CAN-08-0945R

Pericentromeric instability and spontaneous emergence of human neo-acrocentric and minute chromosomes in the alternative pathway of telomere lengthening

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Supplementary Material on line

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1. Supplementary Materials and Methods

TRAP assays

Telomerase activity of cell lysates was analyzed by telomeric repeat amplification protocol (TRAP) assay with a TRAPeze Telomerase Detection kit (Intergen) according to manufacturer's instructions. Approximately 10^6 cells were harvested and lysed in 400µl of 1x CHAPS lysis buffer [Tris-HCl 10 mM, pH 7.5; 1mM EGTA, 1mM $MgCl_2$, 0.5% CHAPS, 10% glycerol, DEPC treated water] on ice for 30 min. Cell debris were spun down for 20 minutes at 12,000 x g at 4°C. Each reaction was carried out by using 2µl of supernatant, 1µl of each primer, 0.5µl of Taq-Polymerase (TAKARA), 10 µl of solution-Q (Qiagen), 5µl of 10x buffer, 2 µl of dNTPs, in DEPC treated water in final volume of 50 µl. The primers used for the TRAP-assay PCR were TS-5'-AATCCGTCGAGCAGAGTT-3' and Cxa-5'-GTGTAACCCTAACCCTAACCC-3'. The PCR program consisted first of an incubation at 30°C for 30 min and then in a thermocycler, 94°C for 2 min; 94°C for 30s, 50°C for 25s, 72°C for 30s (33x); 72°C for 1min. PCR products were electrophoresed in a 10% 19:1 acrylamide gel (Sigma) /0.5x TBE buffer using the mini protean II gel system (Biorad). Gels were stained either with 2µl of SYBR Green (Sigma) for 15 min at room temperature in 50 ml of TBE 0.5 x buffer, and then exposed to UV light and visualized by a Kodak image acquisition station or by radioisotopic detection using P^{32} labeled dCTPs (Amersham). Radio-labelled gels were dried in a Heto Lyo Pro3000 Dryer, and visualised with a Typhoon 9200 Imager.

Telomerase silencing

The telomerase positive SW-480 cell line was transiently transfected using Lipofectamine™ 2000 (Invitrogen) according to the manufacturer's instructions, with ds

siRNA targeted against hTERT (Ambion) (sense 5'-GGCUCUUUUUCUACCGGAAtt-3', antisense 5'-UUCCGGUAGAAAAAGAGCCtg-3'). Cells were seeded in T25 flasks and/or 6-well plates at appropriate cell densities one day before transfection, in order to be sub-confluent the first day of the experiment. The siRNA/Lipofectamine ratio was 20 pmol/1μl. The amount of siRNA, Lipofectamine and OptiMEM medium (Gibco) was proportional to the surface area and the cell number according to manufacturer. SW-480 cells were subjected to sequential transfections with siRNA, separated by 72-96 h intervals. Cells were maintained in culture for 25, 30 or 60 days prior harvest. The experiment was performed 3 times. Lipofectamine treated (control) and telomerase silenced cells were harvested for karyotype analysis with pancentromeric and PNA telomere specific FISH as described in supplementary material on line. Parallel cultures were immunocytochemically stained *in situ* with a rabbit monoclonal antibody against hTERT (Epitomics) and detected by the appropriate secondary anti-rabbit antibody (Santa Cruz). Semi-quantitative Reverse Transcription PCR (RT-PCR) was performed as follows: 2μg of total RNA were isolated from each SW-480 sub-line and incubated at 70°C for 5 min with 0.5μg oligo T17 (Custom-made from the University of Crete). The PCR mix contained 2μl 10xRT Buffer (Promega), 2.4μl MgCl2 25mM, 1μl 4dNTPs (Promega), 5mM, 0.5μl Reverse Transcriptase (Promega) and H_2O to a total of 20μl. The transcription reaction was performed at 25°C for 5 min, 42°C for 1hr, 70°C for 15min and then to 4°C. Amplification of the hTERT cDNA was done using 5μl of the following primers 5'-AGGCTGCAGAGTGCAGAGCAGCGTGGAGAGG-3', and 5'-GCCTGAGCTGTACTTTGTACTTTGTCAA-3' (Custom-made from the University of Crete). The PCR mix contained 10μl of 10x amplification buffer (Invitrogen), 4μl of

5mM dNTPs (Invitrogen), 0.5μl of 2.5 units Taq/DNA Polymerase (Invitrogen) and 70.5μl H_2O. The PCR products were analyzed by electrophoresis in 8% non-denaturing polyacrylamide gels stained with SYBR Green (1:10000), images were acquired in a Dolphin imaging station and bands were quantified using the ImageQuant (MD/APS software).

