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Advances in Brief

Alternative Lengthening of Telomeres Is Characterized by High Rates of Telomeric Exchange

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Abstract

Telomere maintenance activity is a hallmark of cancer. In some telomerase-negative tumors, telomeres become lengthened by alternative lengthening of telomeres (ALT), a recombination-mediated DNA replication process in which telomeres use other telomeric DNA as a copy template. Using chromosome orientation fluorescence *in situ* hybridization, we found that postreplicative exchange events involving a telomere and another TTAGGG-repeat tract occur at remarkably high frequencies in ALT cells (range 28–280/100 metaphases) and rarely or never in non-ALT cells, including cell lines with very long telomeres. Like the ALT phenotype itself, the telomeric exchanges were not suppressed when telomerase was activated in ALT cells. These exchanges are telomere specific because there was no correlation with sister chromatid exchange rates at interstitial locations, and they were not observed in non-ALT Bloom syndrome cells with very high sister chromatid exchange rates.

Introduction

Most human tumors acquire indefinite replicative capacity through the reactivation of telomerase (1). However, some cancers use alternative lengthening of telomeres (ALT; Ref. 2), and recent evidence indicates that this mechanism is common in various types of cancer including osteosarcoma (3) and glioblastoma multiforme, the most common type of primary brain tumor in adults (4). ALT is a recombination-based DNA replication mechanism that results in lengthening of telomeres (5–7). Given the evidence that the telomerase and ALT mechanisms coexist in some tumors (3, 4, 8) and the possibility that treatment of telomerase-positive tumors with effective telomerase inhibitors will result in activation of ALT (9), therapeutic approaches aimed at inhibiting telomerase activity may need to be complemented with others that target ALT. However, the molecular events implicated in ALT remain unexplored, partly because of the lack of a specific biochemical assay. Here, using chromosome orientation fluorescence *in situ* hybridization (CO-FISH), we found evidence for a remarkably high level of telomeric exchange in ALT cells that was seen rarely or not at all in cells that do not use the ALT mechanism.

Materials and Methods

Cell Lines. WI38 VA13/2RA human cells, here referred to as VA13 and GM847 cells are SV40-immortalized human fibroblast cell lines that use the ALT mechanism (8). GM847 expresses the telomerase RNA subunit, hTERC, whereas VA13 does not (10). The construction of their telomerase-positive derivatives was described previously (11, 12). The tumor cell lines are derived from prostate carcinoma (DU145 and LNCaP), breast adenocarcinoma (SKBR-3), ovarian adenocarcinoma (SKOV-3), osteosarcoma (R970–5, U-2 OS, and SAOS-2) and fibrosarcoma (HT1080). All, except U-2 OS and SAOS-2, are telomerase positive. HT1080+POT1 (variant 1/clone 12) are telomerase-positive HT1080 human fibrosarcoma cells that have lengthened telomeres (mean terminal restriction fragment length of 15 kb) after transfection with variant 1 *hPOT1* cDNA (13). JFCF-6/2H+hTERT cells are an SV40-immortalized telomerase-positive human fibroblast line in which the terminal restriction fragments have been lengthened to an average of 20 kb after transfection with a human telomerase reverse transcriptase (*hTERT*) expression plasmid.[5] BJ, a foreskin primary fibroblast cell strain, was obtained at early passage from J. Smith (Baylor College of Medicine, Houston, TX) and was transfected with *hTERT*.[6] WI38+hTERT, a lung fibroblast cell line, was obtained from J. Campisi (Lawrence Berkeley National Laboratory, Berkeley, CA). NIH3T3 cells, 293T cells, three lymphoblastoid cell lines, and peripheral blood lymphocytes (from healthy donors) were obtained from the Centre d'Etude du Polymorphisme Humain collection. GM16859 is a primary fibroblast cell strain from a Bloom syndrome patient (obtained from Coriell Cell Repositories).

Culture Conditions and Metaphase Preparations. Most cell lines were grown in either α-MEM (VA13, R970–5, U-2 OS, and SAOS-2) or DMEM (293T, NIH3T3, HT1080, HT1080+POT1, JFCF-6/2H+hTERT, BJ, BJ+hTERT, WI38+hTERT, GM847, DU145, U-2 OS, SKBR-3, 293T, and GM16859) supplemented with 10 or 15% fetal bovine serum. LNCaP, the lymphoblastoid cell lines and PHA-stimulated peripheral blood lymphocytes were cultivated in RPMI +10% fetal bovine serum. For CO-FISH and sister chromatid exchange (SCE) analyses, cells were incubated with BrdUrd (30 μM) for either one or two doubling times before 1- to 2-h incubation with colcemid (0.1 μg/ml) followed by hypotonic shock and fixation (methanol/acetic acid). Some cell cultures were treated with mitomycin C (10^{-6} M) for 1 h before the addition of BrdUrd (14). Metaphase spreads were obtained by dropping suspensions of fixed cells onto clean glass slides. Preparations were used the next day for telomeric FISH.

