is due to a G/A polymorphism/mutation that results in loss of a CpG. C, cord blood; A, adult.

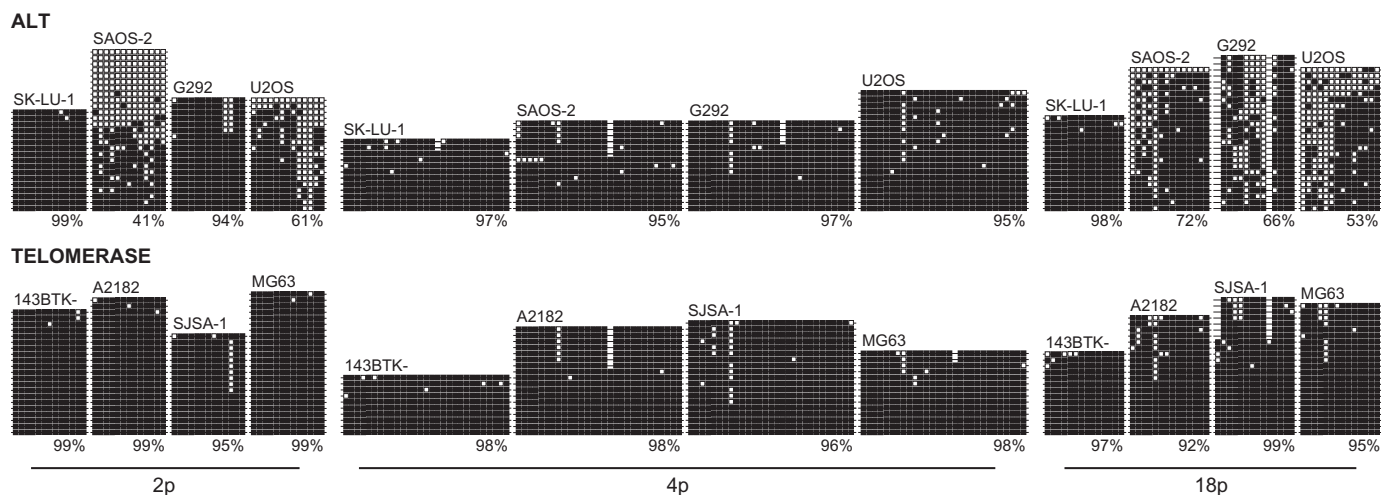


Figure 2. Subtelomeric methylation is heterogeneous in tumour-derived ALT cells and increased in telomerase-positive cells. Bisulphite sequencing maps for subtelomeres at chromosomes 2p, 4p and 18p are shown; those from tumour-derived ALT cells at top and those from tumour-derived telomerase-positive cells at bottom. Within each map, horizontal lines show the methylation pattern of individual alleles; black squares represent methylated CpGs and white squares unmethylated CpGs. The average percent methylation for each individual is shown at the bottom right of each map. The gap in some alleles is due to a G/A polymorphism/mutation that results in loss of a CpG.

Table 1. Total 5-methylcytosine content in telomerase-positive and ALT tumour-derived cell lines

	Cell line	5-methylcytosine content (%) ^a
Telomerase +	143BTK-	4.10 ± 0.28
	A2182	3.35 ± 0.08
	SJSA-1	3.03 ± 0.42
	MG63	3.99 ± 0.18
ALT +	SK-LU-1	3.69 ± 0.08
	SAOS-2	3.04 ± 0.21
	G292	4.09 ± 0.00
	U2OS	3.37 ± 0.24

^aExpressed as the percentage of total amount of cytosine ±SD.

Generalized epigenetic dysregulation is a hallmark of malignant cells, and this is commonly reflected in alterations of normal patterns of DNA methylation. Tumours frequently exhibit hypermethylation of gene promoters that are unmethylated and active in normal tissue (22). This occurs in the context of a generalized loss of methylation across the genome, predominantly at repetitive elements (23,24). Therefore, we sought to determine whether the changes in subtelomeric methylation we observed in tumour-derived cell lines were reflective of global methylation changes. We measured the percentage of 5-methylcytosine in each ALT and telomerase-positive tumour cell line using high-performance liquid chromatography (Table 1). We found no relationship between global methylation levels and either telomere maintenance mechanism ($P = 0.844$) or the level of subtelomeric methylation ($P = 0.922$) indicating that the changes in methylation density we observed at the subtelomere are independent of the generalized epigenetic dysregulation characteristic of cancer cells.

