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Short Communication

Telomerase Activation in Cervical Cancer

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It has been hypothesized that infection with high-risk human papillomaviruses (HPVs), in conjunction with other cellular events, plays a critical role in the development of cervical cancer. Activation of telomerase, a ribonucleoprotein enzyme complex that synthesizes telomere repeats, has been associated with acquisition of the immortal phenotype in vitro and is commonly observed in human cancers. In this study, we have examined 10 high-grade cervical cancers for telomerase activity and for the presence of HPV. Telomerase activity was detected in all of the cancers but in none of the paired histopathologically normal uterine tissues or in normal cervical epithelium. Analysis of these same tissues for HPV nucleic acids by polymerase chain reaction (PCR) using primers from the HPV L1 and E6 open reading frames demonstrated that 7 of 10 cancers were positive for HPV, 3 for HPV type 16 (HPV-16), and 4 for HPV-18. In one case, HPV-16 was detected in histopathologically normal uterine tissue, the same type as that detected in the cancer from the same patient. HPV DNA was not detected in 3 of 10 cancers. These results indicate that telomerase activation is common in high-grade cervical cancers and suggests that telomerase activity may be a useful diagnostic marker for the disease. (Am J Pathol 1997, 151:25–31)

Approximately 500,000 new cases of cervical cancer are diagnosed worldwide each year. The disease is strongly associated with human papillomavirus (HPV) infection with up to 90% of cervical cancers containing detectable HPV nucleic acids.[1] Those HPV types more commonly found in high-grade lesions and invasive carcinomas are termed high risk, with types 16 (HPV-16) and 18 (HPV-18) being the most common. It has been established that the protein products of the E6 and E7 genes of high-risk HPVs are responsible for the transforming properties of the virus.[2–5] When HPVs integrate into the host genome, the E6 and E7 open reading frames (ORFs) remain intact and E6 and E7 proteins are expressed.[6] The E6 protein of high-risk HPVs has been shown to bind to and inactivate the p53 tumor suppressor protein,[7,8] and the E7 protein inactivates the retinoblastoma (Rb) tumor suppressor protein.[9] In the rare cervical cancers that do not contain HPV, mutations have been detected in the p53 and Rb genes,[10] indicating that the interactions of E6 and E7 with p53 and Rb are likely to be involved in the development of malignancy. Other possible functions of HPV E6 and E7 that may play a role in carcinogenesis have also been described.[11–14]

Normal human cervical epithelial cells and other normal human somatic cells have a limited life span both *in vitro* and *in vivo*. It has been hypothesized that this limited capacity for proliferation is regulated by telomere length.[15,16] Telomeres are the 4 to 15 kb of simple DNA repeats (TTAGGG) located at the ends of chromosomes.[17–19] It has been shown that telomeres provide a protective cap at the chromosome ends, stabilizing the structure to prevent genetic instability.[18–21] Furthermore, telomeres can facilitate the replication of chromosomes and in some cases regulate the expression of genes at the chromosome end.[22] Due to the inability of DNA polymerase to com-

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pletely replicate the ends of double-stranded DNA, telomeres progressively shorten with each round of cell division.[23–25] It is hypothesized that cells recognize this stochastic loss of telomeres and, when the telomeres reach a short critical length, enter a program of cellular senescence.[15,26–28] This programmed senescence is believed to be a mechanism utilized by multicellular eukaryotes to prevent uncontrolled proliferation. Unicellular eukaryotes and human germline cells, which must maintain telomere length, have been shown to contain an enzyme activity called telomerase, which adds the six base repeats to the telomere ends.[29–31] Most normal human somatic cells have undetectable levels of this enzyme.[32] Strong evidence suggests that immortalization requires a mechanism to restore telomere length, and this is usually by activation of the normally silent telomerase complex.[33] Human cells that express viral oncogenes such as HPV E6 and E7 generally have an extended life span and then go through a crisis after which a subpopulation of cells emerges that is immortal and expresses active telomerase.[21,34–36] Although telomerase can be detected in most immortal cells and cancers, little is known about how it is activated. The telomerase complex consists of an RNA template component[37,38] and several proteins of unknown function.[39,40] The RNA component of human telomerase has been cloned,[41] but its expression does not correlate with telomerase activity.[42]

