Pericentromeric Instability and Spontaneous Emergence of Human Neoacrocentric and Minute Chromosomes in the Alternative Pathway of Telomere Lengthening

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Abstract

In the alternative pathway of telomere lengthening (ALT), neoplastic cell growth is prolonged by telomere recombination. We show that ALT is unexpectedly characterized by high rates of ongoing pericentromeric chromosomal instability. Combined with telomeric recombination, ALT pericentromeric instability generates neoacrocentric chromosomes. In the present studies, we describe a subgroup of ALT neoacrocentric minute chromosomes, composed of DNA entities two to five times smaller in size than human chromosome 21. The frequencies of ALT minute chromosomes were increased by γ-irradiation and suppressed by telomerase. Continuous growth after telomerase inhibition/depletion was followed by increased rates of telomeric sister chromatid recombination and the emergence of minute chromosomes. We show that ALT minute chromosomes were derived from true centromeric fissions and/or chromosomal breakage/fusion/bridge cycles. They exhibit a two-chromatid structure, carry genomic DNA, centromeric and telomeric repeats, and display regular mitotic functionality. These observations are important in understanding the global genomic instability that characterizes most human advanced malignancies. [Cancer Res 2008;68(19):8146–55]

Introduction

In mammalian tumors and immortalized cell cultures, telomere length is almost exclusively maintained by telomerase (TERT; ref. 1) or through a much less common mechanism termed alternative lengthening of telomeres (ALT), which involves DNA recombination (2). Although ALT is a mechanism of telomere maintenance for only a small proportion of human neoplasias (3, 4), it raises important biological issues: various types of cancer develop in the absence of telomerase (5), whereas telomere elongation and cell proliferation potential can be substituted by recombinatorial mechanisms upon suppression of telomerase (6, 7).

Chromosomal instability in neoplasia (CIN) has been defined as an accelerated rate of continuous gains and losses of whole or large portions of chromosomes during cancer growth (8). Telomere attrition is considered as a causal event of tumorigenesis (1, 9), whereas others consider it an initial potent tumor suppressor mechanism (10). Telomerase deficiency and mutations affecting genes related to telomere protection in mouse and human cells has been associated with elevated rates of chromosome or sister chromatid terminal fusions (9). These may be causal events for the initiation of chromosomal breakage/fusion/bridge (B/F/B) cycles. The latter produce dicentric or polycentric chromosomes that are prone to anaphase bridges, abnormal chromosome condensation, and extreme fragmentation during mitosis (11–13). Hence, the B/F/B cycles are believed to be a major cause of *de novo* structural chromosome rearrangements in neoplasia (11–13). The B/F/B cycles are abundant in the ALT cells due to high rates of endogenous extreme telomere depletion (4).

Excessive telomere length heterogeneity and high frequency of telomeric sister chromatid exchanges (tSCE) are hallmarks of ALT. Such trends indicate an active process of intertelomere or intratelomere recombination (14). Another characteristic of the ALT pathway is the presence of ALT-associated promyelocytic leukemia (PML) protein bodies (APB; ref. 15). These subnuclear compartments are encountered in various numbers and proportions of the ALT nuclei (16). In addition to PML, APBs contain telomeric DNA, telomere binding proteins, helicases, and DNA repair and recombination factors (4). Insufficiency of the APB components RAD51, MRE11, RAD50, or NBS1 ameliorated continuous growth in mammalian ALT cell lines and indicated the important role of DNA recombination in the ALT pathway (17, 18).

Conventional and molecular cytogenetics is the "gold standard" to study intratumor genomic heterogeneity and to reveal underlying patterns of CIN (19, 20). We used detailed cytogenetic approaches to investigate *in vitro* CIN differences between telomerase-dependent and ALT continuous *in vitro* growth. Interestingly, in addition to the well-established increased rates of B/F/B cycles (4), the ALT cells of our study displayed increased pericentromeric instability. Here, we show, for the first time, that both pericentromeric instability and B/F/B cycles, combined to ALT recombinatorial telomere capping, are capable of generating complex karyotypes with continuously emerging pericentromeric rearrangements and mitotically functional human neoacrocentric and minute chromosomes. A better understanding of these processes may help elucidate fundamental mechanisms of genomic instability that characterize most advanced human malignancies.

Materials and Methods

Cell lines and culture conditions. The colon cancer SW-480 and the osteosarcoma Saos-2 cell lines were obtained from American Type Culture

Note: Supplementary data for this article are available at Cancer Research Online (http://cancerres.aacrjournals.org/).

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Collection, cervical cancer HeLa(a) cells were from European Collection of Animal Cell Cultures, HeLa(b) and breast cancer MCF-7 cells were provided by I. Irminger-Finger (Geneva Medical School), and the ductal breast carcinoma T47D and the osteosarcoma cell lines KHOS and U2-OS(b) were a gift from E. Gonos at the Greek National Institute for Research, Athens, Greece. HeLa(c) and U2-OS(b) were provided by S.E. Antonarakis and J. Merla (Geneva Medical School). HCT-116, HT-29, NCI-H-460, SF-268, and T24 cells were donated from C. Dimas and T. Vlahou (Biomedical Research Foundation of the Academy of Athens). The U2-OS(c) cell line was obtained from V. Gorgoulis (University of Athens Greece, Medical School). The ALT cell lines GM-847, VA-13, and IMR-90 were provided by A. Londono-Vallejo (Institute Curie). The VA-13 + hTERC + hTERT (VA-13TA; ref. 21) and the HCT-15 cell line expressing an antimorph against hTERT (7) were provided from J.W. Shay. We obtained in total 10 HCT-15 sublines. Two sublines (SL1, SL2) were TRAP-positive HCT-15 parental cells; the rest (SL3 to SL10) were TRAP-negative and represented in duplicates subsequent population doublings (PD; PD27–PD107) after the stable expression of the hTERT antimorph (7). According to shown cytogenetic results (Fig. 4*A*), the sublines SL5 and SL6 were undergoing crisis. All cell cultures were grown at 37°C in DMEM supplemented with 10% fetal bovine serum, 0.08 mg/mL amphotericin, 25 units/mL penicillin, and 25 pg/mL of streptomycin (Invitrogen).

Construction of representative karyotypes and study of structural CIN. Cell cultures of high mitotic index were exposed to colcemid [0.1 μg/mL; Life Technologies, Bethesda Research Laboratories (BRL)] for 10 min to 12 h at 37°C and harvested according to routine cytogenetic protocols, as described in ref. (22). For the construction of representative karyotypes, we combined inverted 4′,6-diamidino-2-phenylindole (DAPI), locus specific and centromeric fluorescence *in situ* hybridization (FISH), G-Banding, C-Banding, molecular karyotyping by SKY (Spectral Imaging) or Multicolor-FISH (M-FISH; Metasystems), and telomeric PNA FISH (DAKO) results obtained from >100 metaphases per cell line. G-Banding was performed after trypsine (Life Technologies-BRL) and Giemsa (BDH) staining. For C-Banding, we used barium hydroxide (SIGMA) denaturation and Giemsa staining (22). M-FISH was performed according to manufacturer's protocols (Metasystems). Conventional cytogenetic analyses were done using either the PSI karyotype software or the Metasystems Ikaros, both equipped by Zeiss microscopes. Molecular cytogenetic analyses were performed by the aid of Isis software (Metasystems).

