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SHORT REPORT

Reduced telomeric signals and increased telomeric associations in human lung cancer cell lines undergoing p53-mediated apoptosis

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Transduction of a p53-negative H1299 human non-small cell lung cancer cell line with an adenoviral vector containing wild-type *p53* (Ad5p53) induced apoptosis. Analysis of the Ad5p53-infected H1299 cells showed high levels of telomeric association prior to apoptotic nuclear fragmentation. Similar telomeric association was observed in stably transfected clones of the wtH226b cell line, which expressed exogenous wild-type p53 protein and also showed complex chromosomal abnormalities including dicentrics, rings and fragments. Fluorescence *in situ* hybridization (FISH) analysis using a human telomeric DNA probe indicated reductions in telomere signals in both Ad5p53-infected H1299 cells and wtH226b-S cells. In contrast, stably transfected wtH226b-AS clones expressing antisense *p53* cDNA showed no telomeric association and had high levels of telomeric signals associated with a faster growing phenotype. These results suggest that wild-type *p53* is involved in shortening telomeres, a possibly early event in the *p53*-mediated apoptotic process and in the subsequent telomeric association that predisposes a cell to genetic instability and DNA fragmentation resulting in apoptotic cell death. Moreover, loss of telomeric signals may indicate a cell's decision to undergo programmed cell death and, if so, could, serve as a sensitive marker of *p53*-mediated apoptosis.

Keywords: *p53*; lung cancer; apoptosis; telomere; gene expression

The wild-type p53 tumor suppressor, which is encoded by a gene that is mutated or deleted in a wide variety of human cancers (Levine and Momand, 1990), plays a key role in maintaining genome integrity in mammalian cells. p53 does this by eliminating damaged or potentially unstable cells by apoptosis or by inducing their cell-cycle arrest. Several experimental studies have demonstrated that wild-type p53 plays a key role in triggering apoptosis under several different physiological conditions (Levine, 1997). For instance, the wild-type *p53* gene can initiate apoptosis in response to the expression of viral or cellular oncogenes or the absence of a critical tumor suppressor Rb gene product.

A number of factors affect the decision of a cell to undergo p53-mediated cell-cycle arrest or apoptosis. It has been hypothesized that under conditions in which DNA is damaged, survival factors for the cells are limiting, or an activated oncogene is forcing the cell to replicate, p53-mediated apoptosis will prevail. In such cases, the accumulation of wild-type p53 protein may push the cell to enter one of two pathways: cell-cycle arrest (Kuerbitz *et al*., 1992) or programmed cell death (apoptosis) (Yonish-Rouach *et al*., 1991; Ryan *et al*., 1993). The exact mechanism through which wild-type *p53* induces apoptosis is not clearly understood, however. Some studies suggest that *p53* transcriptionally activates other molecules involved in the induction of apoptosis (Yonish-Rouach *et al*., 1996), while other studies suggest it does not (Wagner *et al*., 1994). In any case, it has become apparent that apoptosis is a complex network of pathways that requires multiple genetic and epigenetic stimuli (White, 1996).

In a transient assay, we infected p53-deleted H1299 cells with adenoviral vector carrying a wild-type *p53* cDNA under the control of the CMV promoter (Ad5-p53) at different MOIs and then monitored the growth of the cells for 4 days (Figure 1a). As a result, we found that the growth of these cells was severely inhibited after exogenous wild-type *p53* expression. However, dl312 (empty adenoviral vector) had no effect on the cell growth even when used at 10 MOI in 5 days growth assay as shown in Figure 1b. We also found that Ad5p53 administered at MOI 5 is enough to kill 50% of cells (EC_{50}) in this assay. In addition, we infected H1299 *p53*-negative human lung cancer cells for 24 h with Ad5p53 at MOI 1 and then examined cell lysates for p53 protein by Western blot analysis. As shown in Figure 1c, uninfected H1299 cells were deficient in endogenous p53 (lane 1) but strongly expressed wild-type p53 protein when infected with Ad5p53 at MOI 1 (lane 2) for 24 h. We also noted that the high level of wild-type p53 protein expression after Ad5p53 infection was associated with the tumor cells undergoing apoptosis.