Our laboratory has shown that the HPV E6 oncoprotein may play a role in telomerase activation.[11] Human cervical keratinocytes infected with retroviruses expressing the E6 protein have detectable levels of telomerase. Cells immortalized *in vitro* by HPV all have high levels of telomerase. One would hypothesize that if telomerase activation is associated with the immortalization of human cervical cells *in vitro* that it also should be associated with the malignant conversion of cervical cells *in vivo*. In the present study, we have examined high-grade cervical cancers for telomerase activity and the presence of HPV. We found that all of the tumors had active telomerase whereas 7 of 10 had detectable HPV, indicating that telomerase activation is frequent in the development of cervical cancer and may be independent of HPV status.

Materials and Methods

Collection of Samples

Patients were recruited from the University of Washington Medical Center. All tumor samples were obtained from women who had been previously diagnosed with invasive cervical carcinoma and who had consented to donate tissue for research purposes. During surgery, specimens of malignant cervical cancer were collected and placed in phosphate-buffered saline and kept on ice until processing. Histopathological analysis of specimens confirmed the original diagnosis. Normal uterine tissue, usually of noncervical origin because of widespread dissemination of the disease in the cervix, was also collected from the patients. Normal cervical epithelium was obtained from surgeries performed for reasons other than the diagnosis of malignant cervical cancer. The majority of the underlying dermis was dissected away and the remaining epithelial layer was processed in the same way as the tumor samples. All specimens were snap-frozen in liquid nitrogen and stored at −80°C until analysis. Tissue was homogenized on ice using a sterile dounce homogenizer with 200 to 400 μl of telomere repeat amplification protocol (TRAP) lysis buffer as described.[11,33] The homogenized solution was placed in a sterile Eppendorf tube and incubated on ice for 30 minutes. The lysate was then spun at 14,000 × *g* for 30 minutes at 4°C, and the supernatant was removed, transferred to a new tube, and stored at −80°C. The amount of protein was determined using the Bio-Rad protein detection kit.

TRAP Assay

The TRAP assay was performed as described[11,33,43] using a 0.6-ml Eppendorf tube with 0.1 μg of lyophilized CX primer ([5′-$(CCCTTA)_3$CCCTAA-3′]) on the bottom of the tube sealed with a bead of ampliwax (Perkin Elmer, Norwalk, CT). The telomerase reaction above the wax involves a TS primer (5′-AATCCGTCGAGCAGAGTT-3′) labeled with [γ-^{32}P]ATP using polynucleotide kinase. The TS primer was used at 0.1 μg per reaction at 25 × 10^6 counts per minute per μg of primer. The reaction mix was made as previously described. Five micrograms of protein were used for each reaction. The reaction was incubated for 25 minutes at room temperature. During this portion of the reaction, the TS primer is utilized by telomerase, if present, for the addition of TTAGGG repeats. After this incubation, the sample was subjected to 3 minutes at 90°C to melt the wax barrier and 27 cycles of polymerase chain reaction (PCR) as described. One-tenth of the reaction was run on an 8% nondenaturing polyacrylamide gel, after which the gel was dried and exposed to film for 24 hours at −80°C.

HPV Nucleic Acid Detection

DNA was isolated from tissue lysates by digestion in 3 vol of 50 mmol/L Tris-HCl, pH 8.5, 1 mmol/L EDTA containing 200 μg/ml proteinase K and 0.5% Tween-20 for 3 hour at 55°C. Proteinase K was inactivated at 95°C for 9 minutes. Any remaining debris was pelleted, and the supernatant was used for PCR.

