Telomerase Activity Exclusively in Cervical Carcinomas and a Subset of Cervical Intraepithelial Neoplasia Grade III Lesions: Strong Association with Elevated Messenger RNA Levels of Its Catalytic Subunit and High-Risk Human Papillomavirus DNA


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ABSTRACT

In this study, we investigated telomerase activity and human telomerase reverse transcriptase (hTERT) mRNA expression in relation to high-risk human papillomavirus (HPV) DNA presence in the spectrum of cervical premalignant lesions. Reconstruction experiments revealed that telomerase activity determined by the telomeric repeat amplification protocol assay and hTERT mRNA by reverse transcriptase-PCR could be detected in 96% of squamous cell carcinomas. Despite the fact that hTERT mRNA was found at much higher frequencies, semiquantitative reverse transcriptase-PCR revealed that elevated hTERT mRNA levels were strongly correlated with detectable telomerase activity. Furthermore, telomerase activity and elevated hTERT mRNA levels were only detected in cases that contained high-risk HPV DNA. In contrast, low or undetectable hTERT mRNA levels were demonstrated in both high-risk HPV positive and negative cases. These data indicate that telomerase activity detectable with the assay used and concomitant elevated levels of hTERT mRNA reflect a rather late step in the CIN to squamous cell carcinoma sequence, which follows infection with high-risk HPV.

INTRODUCTION

Infection with high-risk HPV types, particularly HPV 16 and HPV 18, plays a pivotal role in cervical carcinogenesis. It has been reported that cervical SCCs develop from a continuum of premalignant cervical lesions, so-called CIN lesions, graded (grades I-III) on the basis of mildly to severely dysplastic features. Analyses of the natural history of CIN lesions have revealed that persistence of high-risk HPV types is a likely prerequisite for the development of CIN III lesions. Moreover, high-grade CIN lesions display the greatest potential of progression to invasive cancer. Still, CIN lesions of all grades represent a heterogeneous disease, as they can spontaneously regress, persist, or, in a small subset of cases, progress to invasive cancer. This indicates that HPV infection, albeit apparently necessary, is insufficient for malignant growth. Presently, no markers are available that can indicate which of the CIN lesions have reached a point-of-no-return in terms of malignant potential.

In vitro studies have revealed that high-risk HPV genotypes can induce immortalization of primary human keratinocytes by means of their E6 and E7 oncogenic functions. However, the process of immortalization, considered an important step toward malignancy, requires host gene alterations in addition to the expression of these viral oncoproteins. Although the identity of these genes is still unknown, immortalization mediated by full-length HPV 16 or HPV 18 has been shown to be associated with reactivation of the telomerase lengthening enzyme telomerase and arrest of telomere shortening upon culturing. Once telomeres become critically short, this would trigger entry into senescence. Telomerase is a ribonucleoprotein complex that can add six bp repeats to telomeres ends, thereby compensating for telomere shortening and allowing cells to bypass the senescence barrier. Telomerase is normally not active in mortal cells, but strongly activated in the majority of immortal cell populations. Consequently, activation of this enzyme is likely to be the most common mode by which telomere shortening is compensated and an immortal state is acquired. The potential importance of telomerase activity for cervical carcinogenesis has been underlined by the observation of telomerase activity in the far majority, if not all, cervical carcinomas analyzed, but in none or only a minority of paired histomorphologically normal uterine tissue specimens or in normal cervical samples.

Recently, several components of human telomerase have been cloned. In vitro studies have revealed that high-risk HPV genotypes can induce immortalization of primary human keratinocytes by means of their E6 and E7 oncogenic functions. However, the process of immortalization, considered an important step toward malignancy, requires host gene alterations in addition to the expression of these viral oncoproteins. Although the identity of these genes is still unknown, immortalization mediated by full-length HPV 16 or HPV 18 has been shown to be associated with reactivation of the telomerase lengthening enzyme telomerase and arrest of telomere shortening upon culturing. Once telomeres become critically short, this would trigger entry into senescence. Telomerase is a ribonucleoprotein complex that can add six bp repeats to telomeres ends, thereby compensating for telomere shortening and allowing cells to bypass the senescence barrier. Telomerase is normally not active in mortal cells, but strongly activated in the majority of immortal cell populations. Consequently, activation of this enzyme is likely to be the most common mode by which telomere shortening is compensated and an immortal state is acquired. The potential importance of telomerase activity for cervical carcinogenesis has been underlined by the observation of telomerase activity in the far majority, if not all, cervical carcinomas analyzed, but in none or only a minority of paired histomorphologically normal uterine tissue specimens or in normal cervical samples.