CO-FISH and SCE Analysis Slides were treated before hybridization as described by Cornforth and Eberle (15). For telomeric FISH and analysis of fluorescent signals, the procedure we described previously was used (12). In preparations from cells incubated for two doubling times, the concentration of 4′,6-diamidino-2-phenylindole was increased to 4 μg/ml to distinguish light and dark sister chromatids.

Results and Discussion

CO-FISH is a strand-specific hybridization technique commonly used to deduce the orientation of sequences along the chromosomes (16). It is based on the specific degradation, in metaphase chromosomes, of the newly synthesized strand that has incorporated a base substitute (usually BrdUrd). When applied to telomeres, which have a characteristic strand asymmetry, it is expected that CO-FISH will show one signal per chromosome extremity (Fig. 1, *A-B*). What we observed in ALT cells, however, was that a substantial proportion of metaphases had many chromosome extremities bearing "double sig-

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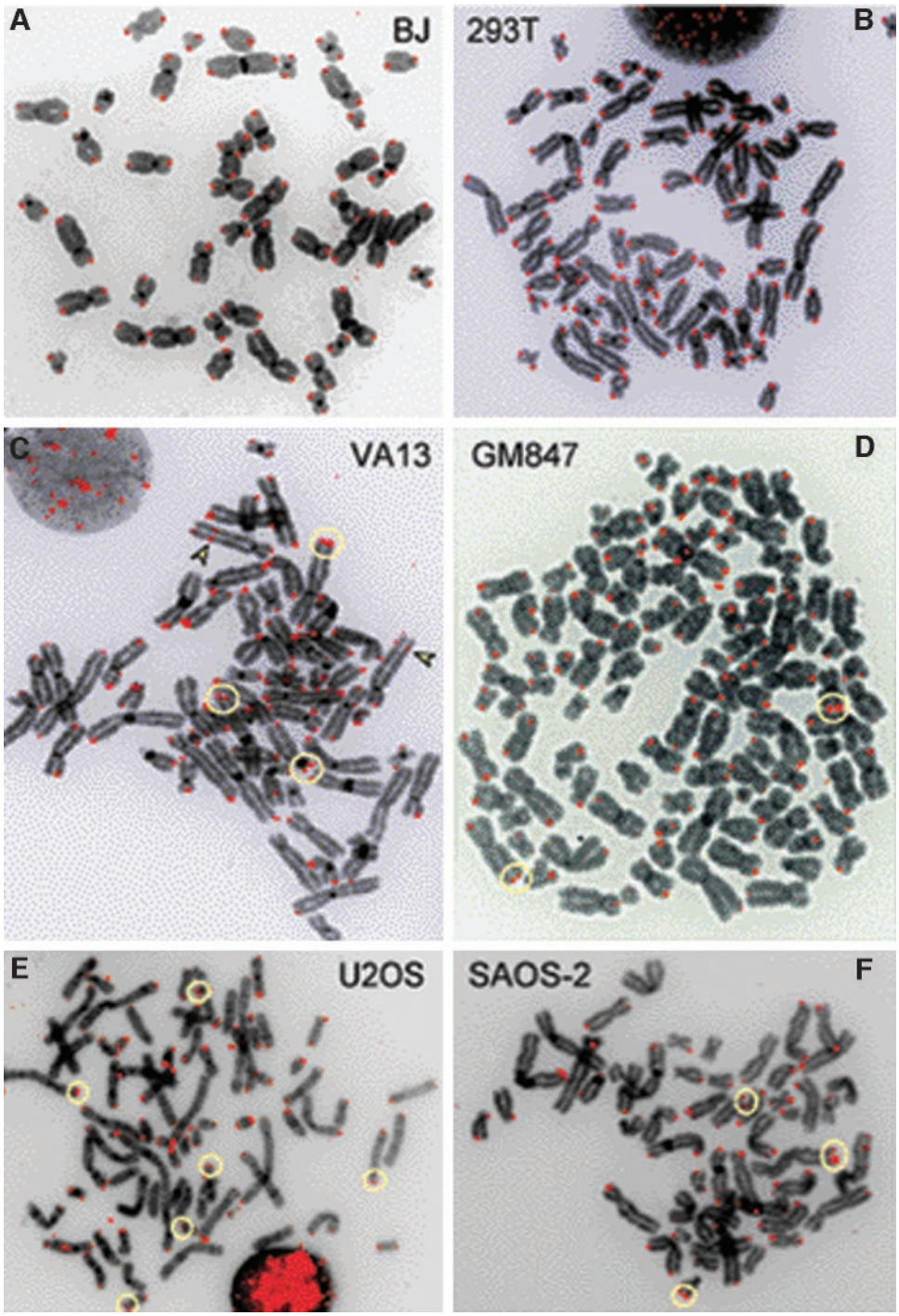

