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hTERT expression as a potential diagnostic marker

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Abstract

Cervical cancer is the second type of women cancers, most cases being reported in the developing countries where it represents the main cause for mortality in women. The aim of this study was to clarify the role of hTERT expression levels in cervical carcinogenesis, in each type of cytological diagnostic group (normal/inflammatory, ASCUS, LSIL, HSIL, cancer groups) like potential diagnostic marker. ***Methods:*** *The smears obtained from 50 women with/without suggestive HPV infection pathology were cytological investigated. The viral testing was based on the presence of HPV DNA using the IINNOLIPA kit and semi-quantitative expression levels of hTERT were estimated in RT-PCR.* ***Results****: HPV was present in 84% of the examined cases, but only in 40.48% of them hTERT expression was observed. hTERT mRNA was detected in 17.65% cytologically normal/inflammatory patients, in 30% patients with ASCUS, 61.50% patients presenting LSIL and 70% patients with HSIL/cancer. hTERT mRNA expression was significantly increased in LSIL (p = 0.035) and HSIL/cancer (p=0.0044) as compared with normal group, but hTERT expression in ASCUS patients group does not present statistical significance as compared with the normal group (p=0.37). The association between the expressions of hTERT, the presence of hrHPV as well as dysplasia grade suggests that the hTERT activation may be a central mechanism by which HPV infections lead to malignant transformation. Analysis of hTERT expression can be used in diagnosis to decrease the false-negative cytology tests but only as an adjuvant, requiring correlation with the results of morphological feature.*

Keyword: *diagnostic marker, papillomavirus, hTERT*

Introduction

Normal somatic cells proliferates a limited number of passages *in vitro*, the maximum being considered Hayflick limit [1]. Hayflick limit determines a permanent cessation of growth being known as replicative senescence or stage 1 of mortality (M1). Cells that escape from senescence by gene inactivation continue to divide and suffer telomeres loss reaching the second proliferative block, stage 2 of mortality (M2); this is characterized by massive cell death caused by critical shortness of telomeres and telomeres dysfunction. Rarely, cells that escape from crisis are able to maintain telomeres length, in most cases by enabling telomerase; this leads to an unlimited capacity of proliferation known as cell immortalization.

Telomerase is a RNA-dependent DNA-polymerase that synthesizes telomeres DNA and provides molecular bases for unlimited proliferative potential. Discovered in *Tetrahymena thermophila* in 1985 [2], telomerase activity is absent in most normal somatic cells but present in more than 90% of tumour cells and immortalized cells *in vitro* [3, 4]. Telomerase contains two essential components: a functional RNA component that serves as a

template for telomeres DNA synthesis (hTR or hTERC) and a catalytic protein (hTERT) with reverse transcriptase activity. hTR is constitutively expressed in all tissues despite telomerase activity, cancer cells presenting an expression generally five fold greater than normal cells [5]. In contrast, the catalytic component hTERT is expressed at less than 1-5 copies per cell [5] and is associated with the telomerase activity. Generally, hTERT is repressed in normal cells but over regulated in immortal cells suggesting that hTERT represent major determinant of enzyme activity. Regarding cervical neoplasia, it is not clear whether telomerase is activated during the progression of this disease. Taking into account that HPV infections have been associated with cervical cancer, telomerase activity may be a central mechanism by which HPV infections can lead to malignant transformation of cervical mucosa [6]. Although hrHPV types were detected in cervical smear, a small percentage of women with abnormal cytology developed carcinoma. Diagnostic criteria to accurately predict clinical progression of cervical lesions are required. As biopsy is an invasive method, and not all women have an indication for biopsy we quantified mRNA hTERT expression in cervical smear from women with normal and abnormal cytology. Using RT-PCR analysis we established a quantitative relationship between the hTERT mRNA expression, HPV infection status and cytology.

Materials and methods

Patients: 50 women (17-54 years) referred to the Gynecology Department of Clinic Hospital Iassy for periodical cervical cytological smears test were included in this study. Informed consent was obtained from each participant. The project had the approval of the Ethics Committee of Stefan S. Nicolau Institute of Virology, Bucharest, Romania. Cervical specimens for molecular analysis were taken during colposcopic examination using a combination of plastic spatula and cytobrush. Cervical specimens were collected in Copan transport medium.