The goal of this study was to find out to what frequency and at what stage telomerase activity and hTERT mRNA expression can be detected in the spectrum of HPV-containing cervical premalignant lesions. For this reason, consecutive sections of frozen CIN and cervical cancer specimens were used for measurement of telomerase activity by the TRAP assay and hTERT mRNA expression analysis by RT-PCR, and histomorphological assessment, respectively. In contrast to cervical carcinomas, 96% of which revealed telomerase activity, telomerase activity was undetectable and hTERT mRNA was absent or present at low levels in histomorphologically normal cervi-
Materials and Methods

Tissue Specimens and Cell Lines. Biopsy specimens were selected from a random group of CIN lesions and SCCs of which both formalin-fixed and frozen tissue biopsies were available. Generally, the stored frozen biopsies had been snap-frozen in liquid nitrogen within half an hour after they were taken. Selection of the samples was based on both of the following criteria: (a) an optimal preservation of morphology of the frozen tissue, as determined by histopathological examination of tissue sections; and (b) the capability to yield sufficient protein of proper quality for TRAP analysis (see below). At the end, 65 samples that fulfilled both criteria were used in this study, comprising 8 histomorphologically normal cervical specimens from patients with CIN disease, 10 CIN I (mildly dysplastic) lesions, 8 CIN II (moderately dysplastic) lesions, 15 CIN III (severely dysplastic) lesions, and 24 SCCs. Of the frozen specimens, consecutive sections were cut, the first and last of which were stained with H&E for careful histomorphological examination. This examination also included a crude estimation of the percentage of dysplastic/neoplastic cells relative to histomorphologically normal epithelial and underlying stromal cells, as well as the relative degree of lymphocytic infiltrate. Series of in-between sections were used for extracting protein, RNA, and DNA for TRAP, RT-PCR, and HPV DNA PCR purposes, respectively.

The immortal cervical carcinoma cell lines SiHa, HeLa, and CaSkii were obtained from the American Type Culture Collection. A cell lineage of primary keratinocytes named EK94-2 has been described before, by Steenbergen et al. (7). EK94-2 cells showed neither telomerase activity nor hTERT mRNA expression, exhibited a finite proliferative capacity, and underwent senescence at passage 7. Cells were grown either in growth medium (EK94-2: Life Sciences, Inc.). From subconfluent cultures, cells were harvested by trypsinization, washed with PBS, and pellets were resuspended in TRAP lysis buffer or RNAzol B (Campro Scientific), for extraction of protein or RNA, respectively.

Measurement of Telomerase Activity. Telomerase activity was measured by the TRAP assay as described (25), except that the PCR was carried out for 31 cycles in a reaction volume of 25 μL. For this purpose, 8–10 tissue sections or pellets of cultured cells were suspended in 50 μL of TRAP lysis buffer, and the suspension was homogenized by mixing vigorously. The suspension was placed on ice for 30 min and subsequently spun down in a microcentrifuge at maximum speed for 30 min at 4°C. The supernatant was transferred to a new tube, snap-frozen in liquid nitrogen, and stored at −80°C. Frozen tissue biopsies were available. Generally, the stored frozen biopsies had been snap-frozen in liquid nitrogen within half an hour after they were taken. Selection of the samples was based on both of the following criteria: (a) an optimal preservation of morphology of the frozen tissue, as determined by histopathological examination of tissue sections; and (b) the capability to yield sufficient protein of proper quality for TRAP analysis (see below). At the end, 65 samples that fulfilled both criteria were used in this study, comprising 8 histomorphologically normal cervical specimens from patients with CIN disease, 10 CIN I (mildly dysplastic) lesions, 8 CIN II (moderately dysplastic) lesions, 15 CIN III (severely dysplastic) lesions, and 24 SCCs. Of the frozen specimens, consecutive sections were cut, the first and last of which were stained with H&E for careful histomorphological examination. This examination also included a crude estimation of the percentage of dysplastic/neoplastic cells relative to histomorphologically normal epithelial and underlying stromal cells, as well as the relative degree of lymphocytic infiltrate. Series of in-between sections were used for extracting protein, RNA, and DNA for TRAP, RT-PCR, and HPV DNA PCR purposes, respectively.