To better understand the role of specific numerical and structural chromosome changes in the multifactorial process of p53-mediated apoptosis, we subjected H1299 cells transiently infected with Ad5p53 and our previously described H226b clones (wild-type *p53* containing H226b cell lines stably transfected with sense or antisense wild-type p53 cDNA) to cytogenetic analysis. The increased levels of expression of antisense *p53* RNA in these stable clones reduced the levels of wild-type p53 proteins (Mukhopadhyay and Roth, 1993). Wild-type H226b-S cells expressing the ectopic wild-type p53 grew slowly in culture (Figure 1d) and showed a higher rate of spontaneous apoptosis due to ectopic expression of

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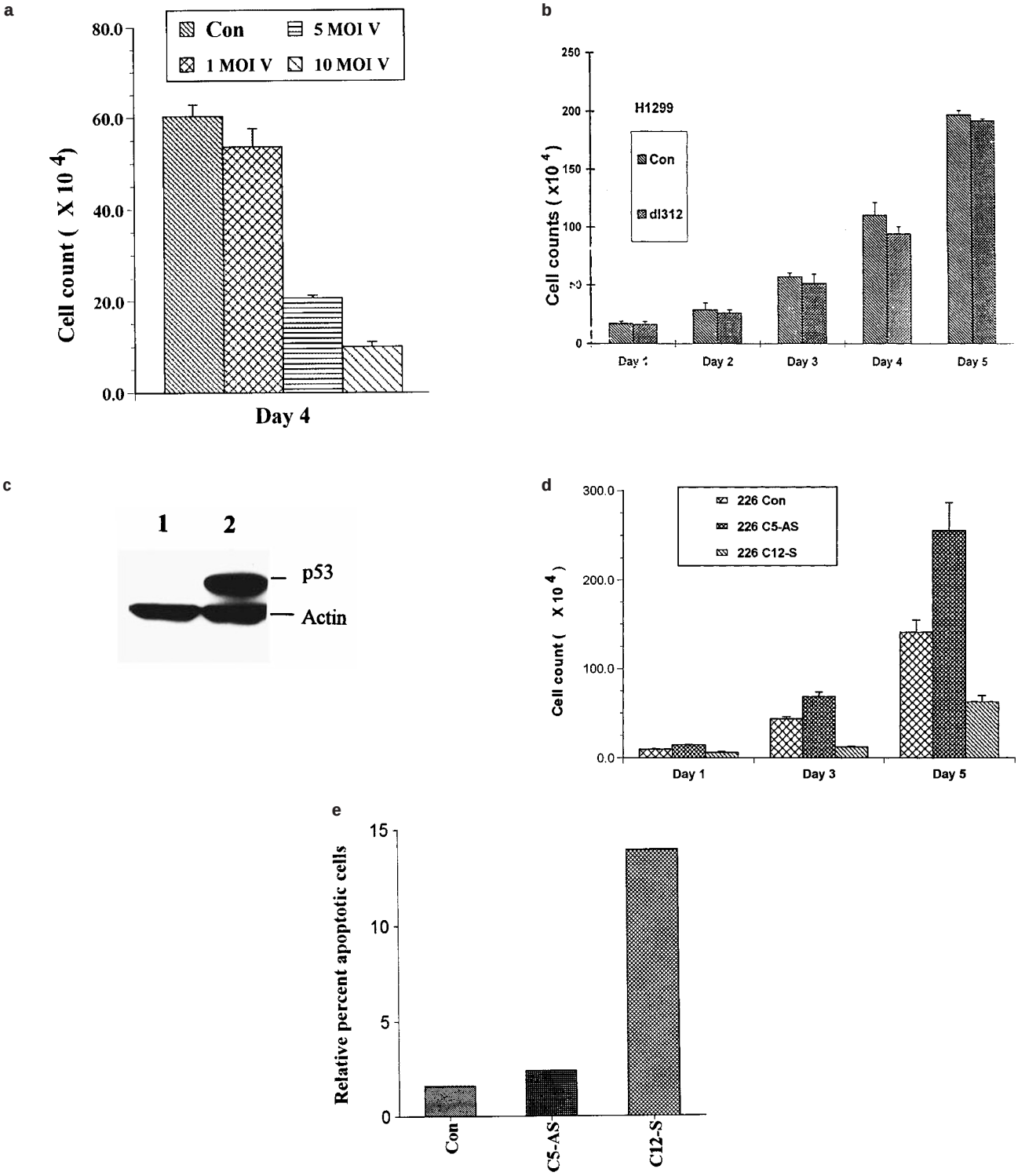

