# Abrogation of Radiation-Inducible Telomerase Upregulation in HPV16 E6 Transfectants of Human Lymphoblasts

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**Background:** Telomerase activity in a human lymphoblastoid cell line with wild-type p53 status (TK6) was previously shown to be rapidly induced by ionizing radiation doses as low as 10 cGy. Since this low-dose response was absent in a closely related cell line overexpressing a mutant form of p53 (WTK1), the putative involvement of p53 was further investigated using stable human papillomavirus 16 (HPV16) E6 transfectants of these cell lines. The E6 product mediates rapid degradation of wild-type p53, but has also been found to upregulate telomerase.
**Material and Methods:** Telomerase activity in HPV16 E6 transfectants of the human lymphoblastoid cell lines TK6 and WTK1 was measured by PCR/ELISA and was quantified using internal standards (titration by cell number) run within each separate assay. Mean telomere length was determined by Southern hybridization of terminal restriction fragments with a biotin-labeled telomeric DNA probe.
**Results:** The TK6E6 and the WTK1E6 cells exhibited higher baseline telomerase activities than the parental cells. This was also accompanied by increased telomere lengths. Radiation exposure (up to 10 Gy) was unable to significantly further enhance telomerase activities, although the dynamic range of the assay would have allowed to record higher signals.
**Conclusion:** The lacking radiation induction of telomerase activities in the E6 transfectants could reflect saturation, if E6 and radiation would share a common pathway of telomerase upregulation. Present evidence from the literature, however, suggests that E6 mediates telomerase reverse transcriptase (TERT) subunit transcriptional activation, whereas radiation signals to posttranscriptional/posttranslational control of telomerase activity. Therefore, the present data enforce the previous hypothesis of a p53 dependence of telomerase upregulation by low doses of radiation and its abrogation, likely due to p53 degradation, in E6-expressing cells.

**Key Words** Telomerase · Radiation · p53 · HPV16 E6 · Lymphoblasts

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**Verlust der strahleninduzierten Telomeraseaktivierung in HPV16-E6-transfizierten humanen Lymphoblasten**

**Hintergrund:** Eine vorhergehende Studie an humanen Lymphoblasten mit Wildtyp-p53-Status (TK6) zeigte eine rasche Induktion der Telomeraseaktivität bereits durch kleine Strahlendosen (10 cGy). Da dieser Effekt in einer eng verwandten Zelllinie mit mutiertem p53 (WTK1) nicht auftrat, sollte die vermutete Rolle von p53 durch Verwendung der mit HPV16 (humanes Papillomavirus 16) E6 stabil transfizierten Zelllinien weiter untersucht werden. Das E6-Produkt vermittelt die rasche Degradation von p53, ist aber auch als Aktivator der Telomerase bekannt.
**Material und Methodik:** Die Telomeraseaktivität in mit HPV16 E6 transfizierten Zelllinien (TK6E6 und WTK1E6) wurde mittels PCR/ELISA gemessen und anhand interner Standards in jedem separaten Assay quantifiziert. Zur Bestimmung der mittleren Telomerlängen diente die Southern-Hybridisierung terminaler Restriktionsfragmente mit einer biotinylierten Telomersonde.
**Ergebnisse:** Die TK6E6- und WTK1E6-Zellen zeigten eine gegenüber den parentalen Zellen deutlich erhöhte Telomeraseaktivität und verlängerte Telomere. Bestrahlung (bis 10 Gy) konnte diese Telomeraseaktivität nicht signifikant steigern, obwohl der dynamische Bereich des verwendeten Assays eine Detektion höherer Werte erlaubt hätte.
**Schlussfolgerung:** Die fehlende Induktion der Telomeraseaktivität nach Bestrahlung in E6-transfizierten Zellen könnte als ein Sättigungsphänomen interpretiert werden, wenn ein gemeinsamer Pfad der E6- und der strahleninduzierten Regulation der Telomerase unterstellt wird. Dies widerspricht aber aktuellen Ergebnissen aus der Literatur, wonach E6 Transaktivierung der Telomerase-Reverse-Transkriptase-(TERT-)Untereinheit vermittelt, während Bestrahlung die Telomeraseaktivität offenbar über posttranskriptionelle/posttranslationale Mechanismen reguliert. Die vorliegenden Daten unterstreichen daher die frühere Vermutung einer p53-Abhängigkeit der bei kleinen Strahlendosen gemessenen Telomeraseinduktion, die in E6 exprimierenden Zellen, vermutlich durch die p53-Degradation, supprimiert ist.