Fig. 1. Detection by chromosome orientation fluorescence *in situ* hybridization (CO-FISH) of chromosome extremities with double signals in ALT cells. CO-FISH analysis was performed after one round of DNA replication in the presence of BrdUrd. In primary BJ cells (*A*) and telomerase-positive 293T cells (*B*), only single signals are detected typically at chromosome extremities, although in very rare metaphases (not shown here), a double signal may be detected at one chromosome extremity (Table 1). In contrast, CO-FISH frequently detects extremities bearing double signals (some of which are indicated by *circles*) in ALT-positive VA13 (*C*) and GM847 (*D*) cell lines. In *C*, *arrowheads* point to interstitial telomeric signals, probably the result of past nonhomologous end-joining events. Two telomerase-negative cell lines derived from human osteosarcomas, U-2 OS (*E*) and SAOS-2 (*F*), also have many CO-FISH-double signals (Table 1).

nals" (Fig. 1, *C-D*; Table 1). The most likely explanation is that an exchange had occurred between a telomere of the chromosomes with double signals and other TTAGGG-repeat sequence subsequent to DNA replication. However, it was not possible to identify the potential donors of the second signal (for example, by finding extremities that had lost their telomeric signal) in the same metaphases, mainly because in ALT cells, chromosome extremities with no detectable telomere signals are common. To circumvent this obstacle, we examined the telomerase-positive derivatives of the GM847 and VA13 cell lines that were generated by transfecting them with expression constructs for TERT (GM847; Ref. 11) or TERT and TERC (VA13; Ref. 12). In these cells, ALT is still active but all extremities have telomeric signals detectable by FISH, indicating that telomerase has lengthened the shortest telomeres (11, 12). Using CO-FISH, we observed telomeric exchanges in an even higher proportion of metaphases than in the parental cell lines (Table 1). However, telomeric signals were detected on all chromosome extremities (not shown), indicating that the double signals do not result from the loss of signals from other chromosomes.

Double signals may be the result of exchange events occurring within the telomere, which both transfer and leave behind enough unsubstituted T_2AG_3 repeats to be detected by CO-FISH. Whether these exchanges take place between sister chromatids or between different chromosomes is not known. An additional possibility is that the exchanges may even involve the interaction of a telomere with the extrachromosomal TTAGGG-repeat DNA that is present in ALT cells

Table 1 *Telomere CO-FISH[a] and SCE analyses in different cell types*

Phenotype cells (total no. of metaphases)	SCEs[b] mean/ metaphase	No. double CO-FISH signals[c]/100 metaphases
Mortal and hTERT-immortalized diploid cell lines		
PBL[d] (50)	10	0
BJ (30)	5.3	2
BJ + hTERT (40)	6.2	3
WI38 + hTERT (35)	4.9	2
GM16859[e] (30)	93.2	0
Transformed (telomerase+)		
LBL[f] (25)	5.1	0
LBL + Mitomycin (25)	30.8	0
293T (30)	19	0
293T + Mitomycin (30)	61.7	0
NIH3T3 (55)	N.D.	5–9
SKOV3 (35)	23.4	0
R970.5 (30)	23.3	0
HT1080 (40)	13.8	0
HT1080 + hPOT1 (25)	10.2	2
JFCF-6T/2H 2FL-6 (20)	22.9	0
Transformed (ALT+)		
VA13[g] (80)	25.3	45–150
VA13 (+telom.)[g] (55)	23.2	170–280
GM847 (70)	20.9	28–65
GM847-C3 (+telom.)[g] (40)	21.2	56–90
GM847-C6 (+telom.)[g] (45)	19.1	85–125
U-2 OS (33)	12	85–105
SAOS-2 (35)	10.1	167–240

[a] CO-FISH, chromosome orientation-fluorescence *in situ* hybridization; SCE, sister chromatid exchange.
[b] The reported number of SCEs corresponds to the sum of events that took place in two rounds of DNA replication in the presence of BrdUrd.
[c] Most results from CO-FISH analyses are from two independent experiments.
[d] Phytohemagglutinin-stimulated peripheral blood lymphocytes from a healthy donor.
[e] Primary fibroblast cell line from a Bloom syndrome patient.
[f] Lymphoblastoid cell line "B" (healthy donor).
[g] Clonal origin.

