DNA Methylation Is Not Likely to Be Responsible for hTERT Expression in Premalignant Cervical Lesions

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Human telomerase reverse transcriptase (hTERT) mRNA expression seems to play an important role in cervical carcinogenesis. Analysis of the hTERT promoter region revealed the presence of a CpG island and a high overall GC content, suggesting a possible role for methylation in the regulation of hTERT gene expression. The present study was designed to evaluate the role of hTERT promoter methylation and E6/E7 human papilloma virus 16 (HPV-16) mRNA expression in hTERT regulation in premalignant cervical specimens. The methylation status of the hTERT promoter gene and hTERT mRNA quantification were investigated in 26 normal and 64 specimens of abnormal cytology using the MethyLight technique, TeloTAGGG hTERT Quantification Kit and LightCycler technology. E6/E7 HPV-16 mRNA expression was also evaluated. No significant correlations were observed between hTERT mRNA expression and hTERT promoter methylation, as well as between telomerase activity and hTERT promoter methylation in normal and in premalignant cervical specimens. E6/E7 HPV-16 mRNA expression was observed in 72% of HPV-16–infected samples and was correlated with hTERT mRNA expression and telomerase activity ($P < 0.05$). This is the first study investigating the role of hTERT promoter methylation in hTERT mRNA expression and telomerase activity in premalignant lesions. The observed lack of correlation suggests that other mechanisms might be involved in the regulation of hTERT expression. The correlation between hTERT mRNA and E6/E7 mRNA expression confirms the role of HPV infection in hTERT regulation. Exp Biol Med 232:881–886, 2007

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Introduction

The role of telomerase, the ribonucleoprotein responsible for maintaining the ends of chromosomes, has been the subject of intense investigation due to its association with cellular aging, immortalization, and cancer (1). Telomerase is composed of two major subunits contributing to its enzymatic activity that are considered essential for the reconstitution of telomerase activity: human telomerase RNA (hTR), a component that serves as the template for the polymerase activity of the enzyme, and human telomerase reverse transcriptase (hTERT), a conserved catalytic subunit with reverse transcriptase activity (2). Although hTR is highly expressed in all tissues regardless of telomerase activity, with cancer cells having higher expression than normal cells, hTERT is presently considered to be the most important factor in the formation of functional telomerase and concomitant immortalization and is strongly associated with the enzyme's activity (3–6). Studies in both tumor cell lines and human tumor specimens have shown that, in contrast to normal somatic cells, more than 90% of malignant cells are characterized by increased telomerase activity. Several studies have suggested that there are quantitative differences in the level of hTERT mRNA expression in premalignant and malignant cervical lesions, indicating the role of hTERT in early cancer detection (7–9).

Regulation of hTERT, however, is a complex and dynamic process that is tightly linked to the regulation of cell proliferation. Several mechanisms, including genetic and epigenetic events, may regulate control of hTERT transcription, leading to repression or reactivation of telomerase activity in normal and cancer cells in a context-dependent manner (10). More specifically, several virus-encoded proteins have been suggested to contribute to human cell transformation and carcinogenesis (2). Among them, human papillomaviruses (HPVs), especially high-risk types 16 and 18, have been suggested to play an important role in immortalization and transformation of human keratinocytes; their oncogenic activities are mainly attributed to the E6 and E7 oncoproteins, which are able to

abrogate the tumor suppressive function of p53 and Rb signaling pathways (10).

Also, analysis of the promoter region of the hTERT gene has revealed the presence of a CpG island and a high overall GC content, suggesting a possible role of methylation in the regulation of hTERT expression (11). It is known that DNA methylation correlates inversely with gene expression, suggesting that modification itself might play a direct role in the inhibition of transcription (12). Until now, only one study has investigated the correlation between hTERT promoter methylation and hTERT expression in cervical cancer, whereas there has been no study investigating the above correlation in premalignant cervical samples (13).

The present study was designed to determine whether DNA methylation of the hTERT promoter is an epigenetic mechanism regulating hTERT expression and furthermore telomerase activity in cervical samples of different cytology. For the first time, we found a lack of correlation between hTERT promoter methylation and both hTERT mRNA expression and telomerase activity in premalignant cervical samples. Moreover, we investigated HPV-16 E6/E7 mRNA expression, and we observed an association between E6/E7 mRNA and hTERT mRNA expression, confirming the fact that HPV infection rather than hTERT DNA promoter methylation plays a key role in hTERT regulation and therefore in cervical carcinogenesis.