**Schlüsselwörter:** Telomerase · Bestrahlung · p53 · HPV16 E6 · Lymphoblasten

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## Introduction

Telomerase is a reverse transcriptase that adds DNA repeats at chromosome ends [11]. With the exception of stem cell populations and primitive precursor cells, human somatic cells lack telomerase activity and telomeres shorten with each division until the cells reach replicative senescence [3, 10, 11, 15, 16]. This growth arrest is mediated by the p53 DNA damage response pathway [1, 14] and is thought to prevent the deleterious effects arising when progressively shortened telomeres fail to prevent chromosomal fusions and rearrangements [32, 50]. By contrast, high levels of telomerase are expressed in the majority of human cancers [11, 17, 18, 35, 47, 48]. Stabilization of telomere length by telomerase, or less frequently by an alternative recombinational (ALT) pathway, appears to be a prerequisite for tumor cells to proliferate indefinitely [2, 6, 18, 36].

In addition to telomere maintenance, telomerase can add telomeric sequences onto chromosome breaks at nontelomeric locations, a process termed chromosome healing, which requires only very short homologies to the telomere repeat [8, 26, 27, 32, 37]. Ionizing radiation-induced DNA double-strand breaks provide a substrate for chromosome healing, but this appears to be a relatively rare event in mammalian cells [37] and its direct demonstration mimicked by I-SceI restriction cutting involved highly selective experimental conditions [38]. Nevertheless, a role of telomerase in the cellular response to radiation damage is supported by the observations from several laboratories that telomerase activity represents a radiation-inducible function [7, 13, 22, 28, 29, 42]. This response is apparently absent in telomerase-negative cells, and distinct mechanisms for telomerase activation or reactivation during oncogenesis and positive modulation of that activity by radiation have been suggested [7].

Oncogenic telomerase activation (in telomerase-negative cells) was first demonstrated for the human papillomavirus 16 (HPV16) E6 gene product [21], which is also known to promote the ubiquitination-dependent degradation of the p53 tumor suppressor protein [33, 45]. The telomerase activation by E6 may be mediated by an upregulation of the Myc transcription factor which binds to specific sequences within the promotor of the telomerase reverse transcriptase (TERT) subunit gene, thus directly activating TERT expression [5, 19, 43]. While these studies have not been proven universal, including diverse mechanisms of E6/Myc interaction [12, 19, 43] as well as other suggested pathways [30, 39, 41], the prevailing view of telomerase activation by the E6 oncogene is that of TERT transcriptional upregulation. The signaling pathway leading to radiation induction of telomerase activity is still unknown but appears to be distinct from TERT transcriptional activation and rather regulates telomerase function on the posttranscriptional level [7]. Recent results from our laboratory with human lymphoblastoid cell lines have demonstrated some role for p53 in radiation-induced telomerase upregulation [28]. While both the p53 wild-type (TK6) and the closely related p53 mutant cells (WTK1) induced telomerase activity to similar levels and with similar kinetics following irradiation with doses > 1 Gy, a significant low-dose response (down to 0.1 Gy) was only detectable in the p53 wild-type cells.

Extending such previous work, telomerase activity was studied in the HPV16 E6 transfectant cell lines TK6E6 and WTK1E6 [49], with the expectations of elevated telomerase activities in the E6 transfectants compared to the parental cell lines and of an abrogation of the response at low doses due to the wild-type p53-negative status of the TK6E6 cells.

## Material and Methods

### Cell Culture and Irradiation

The TK6E6 and the WTK1E6 cells are stable HPV16 E6 transfectants of the closely related human lymphoblastoid cell lines TK6 and WTK1, respectively [49]. The parental TK6 cells have wild-type p53 status, whereas WTK1 cells overexpress a mutated p53 with a single base pair substitution in codon 237 [23]. p53 function is abrogated in TK6E6 cells because of rapid E6-mediated p53 protein degradation via the proteasome pathway [33]. The p53 status of the WTK1E6 cells is not different from the WTK1 cells and allowed to monitor E6 effects other than p53 degradation. The cells were grown at 37 °C in suspension cultures in a humidified 6% $CO_2$ atmosphere in RPMI-1640 medium supplemented with 10% heat-inactivated horse serum (Gibco). The E6 transfectant cell lines were stably maintained in medium containing 400 mg/ml G418 (Gibco). Cell growth in culture flasks was at cell densities between 105 and 106 cell/ml by subculturing at regular intervals. Single photon doses of up to 4 Gy (2.5 Gy/min) were given at room temperature using a medical linear accelerator operating in 6-MV X-ray mode.