(reviewed in Ref. 17). The most likely explanation for the increased detection of telomeric exchanges in telomerase-positive ALT cells is that elongation of short telomeres by telomerase facilitates the visualization of telomeric exchanges by CO-FISH, suggesting that these exchanges also involve relatively short telomeres.

An alternative interpretation for the occurrence of the double signals in CO-FISH experiments could be the presence of repeat tracts in which the G-rich/C-rich asymmetry of telomeric strands has been disrupted. It is conceivable that the double-stranded extrachromosomal TTAGGG-repeat DNA fragments in ALT cells may be substrates for non-homologous end joining, resulting in telomeric repeat tracts being added to unprotected chromosome extremities. Given that such fragments could be ligated in any orientation, the resulting product would be a patchwork of interspersed G-rich and C-rich sequences, readily detectable by CO-FISH on both sister chromatids of particular chromosome extremities. Although several of the cell lines examined here are of clonal origin (Table 1) the chromosomes that exhibited telomeric exchanges varied from metaphase to metaphase (not shown). In contrast, CO-FISH double signals were detected at constant interstitial locations in some aberrant chromosomes carried by VA13 cells (Fig. 1*C*), probably the mark of past nonhomologous end-joining events between two chromosome extremities still carrying telomeric repeats.

As a further and more direct test of whether the double signals detected by CO-FISH result from G-rich repeats being present on the same DNA strand at the opposite extremities of the chromosome caused by putative ligation events, or result from telomeric exchanges, we followed the segregation of CO-FISH telomeric signals after a second round of DNA replication, as above (Fig. 2). In the case of G-rich tracts being present on the same strand at opposite extremities (and in the absence of SCE), the dark sister chromatid would appear to be labeled on both extremities. In fact, CO-FISH signals on both extremities of dark sister chromatids were extremely rare compared with the number of CO-FISH signals linked to the lighter sister chromatid with no detectable SCE event (Fig. 2). Interestingly, we also observed occasional double signals in these experiments, indicating the occurrence of telomeric exchanges after the second round of replication (Fig. 2).

This remarkable level of telomeric exchange was specific for ALT cells, because double signals were observed rarely or not at all in