Primer Selection and RT-PCR for hTERT. On the basis of the cDNA sequences conserved between hTERT (20) and hEST2 (21), hTERT oligonucleotides were to be used for RT-PCR. The PCR products were checked using the PCGene software (IntelliGenetics, Inc.). The nucleotide sequence of the selected oligonucleotides were: Forward PCR primer hTERTF: 5'-GAAGGGACTTGGCACGGTCAACG-3' (nt position 2029/2030–2054/2055 of hTERT/EST2); reverse PCR primer hTERTRF: 5'GGTTCGTGGATCATGCTCGGTAGACC-3' (nt position 2236/2237–2259/2260 of hTERT/EST2); internal oligonucleotide probe hTERT3P: 5'-GCCGGCTCAGCCTACTTTTGTGGAAGTTGA-3' (nt position 2161/2162–2190/2191 of hTERT/EST2). Primers hTERTF1 and hTERTRF2 direct the amplification of a 230-bp cDNA fragment in the PCR. These primers were selected in such a way to flank putative splice junctions, allowing a discrimination between amplification of spliced cDNA and coamplification of traces genomic DNA eventually present in the RNA sample. Indeed, hTERTF1 and hTERTRF2 directed the amplification of a genomic DNA fragment approximately 800 bp in size, indicating that at the mRNA level a fragment(s) of about 570 bp is spliced out.

For RNA quality analysis RT-PCR was performed using intron flanking primers specific for the cDNA encoding the U1 small nuclear ribonucleoprotein specific A protein (snRNP U1A mRNA). The following oligonucleotide was used for this target: Forward PCR primer U1A1: 5'-CAGTATGCGGAAGACCAGACTCAAGA-3'; reverse PCR primer U1A2: 5'-GGCCCGCGCATTTGGTGCATTAAA-3'; internal oligonucleotide probe U1A3 5'-AGAGGAAGAGCCAGACCCGATCAAA-3'. Primers U1A1 and U1A2 direct the amplification of a 215 bp cDNA fragment.

Total RNA was extracted using RNAzol B, according to instructions of the manufacturer (Campro Scientific). First strand cDNA was synthesized using antisense primers for both hTERT and the housekeeping gene snRNP U1A in a single reaction. Subsequent RT-PCR reactions for hTERT and snRNP U1A were performed separately, the latter serving as a control for both proper RNA quality and a successful cDNA synthesis. First strand cDNA synthesis was performed on 100 ng of total RNA. The conditions of cDNA synthesis, PCR amplification for 40 cycles, and oligonucleotide hybridization were essentially as described before (27). After PCR, 10 μL of product was electrophoresed on a 1.5% agarose gel, blotted onto a nylon membrane (Qiagen), and hybridized with (y-32P)ATP end-labeled hTERT3P and U1A3 oligoprobes for hTERT and snRNP U1A targets, respectively, as described previously (27). Autoradiography was performed for 8 h (snRNP U1A) up to 24 h (hTERT) at −80°C, using intensifying screens.

For semiquantitative assessment of hTERT mRNA levels, the CIN lesions and histologically normal samples as well as 100 ng of SiHa RNA were subjected to another RT-PCR round for 30 cycles. At this number of PCR cycles the amplification reaction showed linearity, as was determined on serial dilutions of SiHa RNA. All PCR products were run on the same agarose gel and blotted to the same nylon membrane, before hybridization with the hTERT3P and U1A3 oligonucleotide probes, respectively. Signal intensities were measured after exposure of the hybridized filters to a phosphor imager (Molecular Dynamics). Levels of hTERT mRNA, normalized to the hTERT levels from 100 ng SiHa RNA were calculated according to the following formula: intensity ratio hTERT:snRNP U1A of the sample: intensity ratio hTERT:snRNP U1A of 100 ng SiHa RNA × 100%.