PCR amplification was performed using degenerate consensus primers amplifying a region of the conserved HPV L1 ORF[44] with the following modifications: each 50-μl reaction contained 50 mmol/L KCl, 10 mmol/L Tris, pH 8.3, 4 mmol/L $MgCl_2$, 200 μmol/L each dNTP, 50 pmol of each primer, and 1.25 U of Amplitaq DNA polymerase. Thermocycling conditions for the PE 9600 were as follows: 40 cycles of 95°C for 15 seconds, 56°C for 15 seconds, and 72°C for 75 seconds, with a final extension at 72°C for 4 minutes. Because the L1 region may be lost during viral integration, additional PCR analysis for HPV types 16 and 18 was performed using primers derived from the E6 ORFs of these two viral types. Primer sequences, designed by Denise Galloway at the Fred Hutchinson Cancer Research Center, were 16E6–1, 5′ ATG CAT AGT ATA TAG AGA TGG GAA T; 16E6–2, 5′ ATG CAT GAT TAC AGC TGG GTT TCT C; 18E6–5, 5′ AAG ACA TAG AAA TAA CCT GTG TAT A; and 18E6–6, 5′ GTT GCA GCA CGA ATG GCA CTG GCC T. Reaction and thermocycling conditions used were identical to those above with the substitution of a 55°C annealing temperature. Recombinant plasmids containing HPV types 6, 16, 18, 31, 33, 35, and 45 at 0.1 pg/reaction were used as positive controls; HPV-negative human genomic DNA served as a negative control. All HPV-negative specimens were analyzed for amplifiability of the β-globin gene using primers amplifying 536- and 268-bp products. Eight-microliter aliquots of each PCR were separated by electrophoresis on 2% NuSieve 3:1 agarose and transferred to Hybond N+ nylon membrane in 0.4 mol/L NaOH. Membranes were rinsed briefly in 2X SSC and dried at 80°C for 1 hour. The identity of PCR products was confirmed by Southern hybridization using an L1 consensus probe[45] and probes specific for each of the E6 products (D. Galloway; 16E6–3, 5′ TGT ATG TGA TAA ATG TTT AAA GTT TTA TTC TAA AAT TAG T, and 18E6–3, 5′ CAG ACT CTG TGT ATG GAG ACA CAT TGG AAA AAC TAA CTA A). One hundred picomoles of each oligonucleotide were 3′-end labeled with digoxigenin-11-ddUTP (Boehringer Mannheim, Indianapolis, IN) according to the manufacturer's instructions. After prehybridization at 55°C for 30 minutes in 6X SSC, 5X Denhardt's, 0.5% SDS, and 100 μg/ml denatured salmon sperm DNA, hybridizations were performed for 90 minutes at 55°C in the same solution using the labeled oligonucleotides at 1 pmol/ml. Stringency washes were performed at room temperature (twice for 5 minutes each, twice for 10 minutes each, and twice for 15 minutes each) in 2X, 1X, and 0.5X SSC/0.1% SDS, respectively, with the final wash in 0.5X SSC/0.1% SDS at 55°C. Membranes were blocked at room temperature for 30 minutes in 1% (w/v) casein in 100 mmol/L Tris/HCl, pH 7.5, 150 mmol/L NaCl. Anti-digoxigenin-alkaline phosphatase was added to the blocking solution at 0.075 U/ml, and the membranes were incubated for 30 minutes and then washed twice for 15 minutes each at room temperature in 50 mmol/L Tris, pH 7.5, 75 mmol/L NaCl with 0.3% Tween-20. Approximately 0.8 ml/cm^2 of a 250 μmol/L CSPD chemiluminescent substrate (Boehringer Mannheim) in 100 mmol/L Tris-HCl, pH 9.5, 100 mmol/L NaCl was applied to the membrane. After incubation for 10 minutes at 37°C and then at room temperature overnight, the membranes were exposed to Fuji x-ray film for 8 to 10 minutes.