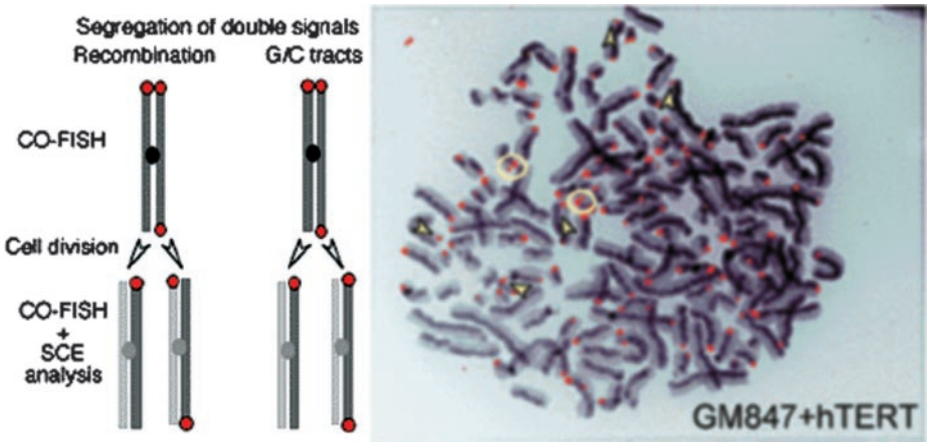

Fig. 2. Detection by chromosome orientation fluorescence *in situ* hybridization (CO-FISH) of postreplicative telomeric events after two rounds of BrdUrd incorporation. *Left*, diagrammatic representation of CO-FISH/sister chromatid exchange (SCE) analysis of ALT cells after one and two rounds of DNA replication in the presence of BrdUrd. The first round of semiconservative DNA replication results in each sister chromatid containing one substituted and one unsubstituted strand. After the second round, one sister chromatid is still unifilarly substituted (appearing *dark*) whereas the other has been bifilarly substituted (appearing *light*). Note that the total number of telomeric signals detected by CO-FISH in a metaphase is reduced approximately by half after cell division. Double signals detected by CO-FISH after the first round may be the result of postreplicative telomeric recombination events between sister chromatids, between different chromosomes, or else involving extrachromosomal TTAGGG repeats. Alternatively, double signals could be the consequence of nonhomologous end-joining events that can ligate extrachromosomal telomeric fragments in both orientations, thus creating a patchwork of interspersed G/C tracts. The segregation analysis in daughter cells of telomere signals belonging to the same sister chromatid can distinguish between these hypotheses. In the case of a recombination event, the strands will be separated during the next round of replication, and the signals will appear on different sister chromatids. In the case of G/C tracts and in the absence of a SCE event, both signals will remain associated with the same dark sister chromatid. *Right*, the simultaneous CO-FISH/SCE analysis in ALT cells (in this case GM847+hTERT) reveals telomeric signals frequently associated with the lighter sister chromatid (*arrowheads*), suggesting that they are the result of recombination events, either subtelomeric or telomeric. Double signals are also detected in this analysis, suggesting that telomeric exchanges also occurred after the second round of DNA replication. Dark sister chromatids with both extremities labeled are very rare, making it unlikely that G/C tracts are the cause of double signals. Similar experiments with the VA13 cell line suggest that SCE rates are not increased at interstitial loci carrying telomeric repeats (not shown).

normal cell strains and in telomerase-positive cell lines (Table 1). To test whether this phenomenon is confined to cell lines immortalized *in vitro*, we analyzed by CO-FISH eight cell lines derived from human tumors, six of which are telomerase positive (HT1080, DU145, SKBR-3, R970–5, SKOV-3, and LNCaP) and two of which use ALT for telomere maintenance (U-2 OS and SAOS-2). We observed frequent telomeric exchanges in metaphase preparations only from the two ALT cell lines (Table 1; Fig. 1, *E-F*), supporting the ALT specificity of this observation. CO-FISH analysis may thus constitute an additional assay for the presence of ALT in tumor cell lines, although it may be difficult to carry out in fresh material from cancer patients because it requires metaphase preparations.

Because ALT cells bear very long telomeres, we considered the possibility that telomeric exchanges may be triggered by these long structures independently from the ALT process. Telomerase-positive human cell lines with telomeres of about 40 kb not being available to test this hypothesis, we used mouse embryonic fibroblasts (NIH3T3), the telomeres of which, as judged by quantitative FISH, were at least as long as in ALT cells (not shown; 18). CO-FISH analyses detected double signals at chromosome extremities in a few NIH3T3 metaphases (Table 1). We also examined human cells with mean telomere lengths over 10 kb (BJ+hTERT and WI38+hTERT not shown), 15 kb (HT1080+POT1; Ref. 13), and 20 kb (JFCF/2H+hTERT, not shown). Again, CO-FISH analyses revealed only occasional double signals in these cells (Table 1). This very low frequency suggests that although long telomeres may be a substrate for telomeric exchange, additional factors are required to trigger the high rate of exchange seen in ALT cells.

Because the telomeric exchanges may be occurring between sister chromatids in ALT cells, they could be the result of the same type of DNA-damage response that leads to interstitial SCEs. Nevertheless, they are not observed in normal cells or telomerase-positive tumor cell lines with high SCE rates even when these rates have been increased by several fold in response to a short exposure to mitomycin C (Table 1). Furthermore, telomeric exchanges were not observed in primary fibroblasts or lymphoblastoid cell lines derived from Bloom syndrome patients, which have SCEs rates 10- or 12-fold higher than normal cells (Table 1). Together, these findings suggest that in most cells, telomeres either are not accumulating DNA damage or are prevented from undergoing exchange events, possibly as a consequence of telomere cap function. In ALT cells, on the other hand, the telomeres may be subject to a significant level of DNA damage (or some other alteration that results in "uncapping" and, therefore, in recognition of the telomere end as a site of DNA damage) and consequently have a strong SCE response. It is important to note that the SCE frequency is increased only at the telomeres of ALT cells, but not at interstitial locations (Table 1), including sites containing telomeric repeats (not shown). This observation is in agreement with a recent report showing that ALT cells do not have increased levels of homologous recombination within chromosomes (19).

