

Absence or Low Number of Telomere Repeats at Junctions of Dicentric Chromosomes

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Human ovarian surface epithelial (HOSE) cells transfected with the *E6* and *E7* oncogenes of the human papilloma virus (PV) do not express measurable telomerase activity. Relative to untransfected control cells, HOSE-PV cells have an extended in vitro lifespan characterized by a very high frequency of telomeric associations (TAs) of chromosomes. In order to study the role of telomere shortening in the formation of TAs, we studied the telomere length in 120 dicentric chromosomes in HOSE-PV cells by using quantitative fluorescence in situ hybridization. Forty percent of the dicentric chromosomes had no fluorescence signal at the junction site, and in the remainder the fluorescence at the junction was less than at corresponding unjoined ends. These observations support a critical role of telomere shortening in the development of TAs and the subsequent genetic instability observed in a majority of tumor cells. *Genes Chromosomes Cancer* 24:83–86, 1999. © 1999 Wiley-Liss, Inc.

The ends of chromosomes consist of a special structure called the telomere (Blackburn, 1994; Zakian, 1995). Like all vertebrate chromosome ends, human telomeres consist of tandem repeats of the sequence TTAGGG (Moyzis et al., 1988; Lejnine et al., 1995). Telomere repeats constitute the final 2–10 kb of normal human chromosomes (Martens et al., 1998) and are synthesized by the enzyme telomerase (Greider and Blackburn, 1989; Lingner et al., 1997). Telomeres are crucial for chromosomal stability, and it has long been known that chromosomes without ends have a tendency to fuse (McClintock, 1941, 1951). More recent studies in yeast and in the mouse have shown conclusively that telomere repeats are indeed essential in maintaining genomic stability (Zakian, 1989; Sandell and Zakian, 1993; Blackburn, 1995; Blasco et al., 1997). In most, if not all, somatic cells, telomeric DNA is gradually lost from chromosome ends every time a cell divides (Harley et al., 1990; Hastie et al., 1990; reviewed in Harley, 1995). The replicative shortening of telomeres has been proposed to limit the life span of normal somatic cells (Harley, 1991) and to cause genetic instability in tumor cells (Hastie and Allshire, 1989; de Lange, 1995). Whereas the role of telomere shortening in cellular senescence has recently been confirmed by the introduction of telomerase in primary human fibroblasts (Bodnar et al., 1998; Vaziri and Benchimol, 1998), the role of telomere shortening in the development of the

genomic instability of tumor cells is still controversial. Because such genomic instability is thought to play a major role in tumor progression (Bishop, 1991; Brison, 1993), a detailed understanding of the factors that trigger this phenomenon is an important goal in cancer research.

Many of the characteristic genetic abnormalities in tumor cells, including gene amplification, aneuploidy, and loss of heterozygosity, can be explained by the chromatid breakage-fusion-bridge (BFB) cycle model (McClintock, 1951; de Lange, 1995). Defective repair of double-strand breaks (DSBs) could initiate BFB cycles, and it has been proposed that DSBs at fragile sites play a key role in the amplification of oncogenes during tumor progression (Coquelle et al., 1997). The cause of the DSB that triggers BFB cycles is not known, but could include radiation, clastogenic drugs (Coquelle et al., 1997), and breakage at anaphase of fused chromatids (Hastie and Allshire, 1989; Counter et al., 1992). In agreement with the latter is that

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end-to-end or telomere associations (TAs) of metaphase chromosomes are frequently observed in human tumors, senescent cells, and various chromosome instability syndromes (reviewed in de Lange, 1995). In most cases, TAs are unstable (Hastie and Allshire, 1989), complicating the characterization of the cytogenetic and molecular abnormalities that gave rise to these events. It has been suggested that loss of telomere repeats may predispose to TAs (Hastie and Allshire, 1989), and this hypothesis gained support with the demonstration that telomeres decrease in length with age (Hastie et al., 1990) and with proliferation *in vitro* (Harley et al., 1990). Recent studies on cells from telomerase (-negative) knockout mice (Blasco et al., 1997) have shown that telomere shortening indeed predisposes to chromosome fusion events. However, several studies, including our own (Saltman et al., 1993; Kirk et al., 1997; Slijepcevic et al., 1997), have shown that the overall telomere length of chromosomes is not the only determinant involved in TA formation.

To examine further the role of telomere length in the development of TAs of human chromosomes, we analyzed the telomere length of chromosomes in human ovarian surface epithelial cells transformed by human papilloma viral oncogenes. A human ovarian surface epithelial cell line (HOSE 6-3) was transformed by infection of the cells with a replication-defective retrovirus construct (LXSN16E6E7) according to procedures previously published (Tsao et al., 1995; Wan et al., 1997). Resistant colonies expressing the viral sequences were selected in G418 and ring-cloned. The selected cells did not express measurable telomerase activity by TRAP assay (Wan et al., in preparation) and display an extended *in vitro* life span characterized by very high frequencies of TAs (Wan et al., 1997). We reasoned that, if the TAs in such cells are indeed the result of progressive telomere shortening, quantitative fluorescence *in situ* hybridization techniques should show less fluorescence at the chromosome junction site than at free ends.

