Molecular Markers Implicating Early Malignant Events in Cervical Carcinogenesis

Hanna-Mari Koskimaa, Kaisa Kurvinen, Silvano Costa, Kari Syrjänen, and Stina Syrjänen

Abstract

Background: Human papillomavirus can induce a stepwise progression of precursor lesions to carcinoma. Sensitive and specific molecular markers are needed to identify the cervical lesions (CIN) at risk for this progression. hTERT activation could be one indicator of a point of no return in malignant progression.

Methods: The UT-DEC-1 cell line is an in vitro model for the study of human papillomavirus-induced progression. Using molecular mining, nine potential genes interlinking hTERT and viral oncogene expression with the phenotypical features of CIN2 were identified. After preliminary testing with real-time PCR, five genes were selected for further analysis: hTERT, DKC1, Bcl-2, S100A8, and S100A9. These proteins were also tested in a series of 120 CIN lesions using immunohistochemistry.

Results: Analysis of the mRNA expression of these genes at different cell passages revealed three time points with significant changes. hTERT, Bcl-2, and S100A9 were also overexpressed in CIN lesions, and the expression pattern changed during the progression toward CIN3 lesions.

Conclusions: These identified time points that were combined with the mRNA overexpression of target genes matched events previously shown to be important in the progression toward malignancy: (a) the viral integration into the cell genome and episome loss; (b) the selection of cells with an acquired growth advantage and ability to maintain telomerase activity; and (c) the final stage of malignancy with permanently upregulated telomerase.

Impact: hTERT, Bcl-2, and S100A9 together might compose a potential prognostic marker panel for the assessment of CIN lesions. These results, however, need further validation in prospective clinical settings.

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Introduction

Human papillomavirus (HPV) is the single most important risk factor for cervical cancer (CC) and its precursor (cervical intraepithelial neoplasia (CIN)) lesions (1, 2). HPV infections are very common in the general population. If left untreated, an estimated 15% of the CIN2 lesions will progress to CIN3, and eventually, approximately 40% of the CIN3 lesions will progress to carcinoma in situ within 12 months (2-4). In countries with insufficient resources for organized screening programs, CC continues to be the second most common type of cancer among women and a significant cause of death (5, 6). Thus, finding a highly specific and sensitive marker or marker set that can be used in routine pathology to identify which CIN lesions have a higher risk for progression will be both cost-effective and important.

High-risk HPV types are detectable in almost 100% of CCs, the most common being types 16, 18, 31, 33, 35, 45, 52, 51, and 58 (7-9). High-risk HPV types induce transformation of epithelial cells mainly through their oncogenes E6 and E7. The key function of the oncoprotein products of these oncogenes in cell transformation is their capability to target the tumor suppressor proteins p53 and pRb, respectively, leading to a disturbance of cell cycle control (10). E6 and E7 are overexpressed in most CC cases (2, 11, 12), which has been suggested to associate with the integration of HPV DNA into the host-cell genome conjoined with the disruption of the viral E2 gene. Its protein product, E2, normally downregulates the expression of E6 and E7 (13-15). In the majority of HPV16- and HPV18-positive CCs, the virus has been detected in an integrated form (15-18), although there are also HPV-positive CCs in which the virus is either in a mixed form or only in episomes (15, 18).

We have used the HPV33-positive UT-DEC-1 cell line (originally established from VAIN1, vaginal intraepithelial lesion) as a model for HPV-induced carcinogenesis in vitro, and our previous studies have revealed the following critical features: (a) the HPV genome is mostly...
episomal in cells until passages 19 and 20, during which the episomes are totally lost by integration; (d) in the subsequent passages, only the integrated form of HPV is detected at the chromosome 5p14 integration site (19); (e) in the UT-DEC-1 tissue culture model, progressive changes in the phenotype of the epithelium are found (20); (f) the cell line loses its response to retinoids at later passages (21); and (g) a shift from the mitogen-activated protein kinase pathway to cell cycle dysregulation (G2-M) is found. Taken together, these results support the progression toward malignancy (22).

Elevated telomerase activity is reported in most cancers (23-25). Importantly, in previous findings, we observed that the mRNA levels of hTERT (encoding the catalytic subunit of telomerase) and telomerase activity in cells carrying episomal virus seemed to be constant during passaging, but in later passages, when cells were carrying integrated HPV, however, the mRNA level of hTERT and telomerase activity seemed to increase sharply (26). HPV E6 has been suggested to induce telomerase activity during the early stages of transformation, giving the cells a growth advantage so that they eventually displace telomerase-deficient cells (27-31). The E7 protein has also been shown to contribute to telomerase activation and maintenance (32).