Figure 1 Human non-small cell lung cancer cell lines H226b (wild-type *p53*) and H1299 (*p53* deleted) were gifts from Drs Adi Gazdar and John Minna (University of Texas Southwestern at Dallas). Both cell lines were grown in RPMI 1640 medium supplemented with 5% fetal bovine serum and 5% CO_2. An Ad5p53 mammalian expression vector was constructed in our laboratory as reported earlier (Zhang *et al*., 1993). Briefly, PCR-generated wild-type human *p53* cDNA was subcloned into a pXCJL. 1 shuttle vector under the control of the human cytomegalovirus (CMV) promoter. The resulting construct and pJM17 were cotransfected into 293 cells. Adenoviral recombinants were then isolated, tested by Western blot analysis, and further purified. Finally, adenoviral titers were determined. Adenovirus preparations were free of replication-competent adenovirus as determined by previously described techniques (Zhang *et al*., 1995). The multiplicity of infection (MOI) was defined as the ratio of the total number of plaque-forming units used in a particular infection to the total number of cells to be infected. For cell-growth measurements, 5×10^4 cells, were plated in each well of six-well plates. Control and treated cells were trypsinized and counted using a hemocytometer. Experiments were done in triplicate. All experiments were done when the cells were 70% confluent. (**a**) Results of dose titration experiments done using different concentrations of Ad5p53 viral vectors. H1299 cells were infected for 24 h with Ad5p53 at different MOI and then measured for cell growth 4 days by counting in a hemocytometer. (**b**) Five days growth assay was performed infecting H1299 cells with 10 MOI dl312 (empty adenoviral vector) to show no effect on cell growth. (**c**) Western blotting analysis of p53 protein levels in H1299 cells infected with Ad5p53 for 24 h. Control uninfected and Ad-p53 infected cells were harvested, placed in $1 \times$ SDS–PAGE loading buffer and boiled for 5 min. A total of 30 μg protein/lane was then electrophoresed through a 10% denaturating polyacrylamide gel and electroblotted onto a membrane as described previously (Mukhopadhyay and

wild-type p53. For comparison, the percentage of wtH226b-AS cells showing apoptosis was measured by FACScan analysis after TdT staining (Figure 1e). The results indicated that antisense clones had fewer apoptotic cells than the control H226b cell line. On the other hand, the H226b sense clone had a higher rate of spontaneous apoptosis and a slower growth rate.

To investigate further the associated chromosomal changes occurring prior to DNA fragmentation, we examined the 30 G-banded metaphase spreads from clonally derived H226b sense and antisense clones. Three clonally derived H226b sense clones C2-S, C9-S and C12-S) showed telomeric associations in more than 90% of metaphases examined, whereas two antisense clones (C5-AS and C6-AS) showed no telomeric associations nor for that matter any chromosomal alterations. However, the parental H226b cell line did show telomeric associations in two of 30 metaphases examined (6.7%). A representative chromosome complements of wtH226b-S cells undergoing apoptosis is shown in Figure 2. Telomeric associations were evident between chromosomes involving both chromatids (Figure 2a) or single chromatids (Figure 2b). Ring configurations were formed due to intrachromosomal restitutions (Figure 2c). In contrast to the parental line, H226b-S transfectants showed several chromosomal abnormalities including dicentrics and ring configurations in a subpopulation of cells supposedly undergoing apoptosis. Moreover, an increased level of telomeric association, which predisposes to chromosome segregation abnormalities, was frequent in these cell lines (Pathak *et al*., 1994a,b, 1996). Similar telomeric association was noticed when H1299 cells were infected with Ad5p53. We were unable, however, to analyse the karyotype of the Ad5p53-infected H1299 cell line due to excessive clumping of chromosomes, which was possibly due to the telomeric association that destined the cells for programmed cell death (Pathak *et al*., 1996). In contrast, the wtH226-AS clones with reduced *p53* expression showed no telomere abnormalities, nor for that matter any chromosomal alterations, when compared with the parental H226b cell line.