Materials and Methods

Patients. A total of 90 patients were studied, including 64 patients with abnormal cytologic findings and 26 normal patients. The mean age of women with abnormal findings was 37.6 years (range, 19–58 years) and of the control group was 36.8 years (range, 24–50 years). Cervical cytology samples were obtained from the colposcopy unit of University Hospital of Larissa. All abnormal samples were collected at the time of colposcopic evaluation for the management of previous abnormal cytology test results, including repeated cytologic diagnosis of atypical squamous epithelial cells of undetermined significance (ASCUS) or previous diagnosis of low-grade squamous intraepithelial lesions (LGSIL) or high-grade squamous intraepithelial lesions (HGSIL).

Two separate cervical specimens were obtained from each woman in appropriate collection vials using an endocervical cytobrush prior to the colposcopic examination and cervical biopsy and immediately after collection of the routine cervical sample. The study was double blind. The samples were kept on ice and immediately processed in the laboratory. The first specimen was collected in STE buffer (pH 7.5; 0.05 *M* Tris-HCl, 0.1 *M* NaCl, 1 m*M* EDTA) and was used for DNA extraction; the second cervical specimen was collected in TRIzol (Invitrogen Corp., Paisley, UK) and was used for RNA extraction and quantitative hTERT mRNA evaluation. Cervical biopsies for histologic examinations were performed at the end of the colposcopy and were evaluated at the pathology laboratory of Larissa University Hospital. The ethics committee of the hospital approved the protocol, and informed consent was obtained from all patients.

HPV Analysis. Human genomic DNA was extracted from the scraped cervical cells using a proteinase K/phenol-chloroform protocol. HPV detection and genotyping were performed as previously described (14).

Total RNA Isolation. Total RNA was extracted using TRIzol according to the manufacturer's instructions.

Telomere Repeat Amplification Protocol (TRAP) Assay. Telomerase activity was evaluated using the TeloTAGGG Telomerase Polymerase Chain Reaction (PCR) Enzyme-linked Immunosorbent Assay Kit (Roche Diagnostics Corp., Indianapolis, IN) in accordance with the manufacturer's instructions, as previously described (14). The TRAP assay can detect 100 cells in a background of 10,000 telomerase-negative cells (15).

Quantification of hTERT mRNA. Real-time PCR amplification and quantification of hTERT mRNA have been previously described using a commercially available TeloTAGGG hTERT Quantification Kit (Roche Diagnostics Corp.) and LightCycler technology (Roche Mol Biochemics, Mannheim, Germany) according to the manufacturers' instructions. The quantitative real-time reverse transcription (RT) PCR method allowed the detection of hTERT mRNA in 10 cells of cytologic-abnormal specimen in a background of 10,000 hTERT mRNA–negative cells (9).

Several hTERT splice variants have been described, including four insertion variants and two deletion site variants (16). The primers used in the TeloTAGGG hTERT Quantification Kit (Roche Diagnostics Corp.) were selected to exclusively reverse transcribe and amplify hTERT mRNA encoding for the functionally active telomerase protein.

E6/E7 mRNA Detection. RT of the extracted mRNA and PCR for the identification of E6/E7 ± E6* transcripts have been previously described (17). A nested RT-PCR was also developed for sensitive detection and type-specific characterization of E6 and E7 transcripts. HPV-16 revealed two different spliced transcripts referred to as E6*I and E6*II. Unspliced E6/E7 transcripts encode the E6 protein (E6/E7 ± E6*), whereas spliced E6* transcripts (E6*I and E6*II) encode the E7 protein. The RT-PCR and nested RT-PCR were tested for their specificity using an HPV DNA–containing plasmid HPV-16, which was kindly provided by TIB (MOLBIOL, Berlin, Germany). The amplification products were analyzed by electrophoresis on a 2% agarose gel stained with ethidium bromide.

MethyLight Analysis. To assess hTERT promoter methylation, DNA was treated with bisulfite as described previously (18). After sodium bisulfite conversion, methylation analysis was performed by the fluorescence-based, real-time PCR assay, MethyLight. Briefly, one set of primers designed specifically for bisulfite-converted DNA

was used: a methylated set for the gene of interest (GENE) and a reference set for β-actin (ACTB) to normalize for input DNA. Specificity of the reactions for methylated DNA was confirmed separately using *Sss* I (New England Biolabs, Hitchin, England, UK)–treated human genomic DNA (heavily methylated). The percentage of fully methylated molecules at a specific locus was calculated by dividing the GENE to ACTB ratio of a sample by the GENE to ACTB ratio of *Sss* I–treated human genomic DNA and multiplying by 100. The percentage of fully methylated reference (PMR) indicates this measurement; a gene was deemed methylated if the PMR value was greater than zero. To verify the reproducibility of each assay, the normalized values (GENE to ACTB ratio) of the standard sample was compared among the different PCR runs. The primers and probes used for the MethyLight reactions have been previously described (19).