We hypothesized that there must be “a point of no return” in the path of the transformed cells to malignancy. A possible point of no return in the malignant progression of HPV-infected lesions could be when an increased activity of hTERT and telomerase is seen. We tested our hypothesis by molecular mining to trace molecules involved in the telomerase and HPV E6/E7 expression. Nine molecules interlinking HPV integration, telomerase activation, dedifferentiation, and the proliferation of epithelial cells were identified. After preliminary testing, the most promising candidates were selected for mRNA quantitation at different passages: two subunits of telomerase (the catalytic protein gene hTERT and the DKC1 gene encoding dyskerin; ref. 33), the proto-oncogene Bcl-2, and two calcium-binding protein genes, S100A8 (calgranulin A, MRP8) and S100A9 (calgranulin B, MRP14). Finally, the protein expression of these selected genes was assessed in different CIN lesions by immunohistochemistry to estimate their potential use in routine diagnostics.

Materials and Methods

Molecular mining of target genes

The target gene selection was based on a hypothesis that viral oncogene expression might trigger telomerase upregulation in an early subpopulation of cells, which could have the advantage of growth and selection during subsequent passaging in which the driving force is telomerase rather than E6/E7 expression. The potential markers should also be involved in cellular proliferation, dedifferentiation, and avoidance of apoptosis, which are the hallmarks of CIN3 or carcinoma in situ. A connection with the Ca²⁺ metabolism of the cell was also assessed, as Ca²⁺ concentration is critical for epithelial differentiation. The molecular mining was done with the ArrayAssist Expression (Stratagene) and PathwayArchitect software (Stratagene).

Cell lines

The UT-DEC-1 cell line has been established from an HPV33-positive mild vaginal dysplasia (VAIN1 lesion) in 1990 and HPV33 DNA has been detected with Southern blot hybridization and PCR (34). The viral integration site has been scored using the amplification of papillomavirus oncogene transcripts assay (19). The cell line is widely characterized also later (20-22, 26). Four other cell lines were also used, two transformed cell lines and two carcinoma cell lines, to control the mRNA expression of the selected genes: the HMK cell line (passage 25), a spontaneously transformed cell line originally established as a normal mucosal keratinocyte cell line isolated from surgical gingival biopsies by explant culture technique (35); the IHGK (immortalized human gingival keratinocyte) cell line, derived from healthy gingival epithelium and immortalized by transfection with the HPV16 E6 and E7 oncogenes (36); the CaSki cell line (CRL-1550, passages 12-15, obtained from the American Type Culture Collection, LGC Promochem), originally established from a CC and reported to carry 600 copies of the integrated HPV16 genome as well as sequences related to HPV18; and a HeLa cell line with approximately 50 copies of the integrated HPV18 genome, established from a cervical adenocarcinoma (CCL-2, obtained from the American Type Culture Collection).

Cell cultures

In the present study, UT-DEC-1 cells from passages 7 to 185 were used. All cells from passages 24 to 185 were taken and grown from cryopreserved stocks. Because the availability of early passage cells is limited, the cryopreserved total RNA isolated earlier from passages 7 to 21 was used. The cells were cultured in 75-cm² bottles in K-SFM supplemented with 5 μg/mL epidermal growth factor and 50 μg/mL bovine pituitary extract (Life Technologies, Inc.). HMK cells were cultured under similar conditions. The IHGK cell line (p19) was cultured in defined K-SFM with a low calcium concentration (0.045 mmol/L). The CaSki and HeLa cell lines were cultured in DMEM supplemented with 2 mmol/L L-glutamine, 1% nonessential amino acids, 50 μg/mL streptomycin, 100 units/mL penicillin, and 10% fetal bovine serum (Life Technologies). Cells were harvested when 80% confluence was attained.

RNA extraction

Total RNA from the test cells was extracted using Trizol reagent (Invitrogen) according to the manufacturer’s guidelines.

Reverse transcriptase real-time PCR

The reverse transcriptase reactions were carried out using the First-Strand cDNA Synthesis Kit (Amersham
Biosciences) and total RNA as a template. Reactions were performed according to the manufacturer’s instructions by using random hexamers for priming.