In situ hybridization analysis of telomeres in normal bronchial epithelial (NHBE) cells or cells infected with adenoviral p53 gene indicated that exogenous p53 had no effect on the telomere signals (Figure 3a–c). This data is consistent with our earlier data that exogenous p53 delivery into these cells did not result in any significant growth inhibition or apoptosis. Our results indicated that at an MOI of 50 p.f.u./cell, AdCMV-p53 infection and expression were detectable in 80% of treated cells. Although on Western blot analysis exogenous p53 protein was detected after infection, but no apoptotic cell death or cell growth inhibition was noticed. (Zhang *et al*., 1995). FISH analysis indicated reduction of telomeric signals in the H1299 cells infected with Ad5p53 as compared to vector infected or uninfected control H1299 cells (Figure 3d–f). In case of stably transfected clones, the wtH226b-S cells (Figure 3i) showed a weaker telomeric signal than the parental H226b cells (Figure 3g). In contrast, the wtH226b-AS clones showed intense telomeric signals (Figure 3h). The total intensity of the telomeric signal was decreased dramatically in Ad5p53-infected H1299 cells undergoing apoptosis. Both the experimental results showed decrease in telomeric signals indicating that the decrease in telomeric signals was probably due to a reduction in the number of $(TTAGGG)_n$ repeats. Apoptotic cell death was further confirmed by *in situ* TdT staining. *In situ* TdT staining of Ad5p53-infected H1299 cells indicated 80–90% apoptosis at 48 h after infection (Figure 4).

Next we wanted to know if any correlation exists between telomerase activity and telomere signals in p53

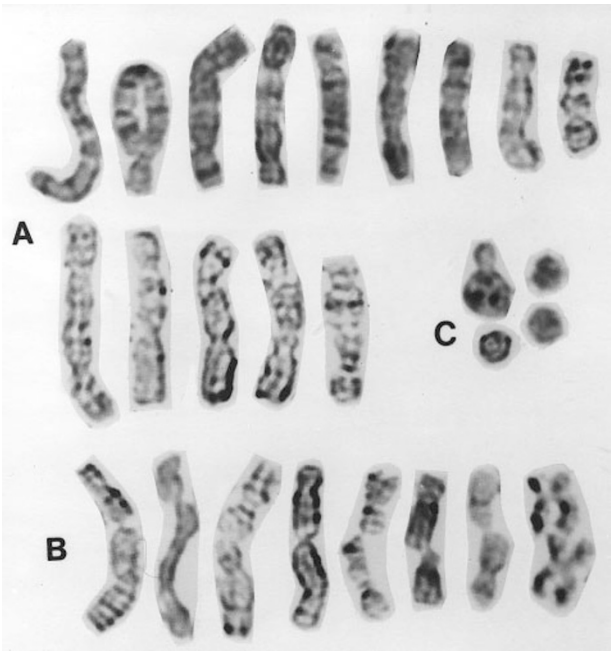

Figure 2 Cytogenetic analysis of Giemsa-banded chromosomes showing increased telomeric association in cells overexpressing ectopic wild-type p53 protein. About 80%-confluent cell cultures were used for chromosome preparations. The cells were fed with a fresh medium 24 h prior to harvesting. The cells were treated 30 min with colcemid (final concentration, 0.04 μg/ml) and 20 min with a hypotonic solution (0.06M KCl) before fixation in acetic acid-methanol (1 : 3 by volume). Slides were prepared following the standard air-drying procedure and allowed to age optimally before being subjected to standard banding techniques (Pathak, 1976). Chromosome analysis of H226b-S clones showing various chromosome abnormalities including telomeric associations between double chromatids (**a**) and between single chromatids (**b**) as well as intrachromosomal restitution in the form of rings (**c**). Chromosome analysis of H226b parental cells and H226b-AS clones very rarely showed such chromosomal abnormalities