### Telomerase Activity and Telomere Length

Telomerase activity was measured using the Telomerase PCR ELISA kit (Roche). The assay allows a nonradioactive detection of telomerase activity, and it was performed exactly as recommended by the manufacturer. In principle, telomerase activity, if present in cell lysates, adds telomeric repeats to a biotin-labeled primer. The elongation products are then amplified by PCR. An aliquot of the PCR product is denatured and hybridized to a digoxigenin-labeled, telomeric repeat-specific detection probe. This hybrid is immobilized by the biotin-labeled primer to a streptavidin-coated microtiter plate. Finally, the digoxigenin labels are detected with an anti-digoxigenin peroxidase. The probe is visualized by virtue of peroxidase-metabolizing tetramethyl benzidine to form a colored reaction product. Color intensities are measured at 450 nm with a microtiter plate reader. In previous experiments, 100 TK6 or WTK1 cells were used per microplate well to observe small increases in telomerase activity after irradiation. Cell number of the E6 transfectants per microplate well was reduced by a factor of 2 (from 100 to 50) because of the higher baseline assay signal (optical density) with the transfectants (see Results). To derive an estimate of relative telomerase activity from the sig-

nal of the assay (optical density) after irradiation, telomerase activity of 50–1,000 unirradiated cells was measured in each experiment, and these data were used for an internal calibration curve. The telomerase activity of 50 irradiated cells was converted to the cell equivalent of unirradiated cells. Telomere length (terminal restriction fragment length [TRFL]) was measured by Southern blotting.

### Results

Baseline telomerase activity in the HPV16 E6 transfectant cell lines TK6E6 and WTK1E6 was higher than in the parental TK6 and WTK1 cells. Using the appropriate comparison of cell numbers (included in the assay) that led to identical densitometric readings [28], relative increase of telomerase activity due to E6 transfection was about tenfold for the TK6 cells and about fivefold for the WTK1 cells, respectively. Notably, the dynamic range of the calibration curves generated from the internal standards (data not shown) would have allowed to record an at least 20-fold increase of enzyme activity (cell number equivalent of 1,000 cells). The functional expression of increased telomerase activity in the TK6E6 cells was assessed by measuring telomere length (Figure 1). While the TK6 cells already exhibited longer telomeres than the WTK1 cells (mean TRFL of about 6 kbp vs. about 3.5 kbp), telomeric DNA in TK6E6 cells was extended dramatically (mean TRFL of roughly 25 kbp). This result was not due to unsuccessful restriction digestion, as this DNA migrated clearly below undigested controls and it was reproducible. The increase of mean TRFL from WTK1E6 was slightly less pronounced than in the TK6E6 cells and exhibited a marked size heterogeneity smearing out between about 5 and 20 kbp, as far as detectable.

Telomerase activity was measured in samples of TK6E6 and WTK1E6 cells 1 h after irradiation with various doses up to 10 Gy (Figure 2). While the previous data with the parental cell lines [28] exhibited a clear radiation induction of telomerase activity (data included in Figure 2, for comparison), a respective dose response of the TK6E6 and WTK1E6 cells transfectant cells was not, or only marginally, expressed. A similar result was obtained for different points of time following exposure with 1 Gy (Figure 3). This lack of a significant telomerase activation includes the low-dose range (down to 0.1 Gy) where the TK6 cells, but not WTK1 cells, had been found to upregulate telomerase upon irradiation.

### Discussion

The present investigation demonstrates that telomerase activity in human lymphoblastoid cell lines is elevated in response to HPV16 E6 gene transfection. Contrary to the parental cell lines, however, a further radiation-induced increase of those baseline activities could not be detected.