Real-time PCR reactions were done in a reaction volume of 20 μL containing 25 to 200 ng of sample cDNA with Universal MasterMix (Applied Biosystems) and TaqMan Gene Expression assays (Applied Biosystems) for hTERT (Hs99999022_m1), DCK1 (Hs00154737_m1), Bcl-2 (Hs00608023_m1), S100A8 (Hs00374263_m1), S100A9 (Hs00610058_m1), BIRC5 (Hs00153353_m1), p300 (Hs00230938_m1), Hsp90 (Hs00743767_sH), and c-Myc (Hs00153408_m1). Real-time PCR reactions were done with the ABI PRISM 7700 Sequence Detection System and 7900HT Fast Real-Time PCR System (Applied Biosystems). The reaction conditions were 2 minutes at 50°C, 10 minutes at 95°C, and a two-step cycle of 95°C for 15 seconds and 60°C for 60 seconds for a total of 40 cycles. Each run included standard curves prepared by amplification of a dilution series of 400 to 3,125 ng of cDNA from the CaSk samples. In addition, three no-template control reaction mixtures were added in every run. All reactions were done in triplicate, and every run was repeated twice. The amplification curves were drawn and the threshold cycles analyzed with the instrument’s software programs, SDS 1.7 and SDS 2.3 (Applied Biosystems). The average of the triplicates was taken, and the results were normalized against the mRNA levels of the housekeeping gene GAPDH (Applied Biosystems). Four pretested genes, BIRC5, P300, Hsp90, and c-Myc, with no notable changes in mRNA expression in different cell passages, were eliminated from the subsequent analyses.

Real-time reverse transcription-PCR analysis was done first with the ABI PRISM 7700 Sequence Detection System and later with the 7900HT Fast Real-Time PCR System from the same manufacturer due to failure of the hardware. The obtained Ct values from both instruments were compared with each other. Due to the high SD of Ct values from the same manufacturer due to failure of the ABI PRISM 7700 Sequence Detection System and 7900HT Fast Real-Time PCR System (0.924-1.703), the analyses were repeated twice with the ABI PRISM 7700 Sequence Detection System and 7900HT Fast Real-Time PCR System (0.938-1.775) and Ct values between the instruments were compared with each other. Due to the high SD of Ct values from the same manufacturer due to failure of the ABI PRISM 7700 Sequence Detection System and 7900HT Fast Real-Time PCR System, the analyses were repeated twice with the ABI PRISM 7700 Sequence Detection System and 7900HT Fast Real-Time PCR System, and every run was repeated twice. The SD of Ct values from the subsequent analyses.

Immunohistochemical staining

The samples used for immunohistochemical staining consisted of 120 cervical biopsies that were diagnosed, graded, and treated earlier at the Department of Obstetrics and Gynecology, University of Bologna (Italy), between June 1986 and March 2002. The same cohort had been previously studied (37, 38). The directed punch biopsy samples were fixed immediately after sampling in 10% neutral formalin and embedded in paraffin. These biopsy samples represent all grades of CC precursors, including normal epithelium (control; n = 30, 25.0%), CIN1 (n = 25, 20.8%), CIN2 (n = 20, 16.7%), and CIN3 (n = 37, 30.8%), as well as invasive CC (n = 8, 6.7%).

Based on the results from the UT-DEC cell line, the following potential markers of the point of no return were selected for immunohistochemistry: Bcl-2, S100A8/ S100A9 (calgranulin), dyskerin, and hTERT. The Bcl-2 protein was detected using the mouse monoclonal anti-human Bcl-2 oncoprotein antibody, clone 124, which recognizes an epitope between amino acids 41 and 54 (dilution 1:75; DakoCytomation). The monoclonal mouse anti-human myeloid/histiocyte antigen, clone MAC 387, was used for identifying the calgranulin protein, a calcium-binding molecule consisting of two major subunits, S100A8 and S100A9 (dilution 1:250; DakoCytomation). The rabbit polyclonal anti-DKCI antibody (HPA000166) was used for detection of dyskerin (dilution 1:100, Atlas antibodies AB, Alba Nova University Center, Stockholm, Sweden). The rabbit polyclonal anti-human hTERT antibody (PC563) was used for detection of the hTERT protein (dilution 1:100; EMD Biosciences, Merck KGaA). This antibody recognizes the amino acids 348 to 358 of human hTERT.