Roth, 1993). p53 was detected immunochemically using an anti-human p53 monoclonal antibody (Biotechnology Inc., Santa Cruz, CA). Then blots were simultaneously probed with actin monoclonal antibody as described previously (Mukhopadhyay and Roth, 1993). Lane 1, control cells; lane 2, cells infected with Ad5p53 (1 MOI) for 24 h. (**d**) Cell growth as measured in control H226b, H226b-S, and H226b-AS clones. Cultures were harvested every other day, and cell numbers were determined after crystal violet staining. Results are representative of three independent experiments. Bars, s.d. (**e**) TdT FACS analysis was done to show the relative apoptotic cells in control H226b (Con), H226b#C5-AS and H226b#C12-S clones

antisense clones. We examined the telomerase activity in wtH226b and wtH226b p53 sense and antisense clones. Cells were lysed in 1 × CHAPS buffer and telomerase activity in cell extracts were measured using a commercially available reagents kit which is based on the PCR – TRAP assay as described previously (Kim *et al*., 1994). Amplification of a 36 bp internal control template ensured that PCR reactions worked optimally. Telomerase positive cell extract was used as a positive control (supplied by the manufacturer) and heat inactivated cell extracts served as negative controls. TSR8 control template served as a standard for estimating the amount of TS primers with telomeric repeats extended by telomerase in a given extract. A significant increase in telomerase activity was noticed in wtH226b p53 antisense clone (Figure 5). As a result, we detected about twofold increase in the level of telomerase activity in the wtH226b antisense clone as compared to control parental wtH226b cells. We repeated the experiment three times and these results are consistent with the higher level of telomere signals displayed by wtH226b p53-AS clones and showed no complex chromosomal configurations.