Cytology: Harvested vaginal secretions have been spread and fixed on the blade by spraying with alcohol (not to dry before fixing). Subsequently preparations have been coloured with Harris hematoxilin (3-5 minutes) and orange-G (3-4 minutes). The diagnostic elements were isolated cell morphology and any significant changes in terms of diagnosis. The test may reveal nonspecific inflammatory lesions caused by various forms of vaginitis, or some significant issues for vaginal infections. It was essential to identify those cellular changes characteristic induced by human papilloma virus. Results were reported according to Bethesda classification.

DNA extraction: Exfoliated cells obtained from the patients were transported in Copan medium. DNA extraction was done with Quiagen kit according to the manufacturer procedures. For handling biochemical processes in molecular diagnostics, DNA was resolubilised in Tris-EDTA buffer (TE^{-1}), pH 7.4-8.0. The amount of DNA was determined both in spectrophotometry reading UV absorber in the micro-titer plate and in fluorimetry with Hoechst 33258, using the device TECAN Genius.

HPV detection / genotyping: viral testing was done with IINNOLIPA kit (*Innogenetics*), according to manufacturer instructions. Briefly, a fragment of L1 HPV region was amplified in a PCR reaction using biotinilated nucleotides; denatured amplicons were hybridized with specific oligonucleotid probes that are fixed on the nitrocellulose membrane. After hybridization and stringent washing, streptavidin conjugated with alkaline phosphatase and chromogen incubation revealed the positive sample as a purple precipitate. Interpretation of the results was done with the specific read card of the INNOLIPA HPV Genotyping kit. A test is considered positive when a specific line of type or a control line is HPV positive. The kit allows the identification of 17 different HPV genotypes.

RNA EXTRACTION: A 0.5 ml aliquot of the sample was mixed with 0.5 ml phosphate buffered saline (PBS) and centrifuged at 60000 x *g* for five minutes. The cell pellet was lysed with 1 ml Trizol reagent (*Invitrogen*). RNA extraction was carried out according to the manufacturer's instructions. The pellet was vacuum dried for 30 seconds and the RNA dissolved in ultra high quality water by heating at 55°C for 10 minutes. Any contaminating DNA was removed by incubating with 1 U of DNAse I (Life Technologies) at room temperature for 15 minutes. The enzyme was denatured at 65°C for 10 minutes and reverse transcription was performed. An aliquote of this sample was stored at -20°C as a control to test for DNA contamination.

Reverse transcription: 2 µg RNA was mixed with 1.5 pmoles of random primers, and incubated at 70°C for 10 minutes, allowed to cool and the reverse transcription (RT) reaction carried out using 200 units Superscript reverse transcriptase (*PROMEGA*), 1 mM dNTP according to the manufacturer's instructions. The cDNAs were stored at -20 °C until use.

RNA PCR analysis: The efficiency of cDNA synthesis from each sample was estimated by PCR using GAPDH-specific primers. The expression of hTERT mRNA was semi-quantitatively evaluated after RT-PCR amplification. Briefly, 2-µl aliquots of the reverse-transcribed cDNA were amplified for 33 cycles in PCR in final volum 50 µl containing buffer (10 mM Tris-HCl (pH 8.3), 2.5 mM $MgCl_2$, and 50 mM KCl) containing 1 mM each of dATP, dCTP, dGTP, and dTTP, 2.5 units of Taq DNA polymerase (*Promega*), and 0.2 µM primers (FW: 5'-CGGAAGAGTGTCTGGAGCAA-3' and R: 5'-TCCAGACTCCGCTTCATCC-3'). Each cycle consisted of denaturation at 95°C for 40 s, annealing at 60°C for 50 s and extension at 72°C for 50 s. The PCR products were resolved by electrophoresis in 2% agarose gels and analysis of the bands intensity was performed using Image J 1.33u.

Statistical analysis: The Student *t* test was performed to compare differences between two groups for quantitative variables. Double-sided *P* values of ≤0.05 were considered significant.