Statistical comparison of hTERT mRNA levels was carried out using the Mann Whitney U test. Differences were considered significant when P < 0.05.

HPV Detection and Typing. HPV DNA was detected by general primer-mediated PCR using the GP5+/GP6+ primer combination (28). Typing was performed on GP5+/GP6+ PCR products for 14 high-risk (HPV 16, 18, 31, 33, 35, 39, 45, 51, 52, 56, 58, 59, 66, and 68) and 6 low-risk (HPV 6, 11, 40, 42, 43, and 44) HPV types using a nonradioactive enzyme immunoassay as described (29).

Results

Analysis of Telomerase Activity and hTERT mRNA Expression in Cervical Carcinoma Cell Lines. Telomerase activity was measured by TRAP analysis on dilution series of protein extracted from the HPV containing cervical cancer cell lines CaSkii, HeLa, and SiHa. Telomerase activity, characterized by a regular step ladder pattern, was clearly detectable in down to 5 ng of protein of all cell lines in a
of SiHa protein are shown. Temperatures and incubation times are indicated above the lanes. O/N, overnight. C. representative results of TRAP analysis on one CIN I, two CIN II, four CIN III, and four SCC samples. Reaction products without (−) and with (+) a prior preheating step to inactivate telomerase are shown. POS, the positive control.

Fig. 1. Telomerase activity measurements using the TRAP assay. A. TRAP analysis on serial protein dilutions of SiHa, CaSk, and HeLa cell lines in a background of 500 ng of BSA. The amounts of cell line protein are indicated above the lanes. B. stability analysis of telomerase complex by preincubating pellets of SiHa cells at several temperatures during various time intervals. TRAP results on 500 ng (left lanes) and 50 ng (right lanes) of SiHa protein are shown. Temperatures and incubation times are indicated above the lanes. O/N, overnight. C. representative results of TRAP analysis on one CIN I, two CIN II, four CIN III, and four SCC samples. Reaction products without (−) and with (+) a prior preheating step to inactivate telomerase are shown. POS, the positive control, comprising 500 ng of SiHa protein.

subsequently, hTERT mRNA expression was analyzed by RT-PCR for 40 cycles on dilution series of SiHa RNA in a background of 100 ng of RNA from primary foreskin keratinocytes (EK94–2, passage 6). Amplified hTERT cDNA was detectable in down to 10 pg of SiHa RNA (Fig. 3, right upper panel). Assuming that 1 cell contains approximately 10 pg of RNA, this indicates that the RT-PCR method allowed the detection of hTERT mRNA in a single carcinoma cell of the SiHa cell line in a background of 10,000 hTERT mRNA negative cells.

Telomerase Activity in Cervical Lesions. Protein extracts of a total of 65 samples did not reveal any evidence for the presence of PCR inhibitors and were considered appropriate for TRAP analysis (data not shown). During the course of this study, samples were rescreened with an internal PCR control in the TRAP assay using the commercially available TRAPEze kit (Oncor), which gave consistent results.

TRAP analysis revealed that none of the histomorphologically normal (n = 8), CIN I (n = 10), and CIN II (n = 8) samples had detectable telomerase activity. However, telomerase activity was detectable in 6 of 15 CIN III lesions (40%). Histomorphological examination revealed no correlation between telomerase activity and the dysplasia/stroma ratio nor the degree of lymphocytic infiltrate in CIN lesions. Examples of H&E-stained sections of CIN III lesions with and without detectable telomerase activity are shown in Fig. 2. Furthermore, 23 of 24 SCC samples (96%) showed telomerase activity. Also in these cases, the relative percentage of neoplastic cells or degree of lymphocytic infiltrate did not correlate with detectable activity. TRAP results on cervical samples are exemplified in Fig. 1C. A summary of the telomerase data is given in Table 1.