Statistical Analysis. Data were analyzed by unpaired *t*-test, Mann-Whitney *U* test, Kruskal-Wallis, as well as ANOVA, Pearson correlation, and Fisher's protected least significant difference; the post hoc test was used to correct for multiple comparisons when applicable. A two-sided $P < 0.05$ was considered statistically significant. Statistical analysis was performed using SPSS 12 software for Windows (SPSS, Inc., Chicago, IL).

Results

Among the 64 abnormal specimens, 18 samples were diagnosed as ASCUS, 28 samples as LGSIL, and 18 samples as HGSIL. Three of 18 (16.7%) ASCUS samples were histologically classified as cervical intraepithelial neoplasia 1 (CIN 1), and one sample was classified as CIN 2 (5.5%). Twenty-five of 28 (89.2%) LGSIL samples were classified as CIN 1, two (7.2%) as CIN 2, and one (3.6%) as CIN 3. Fifteen of 18 HGSIL (83.3%) were classified as CIN 2, and three (16.7%) as CIN 3.

We previously investigated HPV DNA, telomerase activity, and hTERT mRNA expression in premalignant cervical samples (9, 14). In the present study, HPV DNA was detected in 46 of 64 (85.1%) specimens with abnormal cytologic findings and in 5 of 26 (19.2%) normal specimens. High-risk HPV types 16, 18, 31, 33, 45, 61, and 58 were present in 32 of 64 (59.2%) specimens with abnormal cytologic findings and in 1 of 26 (3.8%) normal specimens. Low-risk HPV types 6, 11, 53, 64, CP141, and CP8304 were detected in 14 of 64 (25.9%) patients with abnormal cytologic findings and in 4 of 26 (15.4%) normal samples. Twenty-five samples were infected with HPV-16, which was the most frequent type in this group of patients.

Telomerase activity was observed in 5 of 18 (27%) ASCUS, in 12 of 28 (42.8%) LGSIL, and in 12 of 18 (66.7%) HGSIL; it was not observed in normal specimens.

hTERT mRNA expression was observed in 6 of 26 (23%) normal samples, in 7 of 18 (38.8%) ASCUS, in 17 of 28 (60.7%) LGSIL, and in 16 of 18 (88.8%) HGSIL. The mean levels of hTERT mRNA expression were 0.10 hTERT mRNA copies/PBGD copies (SEM, 0.02; range, 0–0.7) in normal tissue, 0.31 (SEM, 0.04; range, 0–1.6) in ASCUS, 0.98 (SEM, 0.1; range, 0–3.4) in LGSIL, and 2.33 (SEM, 0.17; range, 0–5.2) in HGSIL. A significant correlation was observed between hTERT mRNA expression and telomerase activity ($P = 0.00$).

hTERT promoter DNA methylation was also observed in 7 of 26 (26.6%) normal samples, in 9 of 18 (50%) ASCUS, in 19 of 28 LGSIL (68.2%), and in 14 of 18 (77.8%) HGSIL. Mean normalized quantitative methylation-specific PCR (QMSP) values of hTERT promoter methylation were 0.8 (range, 0–3.9), 1.6 (range, 0–6.7), 3 (range, 0–11.2), 4.6 (range, 0–18.4) in normal samples, ASCUS, LGSIL, and HGSIL, respectively.

To examine the role of hTERT promoter methylation in the regulation of hTERT mRNA expression and subsequently in telomerase activity, we compared PMR values for hTERT promoter methylation with hTERT mRNA expression as well as with telomerase activity in cervical samples of abnormal cytology. No significant correlation was observed between hTERT mRNA expression and hTERT promoter methylation in normal (Pearson correlation, $r = 0.13$, $P = 0.528$), ASCUS (Pearson correlation, $r = –0.09$, $P = 0.722$), LGSIL (Pearson correlation, $r = 0.04$, $P = 0.841$), and HGSIL (Pearson correlation, $r = –0.287$, $P = 0.248$) cervical samples (Fig. 1a–d). Also, no correlation was found between hTERT promoter methylation and telomerase activity in normal, ASCUS, LGSIL, and HGSIL specimens ($P > 0.05$).