Results

Tracking down of cervical lesions

Smears obtained from 50 women were cytological investigated (interpreted in Bethesda system). Group inclusion criteria were: the relationship between cell / nucleus size, features and intensity of nuclear staining, nuclear and chromatin architecture, the nuclear membrane integrity, many of which are signs of acute or persistent HPV infection (figure 1).

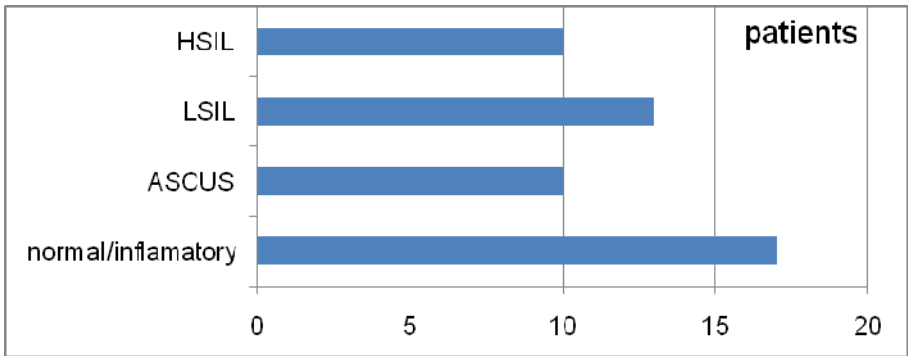

Figure. 1. The cytological characteristics of patients' groups

HPV detection

HPV was present in 84% of the examined cases but only in 40.48% of them hTERT expression was observed. HPV genotypes involved as single infection or co-infections in patients who had hTERT expression are shown in figure 2. Distribution according to cytological diagnostic is show in table 1.

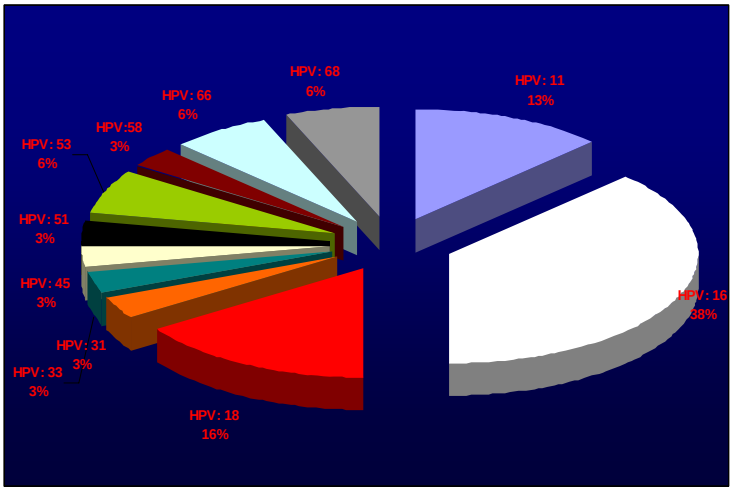

Figure. 2. 3D graphic representation of the presence of HPV infections in patients who presented telomerase activity quantified by mRNA hTERT expression in cervical smears.

Table 1. HPV infection in patients presenting mRNA hTERT expression (distribution according to cytological diagnostic).

	HPV:11	HPV:16	HPV:18	HPV:31	HPV:33	HPV:45	HPV:51	HPV:53	HPV:58	HPV:66	HPV:68
Normal/ Inflammatory	1	3	0	0	0	1	1	1	0	1	0
ASCUS	1	1	1	0	0	0	0	1	0	0	1
LSIL	2	2	4	1	0	0	0	0	1	1	1
HSIL/ Cancer	0	6	0	0	1	0	0	0	0	0	0

hTERT expression

hTERT expression was observed in 42% of studied cases. hTERT mRNA was detected in 3 of 17 (17.65%) cytological normal patients or presenting inflammatory reactions, in 3 of 10 (30%) patients with ASCUS, 8 of 13 (61.50%) patients presenting LSIL and 7 of 10 (70%) patients with HSIL / cancer (figure 3).