Table 1. Telomerase Activity and hTERT mRNA Status in Cervical Lesions

<table>
<thead>
<tr>
<th>Parameter</th>
<th>Number of Samples</th>
<th>Telomerase Activity</th>
<th>hTERT mRNA Status</th>
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<tbody>
<tr>
<td>Normal (n = 8)</td>
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<tr>
<td>CIN I (n = 10)</td>
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<td>CIN II (n = 8)</td>
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<td>SCC (n = 24)</td>
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<td>Positivity</td>
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<td>SCC (n = 23)</td>
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This prompted us to perform a semiquantitative RT-PCR for 30 cycles on the 26 CIN lesions and 6 morphologically normal samples. The hTERT:snRNP U1A RT-PCR signal intensity ratios, normalized to those obtained with 100 ng of SiHa RNA (set to 100%), varied considerably from 0–78%. A hTERT:snRNP ratio value of more than 0% was scored for 1 of the 6 normal samples (17%), 3 CIN I (n = 10) lesions (30%), 4 CIN II (n = 6) lesions (67%), and all CIN III (n = 10) lesions (100%). Among the cases showing telomerase activity (n = 6) these ratios ranged from 19–78% (mean, 45%), whereas cases without telomerase activity (n = 26) displayed a range of 0–78%.
from 0–7% (mean, 2%). The difference in ratio values between samples with and without detectable telomerase activity was statistically significant (P = 0.0002). Semiquantitative RT-PCR results of the 18 CIN lesions that initially revealed a hTERT mRNA signal after 40 cycles of amplification are shown in Fig. 4.

HPV DNA in Relation to Telomerase Activity and hTERT mRNA Expression in Cervical Lesions. As determined by GP5+/GP6+ general primer-mediated HPV PCR, HPV DNA was detectable in 2 normal (n = 8), 5 CIN I (n = 11), 2 CIN II (n = 8), and all CIN III (n = 15) samples and all SCCs (n = 24). All HPV-positive samples contained DNA of one or more of the high-risk HPV types HPV 16, 18, 33, 35, 45, 56, 58, or 59. Except for 1 normal, 1 CIN I, 2 CIN II, 2 CIN III, and 3 SCC samples, all HPV-positive cases contained DNA of HPV types 16 and/or 18. Comparative analysis revealed that telomerase activity was not detectable in cases that were high-risk HPV negative (Table 1). On the other hand, a significant number of high-risk HPV-positive cases also did not show telomerase activity. These comprised all high-risk HPV containing normal, CIN I, and CIN II samples, 9 of 15 CIN III, and 1 of 24 SCC samples.

Conversely, hTERT mRNA expression was not only limited to HPV-positive cases (Table 1). However, elevated hTERT messages, characterized by hTERT:snRNP U1A ratios of 19% or more, were only detected in high-risk HPV-positive cases (Fig. 4). Moreover, when considering only the CIN lesions and normal samples without detectable telomerase activity (n = 26), a significant difference in the ratio value was evident between those that were HPV-positive (range, 0–7%; mean, 3.1%) and the HPV-negative (range, 0–3%; mean, 0.5%) cases (P = 0.017).

DISCUSSION

This study aimed at the analysis of telomerase activity and hTERT mRNA expression in the spectrum of cervical premalignant lesions. For this purpose, the utility of both TRAP and RT-PCR methods was first tested in reconstruction experiments on immortal cells of cervical cancer cell lines. It seemed that in our hands the hTERT RT-PCR method was approximately 100 times more sensitive than TRAP in detecting immortal cancer cells, reaching a sensitivity of approximately 1 SiHa cell in a background of 10,000 keratinocytes without telomerase activity. Moreover, we showed a rather high stability of the telomerase complex after incubating SiHa cells at temperatures up to 37°C during varying time intervals. This feature potentially allows a reliable examination of telomerase activity in clinical samples, notwithstanding the time span between biopsy taking and freezing.