L1 consensus products were typed by restriction fragment analysis[46] with the following modifications: before restriction digestion, the L1 products were excised from 2% NuSieve 3:1 agarose and extracted with QIAEX II resin (Qiagen, Chatsworth, CA) according to the manufacturer's instructions. HPV type was assigned by comparison of restriction patterns with those of the recombinant plasmids described above.

Results

Malignant and normal tissues were obtained from ten women who had previously been diagnosed with invasive cervical cancer. Post-operative histological analysis verified the clinical diagnosis. Five of the ten tumors were classified as adenocarcinomas, three as squamous cell carcinomas, one as adenosquamous mixed, and one as poorly differentiated (Table 1). Two samples of normal cervical epithelium were also obtained from women without cervical cancer. Tissue specimens were analyzed for the presence of HPV DNA and telomerase activity as described in Materials and Methods (above). Using E6 primers for HPV-16 and -18 in a PCR amplification followed by Southern blot with an internal probe, four of the tumor samples were found to be HPV-18 positive (Figure 1) and three HPV-16 positive (Table 1). The relatively high number of HPV-18-positive tumors likely reflects the number of adenocarcinomas analyzed in this

Table 1. *Telomerase Activity and Presence of HPV in Cervical Cancers*

Sample	Pathology	HPV type	Telomerase
44T	Adeno-	HPV-16	+
44N		None detected	–
92T	Adeno-	HPV-18	+
92N		None detected	–
88T	Adeno-	HPV-18	+
88N		None detected	–
77T	Poorly differentiated	None detected	+
77N		None detected	–
97T	Squamous cell	HPV-18	+
97N		None detected	–
66T*	Adenosquamous	None detected	+
58T	Adeno-	HPV-18	+
58N		None detected	–
19T	Adeno-	HPV-16	+
19N		HPV-16	–
71T	Squamous cell	HPV-16	+
71N		None detected	–
18T	Squamous cell	None detected	+
18N		None detected	–
NCE-53		None detected	–
NCE-14		None detected	–

T, tumor; N, normal; NCE, normal cervical epithelium.
*Normal tissue not available from this patient.

study, as HPV-18 is more commonly associated with adenocarcinoma than HPV-16.[47] In one case, number 19, HPV was detected in both tumor and normal tissue from the same individual (Table 1). HPV detection and typing analysis was also performed using consensus primers for the HPV L1 ORF followed by restriction digest to determine HPV type based on polymorphic variations between the different types (data not shown). This analysis verified the findings described above with E6 PCR. In several cases, specimens giving a positive result with the E6 primers were negative with the L1, possibly reflecting a loss of the L1 ORF upon integration of HPV into the genome.

Lysates from the tissues were examined for telomerase activity using the TRAP assay. In this assay, the presence of a 6-bp ladder indicates active enzyme in the sample. We found that all of the tumors were positive for telomerase whereas none of the normal uterine tissue or normal cervical epithelium from other patients had detectable levels of telomerase (Figure 2; Table 1). The level of telomerase activity varied from tumor to tumor but in most cases was easily detectable and comparable to the positive control HPV-16E6/E7 immortalized cell line. Actual quantitation of telomerase activity was not performed due to the difficulty in quantifying products from a PCR reaction. Interestingly, all tumors were positive for telomerase regardless of histopathological type and HPV type. Furthermore, three cancers that did not have detectable HPV were positive for telomerase activity.