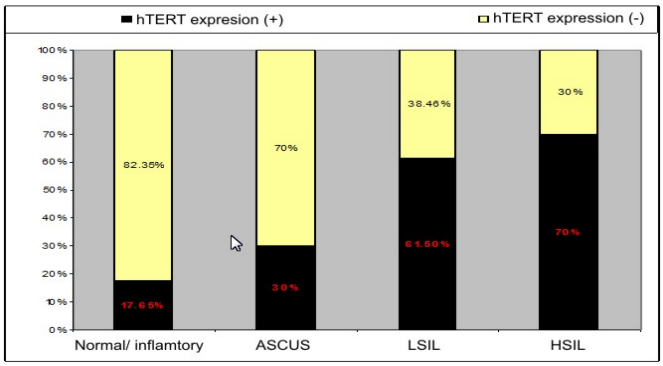

Figure. 3. hTERT mRNA expression in patients with normal / inflammatory, ASCUS, LSIL, HSIL / cancer cytology; Chart shows the percentage of samples with or without hTERT expression.

Our results showed that hTERT expression is an early event in cervical carcinogenesis. The numbers of cases with perceived hTERT expression gradually increased with severity of neoplasic lesion (Figure 4). hTERT mRNA expression was significantly increased in LSIL ($p = 0035$) and HSIL / cancer ($p = 0.0044$) as compared with normal group.

In this study, hTERT mRNA expression was detected in 70% of cancer cases, suggesting that telomerase is essential for malign phenotype maintaining. Alternative mechanisms for telomere length maintaining were described [12], thus sustaining that in 30% of cancers we could not find hTERT expression.

On the other hand, hTERT expression was not observed in 3 patients presenting HPV infection: 2 with LSIL cytology and 1 with HSIL cytology. It is possible that telomerase can be activated in the absence of HPV; there are alternative ways to activate.

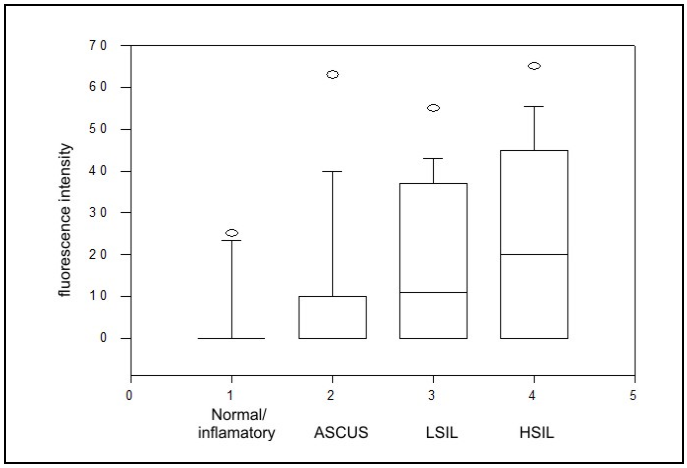

Figure. 4. The dot box distribution of hTERT mRNA intensity in patients (normal/inflammatory, ASCUS, LSIL, HSIL / cancer).

hTERT expression in ASCUS patients group does not present statistical significance as compared with the normal group ($p = 0.37$). Telomerase expression was observed in ASCUS group and in patients with inflammatory disease and it was associated with hrHPV single infection (HPV: 16; HPV: 53) or multiple infections with at least 2 genotypes of increase risk (HPV: 16, 45, 51, 53; HPV: 16, 18, 68, 11; HPV: 11, 16, 66). This expression may be due to transformation, as well as active virus replication with production of inflammatory reactions (thus increasing the amount of activated lymphocytes in the biological sample). In addition, one LSIL case with lrHPV type 11 presented hTERT expression, but at low level. In a careful reassessment of cytological diagnostic, in this case were found markers of inflammation and a rich bacillary flora which could influence our result. Due to *Innolipa* limits detection and genotyping we cannot know whether another hrHPV genotype was present.