FISH. For dual-color interphase or metaphase FISH, we used satellite probes specific for chromosomes 1, 2, 3, 4, 1/5/19, 6, 7, 8, 9, 10, 11, 12, 13/21, 14/22, 16, 17, 18, 20, and X. Probes were purchased from Vysis, Cytocell, Q-Biogen, or Cambio. In brief, our general FISH protocol was based on pepsin pretreatment, formamide or NaOH target denaturation, overnight hybridization, and high-stringency posthybridization washes. Telomere-specific PNA hybridizations were performed using a Cy3-$(CCCTAA)_3$ PNA probe (DAKO) according to manufacturer's instructions. All FISH preparations were mounted and counterstained with VectaShield antifade medium (Vector), containing 0.1 μg/mL DAPI (Sigma). Digital images were captured in either a Metasystems or an Applied Imaging molecular cytogenetics workstation equipped with fluorescent Zeiss and Nikon microscopes, respectively. Telomeric PNA fluorescence quantification of interphase nuclei or individual chromatid ends by Q-FISH was performed using the Isis Software (Metasystems).

Immuno-FISH and immunocytochemistry. Naked nuclei and metaphase preparations were obtained through a modification of the standard cytogenetic harvest technique that excluded acetic acid fixation. Briefly, freshly fed and nearly confluent cell cultures were incubated with colcemid (0.01–0.04 mg/mL) for 0.5 to 16 h at 37°C. Cells were dislodged with trypsine-EDTA solution and centrifuged at 1,000 rpm for 10 min, and the supernatant was discarded. Hypotonic solution (0.075 mol/L KC1, prewarmed to 37°C) was added to the cell pellet, gently mixed by pipetting and incubated for 20 to 30 min at 37°C. The cells were washed with methanol. Cytologic preparations were made by dropping cell suspensions onto wet slides that were left to air dry and immediately were placed to 4°C. Fluorescence immunocytochemistry was performed after PBS washes, permeabilization with Triton X-100 (Applichem), and normal serum blocking. The CENP-A (Stressgen) or H2AX (Upstate) antibodies were applied at dilutions 1:50 to 1:500 for 2 h at room temperature. For antibody detection, we used a suitable Alexa-Fluor^{488} conjugated secondary antibody (1:500–1,000 in PBS; Molecular Probes). Slides were incubated for 1 h and washed with PBS, and then FISH was applied as described above. Dual-color immunocytochemistry was performed *in situ* on cells grown on coverslips or chamber slides. The TRF2 and PML (Santa Cruz Biotechnology) antibodies were applied at dilutions 1:500 and 1:100, respectively, in 1% bovine serum albumin (BSA) overnight at 4°C. For antibody detection, we used Alexa-Fluor^{568} (Molecular Probes) and IgG FITC (Santa Cruz Biotechnology) conjugated antibodies, respectively (1:500 in 1% BSA).

Chromatin immunoprecipitation. Chromatin immunoprecipitations (ChIP) were performed as described in Guan and colleagues (23). Briefly, cells were fixed by 1% formaldehyde for 10 min at room temperature. After fixation, glycine was added to a final concentration of 0.125 mol/L. Samples were washed with PBS and lysed with 0.25% Triton X-100 (Sigma), 0.5% NP40, 10 mmol/L EDTA, 0.5 mmol/L EGTA, 10 mmol/L Tris (pH 8.0), and 1 mmol/L phenylmethylsulfonyl fluoride (PMSF; Bio-Rad). Nuclei were collected and resuspended in 1 mmol/L EDTA, 0.5 mmol/L EGTA, 10 mmol/L Tris-HC1 (pH 7.5), and 1 mmol/L PMSF. Then the samples were sonicated, and chromatin was purified by cesium chloride centrifugation and dialyzed against 10 mmol/L Tris-HCl (pH 8.0), 1 mmol/L EDTA, 0.5 mmol/L EGTA, and 5% glycerol (Bio-Rad). The average size of DNA fragments was 500 to 1,000 bp. Immunoprecipitations were performed with or without the PML-19, RAD-50 (Santa Cruz), MRE-11 (Cell Signaling), or γ-H2AX (Upstate) antibodies. Centromeric binding of these proteins was evaluated after subsequent semiquantitative PCR. Suitable centromere-specific PCR primer pairs for ChIP were designed using the Vector NTI software into the amplification regions of single PCR primers previously shown to specifically amplify human centromeres of chromosomes 7 and X (5′-AGCGATTTGAGGACAATTGC-3′ and 5′-GTTCAGCTCTGTGAGTGAAA-3′, respectively; ref. 24). The dual primer sets were FW, 5′-AGCGATTTGAGGACAATTGC-3′ and REV, 5′-CTGTTGGCA TCAAATGGCTAGC-3′ (for centromere 7) and FW, 5′-CGAAC GAAGGCCACCCAGTGG-3′ and REV, 5′-CAACTCATGGAGTTGAACAATCC-3′ (for centromere X). Specificity of amplification was checked by the Primer3 software according to ENSEMBL (25). Quantitation of PCR band intensities was performed after image acquisition in a Dolphin imaging station using the ImageQuant (MD/APS software).

γ-Irradiation. The VA-13 and VA-13TA cell lines grown as subconfluent monolayers in six T-75 flasks were irradiated with 2.4 Gy of γ-rays in a γ-cell 220 irradiator (Atomic Energy of Canada Ltd.) with a dose rate of 1 Gy/min. Cells were incubated for 24 h and subcultured, and metaphases were collected after 72 h of culture and three consecutive intervals of 48 h. The whole experiment was performed twice. Dicentric and minute chromosomes per metaphase were counted after combined staining with pancentromeric and telomere-specific FISH as indicated above.

Chromatid orientation FISH. Analysis of tSCEs was done according to Bailey and colleagues (26), with minor modifications. In brief, subconfluent cell monolayers were cultured for 24 h into medium containing 3×10^{-3} mg/mL 5′-bromo-2′-deoxyuridine (BrdUrd; Sigma). Colcemid (0.1 μg/mL; Life Technologies) was added for 1 h before cell harvest. Metaphase spreads were prepared by conventional cytogenetic methods. Chromosome preparations were treated with 0.5 mg/mL RNase-A (Roche) for 10 min at 37°C, stained with Hoechst 33258 (0.5 μg/mL; Sigma), incubated in 2× SSC (Invitrogen) for 15 min at room temperature and exposed to 365-nm UV light (Stratalinker 1800 UV irradiator) for 30 min. The BrdUrd-substituted DNA was digested with Exonuclease III (Promega) in a buffer supplied by the manufacturer [5 mmol/L DTT, 5 mmol/L $MgCl_2$, and 50 mmol/L Tris-HCl (pH 8.0)] for 10 min at room temperature. Digested strand denaturation was performed at 70°C with 70% formamide (Applichem) in 2× SSC for 1 min. The slides were then dehydrated through a cold ethanol series (70%, 85%, and 100%) and air-dried. PNA-FISH was performed as above.