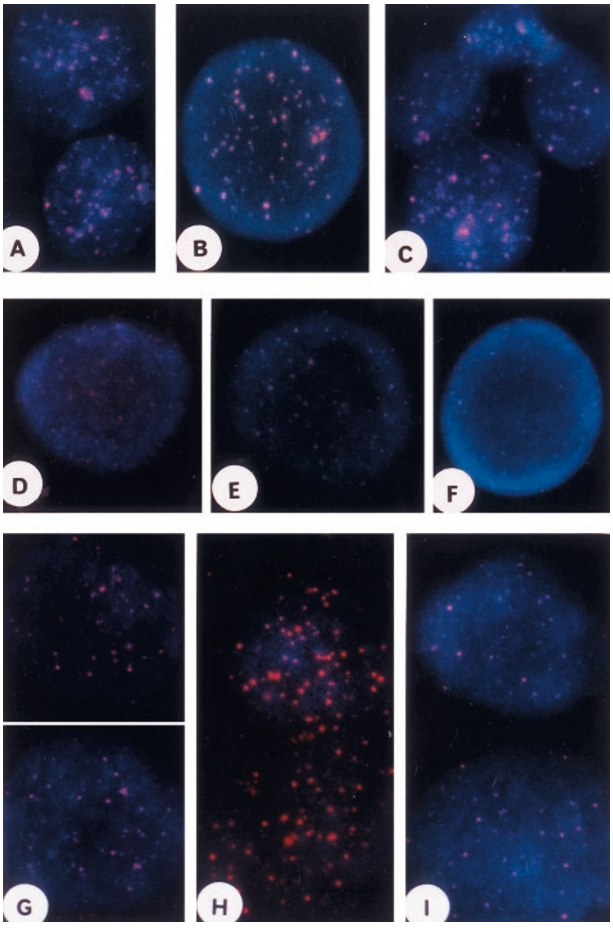

Figure 3 Presence of telomere in the interphase nuclei of cells analysed by fluorescence *in situ* hybridization (FISH). A digoxigenin-labeled all-human telomere probe (Oncor Inc, Gaithersburg, MD) was used for FISH analysis. Briefly, the slides were pretreated in 2 × standard saline citrate (SSC), pH 7.0, at 37°C for 30 min, dehydrated in alcohol of increasing grades (70, 80 and 95%, 2 min each), and air dried. The slides were then denatured in 70% formamide/2 × SSC at 70°C for 2 min, immediately dehydrated in ice-cold alcohol of increasing grades (70, 80 and 95%) (2 min each), and dried. DNA probes (30 μl per slide) were denatured at 70°C for 2 min and then quickly chilled on ice. Hybridization was conducted under a 22- × 50-mm coverslip at 37°C for 16 h in a humidified chamber. Posthybridization washing was carried out in 2 × SSC at 72°C for 5 min, after which the slides were treated with 1 × PBD (Oncor Inc., Gaithersburg, MD). Hybridization signals were detected by applying 60 μl rhodamine-labeled antidigoxigenin at 37°C for 5 min, followed by three washes in 1 × PBD (2 min each). Signals were amplified by first applying 60 μl of rabbit anti-sheep antibody I under a plastic coverslip at 37°C for 15 min and then washing the slides in three changes of 1 × PBD (2 min each). Then, 60 μl of rhodamine labeled anti-rabbit antibody II was applied to the slide and incubated for 15 min at 37°C. The slides were then washed in three changes of 1 × PBD (2 min each) and counterstained with DAPI/antifade (0.1 μg/ml). Finally, the slides were observed under a Nikon photomicroscope. A minimum of 10 – 15 cells were photographed using a triple-band-pass filter (Omega Opticae, Inc., Brattleboro, VT). (**a**) NHBE control cells; (**b**) NHBE cells infected with dl312 (adenoviral vector only); (**c**) NHBE cells infected with (1 MOI) Ad5p53; (**d**) H1299 control cells; (**e**) H1299 cells infected with dl312; (**f**) H1299 cells infected with (1 MOI) Ad5p53; (**g**) H226b control cells; (**h**) H226b-AS clones and I, H226b-S clones

When cultured *in vitro*, normal somatic cells have a strictly limited proliferative potential and undergo senescence or apoptosis, whereas cancer cells program themselves for an extended yet finite life span. The molecular mechanism by which cancer cells manage to do so, however, is not known. Several cytogenetic alterations involved in apoptosis, such as shortening of the telomeres, formation of multicentric chromosomes, malfunction (inactivation) of the centromeres, endo-reduplication of chromosomes, clumping of metaphase chromosomes, and other structural and numerical chromosomes, have been well documented (Pathak *et al*., 1994a,b, 1996). In our present study, complex rearrangements including dicentric and ring configurations were observed in lung cancer cell lines undergoing p53-mediated apoptosis.

The correlation between the increased expression of wild-type *p53* and the loss of telomeric signal is intriguing. Being redundant, eukaryotic telomeres can compensate for the incomplete replication of chromosome ends. In multicellular eukaryotes, however, these telomeres eroded away in varying base-pair increments at every cell division. According to this hypothesis, when telomere repeats are critically shortened, DNA-damage response pathways involving p53 (and in some cell types, retinoblastoma protein) are invoked, leading to M1 senescence in normal cells; cancer cells, which frequently lack normal p53 functions, ultimately become genetically unstable and activate telomerase in order to avoid telomeric associations and breakage-fusion-bridge cycles and so become immortal (Pathak *et al*., 1994a).

Our results suggest that overexpression of wild-type p53 augments this process of telomeric association, thus inducing apoptosis in cancer cell lines that are already genetically unstable. Perhaps this p53-mediated apoptosis is specific for cells with multiple genetic abnormalities: we have previously shown that when a high level of wild-type p53 expression is induced in normal human bronchial epithelial cells, no apoptosis is observed, but that when p53 expression is abnormally low or missing, as in lung cancer cell lines or transformed NIH3T3 cells, apoptosis is frequent (Mukhopadhyay and Roth, 1997; Seegers *et al*., 1997). Loss of telomeric signals in Ad5p53-infected H1299 cells undergoing apoptosis indicates the erosion of telomeric DNA sequences, with the consequence of