Four-micrometer-thick sections were cut from formalin-fixed and paraffin-embedded biopsy samples on ChemMate Capillary Gap Microscope slides (Dako A/S) and incubated overnight at 55°C. The sections were deparaffinized in xylene and rehydrated in graded alcohol.

Before immunohistochemistry, Bcl-2 and dyskerin antigen retrieval was done by heating the tissue sections in a buffer of 10 mmol/L Tris and 1 mmol/L EDTA (pH 9.0) in a microwave oven for 10 minutes (600 W). The same pretreatment was used for hTERT antigen retrieval, but instead using a 10 mmol/L citric acid buffer (pH 6.0). Epitope retrieval of S100A8/S100A9 was done with proteinase K. Immunohistochemical staining was done with the Dako TechMate 500 Plus Autostainer (Dako) using reagents from Dako REAL-kit. The sections were washed with distilled water and TBS buffer. The sections were stained with primary antibody and then the secondary biotinylated antibody (antimouse IgG) for 30 minutes each. Endogenous peroxidase activity was blocked using 5% hydrogen peroxide for 3 × 2.5 minutes. Blocking was followed by incubation with streptavidin peroxidase for 30 minutes. Counterstaining was done with hematoxylin for 1 minute, and the immunoperoxidase reaction was developed using 3,3′-diaminobenzidine for 3 × 5 minutes. Finally, the sections were washed with distilled water and mounted with Aquamount (BDH Laboratory Supplies).

Statistical analysis

SPSS v.16.0.2 for Windows (SPSS, Inc.) was used for statistical analyses. Frequency tables were analyzed using χ² test or Fisher’s exact test (when appropriate) with Pearson’s R or likelihood ratio (LR) statistics to assess the significance of the correlation between categorical variables. Differences in the means of continuous variables between the groups were analyzed using ANOVA (when appropriate) or nonparametric tests (Mann-Whitney, Kruskal-Wallis). For all analyses, P < 0.05 was regarded as statistically significant.
Results

Real-time reverse transcription-PCR

We identified the following nine target genes by molecular mining: telomerase catalytic subunit hTERT (telomerase reverse transcriptase), telomerase subunit small nucleolar ribonucleoprotein DKC1 (dyskerin, dyskeratosis congenita), the proto-oncogene Bcl-2 (B-cell CLL/lymphoma 2), calcium-binding protein genes S100A8 (calgranulin A, MRP8) and S100A9 (calgranulin B, MRP14), apoptosis inhibitor BIRC5 (surviving, baculoviral IAP repeat-containing 5), p300 (E1A binding protein p300), Hsp90 (heat shock protein 90 kDa α), and c-Myc [v-myc myelocytomatosis viral oncogene homologue (avian)].

The mRNA expression levels of hTERT, DKC1, Bcl-2, S100A8, and S100A9 in UT-DEC-1 cells at analyzed passages between 7 and 185 are summarized in Fig. 1. All mRNA levels were standardized to the mRNA values of the cells from passage 17 because those cells were most representative of the normal phenotype with HPV33 episomes. All reverse transcription-PCR analyses were repeated in triplicate, and the changes in expression discussed below could be consistently reproduced.

There were three time points with obvious changes in mRNA expression of the selected genes during passaging (Fig. 1). The first was passage 21, in which the expression ratios of S100A8 and S100A9 were increased to 5- and 13-fold, respectively. At passage 24, hTERT, DKC1, and Bcl-2 expression were upregulated. These passages represent the stage in early progression where the episomes are lost and integrated viruses remain. From passages p33 to p94, the expression of all target genes were expressed at the same or lower level than those at p17, with the exception of hTERT, which remained slightly upregulated at approximately 1.5- to 2.5-fold.

The second point involved passages 100 and 106, in which the expression rates of Bcl-2 and hTERT increased significantly, up to 11- and 9.5-fold, respectively. DKC1 was also upregulated at p100, nearly 4-fold as compared with passage 17. Bcl-2 and DKC1 expression were elevated from p95 through p106. From passages 107 to 183, the expression of all markers was approximately at or below the level of p17.

The third point was at the late passages of 183 and 185, in which the expression of hTERT mRNA increased 15- to 19-fold, the expression of Bcl-2 increased 5- to 9-fold, and the expression of S100A9 and DKC1 increased 4- to 5-fold when compared with p17 expression levels.