Statistical analysis. One-way ANOVA and Mann-Whitney analyses were performed using the MINITAB software. In all bar graphs, error bars represent the SE.

Results

The ALT pathway is characterized by ongoing pericentromeric instability. To investigate chromosomal anomalies in ALT cells, we combined centromere-specific and telomere-specific FISH with classic and molecular karyotyping in 10 telomerase-positive and 5 ALT continuous human cell lines (Supplementary Fig. S1*A*; Supplementary Table ST1). The ALT cell lines showed an extensive involvement of the acrocentric chromosomes in pericentromeric structural rearrangements ($P < 0.005$ by ANOVA; Fig. 1*A*). In addition, the ALT cells had a significantly higher propensity to form clonal neoacrocentric chromosomes (acrocentric derivatives of nonacrocentric chromosomes; $P < 0.0001$ by ANOVA) compared with the telomerase-positive cell lines (Fig. 1*B*). In all five ALT cell lines, neoacrocentric chromosomes were capped by telomeric repeats and were clonally maintained in subsequent and long-term (>20) passages (Supplementary Figs. S1*B*–S3). To evaluate the extent of pericentromeric instability, we mapped the recombination breakpoints of all structural chromosomal rearrangements detected by M-FISH in the five ALT cell lines. An unexpected clustering of centromeric and pericentromeric breakpoints was noted (Fig. 1*C*). To address if ALT pericentromeric instability is a continuous CIN process, we compared the molecular karyotypes of three sublines of the ALT human osteosarcoma cell line U2-OS (termed a, b, and c). These cells were grown in different laboratories for an undetermined number of population doublings (Supplementary Table ST1) and share significant karyotypic similarities. However, all three U2-OS sublines can be distinguished from each other because of unique clonal structural chromosomal rearrangements. Whereas many of the U2-OS neoacrocentric chromosomes persisted in clonal evolution, the karyotypes of the three U2-OS sublines were characterized by distinct pericentromeric chromosomal fissions or fusions (Fig. 1*D* and Supplementary Fig. S2). Analogous phenomena were present between different coexisting subclones of all the ALT cell lines of our study (Supplementary Fig. S3). Combined together, these observations support the view that pericentromeric instability is an ongoing CIN process that shapes stochastically the genomic evolution of ALT continuous growth.

ALT neoacrocentric chromosomes derived from true centromeric fissions due to increased rates of DNA double-strand breakage at centromeres. To further explore the mechanism(s) responsible for the emergence of mitotically proficient ALT neoacrocentric chromosomes, we examined the structural integrity of 71 clonal neoacrocentric chromosomes as detected by M-FISH, inverted DAPI, or G-banding in consecutive passages of the five ALT cell lines of our study. From these entities, 66 were solely composed from genomic material originating from a particular chromosome, whereas only five displayed complex interchromosome or intrachromosome rearrangements. In addition, from the 71 neoacrocentrics, 57 were the products of single centromeric breakpoints whereas 14 were derived from double pericentromeric breakage. These observations suggest that only 19 (5 + 14) of 71 ALT neoacrocentric chromosomes could be by-products of increased chromosome recombinogenicity and extreme fragmentation induced by the B/F/B cycles. The remainder 73% could be possibly attributed to increased rates of centromeric double-strand breaks (DSB). Interestingly, molecular karyotyping and centromeric FISH showed that the majority of clonal ALT neoacrocentric chromosomes of this study were derived from true centromeric fissions of metacentric or hypometacentric chromosomes (Fig. 2*A*). These phenomena were not present in the 10 telomerase-positive cell lines examined (data not shown). To investigate differences in centromeric instability between telomerase and ALT telomere restoration, we performed ChIP with antibodies against the MRE11, RAD50, PML, and γ-H2AX proteins. Immunoprecipitation was followed by semiquantitative PCR with dual primers designed to amplify into single-primer amplification regions specific for human metacentric centromeres 7 and X (24). The ALT cell lines U2-OS, Saos-2, and GM-847 exhibited a significantly higher enrichment in centromeric coprecipitation with all four of these DNA breakage sensor molecules (18, 27) compared with three telomerase-positive cell lines that showed no or lower enrichment (Fig. 2*B* and Supplementary Fig. S4*A*). In the same cell line group, we also performed immuno-FISH to investigate nuclear colocalization of the γ-H2AX antibody and DNA probes specific for centromeres 7 or X. These experiments showed again that the ALT context is characterized by significantly increased rates of γ-H2AX recruitment at centromeres (Supplementary Fig. S4*B* and *C*). Although correlative, these data suggest that the ALT pathway is characterized by extensively higher rates of DSBs at centromeres leading to highly elevated frequencies of true centromeric fissions compared with telomerase-dependent continuous growth.

The ALT pathway karyotypes exhibit a minute chromosome phenotype that is induced by exogenous chromosome breakage and suppressed by telomerase. A particular group of small neoacrocentric chromosomes was observed in all ALT cell lines of our study. These minute chromosomal entities were two to five times smaller than human chromosome 21 (Fig. 3*A*). The telomerase-positive cell lines of our panel did not show frequent formation of minute chromosomes (Fig. 3*B*). Minute chromosomes were present in virtually all ALT mitoses examined. They exhibited two-chromatid morphology, showed C-G and inverted DAPI-positive primary constrictions, were enriched in alphoid repeats, and contained telomere repeats. M-FISH showed that the vast majority of minute chromosomes also contained nonheterochromatic genomic DNA (Fig. 3*C*). In all five ALT cell lines, several minute chromosomes were composed from the short arms of small chromosomes, such as 17, 18, 19, or 20. However, even smaller minute chromosomes originated from bigger chromosomes, such as 1, 2, 3, 5, 7, 10, or 12 (Supplementary Figs. S2 and S3). Based on these observations, we distinguished ALT minute chromosomes in two main categories: (*a*) those produced by single pericentromeric breakpoints on acrocentric or small hypometacentric chromosomes (42%) and (*b*) minute entities originating from double pericentromeric breakpoints occurring in large metacentric or hypometacentric chromosomes (58%; Supplementary Fig. S5). The minute chromosomes of the second category were possibly by-products of B/F/B induced extreme chromosome breakage (12, 13). Chromosomal fragmentation was significantly more frequent in the ALT cells compared with the telomerase-positive cell lines of our panel and was suppressed upon reconstitution of telomerase activity (Supplementary Fig. S6). Moreover, telomere capping of fragmented chromosomes was observed only in the ALT cell lines (Supplementary Fig. S5*A*). Because the minute chromosomes were frequent in the ALT cells, we reasoned that telomerase activity might suppress this phenotype. We found that the TRAP-positive VA-13TA cells (21) showed a significant reduction in the percentage of metaphases with random dicentric chromosomes and significantly lower frequencies of minute chromosomes compared with their parental ALT VA-13 cell line (Fig. 3*D*). To investigate the effects of exogenously induced random chromosome breaks in the *de novo* emergence of minute chromosomes, we exposed both