The telomere fluorescence at the junction of 120 dicentric chromosomes was analyzed in relation to the telomere fluorescence at the free chromosome ends by quantitative fluorescence *in situ* hybridization (Lansdorp et al., 1996; Martens et al., 1998). Briefly, HOSE 6-3 cells after 15 population doublings were treated with Colcemid at a final concentration of 0.1 $\mu\text{g/ml}$ and harvested 6 hours later. After washing and hypotonic swelling in 0.075 M KCl, cells were fixed in methanol/acetic acid (3:1 v/v) and slides were prepared using standard procedures. The slides were dried overnight in air before

proceeding to the telomere hybridization procedure using a Cy3-conjugated $(\text{C}_3\text{TA}_2)_3$ peptide nucleic acid probe, as described previously (Lansdorp et al., 1996). Digital images of chromosomes stained with DAPI and telomeres labeled with Cy3 were recorded with a Microimager MI1400-12 camera (Xillix, Vancouver, BC, Canada) and analyzed using a dedicated computer program (TFL-TELO) as described previously (Zijlmans et al., 1997). With the TFL-TELO program the integrated fluorescence intensity of individual telomeres is expressed in a table for each chromosome which can be subjected to editing and entry of karyotype information. Data are expressed in telomere fluorescence units (TFU), with each unit corresponding to 1 kb of T_2AG_3 repeats. Details of calibration steps using plasmids and fluorescence beads were reported previously (Zijlmans et al., 1997; Martens et al., 1998).

For each dicentric chromosome, the spot intensities from sister chromatids were recorded two by two and summarized. Cells with TAs were almost always aneuploid, showing the acquisition or loss of whole copies of other chromosomes, similar to previously described observations on these cells (Wan et al., 1997). All of the 120 dicentric chromosomes analyzed in this study showed decreased numbers or lack of detectable telomere repeats at the chromosome junction site (Fig. 1). The p arms of chromosomes were slightly more frequently involved than were the q arms (123 vs. 117), and this preferential involvement of p arms was more pronounced for the larger chromosomes, 1-12 and X (70 vs. 43), perhaps in part reflecting a decreased stability of dicentric chromosomes with distantly positioned centromeres (de Lange, 1995). Interestingly, the frequency of specific chromosome arms involved in dicentric chromosomes was significantly correlated (Wilcoxon Rank Sum test; $P = 0.04$) with the average telomere length on specific chromosome arms as recently reported for normal individuals (Martens et al., 1998).

Taken together, our observations support the model that telomere shortening plays a critical role in the formation of dicentric chromosomes in HOSE-PV cells. In all dicentric chromosomes analyzed in this study, fewer telomeric repeats were detected at the junction site than at free ends. We speculate that the dicentric chromosomes observed arose by at least two different mechanisms. The first mechanism involves the formation of TAs in which telomeres, each with very short telomere repeats (<0.5 kb), are noncovalent associated or covalent fused. Such fusions are expected prefer-