One hypothesis to explain the high frequency of telomeric exchanges in ALT cells would be that several components of the mitotic homologous recombination apparatus have been recruited to the telomere for ALT-mediated telomere lengthening. Presumably, one key outcome of this recruitment is that the proteins required for SCE are present at ALT telomeres. If the telomeric exchanges in ALT cells involve sister chromatids, they may be a side effect of a high concentration of SCE proteins at the telomere, rather than being a part of the ALT lengthening. On the other hand, the data do not exclude the possibility that the telomeric exchanges are interchromosomal or involve extrachromosomal TTAGGG-repeat fragments, in which case they may involve additional mechanisms specific to ALT. Additional experiments are needed to explore the relationship between telomere exchange events detected by CO-FISH and the telomere-lengthening mechanisms operating in ALT cells.

References

1. Shay JW, Bacchetti S. A survey of telomerase activity in human cancer. Eur J Cancer 1997;33:787–91.
2. Bryan TM, Englezou A, Dalla-Pozza L, Dunham MA, Reddel RR. Evidence for an alternative mechanism for maintaining telomere length in human tumors and tumor-derived cell lines. Nat Med 1997;3:1271–4.
3. Ulaner GA, Huang HY, Otero J, et al. Absence of a telomere maintenance mechanism as a favorable prognostic factor in patients with osteosarcoma. Cancer Res 2003;63:1759–63.
4. Hakin-Smith V, Jellinek DA, Levy D, et al. Alternative lengthening of telomeres and survival in patients with glioblastoma multiforme. Lancet 2003;361:836–8.
5. Murnane JP, Sabatier L, Marder BA, Morgan WF. Telomere dynamics in an immortal human cell line. EMBO J 1994;13:4953–62.
6. Dunham MA, Neumann AA, Fasching CL, Reddel RR. Telomere maintenance by recombination in human cells. Nat Genet 2000;26:447–50.
7. Varley H, Pickett HA, Foxon JL, Reddel RR, Royle NJ. Molecular characterization of inter-telomere and intra-telomere mutations in human ALT cells. Nat Genet 2002;30:301–5.
8. Bryan TM, Englezou A, Gupta J, Bacchetti S, Reddel RR. Telomere elongation in immortal human cells without detectable telomerase activity. EMBO J 1995;14:4240–8.
9. Neumann AA, Reddel RR. Telomere maintenance and cancer—look, no telomerase. Nat Rev Cancer 2002;2:879–84.
10. Bryan TM, Marusic L, Bacchetti S, Namba M, Reddel RR. The telomere lengthening mechanism in telomerase-negative immortal human cells does not involve the telomerase RNA subunit. Hum Mol Genet 1997;6:921–6.
11. Perrem K, Colgin LM, Neumann AA, Yeager TR, Reddel RR. Coexistence of alternative lengthening of telomeres and telomerase in hTERT-transfected GM847 cells. Mol Cell Biol 2001;21:3862–75.
12. Cerone MA, Londono-Vallejo JA, Bacchetti S. Telomere maintenance by telomerase and by recombination can coexist in human cells. Hum Mol Genet 2001;10:1945–52.
13. Colgin LM, Baran K, Baumann P, Cech TR, Reddel RR. Human POT1 facilitates telomere elongation by telomerase. Curr Biol 2003;13:942–6.
14. Bianchi MS, Larramendy ML, Bianchi NO. Mitomycin C-induced damage and repair in human and pig lymphocytes. Mutat Res 1986;160:27–32.
15. Cornforth MN, Eberle RL. Termini of human chromosomes display elevated rates of mitotic recombination. Mutagenesis 2001;16:85–9.
16. Meyne J, Goodwin EH, Moyzis RK. Chromosome localization and orientation of the simple sequence repeat of human satellite I DNA. Chromosoma 1994;103:99–103.
17. Henson JD, Neumann AA, Yeager TR, Reddel RR. Alternative lengthening of telomeres in mammalian cells. Oncogene 2002;21:598–610.
18. Zijlmans JM, Martens UM, Poon SS, et al. Telomeres in the mouse have large inter-chromosomal variations in the number of T_2AG_3 repeats. Proc Natl Acad Sci USA 1997;94:7423–8.
19. Bechter OE, Zou Y, Shay JW, Wright WE. Homologous recombination in human telomerase-positive and ALT cells occurs with the same frequency. EMBO Rep 2003;4:1138–43.

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