telomerase-positive and telomerase-negative VA-13 cells to 2.4 Gy of γ-radiation. Telomerase activity affected the karyotype of irradiated cells, as evidenced by a significant increase of the relative numbers of minute chromosomes only in the ALT VA-13, but not in VA-13TA, cells (Fig. 3*D*). Collectively, these data can be interpreted to suggest that, in the ALT pathway, centromeric fissions and B/F/B cycle–driven extreme chromosome fragmentation, coupled to stochastic telomere capping of DNA DSBs, are

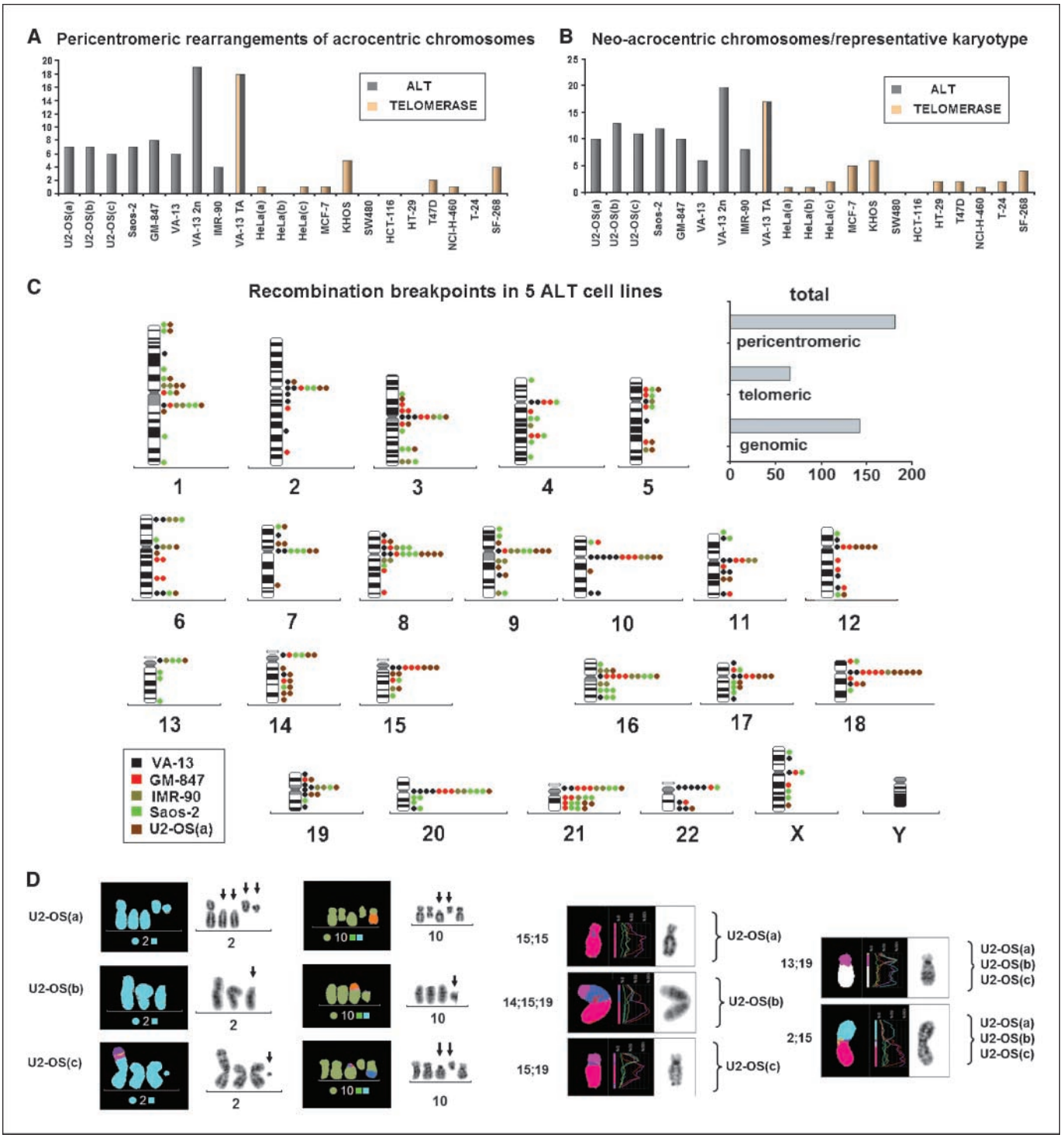

Figure 1. The ALT pathway is characterized by ongoing pericentromeric instability. Pericentromeric rearrangements of acrocentric chromosomes (*A*) and numbers of neoacrocentric chromosomes (*B*) in the representative karyotypes of 5 ALT and 10 telomerase-positive cell lines. The telomerase-positive VA-13TA and an endoreduplicated clone of the ALT VA-13 cell line are also depicted (*A, B*). Clustering of pericentromeric recombination breakpoints in the major clones of the five ALT cell lines of our study, as detected by molecular and conventional karyotyping (*C*). M-FISH shows random emergence, nondisjunctions, or losses of neoacrocentric chromosomes (*arrows*), deriving from both arms of chromosomes 2 and 10, in the clonal evolution of the U2-OS cell line. Examples of common and unique pericentromeric rearrangements in the clonal evolution of three karyotypically distinct U2-OS sublines; inverted DAPI banding patterns and minimal values in the adjacent fluorescence intensity graphs indicate that centromeric heterochromatin is localized at the recombination junction points (M-FISH and inverted DAPI ×1,000; *D*).