Sister Chromatid Exchange (SCE) assays

Sub-confluent cell monolayers were cultured into medium containing 5′-bromo-2′-deoxyuridine (BrdU) (Sigma) in a total concentration of $3x10^{-3}$ mg/ml for 48h. Colcemid (0.1μg/ml) (Gibco) was added for an hour prior cell harvest. Metaphase spreads were prepared by conventional cytogenetic methods. Chromosome preparations were stained with 0.5μg/ml Hoechst 33258 (Sigma) in 1xPBS (Sigma) for 20min at RT, rinsed with dH_2O and air-dried. Slides mounted with coverslips in 2xSSC, were then exposed to 365-nm UV light (Stratalinker 1800 UV irradiator) for 40min. Slides were further exposed to 2xSSC at 60^oC for 30min, rinsed with dH_2O and allowed to air-dry. Preparations were stained with Giemsa (BDH) in pH 6.8. Images were acquired using a Zeiss J1 Imager microscope and the Ikaros software (Metasystems).

2. Supplementary Figure Legends

Supplementary Figure S1: Telomere Repeat Amplification Protocol (TRAP) assays in the cell lines of our panel. DNA ladders reveal telomerase activity. Poly-acrylamide gels were radio-isotopically stained using P^{32} labeled dCTPs. Internal reference band is indicated at 36 bp (A). A telomeric/pancentromeric FISH/DAPI karyotype from the ALT VA-13 cell line: Our criterion for neo-acrocentric chromosomes (arrows) was based on FISH co-localization (yellow) of centromeric (green) and telomeric repeats (red) at the tips of rearranged chromosomes. In a 350-400 band state, these events represent distances among centromeric and telomeric repeats ranging between 12-17Mb (by ENSEMBLE) (25) (Inverted DAPIx1000) (B).

Supplementary Figure S2: Pseudo-colored M-FISH and inverted DAPI karyotypes of the major clones of 3 U2-OS sub-lines that represented stochastic outcomes of chromosomal evolution in vitro (see table ST1). The presence of common structural anomalies (signature aberrations) indicates the monoclonal origin of all three sub-lines (red asterisks). However, U2-OS (a, b and c) could be distinguished from each other, on the basis of independent evolution of clonal structural chromosomal rearrangements (blue asterisks). Note the increased random structural instability of the acrocentric chromosomes (13-15, 21-22) and high rates of unsystematic pericentromeric rearrangements. Several neo-acrocentrics (black arrows) and minute chromosomes (red arrows) were stably preserved through continuous growth indicating that those entities maintained classical mitotic functionality.

Supplementary Figure S3: Pseudo-colored M-FISH and inverted DAPI karyotypes of three cytogenetically distinct VA-13 side-lines. A predominant VA-13 clone is depicted in the upper part of the figure. After endoreduplication (middle) and in the presence of telomerase activity, many pre-existing VA-13 neo-acrocentrics (black arrows) and several of the mini-chromosomes (red arrows) were clonally retained in duplicated copies. Note the spontaneous emergence of minute or neo-acrocentric chromosomes in the endoreduplicated ALT VA-13 subclone (VA-13 2n).

Supplementary Figure S4: Chromatin Immunoprecipitation (ChIP) demonstrates significant differences in the enrichment of γ-H2AX at the interstitial centromeres 7 and X, between three ALT and three TRAP positive cell lines ($p=0.006$ by ANOVA) (A). Increased rates of nuclear co-localization of centromeric repeats specific for centromeres 7 or X, and an antibody against the DNA DSB binding γ-H2AX are detected only in the ALT context: Immuno-fluorescence microscopy images illustrate *in situ* cultures of 3 telomerase positive and 3 ALT cell lines immuno-stained with an antibody specific for γ-H2AX (green-FITC) and hybridized with a DNA probe specific for human centromere 7 (Texas-Red). Signal co-localization is yellow (arrows). Graphs show that the differences in the total numbers of co-localized spots, between telomerase and ALT are statistically significant (by One-way-ANOVA) (error bars represent standard deviation between two microscopists and two *in situ* cultures) (B).

Supplementary Figure S5: Stochastic telomere capture in fragmented chromosomes leads to the emergence of ALT minute chromosomes: Example of random telomere

capping in the ALT VA-13 cell line (A). Minute chromosomes in the size of the short arms of small metacentric or hypo-metacentric chromosomes (i.e.: chromosomes 16, 17, 18, 19 and 20) emerge through true centromeric fissions followed by subsequent recombinatorial telomere capture (B). Pericentromeric DSBs and the generation of human minute chromosomes deriving from acrocentric chromosomes (i.e.: chromosomes 13, 14, 15, 21 and 22) (C). Extreme chromosome fragmentation due to B/F/B cycles, anaphase lagging or delayed entry into mitosis, generates ALT minute chromosomes derivatives of big metacentric or hypo-metacentric chromosomes such as 1-12, or the X chromosome (D). In all cases, recombinatorial telomere capping will ensure clonal persistence only to the fragments that retain centromeric repeats and mitotic functionality.

Supplementary Figure S6: An example of centromere repeats in fragmented chromosomes from a Saos-2 metaphase (pancentromeric FISH=green, inverted DAPIx1000) (A). The ALT pathway is characterized by high rates of extreme chromosome fragmentation ($p=0.006$ by one-way ANOVA). Note that telomerase activity suppresses chromosome fragmentation in the VA-13 TA cell line. Induction of chromosome fragmentation is achieved in both VA-13 and VA-13TA cells after exposure to 2.4 Gy of γ-irradiation (B).