To further investigate the association between telomere maintenance and the epigenetic changes at the subtelomere, and to examine their temporal relationship, we performed bisulphite allelic sequencing on a panel of simian virus 40 (SV40)-transformed cell lines. These cell lines were clonally derived and independently immortalized from the fibroblasts of a single individual; approximately half of SV40-immortalized fibroblast lines maintain telomere length via ALT, and the other half activate telomerase (25). We examined methylation patterns at the same three subtelomeric loci (2p, 4p and 18p) in SV40-transformed immortalized clones (three telomerase-positive and three ALT), and compared these with patterns in pre-immortal SV40-transformed clones with no detectable telomere maintenance mechanism (Figure 3). We found that subtelomeric methylation patterns in pre-immortal and immortalized fibroblasts largely mirrored the patterns we observed in normal PBMC and tumour-derived cell lines, respectively. Pre-immortal fibroblasts demonstrated methylation patterns that were similar to those seen in normal primary PBMC, with the exception of one subtelomere in one cell line (JFCF-6/T.5B at chromosome 2p) which exhibited a considerable loss of methylation (Figure 3). Subtelomeric methylation was significantly increased in telomerase-positive SV40-transformed immortalized cells compared with pre-immortal cells ($93 \pm 3\%$ versus $80 \pm 5\%$, $P < 0.001$; Figure 3 and Table S3); ALT-positive immortalized cells showed heterogeneous subtelomeric methylation between clonal lines and between loci, with average percentage methylation ranging from 53% to 91%. The methylation patterns from pre-immortal cells suggest that, in general, changes in subtelomeric methylation do not precede immortalization by a significant extent, but are related to the activation of a telomere maintenance mechanism. However, our observation of reduced subtelomeric methylation at one locus suggests that losses