The mRNA levels from the control cell lines HMK, IHGK, CaSki, and HeLa are also shown in Fig. 1. DKC1 was expressed at slightly higher levels (1.5- to 2.0-fold) in all cells except for CaSki. The Bcl-2 mRNA levels were increased ~7-fold in HeLa cells and 1.7-fold in IHGK cells. In IHGK cells, both S100A8 and S100A9 showed higher expression rates, with S100A9 as high as 7.7-fold.

Immunohistochemical staining

The significance of the protein levels expressed from the hTERT, DKC1, Bcl-2, and S100A8/S100A9 (calgranulin) genes in discriminating different CIN lesions were tested by immunohistochemistry (Table 1). All eight CC samples for hTERT and calgranulin were excluded for technical reasons, resulting in 112 samples that were used for statistical analyses. In addition, nine samples with different CINs were technically deficient for Bcl-2 immunohistochemistry, resulting in 103 samples available for statistical analysis. All 120 samples for dyskerin were technically sound. All immunohistochemically stained slides were analyzed by one person, and the results were rechecked by another observer with 94.3% agreement.

Signal intensity was graded from − (negative) to ++++...
Table 1. Expression of S100A8/S100A9, hTERT, Bcl-2, and dyskerin in relation to CIN grade

<table>
<thead>
<tr>
<th>Protein</th>
<th>Expression intensity</th>
<th>HPV/NCIN, n = 30* (%)</th>
<th>CIN1, n = 25* (%)</th>
<th>CIN2, n = 20* (%)</th>
<th>CIN3, n = 37* (%)</th>
<th>SCC, n = 8* (%)</th>
<th>P</th>
</tr>
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<tbody>
<tr>
<td>S100A8/S100A9</td>
<td>+</td>
<td>0</td>
<td>3 (12.0)</td>
<td>2 (10.0)</td>
<td>2 (5.4)</td>
<td>1 (12.5)</td>
<td>&lt;0.001†</td>
</tr>
<tr>
<td>hTERT</td>
<td>++</td>
<td>10 (33.3)</td>
<td>10 (40.0)</td>
<td>6 (30.0)</td>
<td>8 (21.6)</td>
<td>&lt;0.001†</td>
<td></td>
</tr>
<tr>
<td>Bcl-2</td>
<td>++</td>
<td>5 (17.2)</td>
<td>2 (9.5)</td>
<td>2</td>
<td>2 (5.7)</td>
<td>0.616</td>
<td></td>
</tr>
<tr>
<td>dyskerin</td>
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<td>13 (43.3)</td>
<td>5 (20.0)</td>
<td>6 (30.0)</td>
<td>6 (16.2)</td>
<td></td>
<td></td>
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<tr>
<td>S100A8/S100A9</td>
<td>+++</td>
<td>11 (36.7)</td>
<td>10 (40.0)</td>
<td>8 (40.0)</td>
<td>2 (5.4)</td>
<td>2 (25.0)</td>
<td></td>
</tr>
<tr>
<td>hTERT</td>
<td>+++</td>
<td>5 (16.7)</td>
<td>3 (12.0)</td>
<td>7 (35.0)</td>
<td>8 (21.6)</td>
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<tr>
<td>Bcl-2</td>
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<td>15 (51.7)</td>
<td>12 (57.2)</td>
<td>10 (55.6)</td>
<td>14 (40.0)</td>
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<td>11 (44.0)</td>
<td>12 (60.0)</td>
<td>20 (54.1)</td>
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<tr>
<td>S100A8/S100A9</td>
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<td>14 (46.7)</td>
<td>10 (40.0)</td>
<td>8 (40.0)</td>
<td>17 (45.9)</td>
<td>1 (12.5)</td>
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<tr>
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<td>1 (4.0)</td>
<td>2 (10.0)</td>
<td>10 (27.0)</td>
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<td></td>
</tr>
<tr>
<td>Bcl-2</td>
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<td>9 (31.0)</td>
<td>7 (33.3)</td>
<td>6 (33.3)</td>
<td>17 (48.6)</td>
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<td>12 (48.0)</td>
<td>9 (45.0)</td>
<td>19 (51.4)</td>
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<td>4 (16.0)</td>
<td>8 (40.0)</td>
<td>18 (48.6)</td>
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<td>4 (16.0)</td>
<td>5 (25.0)</td>
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<td>19 (51.4)</td>
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<td>18 (90.0)</td>
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<tr>
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<td>15 (60.0)</td>
<td>14 (70.0)</td>
<td>31 (83.8)</td>
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<tr>
<td>Bcl-2</td>
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<td>29 (96.7)</td>
<td>21 (84.0)</td>
<td>18 (90.0)</td>
<td>9 (24.3)</td>
<td></td>
<td>&lt;0.001†</td>
</tr>
<tr>
<td>dyskerin</td>
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<td>28 (93.3)</td>
<td>21 (84.0)</td>
<td>19 (95.0)</td>
<td>25 (67.6)</td>
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<tr>
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<td>0</td>
<td>0</td>
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<tr>
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<td>14 (70.0)</td>
<td>31 (83.8)</td>
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<tr>
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<tr>
<td>S100A8/S100A9</td>
<td>Nuclei</td>
<td>22 (73.3)</td>
<td>17 (68.0)</td>
<td>13 (72.2)</td>
<td>35 (94.6)</td>
<td>0.002†</td>
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</table>