The results on telomerase activity in cervical carcinomas are in accordance with the data obtained by other groups (9–17). The single SCC devoid of enzyme activity, which was encountered in this study, is likely to represent one of the uncommon cases in which telomere compensation is achieved by an alternative, telomerase-independent mechanism (30). In contrast, telomerase data on premalignant lesions and normal cervical epithelium seem to contradict results obtained by some other investigators. Several groups showed already telomerase activity in a subset of normal cervical samples and often a progressive increase in frequency from low-grade to high-grade lesions (10–13, 15, 16). The discrepancy may, in part, be explained by differences in sensitivity of the TRAP protocols used. In our study, 500 ng of input protein was used for TRAP, and autoradiography was performed for 24 h. Kyo et al. (13) have reported that telomerase activity in normal cervical samples and low-grade lesions, when present, was only detectable when using 1–5 μg of input protein, but not 10-fold dilutions of these amounts. Moreover, using 6 μg of input protein, Shroyer et al. (16) could detect telomerase activity in a significant subset of normal and low-grade cervical samples only after 7 days of autoradiography. In addition, differences in morphological assessment may effect the outcome of telomerase studies. In the majority of studies reported, histomorphological assessment was performed on samples different from those used for TRAP analysis. In our study, histomorphological assessment was most optimal, because this was performed on adjacent tissue sections flanking the sections that were used for protein and RNA analysis. Reconstruction experiments we performed on frozen pellets of SiHa cells revealed that sectioning before cell lysis does not lead to a decreased sensitivity in detecting telomerase activity, due to possible air-drying artifacts. Finally, some studies have used exfoliated cervical cells rather than tissue for TRAP analysis (10, 11, 13, 15). Pilot studies we performed on accompanying cervical scrapes learned that TRAP scores in the scrapes do not always correspond with the scores obtained from the biopsy specimens. Apparently, exfoliated superficial epithelial cells do not always represent a reliable indicator of telomerase activity in the underlying lesion, as has been found by Gorham et al. (11), as well.

Recent studies have shown that in vitro the HPV 16 E6 oncoprotein is capable to induce telomerase activity in monolayer cultures of proliferating keratinocytes (31). Consequently, the observed patterns of telomerase activity in CIN lesions, the majority of which contained HPV 16 DNA, may be due to differences in levels of E6 expression. Moreover, because telomerase activity in immortal cells is influenced by factors like the phase of the cell cycle or status of differentiation

Fig. 2. Examples of H&E-stained sections of the frozen tissue from C1N III lesions with (A) and without (B) detectable telomerase activity. Magnification: × 400 (A) and × 200 (B).
Table I Telomerase activity and hTERT mRNA expression in relation to HPV DNA presence in cervical premalignant lesions

<table>
<thead>
<tr>
<th>Lesion</th>
<th>Telomerase activity</th>
<th>hTERT mRNA expression</th>
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<tr>
<td></td>
<td>Telomerase positive</td>
<td>Telomerase negative</td>
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<tr>
<td>Normal</td>
<td>8</td>
<td>2</td>
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<tr>
<td>CIN I</td>
<td>10</td>
<td>4</td>
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<tr>
<td>CIN II</td>
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<td>CIN III</td>
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<td>SCC</td>
<td>24</td>
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Telomerase activity was determined on a total of 65 samples.

hTERT RT-PCR for 40 cycles was applied to a total of 56 samples.

(32–34), also topological differences of E6 expression within the stratified squamous epithelium may underlie the observed heterogeneity in telomerase activity. Particularly in low-grade CIN lesions, transcription of the E6 and E7 oncogenes is primarily observed in differentiated cells (35, 36). Because these cells have withdrawn from the cell cycle, telomerase complex, when present, is likely to be silent. In contrast, high-grade CIN lesions often display elevated E6/E7 transcripts in the proliferating basal-like cells that occupy much or all of the epithelium. In these cases, the eventual telomerase induction by E6 may become manifest. However, pilot E6/E7 mRNA in situ hybridization experiments on a small series of HPV 16 containing CIN III lesions with and without detectable telomerase activity revealed no topological differences in E6/E7 transcription; the presence of E6/E7 transcripts in the proliferating cell layers could be demonstrated in both cases with and without detectable telomerase activity. Consequently, it is unlikely that expression of telomerase activity in CIN lesions is solely a reflection of E6 expression in proliferating cells. Instead, it is more likely that E6/E7 transcription in proliferating cells is a prerequisite for induction of telomerase activity, a process that apparently requires additional alterations within the host cell. This hypothesis fits very well with recent data on HPV-mediated immortalization in vitro (37).