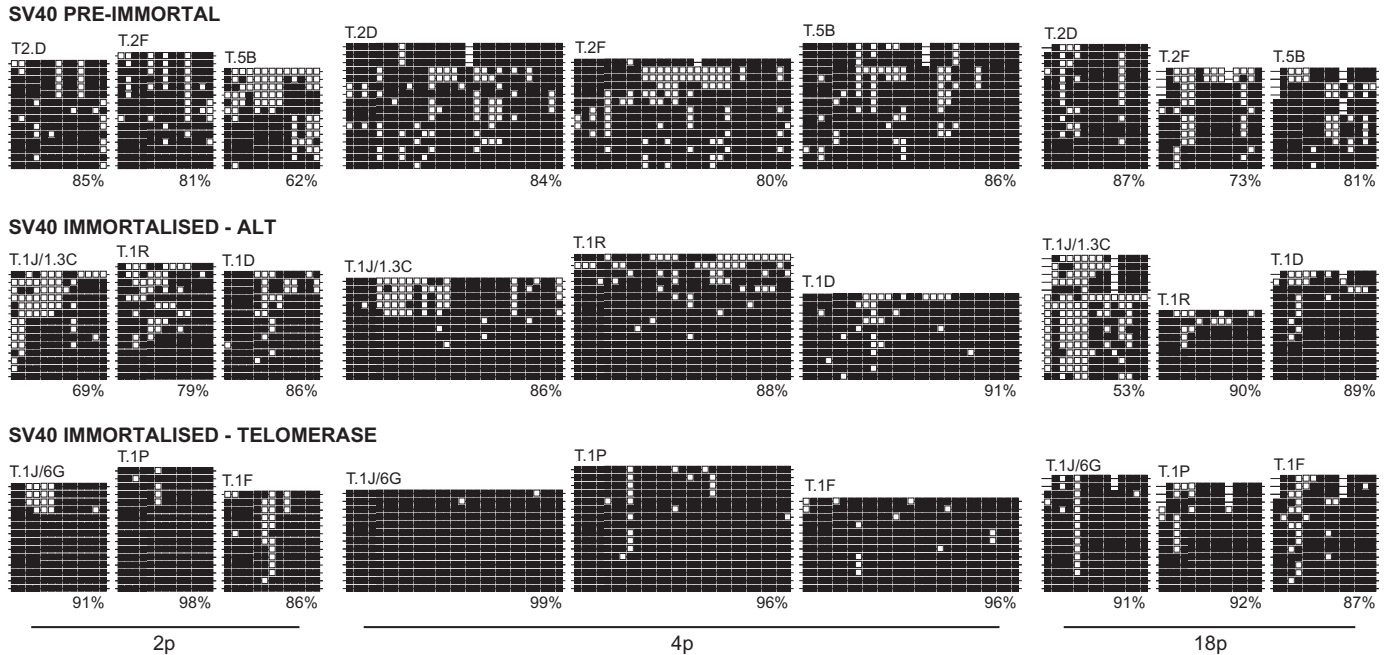


Figure 3. Subtelomeric methylation in SV40-transformed immortalized cell lines mirrors that of tumour-derived cells. Bisulphite sequencing maps for subtelomeres at chromosomes 2p, 4p and 18p are shown; those from pre-immortal cells at top, immortalized ALT cells at middle, and immortalized telomerase-positive cells at bottom. Within each map, horizontal lines show the methylation pattern of individual alleles: black squares represent methylated CpGs and white squares unmethylated CpGs. The average percent methylation for each individual is shown at the bottom right of each map. The gap in some alleles is due to a G/A polymorphism/mutation that results in loss of a CpG.

of subtelomeric methylation may occur prior to activation of telomere maintenance; whether or not this cell line would be more likely to use ALT if immortalized cannot be ascertained.

Taken together, our observations in tumour-derived and SV40-transformed cells indicate that ALT cells can exhibit stochastic changes in subtelomeric methylation, whereas telomerase cells consistently show an increase compared to normal. Recent studies have reported heterogeneous telomere lengthening reminiscent of ALT in mice deficient in the DNA methyltransferase DNMT1 (17). These mice show drastic reductions in methylation at four subtelomeric loci as a consequence of their inability to maintain genomic cytosine methylation. However, the loss of subtelomeric methylation we observed in ALT does not occur at all the subtelomeres we examined; in one ALT cell line (SK-LU-1), we did not observe any loss of methylation but saw gains of methylation at all three subtelomeres. Since methylation patterns do not tend to change from one cell division to the next, but ALT cells undergo frequent telomeric recombination (8,26), our data suggest that recombination occurs at telomeres that carry heavy subtelomeric methylation. Thus, widespread loss of subtelomeric methylation does not appear to be required for ALT. The question remains as to why some subtelomeric loci in ALT cells showed dramatic losses of methylation: perhaps they are merely reflective of the stochastic losses of methylation characteristic of tumours and immortalized cells. This suggestion is supported by recent data from a panel of human tumour cell lines, in which losses of methylation at the subtelomeric

D4Z4 repeat correlates with the global reductions in 5-methylcytosine (18).

Our findings from telomerase-positive cell lines have a clear implication: the significant and consistent increase in subtelomeric methylation we observe at all loci indicates that a heterochromatic structure is maintained at telomeres in cells that utilize telomerase. This suggests that a strong selection pressure for telomeric heterochromatin exists in telomerase-positive cells.

Telomeric transcription is high in ALT cells but low in telomerase-positive cells

Heterochromatic states and dense cytosine methylation are tightly linked to transcriptional repression. Although it has been assumed that telomeres are transcriptionally silent, telomeric transcription has recently been described in mammalian cells (27,28). The telomeric repeat-containing transcript (TERRA) is transcribed from the C-rich strand using promoters situated in the subtelomeric region. Because ALT and telomerase cells exhibit different methylation profiles at the subtelomere, we investigated the levels of TERRA transcripts in ALT and telomerase-positive cells by RNA slot-blot. Total RNA from each of the cell lines described above, as well as from primary human fibroblasts derived from five individuals, was hybridized to probes directed against the sense (UUAG GG) and antisense (CCCUAA) TERRA sequence; signals were normalized to the signal for GAPDH. We observed no significant signal in any cell line with the CCCUAA-directed probe (data not shown). The TERRA:GAPDH ratio was higher in ALT cell lines than in any other