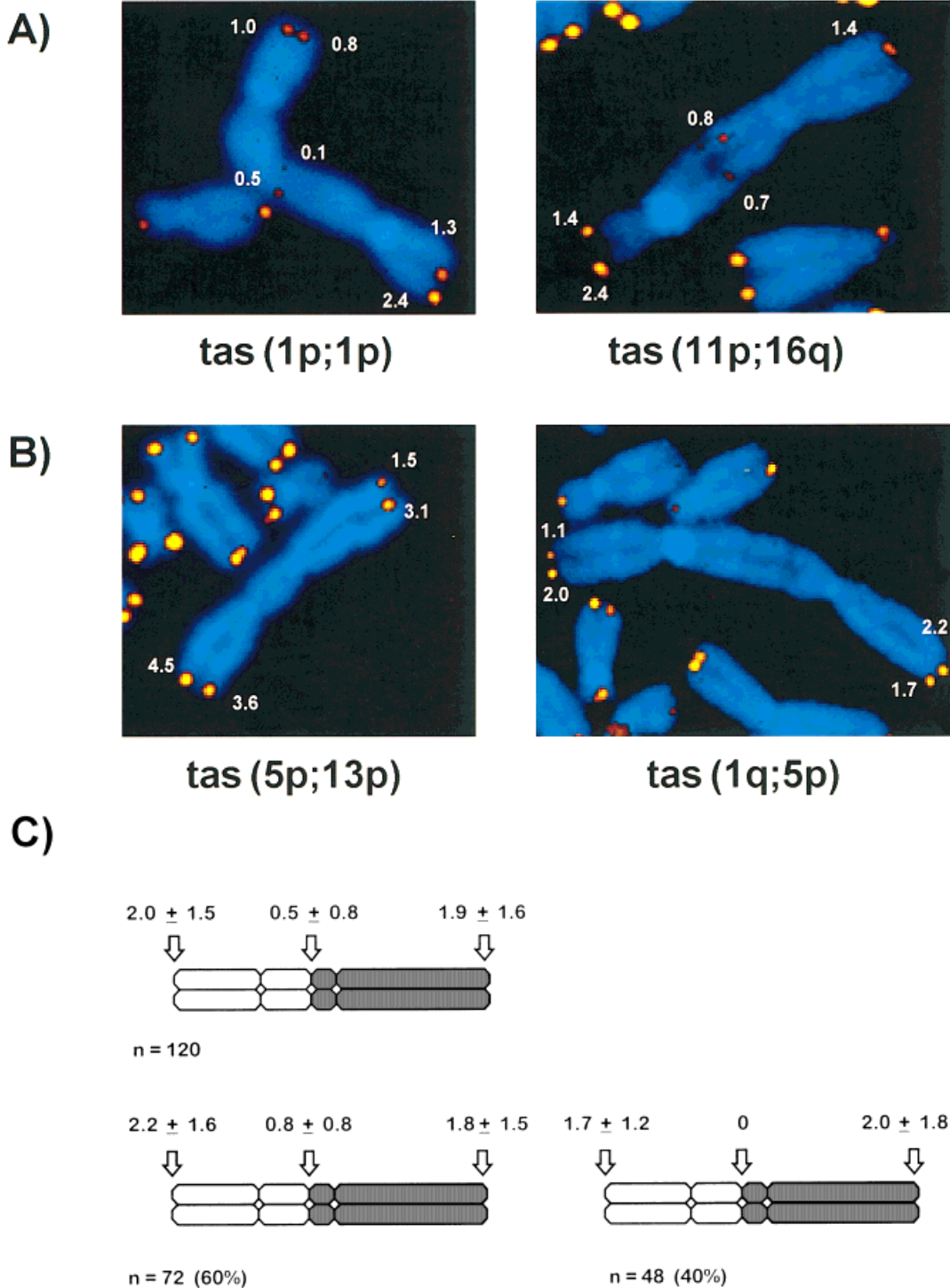


Figure 1. Absence or low numbers of telomere repeats at the junction of dicentric chromosomes. The estimated length of T₂AG₃ repeats in dicentric chromosomes in human ovarian surface epithelial cells transformed by human papilloma viral oncogenes (Wan et al., 1997) was estimated using quantitative fluorescence with directly labeled peptide nucleic acid telomere probe as described (Martens et al., 1998). Examples of dicentric chromosomes with faint fluorescence at the

junction site (A, less than half the fluorescence observed at corresponding free ends = less than a quarter of expected fluorescence) and (B) without detectable telomere fluorescence were found at the frequencies indicated in (C). The average telomere fluorescence expressed in telomere fluorescence units is shown in (C), with each unit corresponding to 1 kb of T₂AG₃ repeats based on calibration data with plasmids of known T₂AG₃ size inserts (Martens et al., 1998).

ably involve sister chromatids, but could also involve different chromosomes, each with short telomeres. Most likely, the chances of such TA formation will increase with the frequency of telomeres containing less than a threshold minimum of telomeric repeats. Furthermore, chromosome arms with short telomeres (Martens et al., 1998) are expected to be involved first. This type of TA, in which no genetic material is lost, is expected to be the first to appear in proliferating cells that are subject to continued telomere shortening. After anaphase bridge formation of such dicentric chromosomes, chromatids may be lost or may break (McClintock, 1941). The broken chromosomes are expected again to give rise to dicentric chromosomes by fusion or association with either other broken ends or other chromosome arms with short telomeres. Such dicentric chromosomes will be characterized by loss of (subtelomeric) DNA and very few, if any, telomeric repeats at the junction site. We speculate that upregulation of telomerase after a variable number of BFB cycles in such (pre-) malignant cells contributes to tumor progression by stabilizing fusogenic chromosome ends and broken chromosomes in cells that have acquired a growth advantage as a result of the prior telomere-mediated genetic instability.

In general, our observations strongly support the role of telomere shortening in the formation of dicentric chromosomes. This mechanism of genetic instability should be considered next to defective repair of mismatched bases and related microsatellite instability (Perucho, 1996) and mutations in mitotic checkpoint genes (Cahill et al., 1998). Because both chromosome-specific and donor-specific factors contribute to differences in T₂AG₃ repeat length at individual chromosome ends (Martens et al., 1998), further studies on the role of chromosome-specific telomeres in telomere-mediated genetic instability are warranted.

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