All 25 HPV-16–infected samples were positive for hTERT mRNA expression. The detection rate of E6 and E7 oncogene transcripts was 72% (18 of 25) when both unspliced E6/E7 transcripts encoding the E6 protein and spliced E6* transcripts encoding the E7 protein were considered (E6/E7 ± E6*) and 48% (12/25) for E6* (I and II). A correlation was observed between hTERT mRNA expression and E6/E7 mRNA expression ($P < 0.05$).

Discussion

Studies concerning the hTERT gene have been mostly focused on hTERT expression and its association with telomerase activity (5, 9, 14, 15, 20, 21). In the present study, a correlation was observed between hTERT mRNA expression and telomerase activity, which has been previously reported by us (14) and confirms the major role of hTERT in the regulation of the enzyme. The fact that 23% of the normal samples were found positive for hTERT transcriptional expression could be attributed to the sensitivity of the quantitative real-time RT-PCR method used, which allowed the detection of hTERT mRNA expression in specimens that were found negative for telomerase activity, and also probably to the cytologic and molecular heterogeneity of the specimens.

Research in the last few years has clearly proved that

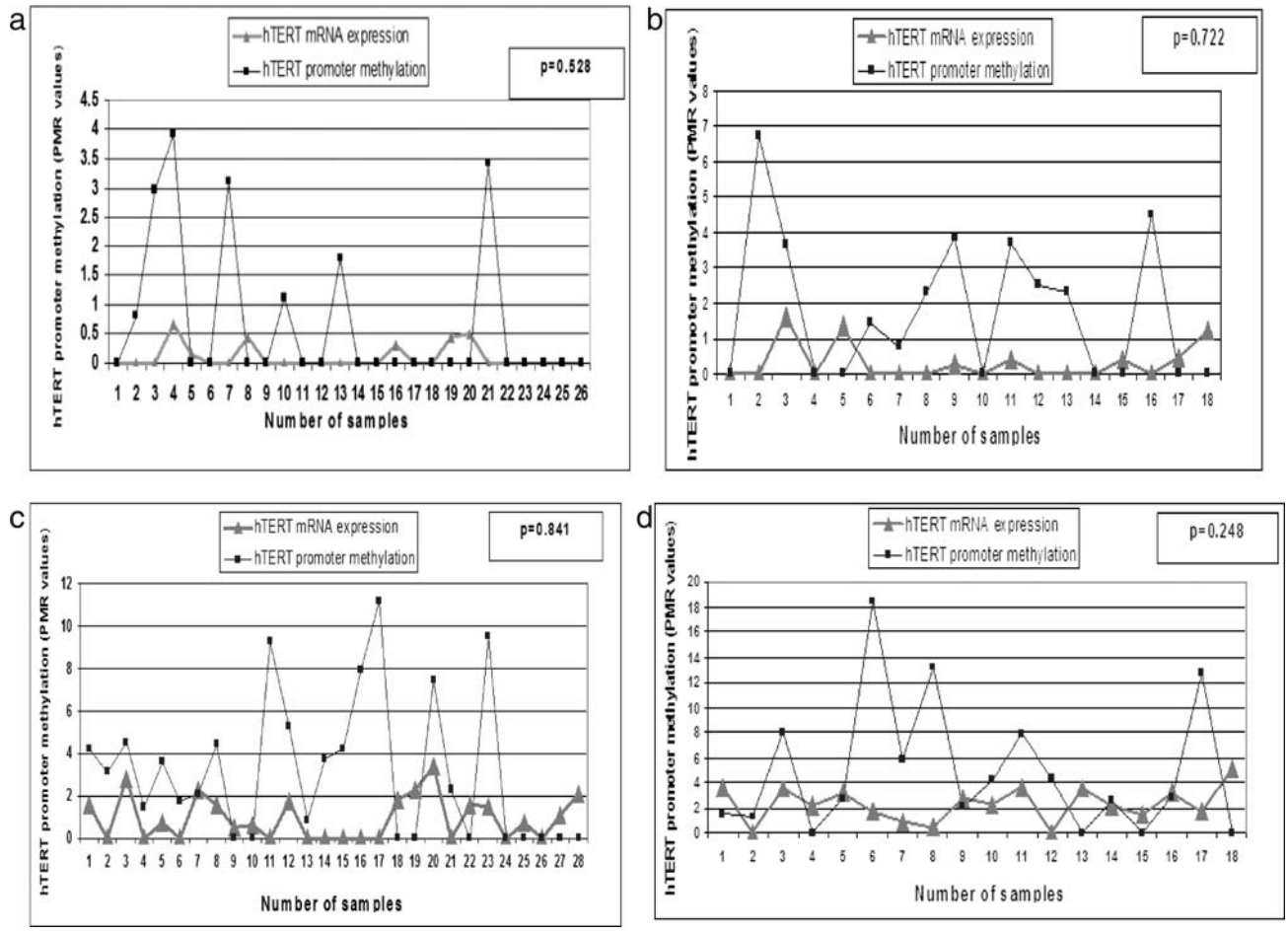

Figure 1. (a) hTERT mRNA expression and hTERT promoter methylation in normal cervical specimens. (b) hTERT mRNA expression and hTERT promoter methylation in ASCUS cervical specimens. (c) hTERT mRNA expression and hTERT promoter methylation in LGSIL cervical specimens. (d) hTERT mRNA expression and hTERT promoter methylation in HGSIL cervical specimens.

several molecular mechanisms, including genetic and epigenetic events, are involved in the regulation of hTERT expression, suggesting a complex manner of hTERT regulation (11, 22–24). The hTERT gene has a typical CpG island domain overlying a putative transcription start site and a number of potential transcriptional regulatory sequences (11). More specifically, this CpG island lies 664 bp upstream of the putative transcription start site to 510 bp downstream of the transcriptional start site, ending 56 bp after the start of the first intron, and points toward a possible role of methylation in the regulation of hTERT gene expression. Methylation-mediated control of gene expression is most often associated with gene silencing, which may result from randomly dispersed or region-specific CpG methylation (25). Previous studies investigating the possible role of promoter methylation in hTERT gene expression have not revealed a generalized mode of methylation-based regulation (26–30).