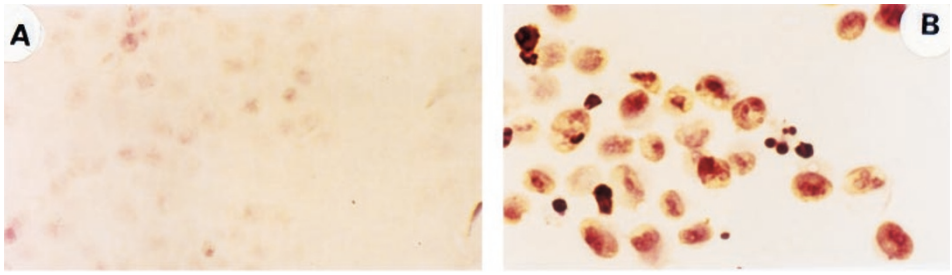

Figure 4 (**a**) TdT staining of apoptotic H1299 cells after 24 h of Ad5p53 transduction (1 MOI). TdT-mediated dUTP-biotin nick end-labeling (TUNEL) assay was performed as described previously (Mukhopadhyay and Roth, 1993). Briefly, cells fixed on the slide and centrifuged. The slides were then incubated in TdT buffer (30 mM Tris HCl, pH 7.2; 140 mM cacodylate; 1 mM cobalt chloride) and incubated with biotinylated dUTP (Boehringer-Mannheim, Indianapolis, IN) and 100 U/ml TdT enzyme (Bethesda Research Laboratory, Gaithersburg, MD) for 1 h at 37°C. The avidin-biotin complex was detected using the Vectastain Elite kit (Vector Laboratories, Burlingame, CA) and the diaminobenzidine-H_2O_2 method. By this method, apoptotic cells are labeled *in situ* with biotinylated dUTP, which in turn binds fluorescent avidin; thus, the apoptotic cells can be visualized. (**a**) Control H1299 cells; (**b**) Ad5p53-transduced H1299 cells

telomeric association followed by cell death (Pathak *et al*., 1996, 1994a). Cytogenetic evidence suggests that induction of wild-type p53 expression leads to extensive telomeric association before cells undergo apoptosis and that the loss of telomeric repeat sequences $(TTAGGG)_n$ may be an early event in the onset of apoptosis, as suggested previously (Pathak *et al*., 1994b). The loss of telomeric repeat sequences makes the chromatin vulnerable to nucleases and pushes the cell to activate the ribonucleoprotein enzyme telomerase, which adds new telomeric repeats at chromosome ends. Therefore, telomerase activation plays a crucial role in malignant transformation and carcinogenesis (Kim *et al*., 1994).

Unfortunately, however, the temporal and pathophysiological aspects of this activation *in vivo* are poorly understood. In vertebrates, telomerase is active only in the germ line and the early embryo. Somatic cells that reactivate or upregulate telomerase *in vitro* or *in vivo* become immortal. Interestingly, telomerase activity has been found in about 25% of lung cancers and in many advanced and metastatic human cancers, suggesting that telomerase-dependent immortalization may contribute to the metastatic potential of cancer cells (Broccoli *et al*., 1996; Healy, 1995). Moreover, immortalization of cells has been associated with the loss of wild-type p53 function, which also often leads to the telomerase activation required for elongation of the telomere (Rogan *et al*., 1995). In our study, the stable transfectants expressing *p53* antisense RNA and showing reduced levels of endogenous p53 protein (Mukhopadhyay and Roth, 1993) showed intense telomere signals consistent with higher levels of telomerase activity, suggesting that these clones bypassed telomeric associations by reactivating telomerase enzyme, thereby avoiding apoptotic cell death. In H226b p53 sense clone, the telomerase activity was significantly reduced. However, role of wtp53 on telomerase expression needs to be studied in detail. It has been previously documented that the level of p53 protein expression is an important determinant of apoptosis (Chen *et al*., 1996). Alterations in telomeric repeat length in lung cancer have been shown to be associated with loss of heterozygosity in p53 (Hiyama *et al*., 1995). There-