NOTE: Frequency tables were analyzed using χ² test or Fisher’s exact test with Pearson’s R or likelihood ratio (LR) statistics. Differences in the means of continuous variables between the groups were analyzed using ANOVA or the Mann-Whitney and Kruskal-Wallis tests. In all analyses, P < 0.05 was regarded as statistically significant. −, negative; +, low; ++, moderate; ++++, strong; ++++, intense immunoreactivity.

Abbreviation: SCC, squamous cell carcinoma.

*The numbers of representative samples were 112, 112, 103, and 120, respectively.
†P value regarded statistically significant.
‡If the expression is detected in several layers in one sample, this sample is listed multiple times.
§The expression detected in the cytoplasm or/and nuclei of the cells.
∥Nuclear expression in all layers of epithelia.
(intense) with a subjective four-stepped scale: Fig. 2B illustrates moderate staining (++), Fig. 2A illustrates strong (+++) staining, and Fig. 2C and D illustrate intense (++++) staining. Both the lateral and the vertical patterns of the protein expression in epithelial layers were determined. In addition, the intracellular location of the protein in epithelia was categorized as cytoplasmic or nuclear.

The expression of hTERT was significantly different among the different histologic grades. The intensity of hTERT expression was mostly weak or moderate but intensified with increasing CIN \( (P = 0.001) \). The hTERT protein expression in a CIN3 lesion is shown in Fig. 2A. Although hTERT was expressed mostly in the basal and parabasal cells, it was also expressed in the intermediate and even superficial layers of epithelia in CIN3 lesions. The change in expression pattern was significantly different among the different lesions \( (P < 0.001) \). hTERT was detected mainly in the nuclei of epithelial cells.

Dyskerin expression was most prominent in the basal cells (69.2%) or parabasal cells (45.8%) in all histologic grades. Any changes in dyskerin expression, either in location or intensity, were not significantly associated with lesion grade. Dyskerin expression in a CIN3 lesion is shown in Fig. 2B. Dyskerin was expressed both in the cytoplasm and in the nuclei as distinct spots.

The intensity of cytoplasmic Bcl-2 expression showed no significant differences between the CIN grades \( (P = 0.616) \). In contrast, the location of Bcl-2 expression was significantly different among the different histologic grades \( (P < 0.001) \). The intensity was most prominent in the basal cells in all histologic grades \( (77 \text{ of } 103 \text{ samples}, 74.7\%) \), and expression was also detected in the intermediate and superficial layers of the epithelia in CIN3 (Fig. 2C).

The intensity of calgranulin (S100A8/S100A9) expression was significantly associated with histologic grade \( (P < 0.001) \), increasing in parallel with the increasing CIN grade. Similarly, calgranulin expression extended from the intermediate layers to superficial cells as the CIN grade progressed \( (P < 0.001) \). In addition, nuclear expression and lesion grade were significantly related.