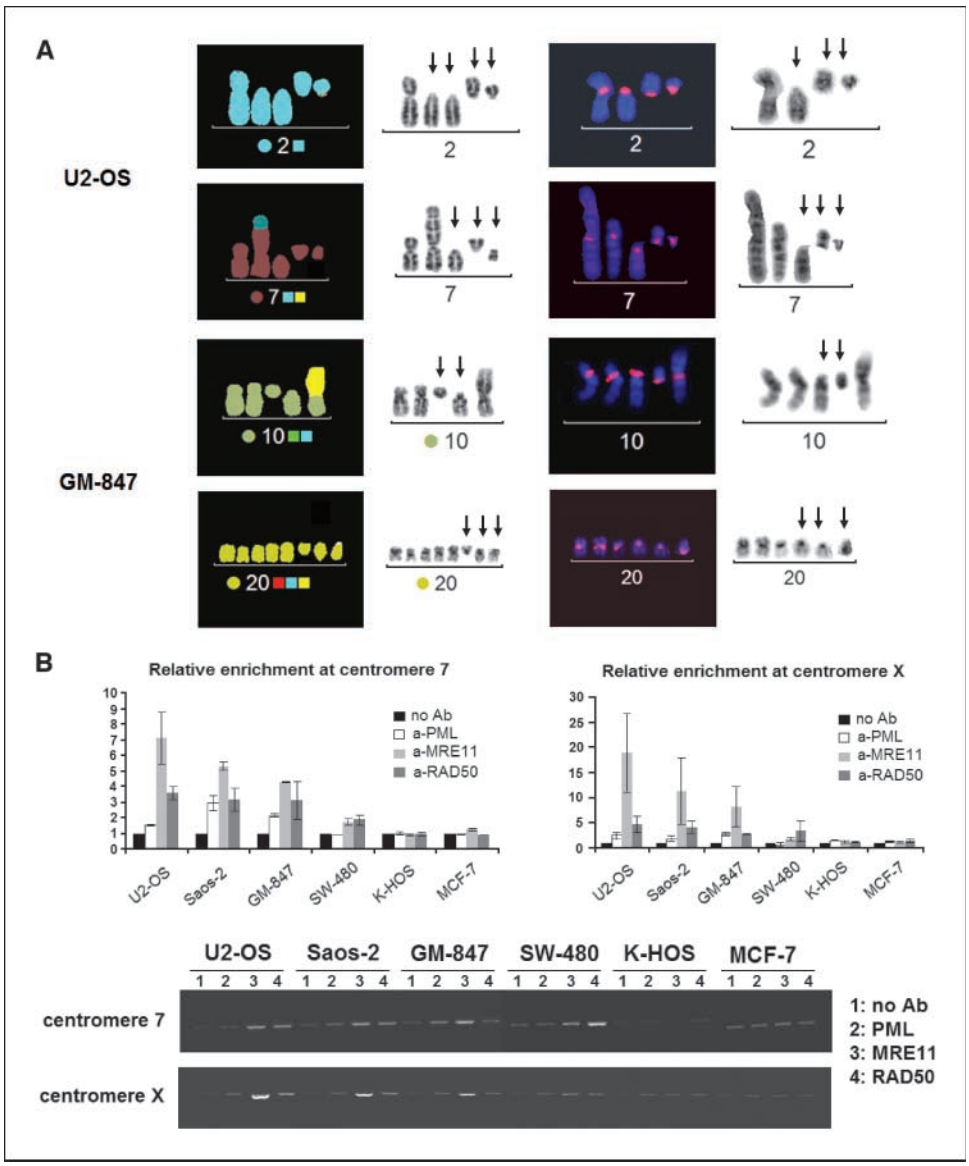

Figure 2. Clonal ALT neoacrocentric chromosomes derived from true centromeric fissions due to increased rates of DNA double-strand breakage at centromeres. Examples of true centromeric cleavage in partial karyotypes of the ALT cell lines U2-OS and GM-847. Red color spots represent specific probes for centromeres 2, 7, 10, and 20 (multicolor karyotyping/centromere-specific FISH and inverted DAPI ×1,000). Note that both pericentromeric breakpoints maintain α-satellite repeats deriving from their original centromere. M-FISH shows no additional structural rearrangements of cleaved chromosomes and rules out classic B/F/B cycles as a main mechanism for the generation of ALT neoacrocentrics (*A*). ChIP showed significant differences in the rates of DNA DSBs at the interstitial centromeres 7 ($P < 0.0001$ by one-way ANOVA) and X ($P = 0.021$), between three ALT and three TRAP positive cell lines, as shown by the highly elevated relative enrichment of ALT centromeric DNA to cross-linked chromatin precipitates of the MRE11, RAD50, and the PML antibodies (*B*).

capable to generate human minute chromosomes. The emergence of minutes is induced by γ-radiation and suppressed by the introduction of telomerase activity.

Continuous growth after telomerase inhibition leads to spontaneous emergence of human minute chromosomes. Because telomerase activity suppressed the frequencies of endogenously and exogenously emerging ALT minute chromosomes, we were interested to examine the effects of telomerase inhibition on this phenotype. It has been shown that the mismatch repair (MMR)–deficient human colon cancer cell line HCT-15 is characterized by an episodic ALT-like elongation of telomeres and increased rates of telomeric recombination after dominant-negative inhibition of telomerase (7). We used multiple subcultures of those cells to test if chromosomal instability coupled to telomeric recombination can lead to spontaneous emergence of minute chromosomes. We found that the expression of the hTERT antimorph (7) resulted in highly diminished TRAP activity and a dramatic increase in the frequencies of dicentric chromosomes due to extreme telomere erosion. Spontaneous emergence of random and clonal minute chromosomes was evident only in the TRAP-negative clones (Fig. 4*A*). We then examined the effects of serial transient telomerase silencing in the MMR-proficient, TRAP-positive, human colon cancer cell line SW-480. According to our observations, this cell line exhibits relatively increased numbers of nuclear colocalization of the telomere binding factor TRF2 and an antibody against the PML protein in structures apparently resembling APB bodies. In addition, we found that the SW-480 cells display elevated frequencies of endogenous tSCEs. One interpretation of these observations is that the SW-480 cells might represent an intermediate condition between ALT and telomerase activity (Supplementary Fig. S7). The efficiencies of our telomerase knockdown experiments, as estimated by the use of *in situ* immunocytochemistry against hTERT and reverse transcription–PCR for the cDNA of hTERT, varied between 10% and 57% (Fig. 4*B* and Supplementary Fig. S7*B*). In SW-480 cells, prolonged telomerase depletion increased significantly the telomeric length and deviation,[6] as well as the total numbers of dicentric

[6] A. Christodoulidou, M. Chiourea, C. Raftopoulou, and S. Gagos, unpublished data.