Furthermore, we showed that the presence of hTERT mRNA per se was not indicative for telomerase activity. However, when considering the amounts of hTERT mRNA, it became clear that relatively elevated hTERT transcript levels are strongly correlated with detectable telomerase activity. Similar results have been obtained after analysis of human liver tissue samples (24). Therefore, the results may reflect a higher sensitivity of the RT-PCR method compared with the TRAP method we used. One caveat to this conclusion is the possibility that hTERT mRNA presence does not necessarily indicate telomerase activity, because posttranscriptional regulation may take place or, alternatively, a certain threshold level of hTERT mRNA may be required for enzyme activity.

An additional tendency became evident that even after exclusion of cases with telomerase activity, hTERT mRNA levels were significantly higher in the HPV-positive group than in the HPV-negative group of samples. Whether this would be due to any effect of HPV-
encoded genes (e.g., E6), eventually in combination with the topology of its expression, remains to be examined. One aspect that should be considered, particularly in the case of low-level hTERT expression, is that it is still unknown to what extent the detected mRNAs are derived from the atypical cells present in the lesion. It has been described that both activated lymphocytes and epithelial stem cells may express telomerase activity at low levels (38-41), and consequently also hTERT. Although in this study no correlation was found between the degree of lymphocytic infiltrate and detectable telomerase activity or hTERT transcripts, it cannot be excluded that low hTERT levels represent low quantities of these cell types expressing telomerase activity. This may result in telomerase activities that fall beyond the detection level of TRAP but not of the hTERT RT-PCR. Therefore, in situ techniques are required to address hTERT expression in CIN lesions in more detail. Currently, we are in the process of developing an RNA in situ hybridization assay for hTERT. Moreover, work is in progress to generate monoclonal antibodies against hTERT, which may have implications for future immunohistochemical approaches.

Assuming that in addition to high-risk HPV functions host cell alterations are necessary to gain telomerase activity of CIN lesions, one would expect a different genetic make-up of CIN lesions with and without telomerase activity. In vitro, telomerase reactivation and immortalization of primary keratinocytes mediated by HPV 16 or HPV 18, was associated with several cytogenetic and molecular genetic alterations (7, 42). The alterations included allelic losses at 3p, 10p, 11, 13q, and/or 18q, each of which may potentially underlie activation of telomerase. Interestingly, allelic losses at several of these loci have been found in both cervical carcinomas and to different degrees in their precursor lesions (43-45). Loss of heterozygosity studies with a panel of markers specific for these loci are currently underway in an attempt to discover molecular genetic differences between CIN lesions with and without telomerase activity.

Also, specific cytogenetic changes may mark CIN lesions with telomerase activity. Of particular interest are comparative genomic hybridization data reported by Heselmeyer et al. (46), showing that a gain at 3q defines the transition from CIN III to invasive carcinoma. More recent studies have revealed that the gene encoding human telomerase RNA resides at 3q, and chromosomal gains at this locus were associated with overexpression of the human telomerase RNA gene (47). Overexpression of hTert was detectable in 43% of cervical carcinomas (48). Another potentially intriguing finding is that approximately one-third of cervical carcinomas are characterized by a gain, if not amplification, at 5p (49), the locus where the hTERT gene is encoded. Of particular interest are comparative genomic alterations (7, 42). The alterations included allelic losses at 3p, lOp, l1q and/or 18q. Similar studies with a panel of markers specific for these loci are currently underway in an attempt to discover molecular genetic differences between CIN lesions with and without telomerase activity.

The establishment of telomerase parameters as late progression markers for CIN lesions.

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REFERENCES

TELOMERASE AND hTERT mRNA STATUS IN CERVICAL LESIONS
Telomerase Activity Exclusively in Cervical Carcinomas and a Subset of Cervical Intraepithelial Neoplasia Grade III Lesions: Strong Association with Elevated Messenger RNA Levels of Its Catalytic Subunit and High-Risk Human Papillomavirus DNA

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