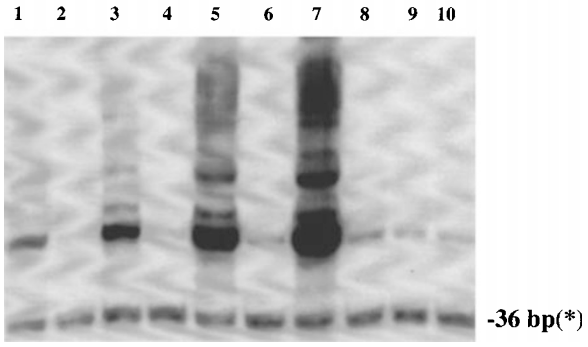

Figure 5 Telomerase activity was measured in the wtH226b control, wtH226b p53 sense and antisense clones using TRAP-assay telomerase detection kit (Oncor Inc., Gaithersburg, MD), which is based on the TRAP assay as described previously (Kim *et al*., 1994). Briefly, a frozen cell pellet was dissolved in 100 μl of 1 × CHAPS buffer (10 mM Tris-HCl (pH 7.5), 1 mM $MgCl_2$, 1 mM EDTA, 0.5% 3-[(3-cholamidopropyl)-dimethyl ammonium]-1-propane sulfonate, 10% glycerol, 5 mM β-mercaptoethanol and 0.1 mM benzamidine], incubated on ice for 30 min and centrifuged at 12 000 r.p.m. at 4°C for 30 min. The supernatant was aliquoted and frozen in liquid nitrogen for TRAP assay and for the determination of protein concentration. Telomerase reaction mixture was prepared by adding 2 μl of cell lysate containing 10 ng of cellular protein to 48 μl of solution comprising 1 × TRAP reaction buffer (20 mM Tris-HCl (pH 8.3), 1.5 mM $MgCl_2$, 63 mM KCl, 0.005% Tween 20 and 1 mM EGTA], 50 μM each deoxynucleotide triphosphate, 0.05 μg of TS primer end-labeled with 20 μCi of [Y-^{32}P]dATP (4500 Ci/mmol; ICN, Costa mesa, CA), 1 μl primer mix and 0.4 unit Taq DNA polymerase (Promega Corp, Madison, WI). The mixture was incubated at 30°C for 30 min, and telomerase reaction product was amplified using a DNA Thermal Cycler (Perkin-Elmer). The following conditions were used for the PCR cycle: 30 cycles at 94°C for 30 s and 55°C for 30 s, followed by one delayed extension cycle at 72°C for 10 min. The PCR products were run in 12.5% nondenaturing polyacrylamide gel in 1 × Tris-borate EDTA buffer for 90 min at 60 W. After drying the gel, the radioactive signal was detected by autoradiography. Alternatively, radioactive signals were quantitated using Phosphoimager (Molecular Dynamics). Lane 1, TSR8 control template; lane 2, TSR8 control template heat inactivated. Lane 3, positive control; lane 4, positive control heat inactivated; lane 5, H226b control, lane 6, H226b control heat inactivated; lane 7, H226b #C5-AS p53 antisense clone; lane 8, heat inactivated antisense cell extract; lane 9, H226b#C12-S p53 sense clone; lane 10, heat inactivated sense clone extract*, amplification of 36 bp internal control band to ensure that PCR amplification worked optimally

fore, it may be that when a normal cell receives a genetic insult, it can induce wild-type p53 protein expression, which in turn induces stimuli for telomeric erosion and subsequent apoptosis. However, the exact mechanism by which wild-type p53 induces telomeric erosion is yet to be identified. Recently it has been shown that wild-type p53 but not mutant p53 possesses an exonucleolytic activity (Mummenbrauer, 1996). Thus, it is possible that a high level of wild-type p53 protein could degrade the telomeric DNA and thereby induce telomeric association and subsequent apoptosis. In contrast, cells that are unable to elevate their wild-type p53 protein levels accumulate genetic damage progressively, lose the *p53* gene, and so avoid apoptosis. When wild-type p53 function is restored into these cells, however, they rapidly undergo telomeric disintegration followed by apoptosis. Thus, we conclude that p53-mediated apoptosis is preceded first by telomere loss and then by telomeric association, which together abrogate cell-cycle progression and result in apoptosis. Consequently, loss of telomere signal could be a sensitive marker of the onset of p53-mediated apoptosis.

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