Induction of telomerase by HPV16 E6 [21, 43] is well known, and is thought to occur via activation of TERT subunit transcription. This may depend on E6-mediated upregulation

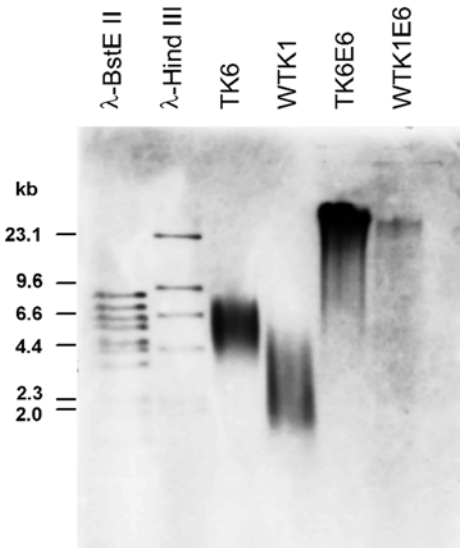


**Figure 1.** Telomere lengths (terminal restriction fragment length) of untreated TK6, WTK1, TK6E6 and WTK1E6 cells. The numbers indicate the molecular lengths of DNA size markers (λ-Hind III).

**Abbildung 1.** Telomerlängen (Längen terminaler Restriktionsfragmente) von unbehandelten TK6-, WTK1-, TK6E6- und WTK1E6-Zellen. Die Zahlen bezeichnen die molekularen Längen von DNA-Standards (λ-Hind III).

of the Myc transcription factor [5, 19, 43], or other mechanisms [30, 41]. Although telomerase activation is not dependent on the E6 function to augment p53 degradation [4, 20, 25, 31, 46], the higher telomerase baseline levels in the p53-negative TK6E6 cells when compared with the mutant p53-overexpressing WTK1E6 cells (see Results) need to be addressed, briefly. The selective pressure, due to the presence of Geneticin, for high levels of retroviral vector gene expression (neo and E6) may be less stringent with the WTK1 cells, because the p53 mutants are much less apoptosis-susceptible than the TK6 cells and also the TK6E6 cells [40, 44, 49]. A respective difference in E6 protein levels was not tested, but the more heterogeneous increase of telomere lengths (TRFL) in the WTK1E6 cells (not shown) would be compatible with the idea that these cells are more tolerable to decreased or occasional losses of transgene expression.

In our previous study, telomerase activity of the parental TK6 and WTK1 cells was increased by ionizing radiation [28]. Telomerase is able to add telomeric sequences onto radiation-induced DNA double-strand breaks. This process could be an important cofactor in the conversion of radiation-induced DNA double-strand breaks into chromosome breaks, thus preventing double-strand break rejoining or, more importantly, misrejoining that could give rise to unstable chromosome aberrations. Baseline telomerase activity in the E6 transfectants was not further increased by ionizing radiation. This could be taken to suggest a simple saturation phenomenon, or overlapping pathways of E6- and radiation-induced telo-

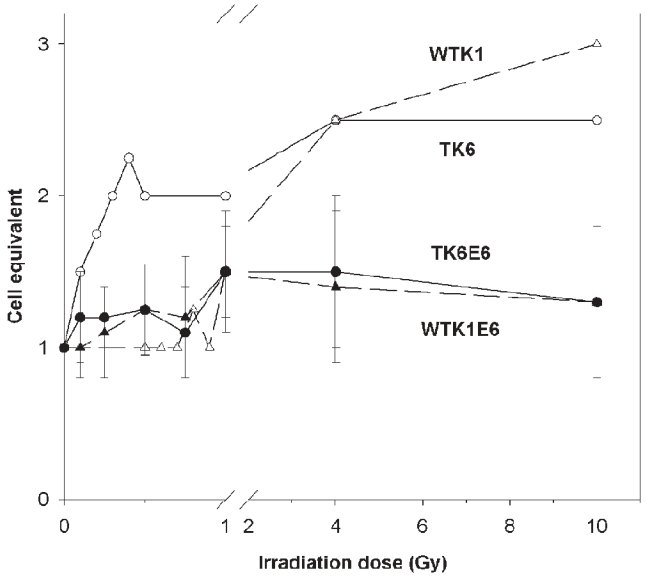


**Figure 2.** Effect of different radiation doses on telomerase activity measured 1 h after exposure of TK6E6 cells (circles) and of WTK1E6 cells (triangles), given as cell number equivalents relative to unirradiated controls. Data points are mean values (and standard deviations) from at least three independent determinations. Previous data [28] with the parental TK6 (solid line) and WTK1 cells (dashed line) are included, for comparison.