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**Figure 2.** Immunohistochemical staining of cervical CIN3 lesions. A, epithelial cell nuclei immunopositive for hTERT. B, immunostaining for dyskerin. Both the basal and parabasal cells express the protein in nuclei. C, immunostaining for Bcl-2. The protein is strongly expressed in whole epithelium. D, immunostaining for calgranulin. The protein is expressed across the whole epithelium, excluding basal cells, and strongly in nuclei. Among all samples, the expression is most intense in the basal layer.
were surprisingly low, even in HeLa cells, which show mRNA analyses of endogenous promoter by E6 is no longer required. The transcription of the hTERT gene in association with Myc (27-30, 32), and E7 contributes to telomerase activation and maintenance concurrently with pRb and E2F through regulation of the hTERT promoter (39). However, we have previously observed enhanced telomerase activity after the integration event while the expression rates of oncogenes E6 and E7 decreased (26). This observation indicates that telomerase activity is not only induced by the viral oncogenes E6 and E7, as is also suggested by Baege et al. (31). The suggestion that E6/E7 expression is not required for continued malignancy, however, is contrary to other wide evidence that E6/E7 expression is required for continued malignancy (40-42).

In line with our findings, James et al. (43) described that E6 in conjunction with E6AP activates hTERT transcription and telomerase by acetylation and activation of the endogenous promoter during both early and late passages of HPV16 E6– and E7–transformed cells. In later passages, however, hTERT transcription is activated through the core promoter of hTERT, and the activation of endogenous promoter by E6 is no longer required. The mRNA analyses of hTERT expression herein are supported by the immunohistochemical staining of CIN lesions, in which both the protein location and staining intensity were significantly correlated with lesion grade. In all control cell lines, hTERT mRNA levels were surprisingly low, even in HeLa cells, which show elevated telomerase activity (44). This observation might indicate that high hTERT levels are needed in early malignancy but not after the point when several genomic changes have occurred, which is presumably the case in the HeLa and CaSki cell lines, which are established from progressed cervical carcinomas (45, 46). In contrast, the two other cell lines, HMK and IHGK, are transformed cells characterized by immortality and numerous progressive chromosomal abnormalities without still being tumorigenic (35, 36). These cells may need additional genetic and/or chromosomal changes for acquiring malignant characters.

The expression of DKCI mRNA was slightly overexpressed, which was congruent with the expression of hTERT. The DKCI gene product dyskerin, a subunit of telomerase, has been suggested to stabilize the telomerase complex by interaction with the hTR subunit. Reduced levels of dyskerin may also be associated with decreased telomerase activity (47-49). In breast cancer studies, dyskerin expression and its activity have been associated with tumor progression, with low expression indicating a better prognosis (50). Here, no changes in dyskerin expression or location were found to be associated with the severity of the CIN lesions. In control cell lines, the expression levels of DKCI were slightly increased, even somewhat more than those of hTERT. This lack of correlation between the mRNA expression in model cells and immunohistochemical tissue staining can be explained by several factors. In this case, the major reason probably involves the control mechanisms for mRNA translation into protein, taking into account that not all mRNAs are always translated to proteins. Other factors such as the properties of the antibody, the stability of dyskerin in old formalin-fixed samples, and the half-life of dyskerin might also affect the immunohistochemical staining results.

Overexpression of Bcl-2 increased during passaging of the UT-DEC-1 cells, in contrast to previous studies that reported its disappearance when cervical lesions progressed to higher grades (51). Bcl-2 functions as an inhibitor of proapoptotic Ca2+ signals, but it also increases Ca2+ signaling, which positively contributes to cell proliferation and survival (52). The viral E6 protein participates in the upregulation of Bcl-2 through p53 degradation. Kurvinen et al. (53) were the first to show that abnormal Bcl-2 expression in suprabasal layers was more common in high-grade CIN lesions (high-grade squamous intraepithelial lesions). The combined p53/Bcl-2 phenotype, which showed a low percentage of p53-positive cells with Bcl-2 overexpression in the upper epithelial layers, was found to be involved in the progression of HPV lesions. In the present study, immunohistochemical staining of Bcl-2 protein was localized mostly to the basal epithelial layer in all CIN lesions, but its expression extended to the upper layers concurrently with progressing lesions. In HeLa cells, Bcl-2 showed distinct overexpression as compared to other control cells and UT-DEC-1 cells.