Previous study on hTERT DNA promoter methylation in normal and cervical cancer specimens showed that the levels of hTERT promoter methylation were not correlated with levels of hTERT mRNA expression (13). However, to date there are no data available on whether hTERT DNA promoter methylation correlates with hTERT mRNA expression in premalignant lesions. We quantified the methylation status in premalignant lesions using real-time QMSP, a more sensitive and specific method than conventional PCR, and correlated the levels of DNA promoter methylation with hTERT mRNA expression in normal, ASCUS, LGSIL, and HGSIL cervical specimens. We observed a lack of correlation between hTERT promoter methylation and hTERT mRNA expression in premalignant specimens, suggesting that hTERT CpG island methylation in cervical specimens is unlikely to play a substantial role in the regulation of hTERT. The observed CpG island methylation could probably reflect the *de novo* CpG island methylation that frequently accompanies tumorigenesis (25, 31), whereas according to previous reports, CpG island regions are generally flanked by less CpG-rich DNA sequences, which are heavily methylated and do not seem to play any role in gene expression (32). It is obvious that one or more methylation-independent mechanisms exist for certain types of cells, including cervical epithelial cells, involving the overlapping of binding or fingerprint motifs,

multiple transcriptional factors, viral proteins, and acetylation-dependent chromatin restructuring (13, 31).

Furthermore, in cervical tissues, viral activation seems to be one of the most important factors that triggers hTERT expression. It has been suggested that viral HPV proteins may directly contribute to hTERT upregulation (7). HPV-16 E6 and E7 proteins seem to activate hTERT mRNA expression and telomerase activity (33–35). Many studies have been performed to detect E6/E7 HPV mRNA transcripts and their role in cervical neoplasia (36–38). In continuation of our previous study, in which we correlated HPV DNA viral load with hTERT mRNA expression (9), we proceeded with E6/E7 mRNA expression in HPV-16–infected samples to draw firm conclusions regarding the potential role of HPV infection in hTERT expression and furthermore in cervical carcinogenesis. We found a correlation between E6/E7 HPV mRNA expression and hTERT mRNA expression in HPV-16–infected cervical specimens, confirming the potential role of HPV infection in hTERT regulation and in cervical carcinogenesis.

Based on the fact that genetic and epigenetic events play an important role in hTERT gene regulation, we focused our study on hTERT promoter methylation. The lack of association observed between hTERT DNA promoter methylation and hTERT mRNA expression suggests that methylation status in the hTERT gene promoter in cervical premalignant specimens might not play an important role in hTERT regulation. It seems that E6/E7 HPV-16 mRNA expression or epigenetic mechanisms other than DNA methylation may regulate hTERT expression in premalignant cervical samples.

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