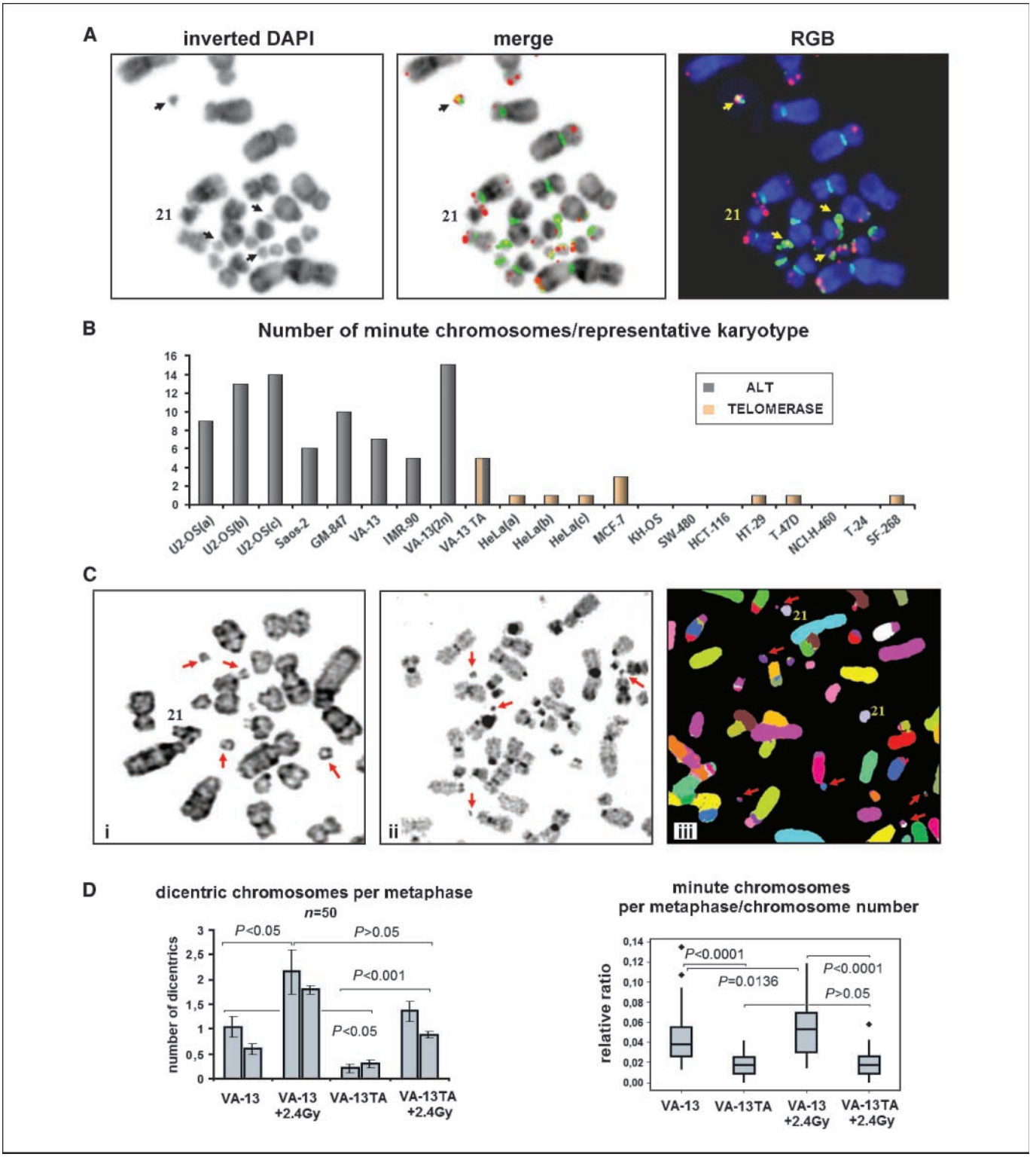

Figure 3. ALT karyotypes exhibit a minute chromosome phenotype that is induced by exogenous chromosome breakage and suppressed by telomerase. ALT minute chromosomes (*arrows*) bear alphoid and telomeric repeats. Partial metaphase spread from the U2-OS cell line hybridized with telomeric PNA (*red*)/pancentromeric FISH (*green*) and counterstained with DAPI (*blue gray*) ×1,000 (*A*). The numbers of minute chromosomes per representative karyotype were significantly elevated in the ALT cell lines ($P < 0.0001$ by one-way ANOVA; *B*). GTG-Banding (*i*), C-Banding (*ii*), and M-FISH (*iii*) show that ALT minute chromosomes (*arrows*) are significantly smaller than human chromosome 21, retain a double-chromatid structure (*i*), are mainly composed from constitutive heterochromatin (*black; ii*), and carry nonheterochromatic genomic DNA (*iii*; U2-OS cells ×1,000; *C*). Reconstitution of telomerase activity inhibits the formation of spontaneous or γ-irradiation induced minute chromosomes. The VA-13 cells show elevated rates of dicentric and minute chromosomes compared with the TRAP+ VA-13TA cells (Mann-Whitney test). The frequencies of dicentric chromosomes were significantly elevated in both telomerase-positive and telomerase-negative VA-13 cells after 2.4 Gy of γ-radiation (Mann-Whitney test). The number of minute chromosomes per metaphase and per chromosome number was significantly increased in the ALT cells, whereas the VA-13TA cell line showed no differences before and after exposure to γ-irradiation (*D*).