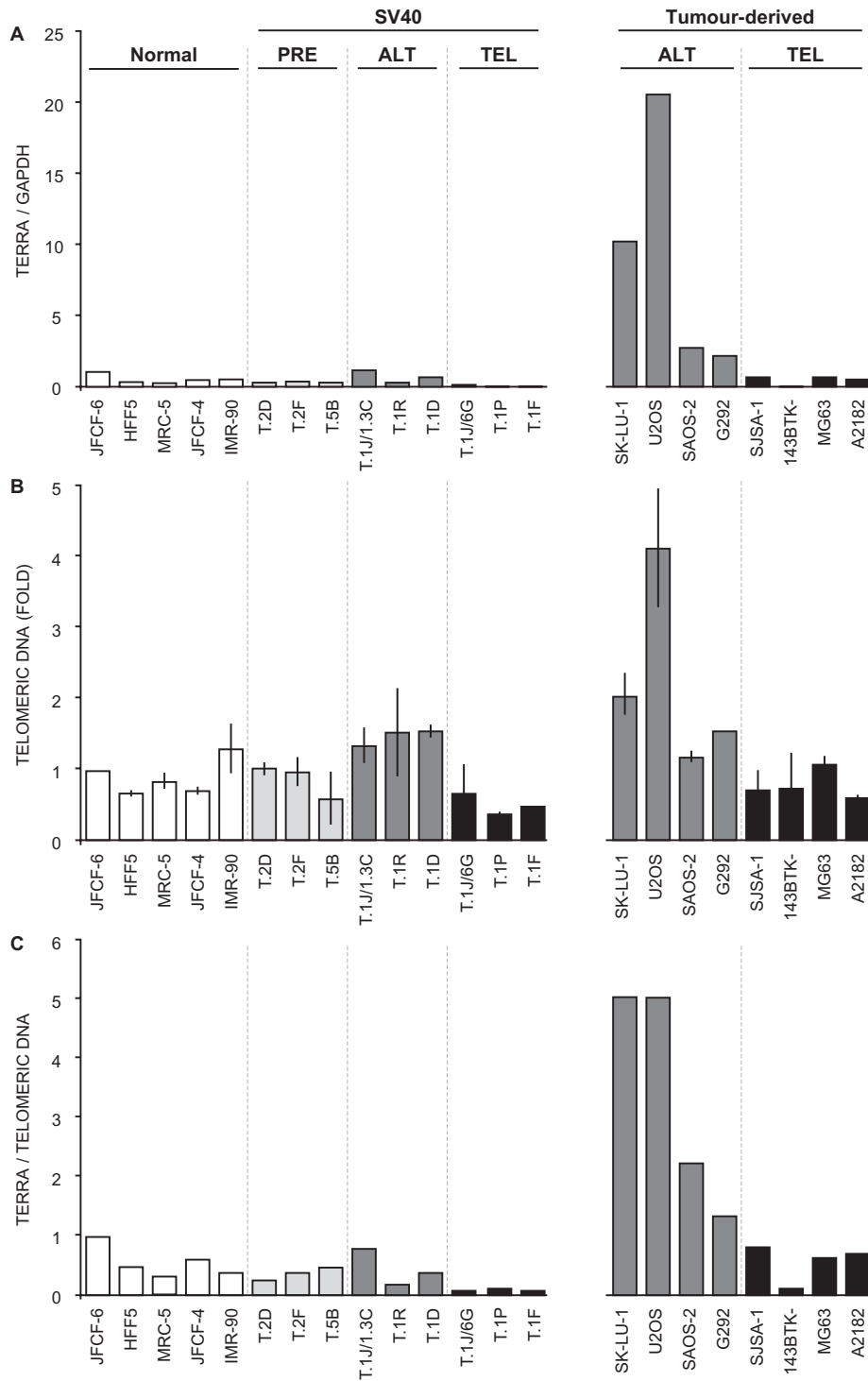


Figure 4. Telomeric transcription is low in telomerase-positive cells compared with matched ALT cells. (A) Quantitation of TERRA transcripts in the normal fibroblasts and cell lines indicated. The intensity of each TERRA sense strand signal was measured by densitometry and normalized to the GAPDH signal. (B) Quantitation of telomeric DNA from the same cells. The intensity of telomeric signal was measured by densitometry and normalized to the Alu repeat signal. Error bars represent standard deviation between three separate experiments. (C) TERRA levels corrected for the amount of telomeric DNA, as shown in (B). In each case, levels for each cell line are expressed relative to the first normal fibroblast sample, JFCF-6.