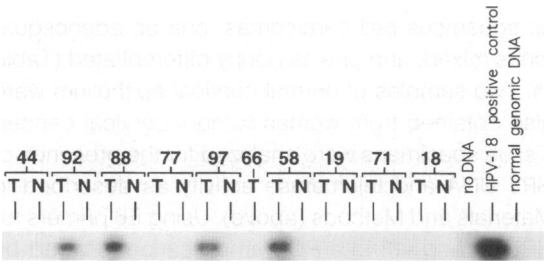

Figure 1. *HPV-18-positive cancers. DNA from tumor (T) and normal (N) tissue was subjected to PCR using HPV-18 E6 primers. After PCR, the reaction was run on an agarose gel, Southern blotted, and probed with an HPV-18 E6 internal fragment as described in Materials and Methods. Control lanes are PCR amplifications using HPV-18 E6 primers of reactions containing no DNA, an HPV-18 recombinant plasmid, and HPV-negative genomic DNA.*

Discussion

In this study, we have shown that high-grade cervical cancers have active telomerase, an enzyme that adds telomeric repeats to the ends of chromosomes. Activation of telomerase is highly associated with cellular immortalization *in vitro*, indicating that high-grade cervical cancers contain immortal cells. One might expect this finding based on the studies that show that high-risk HPV types are efficient at immortalizing cells in culture.[48] We have also previously shown that expression of the E6 protein from HPV-16 may play a role in telomerase activation *in vitro*.[11] Seven of ten cancers contained an identified high-risk HPV type, but all ten were positive for telomer-