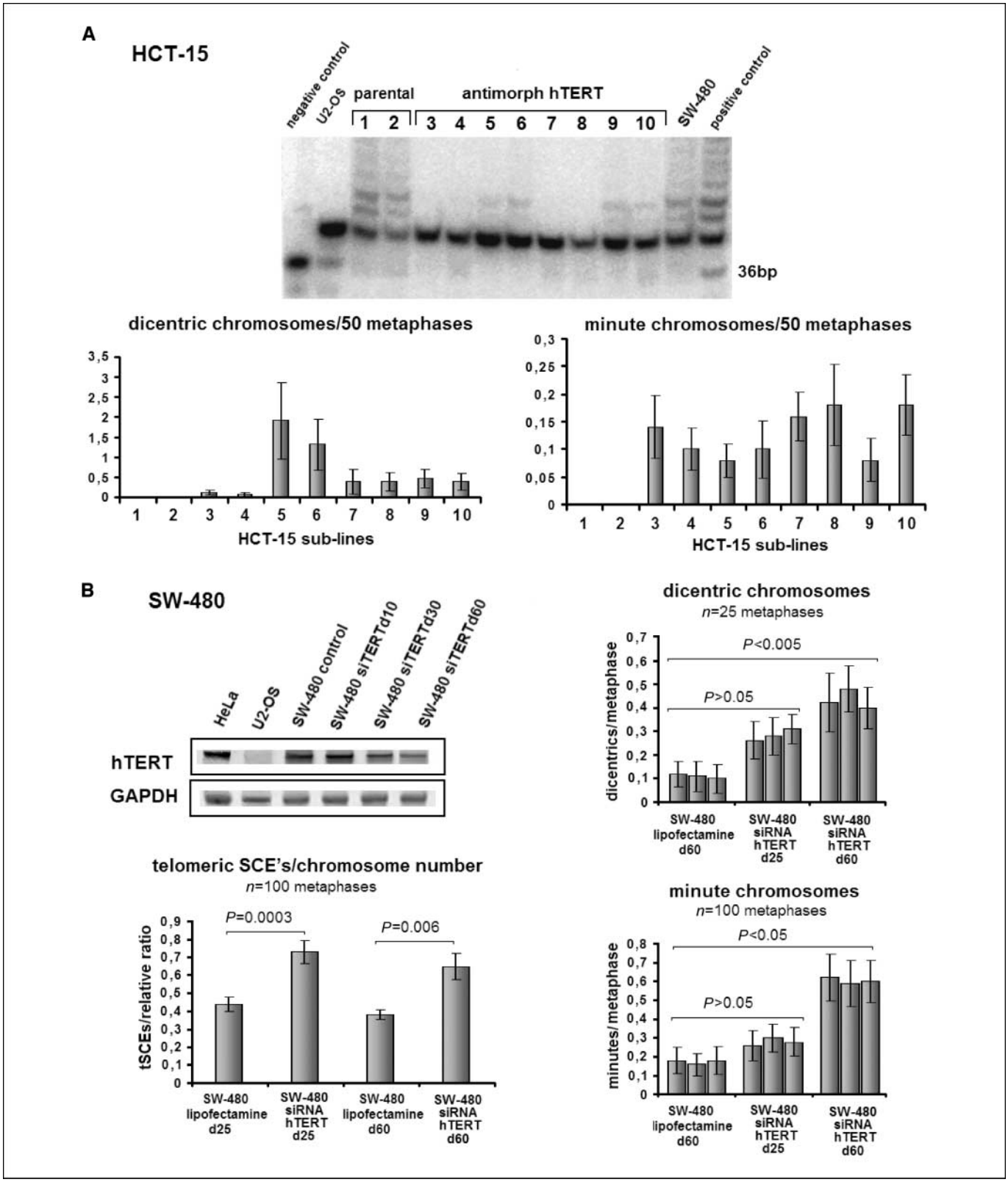

Figure 4. Telomerase inhibition/depletion leads to spontaneous emergence of human minute chromosomes. Engagement of ALT-like recombinatorial telomere restoration after introduction of an antimorph against hTERT leads to the emergence of minute chromosomes. The TRAP+ HCT-15 cell line (SL1-SL2) shows highly diminished or completely eliminated telomerase activity in consecutive population doublings (SL3-SL10; PD27-PD107) after the expression of the hTERT antimorph (gel). Internal reference band is indicated at 36 bp. Note that the frequencies of dicentric chromosomes are highly elevated throughout telomerase-independent continuous growth and especially during first crisis (SL5-SL6). Crisis is immediately accompanied by an increase in the numbers of spontaneously induced minute chromosomes (*A*). Various efficiencies of hTERT RNA interference (see Supplementary Fig. S5) led to higher frequencies of dicentrics, tSCEs, and minute chromosomes in the SW-480 cells (*B*).

chromosomes, APB bodies, tSCEs, and minute chromosomes, compared with Lipofectamine-treated controls (Fig. 4*B* and Supplementary Fig. S7*D*). The above findings suggest that continuous growth after telomerase inhibition in two human colon cancer cell lines is accompanied by the engagement of an ALT-like phenotype and the spontaneous emergence of neoacrocentric and minute chromosomes.

Clonal ALT minute chromosomes are mitotically functional. To verify that neoacrocentric and minute chromosomes are characteristic cytogenetic findings of the ALT pathway, we examined three additional human ALT cell lines (kindly donated by D. Broccoli and A. Godwin). This analysis confirmed high incidences of neoacrocentric and minute chromosomes (Supplementary Table ST1). We then used the U2-OS cell line as a model to investigate the origin and mitotic stability of the centromeres that participate in the formation of minute chromosomes. α-Satellite probes specific for chromosomes 2, 3, 7, 12, 17, 18, 20 and the acrocentrics hybridized on U2-OS(a) minute chromosomes, whereas similar probes specific for centromeres 1, 4, 6, 8, 10, 11, and X did not. Centromere of chromosome 18 was the most frequently found (2–4 minute chromosomes per metaphase), followed by that of chromosomes 3 (1–3 minute chromosomes per metaphase), chromosomes 2, 7, 12, 19, 20, and that of acrocentrics (1–2 minute chromosomes per metaphase; Fig. 5*A*). Several-minute chromosomes persisted through continuous evolution of all five ALT cell lines and were present in the distinct karyotypes of U2-OS and VA-13 sublines (Supplementary Figs. S2 and S3). These findings suggest that the centromeres of ALT minute chromosomes maintain a regular mitotic functionality. To further investigate this, we performed immuno-FISH in U2-OS(a) cells against the CENP-A protein that associates with mitotically active centromeres (28). Colocalization of double CENP-A dots with all specific fluorescent spots representing centromeres of chromosomes 3 or 18 per interphase nucleus was observed in all 200 nuclei examined (Fig. 5*B*). Therefore, most of the U2-OS(a) minute chromosomes were mitotically functional. These results suggest that the minute chromosomes observed in human ALT cell lines are mitotically proficient.

Discussion

ALT telomere dysfunction due to extreme telomere shortening has been associated with chromatid or chromosome end-to-end fusions and the initiation of B/F/B cycles (11, 12). In this context, it is expected that chromosome recombination breakpoints should be mostly telomeric (29). In contrast to these expectations, we observed a clustering of centromeric and/or pericentromeric breakpoints in five ALT cell lines of our study. Moreover, the frequent emergence of random pericentromeric chromosomal fissions or fusions, in the clonal evolution of ALT sublines, indicated that these lesions reflect a continuous CIN process that is characteristic of the ALT cells. Human osteosarcomas that display a high prevalence of the ALT pathway (4) exhibit increased frequencies of centromeric recombination (13, 30) and generally have a very poor prognosis.

The structural integrity of most clonal neoacrocentric chromosomes encountered in the ALT karyotypes of this study indicated that the majority of ALT centromeric fissions are not typical