**Abbildung 2.** Einfluss unterschiedlicher Bestrahlungsdosen auf die Telomeraseaktivität (1 h nach Exposition) in TK6E6-Zellen (Kreise) und in WTK1E6 -Zellen (Dreiecke). Die Symbole repräsentieren Mittelwerte (und Standardabweichungen) aus mindestens drei unabhängigen Experimenten. Frühere Daten [28] mit den parentalen TK6- (durchgezogene Linie) und WTK1-Zellen (gestrichelte Linie) sind zum Vergleich mit eingetragen.

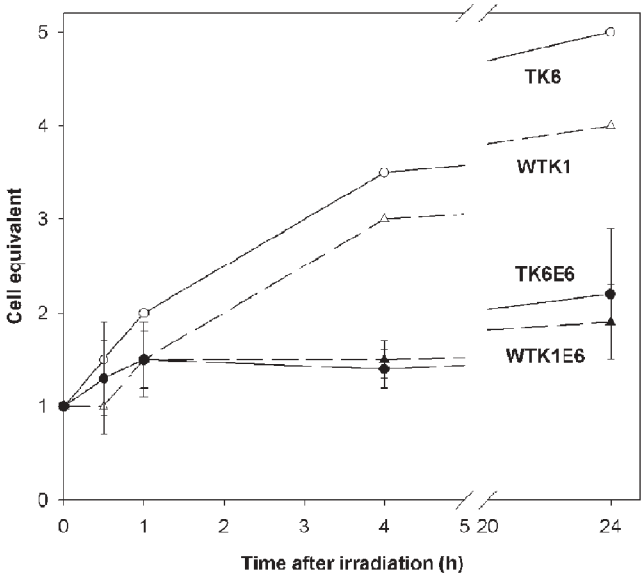


**Figure 3.** As in Figure 2, but for different time points after exposure to 1 Gy.

**Abbildung 3.** Wie in Abbildung 2, jedoch für verschiedene Zeiten nach Bestrahlung mit 1 Gy.

merase activation. Ionizing radiation induces telomerase activity in telomerase-positive cells [7, 13, 22, 29, 42]. On the contrary, telomerase-negative cells (i.e., normal human fibroblasts), when tested in these studies, could not be induced by radiation. The TERT subunit is limiting for telomerase function [24, 34], but no increased TERT transcript levels were observed in irradiated telomerase-positive mouse cell lines, although telomerase activity was induced [7]. Taken together, these findings provide compelling evidence for a posttranscriptional mechanism of telomerase activation by radiation. Considering that telomerase induction by E6 reflects TERT promotor transactivation, one should expect that such increased baseline telomerase levels are still amenable to a positive functional modulation by radiation. This is not found, and other mechanisms must be inferred. In principal, two possibilities could be envisaged. Either an elevated abundance of TERT transcripts could by itself foster a negative regulation of the posttranscriptional/posttranslational activation mechanism, but no further evidence appears available to support this speculation. Alternatively, E6 may directly intersect with the radiation-induced mode of telomerase activation, and the abrogation of the p53-dependent low-dose response (< 1 Gy) of TK6 by E6 transfection cells could be readily explained by the ability of E6 to trigger p53 degradation. But also for higher doses (> 1 Gy) and for extended postirradiation incubations (up to 24 h), a respective telomerase activation was lacking, or only marginally expressed, in both TK6E6 and WTK1E6 cells (Figures 2 and 3). Given the absence of further information, one may only speculate that the primary function of E6 to target various proteins for degradation [9, 35] could also include some other radiation-inducible positive regulator(s) of telomerase activity.

## Conclusion

In summary, the present study with human lymphoblasts demonstrates that HPV16 E6 intersects with the radiation-induced activation of telomerase. Abrogation by E6 of the previously described response of p53 wild-type cells to low radiation doses enforces the existence of a p53-dependent pathway of telomerase activation. But the general suppression of radiation-inducible telomerase activation, also at higher doses, suggests an additional mechanism through which E6 interferes with the upregulation of telomerase by ionizing radiation, and which was previously shown to be independent of p53 at such higher doses.

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