sample (Figure 4A); telomerase-positive cell lines gave a much lower TERRA:GAPDH ratio. ALT cells tend to have longer telomeres than telomerase-positive cells or normal cells and this could account for their higher TERRA signal. Therefore to control for telomere length

we measured the amount of telomeric DNA in each sample (Figure 4B). When corrected for the total amount of telomeric DNA, tumour-derived ALT cells still exhibited high TERRA levels compared to tumour-derived telomerase-positive cells (Figure 4C). The series of

fibroblasts, ranging from normal through to immortalized, allowed us to compare TERRA levels in matching cell types and determine its normal abundance. In this series, the most striking feature is a dramatic reduction in TERRA levels in the telomerase-positive immortalized cells (Figure 4C); normal fibroblasts, SV40 transformed, non-immortal fibroblasts and immortalized ALT cells all show comparable levels of TERRA. Taken together, our data indicate that TERRA transcription is dampened in telomerase-positive cells, but permitted in ALT cells.

The abundance of TERRA in the two series of cell lines examined (fibroblasts and tumour-derived cell lines) appears more closely linked to the mechanism of telomere maintenance than to the level of subtelomeric methylation. While telomerase-positive cells, with high levels of subtelomeric methylation, displayed low levels of TERRA relative to matched ALT or normal cells, the SK-LU-1 ALT cell line had a high abundance of TERRA transcript despite having high subtelomeric methylation levels. However, we cannot rule out the possibility that the SK-LU-1 cell line carries losses of methylation at other unexamined subtelomeric loci.

In telomerase-positive cells, low TERRA transcript levels and high levels of subtelomeric methylation together indicate that consolidation of telomeric heterochromatin represses telomeric transcription. *In vitro* studies suggest that TERRA transcripts can inhibit the action of telomerase, perhaps by blocking the complementary template region of the RNA component, hTR: when HeLa and ES cell extracts were incubated with an excess of synthetic RNA oligonucleotides mimicking TERRA, telomerase activity was abolished (28). Maintenance of telomeric heterochromatin may be necessary in telomerase-positive cells because the presence of TERRA inhibits telomerase activity. In cells with significant TERRA production, telomerase may be unable to act efficiently at the telomere. Interestingly, TERRA localizes to the telomere (27,28) to form part of the telomeric chromatin, and so it may inhibit telomerase activity only at the telomeres from which it is produced. This may explain previous observations of heterogeneous telomere lengthening in DNMT-deficient mice with mosaic subtelomeric methylation patterns (17). Alternatively, it remains a possibility that TERRA produced from a subset of telomeres may act globally within a cell to inhibit telomerase activity. Regardless of whether TERRA acts *in situ* or *in trans* to inhibit telomerase, its existence is closely linked to the mechanism of telomere maintenance: reduced TERRA levels accompany the presence of telomerase activity.

Finally, although it has been suggested that the ALT mechanism of telomere maintenance is facilitated by a loss of methylation at the subtelomere (17), our data indicate that a loss of methylation at all subtelomeres is not necessary for ALT. Furthermore, recent findings from individuals with ICF syndrome (who lack DNMT3B and hence the ability to methylate subtelomeric regions) indicate that subtelomeric hypomethylation does not lead to recombination at the telomere (29). Rather, our results suggest that it is telomerase activity which is tightly linked to a change in subtelomeric methylation. It may be that in ALT cells there is no requirement for the

telomere to be heterochromatic, and telomeres stochastically lose marks of heterochromatin such as subtelomeric methylation. Stochastic loss of telomeric heterochromatin prior to immortalization could increase TERRA production and prevent the action of telomerase; although most such cells would undergo apoptosis at this point, cells that became immortalized would need to activate an alternative mechanism of telomere maintenance. In any case, it appears that consolidation of the heterochromatic state of all telomeres and reduction of TERRA transcription is a necessary requirement for telomerase-mediated telomere lengthening, raising the possibility that there is an underlying epigenetic difference between telomerase-positive and ALT cells.

SUPPLEMENTARY DATA

Supplementary Data are available at NAR Online.

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