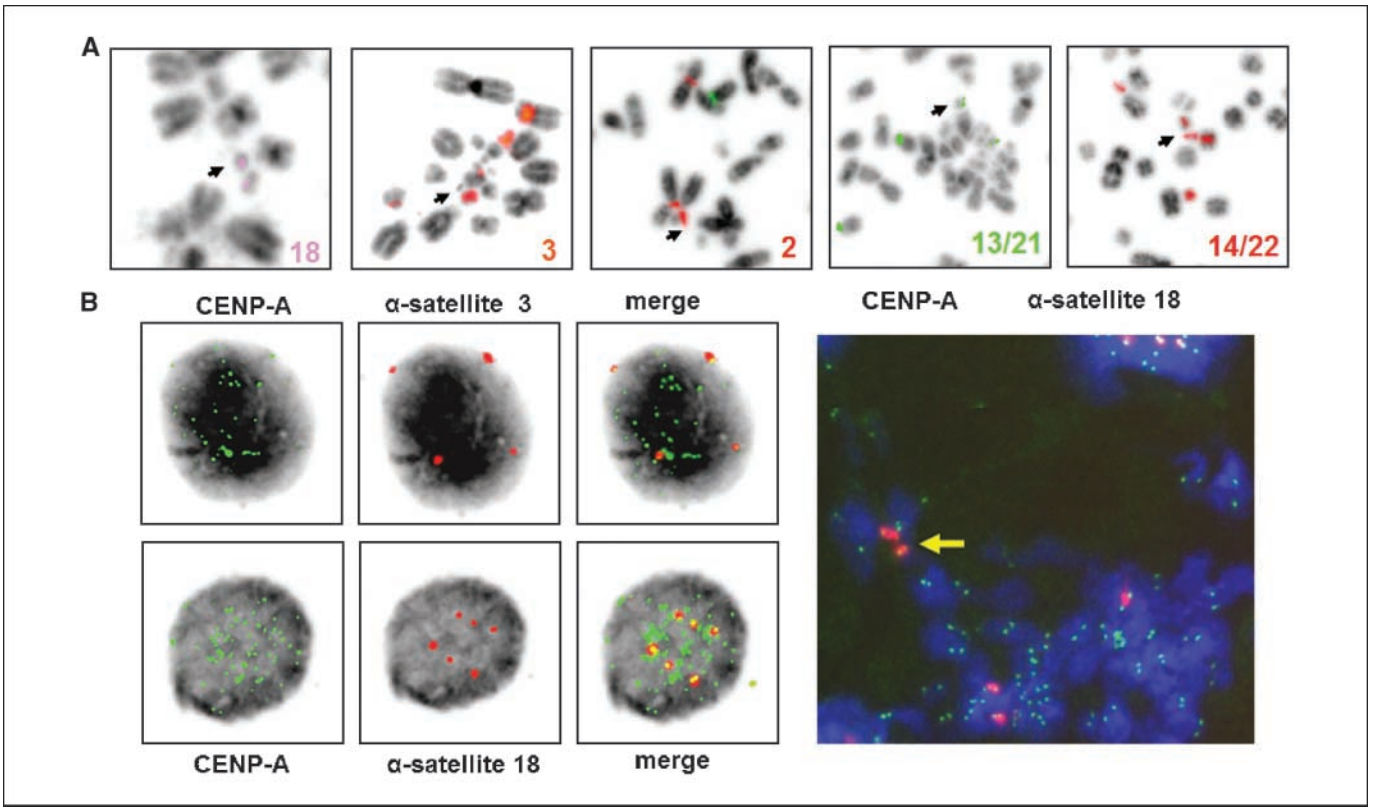

Figure 5. Clonal ALT minute chromosomes are mitotically functional. Centromere-specific FISH in the U2-OS(a) subline shows that centromeres from chromosomes 2, 3, 12, and 18 and the acrocentrics (13/21, 14/22) were involved in the formation of minute chromosomes (*A*). Mitotic functionality of most of the U2-OS(a) minute chromosomes is indicated by 100% nuclear colocalization of the CENP-A protein (*green*) with all centromeres 3 and 18 (*red*; inverted DAPI is gray; *B*).

consequences of B/F/B cycles that usually produce inverted duplications or complex interchromosomal rearrangements (12). Although centromeric cleavage is considered exceedingly rare between both constitutional and acquired chromosome aberrations (31), our cytogenetic findings strongly support the hypothesis that the ALT pathway is characterized by elevated rates of DNA double-strand breakage at centromeres. We confirmed this hypothesis by ChIP-PCR and immuno-FISH, because metacentric α-satellite DNA was found to be highly enriched for γ-H2AX, MRE11, and RAD50 and, to a lesser extent for the PML protein, only in the ALT cell lines. All of these four proteins are engaged in DNA repair of DSBs (18, 27, 32, 33).

Although small marker chromosomes have been recorded in chromosome preparations from human tumor biopsies and immortalized cell cultures (National Center for Biotechnology Information Cancer Chromosomes database),[7] up to the present report, there are no specific links of those entities to a particular biological mechanism. Our data suggest that the ALT pathway is capable of producing various numbers and sizes of neoacrocentric or minute derivatives of virtually all human chromosomes through centromere fissions or B/F/B cycles. Spontaneously evolving minute chromosomes with telomeric DNA have been reported in mouse cells deficient for the recombinatorial proteins ERCC1 and XPF (34), whereas telomere capped minute chromosomes were present in DAPI metaphases of mouse cells lacking the RNA component of telomerase (5, 35).

The basic elements required for mitotic stability of linear chromosomes, centromeres, telomeres, and origins of replication (36) have been herein, directly or indirectly, shown to be included into human ALT minute chromosomes. The consistent binding of CENP-A and the clonal presence of ALT minute chromosomes in consecutive passages and evolutionarily distant sublines of this study denoted that ALT minutes use classic mitotic functionality to perpetuate in culture (28). Therefore, ALT minute chromosomes are different from the well-characterized double-minute chromosomes encountered between dividing cells of many cancer biopsies and cell lines (37). According to International System of Cytogenetic Nomenclature (38), double minutes represent neoplasia-specific, acentric structures.

Telomerase exerted critical effects in altering the emergence of ALT minute chromosomes. Reconstitution of telomerase activity in ALT cells stabilized the karyotype, maintained several preexisting clonal neoacrocentric and minute chromosomes, but reduced the overall number of dicentrics and minutes within the cell population. In addition, whereas γ-irradiation induced elevated rates of dicentric chromosomes or extreme chromosome fragmentation in both telomerase-positive and parental ALT cells, the emergence of minute chromosomes was significantly elevated only in the ALT background. Telomerase is confined to act at the telomeres, whereas in the ALT pathway, every DNA DSB comprises a substrate for recombinatorial telomere capture (39). Moreover, the presence of functional centromeres in the broken ALT chromosomal segments is imperative for mitotic continuation and clonal selection. Telomere capped, but acentric, DNA fragments will be lost during continuous cellular proliferation. It is possible that telomerase activity suppresses the minute chromosome phenotype in two ways; first, as a reverse transcriptase, by ameliorating the consequences of extreme telomere attrition, such as the dicentric formations and extended chromosome fragmentation (1), and second, by inhibiting telomeric recombination. Telomere recombination in yeast and human cells has been shown to be suppressed by reconstitution of telomerase activity (21, 40). In contrast, continuous growth after telomerase inhibition/depletion in either MMR-deficient or MMR-proficient telomerase-positive, human colon cancer cell lines resulted in an episodic ALT-like phenotype and an increase in tSCEs, dicentrics, chromosome fragmentation, and spontaneous emergence of random and clonal minute chromosomes.

Our data establish for the first time a relation between increased heterochromatic instability and the ALT pathway. In yeast, flies, and mice, a defective heterochromatin state leads to abnormal telomere function and length regulation (41, 42). Importantly, the description of spontaneous emergence of ALT human minute chromosomes provides the grounds for further research on these autonomous DNA entities that contain pericentromeric genomic material and maintain regular mitotic functionality. ALT minute chromosomes can provide important information for the function of normal and cancer genome and could be exploited as putative nonintegrating large-scale cloning vectors for gene therapy.

Disclosure of Potential Conflicts of Interest

No potential conflicts of interest were disclosed.

Acknowledgments

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[7] NCBI Cancer Chromosomes (http://www.ncbi.nlm.nih.gov).

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