Supplementary Figure S7: A comparison of SCE and tSCE assays in the TRAP positive SW-480 cells reveals increased rates of endogenous sister chromatid telomeric recombination (Mann-Whitney test). An example of normal and recombinant chromosomes by both methods is depicted (A). Immunocytochemistry for hTERT

(green), after serial transient transfections with siRNA against hTERT: U2-OS cells (negative control), SW-480 (lipofectamine control), SW-480+siRNA hTERT/30days, and SW-480+siRNA hTERT/60days are depicted (counter-stain DAPIx400). Serial transient transfections with siRNA against hTERT, were capable to reduce the total amount of telomerase up to 57% (B). Confocal microscopy in dual color immuno-cytochemistry with antibodies specific for the proteins PML (green) and TRF2 (red) reveals endogenous APB bodies in the SW-480 cell line (arrows). Images were acquired in a Leica TCS SP5 confocal microscope and superimposed with the aid of the Image-J Software (C). According to our unpublished observations, immuno-fluorescence microscopy tends to over-estimate the total numbers of APBs in a cell population. However, it is less laborious than confocal microscopy, does not suffer from rapid signal bleaching, and since its error is randomly distributed in every specimen, it can provide secure comparative results. Merged immuno-fluorescence microscopy images (DAPI=blue, PML=green and TRF2=red), indicate elevated rates of APBs in SW-480 cells after telomerase depletion (arrows). Graphs display significant increase in the numbers of APB bodies only in the siRNA treated cells (Mann-Whitney test). U2-OS and HeLa cells serve as positive and negative control respectively (D).

3. Supplementary Table ST1:

The continuous human cell lines of our panel; telomerase activity (TA) is indicated as (+) or (-) by TRAP assay. Numbers of structural chromosome anomalies (CA), neo-acrocentric chromosomes (NA) and minute chromosomes (Min) per representative karyotype are indicated (*=TRAP assays in Supplementary Ref.1; **= Supplementary Ref.2; TRAP negativity was confirmed by personal communication from D. Broccoli; HOSE=Human Ovarian Surface Epithelium).

Cell line	Origin	Source	TA	CA	NA	Min
HeLa(a)	Cervical cancer	Thanos lab (ECACC)	+	16	1	1
HeLa(b)	Cervical cancer	Irminger-lab (DSMZ)	+	23	2	1
HeLa(c)	Cervical cancer	Antonarakis lab (ATCC)	+	13	2	1
T47D	Breast cancer	Gonos lab (ATCC)	+	17	4	1
SW480	Colon cancer	ATCC	+	14	1	0
MCF-7	Breast cancer	Dimas lab (ECACC)	+	36	6	3
HCT-116	Colon cancer	Dimas lab (ECACC)	+	3	0	0
HT-29	Colon cancer	Dimas lab (ECACC)	+	9	4	1
NCI-H-460	Lung cancer	Dimas lab (ECACC)	+	8	2	0
SF-268	Glioblastoma	Dimas lab (ECACC)	+	18	3	1
KHOS	Osteosarcoma	Gonos lab (ATCC)	+	26	4	0
T-24	Bladder Cancer	Vlahou lab (ATCC)	+	8	0	0
HCT-15	Colon cancer	Shay lab (ATCC)	+	3	0	0
HCT-15 antiTERT	Colon cancer	Shay lab (ATCC)	-	9	1	1
U2-OS(a)	Osteosarcoma	Antonarakis lab (ATCC)	-	48	24	9
U2-OS(b)	Osteosarcoma	Gonos lab (ATCC)	-	52	21	13
U2-OS(c)	Osteosarcoma	Gorgoulis lab (ATCC)	-	51	26	14
Saos-2	Osteosarcoma	ATCC	-	36	8	5
HIO-107	Transformed HOSE	Godwin lab	-*	66	34	6
HIO-118	Transformed HOSE	Godwin lab	-*	30	10	3
LiSa2	Liposarcoma	Broccoli lab	-**	73	38	11
GM-847	Transformed fibroblasts	Londono lab (Reddel lab)	-	53	31	10
VA-13	Transformed fibroblasts	Londono lab (Reddel lab)	-	44	22	7
IMR-90	Transformed fibroblasts	Londono lab (Reddel lab)	-	26	10	5
VA-13 TA	Transformed fibroblasts	Shay lab	+	60	23	5

4. Supplementary References

Supplementary Reference S1: Grobelny JV, Godwin AK, Broccoli D. ALT-associated PML bodies are present in viable cells and are enriched in cells in the G(2)/M phase of the cell cycle. J Cell Science 2000;113:4577-85

Supplementary Reference S2: Wabitsch M, Brüderlein S, Melzner I, et al. LiSa-2, a novel human liposarcoma cell line with a high capacity for terminal adipose differentiation. Int J Cancer 2000; 88(6):889-94