Discussion

We hypothesized that hTERT activation and the decline of viral E6/E7 mRNA expression (26) could present a point of no return in the progression toward malignancy. Using molecular mining, nine potential genes interlinking hTERT and viral oncogene expression with the phenotypical features of CIN3 were initially identified. After preliminary testing, five potential genes were selected for further analyses: hTERT, DKCI, Bcl-2, S100A8, and S100A9. Analysis of the mRNA expression of these genes during different passages of UT-DEC-1 cells revealed three time points with significant changes. These time points, when the mRNA of target genes was overexpressed, matched with events previously shown to be important in the progression toward malignancy: (a) viral integration into the cell genome and episome loss; (b) the selection of cells with an acquired growth advantage and the ability to maintain telomerase activity; and (c) the final stage of malignancy with permanently upregulated telomerase activity.

The mRNA expression levels of hTERT herein are similar to those found in two previous studies (26, 31). An even more prominent overexpression was seen during the late passages 183 to 185. HPV E6 increases the transcription of the hTERT gene in association with Myc (27-30, 32), and E7 contributes to telomerase activation and maintenance concurrently with pRb and E2F through regulation of the hTERT promoter (39). However, we have previously observed enhanced telomerase activity after the integration event while the expression rates of oncogenes E6 and E7 decreased (26). This observation indicates that telomerase activity is not only induced by the viral oncogenes E6 and E7, as is also suggested by Baege et al. (31). The suggestion that E6/E7 expression is not required for continued malignancy, however, is contrary to other wide evidence that E6/E7 expression is required for continued malignancy (40-42).

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Interestingly, the S100A8 and S100A9 genes showed the most increased expression levels at the passages of the presumed point when episomes are lost. S100A8 (calgranulin A, MRP8) and S100A9 (calgranulin B, MRP14) are members of the calcium-binding protein family S100 genes (54), and these two proteins form a heterodimeric complex (55). They are expressed at very low levels in normal epithelium, but increased expression levels have been observed in activated keratinocytes during their proliferation and differentiation (56). In addition to the important role of calcium as a secondary messenger in the apoptotic and survival processes of the cell, calcium contributes to the inhibition of telomerase activity. In differentiating epithelial cells, Ca\textsuperscript{2+} levels increase and calcium binds S100A8, protein temporarily releasing it from its complex with S100A9. This action, in turn, allows S100A8 to bind to telomerase and inhibit its activity (57).

Thus, the overexpression of these genes might reflect the overall intracellular alterations that the integration event induces in the host cell. However, increased levels of S100A proteins might contribute to the possible appearance of a growth advantage among UT-DEC-1 cells after the integration event. Both genes, especially S100A9, showed distinct overexpression in HPV16 E6/E7-immortalized IIHK control cells. In CIN lesions, the calgranulin protein (with both S100A8 and S100A9 subunits) had a defined expression pattern shift during the progression from normal to high-grade CIN. Indeed, the overexpression of both S100A proteins has been detected in epithelial cancers (58, 59). Recently, Zhu et al. (60) found that the S100A9 protein was overexpressed in CINs, where it has been suggested to correlate with the cellular differentiation of epithelial cancer cells as well as with the molecular mechanisms involved in CC invasion and migration.

Thus, HPV likely induces various alterations in the vital functions of the host cell, first by introducing viral oncogenes into the cells. Over the long term, these changes might result in the activation of telomerase in cancer cells. According to our hypothesis, this point in the transformation process, when the UT-DEC-1 cells are suggested to attain independence from viral oncogenes E6 and E7 as their expression decreases and telomerase activity continues to increase, may have a substantial value in understanding the cellular mechanisms of HPV-induced carcinogenesis.

In conclusion, we hypothesized that a selection of nine genes could be critical in malignant progression. Of these genes, five \(htERT, DKC1, Bcl-2, S100A8, \) and \(S100A9\) (calgranulin) were shown to be potential markers of different steps involved in HPV-induced carcinogenesis in vitro. In particular, \(htERT, Bcl-2, \) and \(S100A9\) (S100A8/S100A9) were overexpressed in cervical CIN lesions, and more importantly, changes in their expression patterns were associated with higher-grade lesions. Thus, \(htERT, Bcl-2, \) and \(S100A9\) might be potential prognostic markers for elucidating high-risk CIN lesions. The results obtained in the UT-DEC-1 cell line need, however, further evaluation in additional cell lines (e.g., the HPV16-positive W12; ref. 61) to establish whether these characteristics are specific to the UT-DEC-1 cell line or are also found in other in vitro models of HPV-induced carcinogenesis and, subsequently, further investigation in prospective clinical settings.

**Disclosure of Potential Conflicts of Interest**

No potential conflicts of interest were disclosed.

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**References**

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