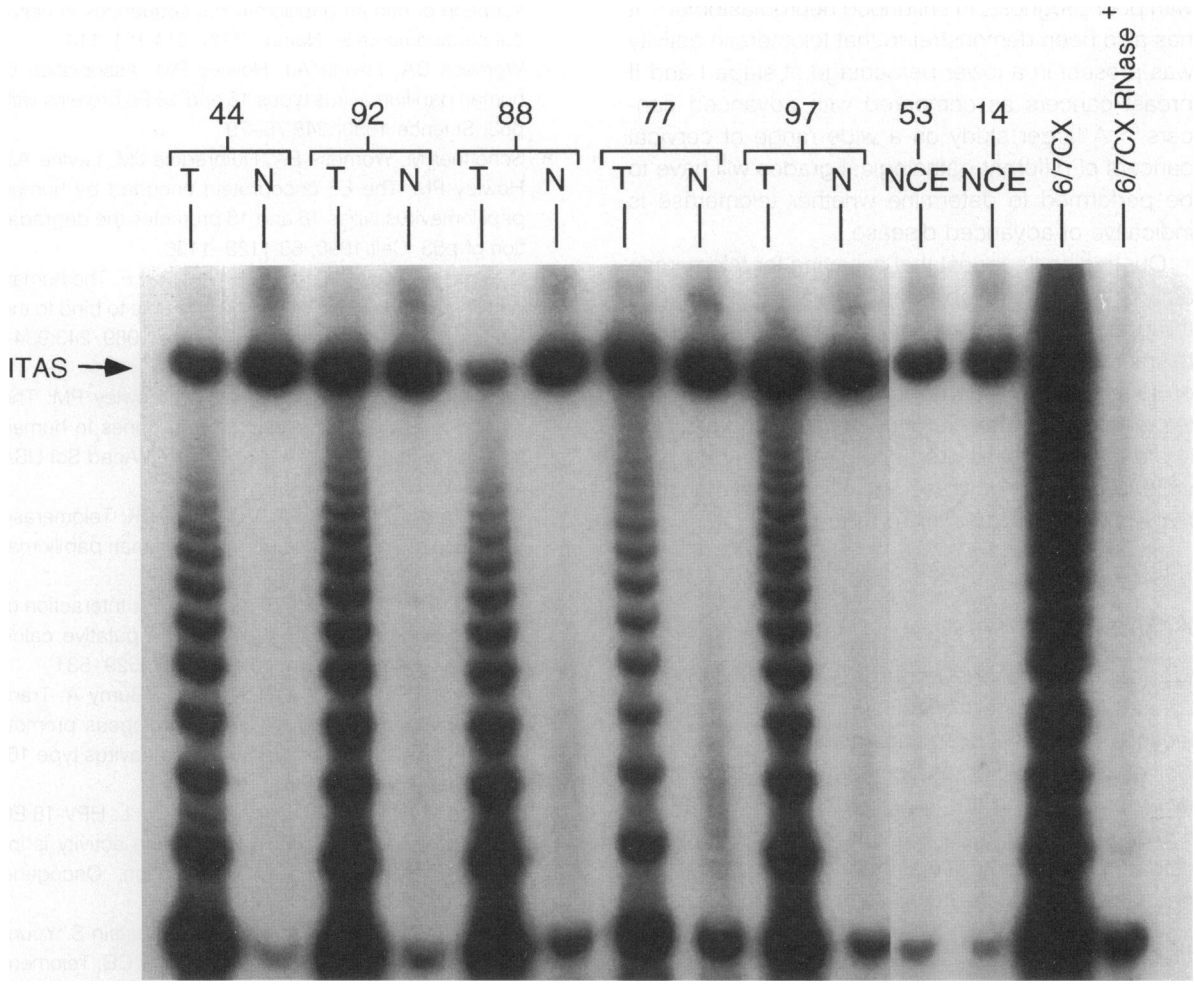

Figure 2. *An example composite gel showing telomerase activity in five tumors (T) but not in normal (N) tissue from the same patient or in normal cervical epithelium (NCE). The ladder represents 6-bp repeats added onto labeled substrate by the telomerase complex. The ITAS band is an internal control for the PCR amplification.*[53] *Extract from an immortal HPV E6/E7 cervical keratinocyte cell line (6/7CX) was used as positive control. The lane labeled 6/7CX RNase*$^{+}$ *is 6/7CX extract treated with RNAse to demonstrate that telomerase activity depends on an RNA component as described.*[37,38]

ase. This suggests that telomerase activation is a regular feature of invasive cervical cancers and can occur in the absence of HPV as has been found with other cancer types. It is possible, however, that the HPV-negative cancers contained HPV types undetected in the PCR assays. Additional analyses using primers derived from other high-risk HPVs might resolve this question.

Whether telomerase activity is affected by the presence of HPV *in vivo* is unclear. It would be of interest to examine low-grade lesions that contain high-risk HPVs to determine whether they are also telomerase positive. In the present study, telomerase activity was not detected in an apparently normal sample that contained HPV DNA, suggesting that infection with HPV *in vivo* does not necessarily cause telomerase activity. In this case, however, the normal tissue was of noncervical origin, and our previous studies have indicated that activation of telomerase by HPV E6 is cell-type specific.[11] Histopathologically normal or precancerous, HPV-positive cervical epithelial samples must be examined to address this issue further. If telomerase activity is found to be exclusive to cancerous tissue, the assay could provide a means to determine whether tumor cells have invaded into lymph nodes or whether apparently normal margins are tumor-free.

The telomerase assay may be useful in the detection of early-stage cervical cancers, particularly if telomerase activity is specific for malignant tissue. Several recent studies indicate that telomerase activity is a marker of cancer progression in some cancer types.[49] For example, it has been shown that adenomatous polyps of the colon did not have active telomerase whereas colorectal cancers were positive.[50] Telomerase activity has also been associated

with poor prognosis in childhood neuroblastoma.[51] It has also been demonstrated that telomerase activity was present in a lower percentage of stage I and II breast cancers as compared with advanced cancers.[52] A larger study on a wide range of cervical cancers of different pathological grades will have to be performed to determine whether telomerase is indicative of advanced disease.

Our findings suggest that assaying for telomerase activity may be a reliable and definitive test to detect the presence of malignancy. One caveat is that the telomerase assay requires fresh or snap-frozen cells or specimens. Once the protein components of the telomerase complex are characterized and antibodies to these proteins are generated, it may be possible to detect telomerase activation by immunohistochemical methods in fixed tissue.

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