To clarify the role of hTERT in cervical carcinogenesis the expression levels in each type of cytological diagnostic group (normal / inflammatory, ASCUS, LSIL, HSIL, cancer groups) were compared. We found significantly different levels of hTERT expression in investigated subgroups. hTERT expression was observed in 42% of cases, increasing with the severity of intraepithelial lesion (30% in ASCUS, 61.50% LSIL and 70% HSIL / cancer). Increased hTERT expression showing a tendency toward cervical lesions severity was reported by other authors.

Discussion

In this study, we quantified mRNA hTERT expression in cervical smears from women with normal and abnormal cytology in order to establish a semiquantitative relationship between the hTERT mRNA expression, HPV infection status and cytology. Our results have shown that hTERT expression is induced in the advanced stages of CIN and invasive cancers, this being concordance with other results, demonstrating that excessive shortness of telomeres occurs predominantly in the early stages of CIN, preceding telomerase activation and therefore [7]. Immunohystochemical studies on biopsy specimens have shown that normal epithelium is completely negative for hTERT or presents a profile scoring positive cells in parabasal layer. More positive cells were observed in the squamous metaplasia epithelium, but only in single cells in layers and sometimes in suprabasal metaplasia proliferating cells. Positive immunostaining was nuclear and limited to a few cells, and stromal immunoreactivity was strictly associated to lymphocytes which have constitutive hTERT expression. HSIL lesions and cancer present a different pattern: hTERT-positive nuclei are present in all layers of epithelium, including many typical koilocytea, and appear as a cytoplasm staining [8]. Paradoxically, there was a decrease in the nuclear staining intensity of hTERT in squamous cell carcinomas (hTERT protein appears to be diffusely distributed in the cells with loss of the discrete nuclear pattern observed in benign and pre-malignant lesions) [9, 10, 11], with an increasing cytoplasm staining [12]. Disturbance of the normal translocation mechanisms of hTERT to the nucleus, associated with cervical mucosa malignant transformation may be responsible for these differences in the expression pattern. Nuclear immunoreactivity is considered a marker of hTERT activation, and this deregulated translocation may decrease the hTERT nuclear staining intensity. Although it has been demonstrated that the loss of hTERT immunostaining may be associated with the deregulation of normal translocation mechanisms of hTERT to the nucleus, we suggest another mechanism involved in a reduce hTERT expression in human cancers: inactivation at the transcription level. In this study, in 30% of cancer cases mRNA hTERT expressions were not detected, suggesting that telomerase is essential in maintaining malign phenotype. Anyway, some authors have been described alternative mechanisms for maintaining telomeres length [6].

A number of authors considered that telomerase is a marker of cancer in cervix malignization that correlates with histological grade; it can be also a useful marker for detecting residual neoplastic lesions [13], but telomerase activity is not detected in all cervical cancers [14], our results confirming this. Trying to determine the hTERT expression significance in prognostic of biological conditions associated with increased risk of tumorigenesis we observed hTERT expression, depending on the state of cervical cells dyscariosis in 42% women with ASCUS, LSIL, HSIL or cancer. The observations show that telomerase activation is an early event in cervical carcinogenesis and appears to be associated with the initiation and progression of cervical neoplasia. The heterogeneity of sensitivity and specificity in telomerase detection seen between different studies is due to: sample size (smear / lavage versus cervical biopsy), contamination test with blood or necrotic cells (including telomerase inhibitors, which can lead to false-negative results [15], or haemoglobin (a powerful inhibitor of PCR reaction). False positive in strong inflammatory reactions may occur due to inflammatory infiltration. In normal cells, telomerase activity appears to be strictly regulated during development, but is inhibited during embryonic differentiation in most somatic cells and remains active only in certain tissues, such as male germinal cells, lymphocytes and activated stem cell populations [16]. Using smear / cervical lavage like biological samples, telomerase activity detection is influenced by the amount of normally

cells present in the examined sample [17]. There are differences in the nature of these normal cells: if they are differentiated keratinocyte (decrease overall level of expression) or if infiltration of inflammatory lymphocytes exists (increase expression level).

Although it is considered that telomerase activity is not associated with the hrHPV detection in any cytology categories [12] or the resection border [13], we observed the expression of hTERT in samples with normal cytology associated with the presence of hrHPV. In this study we detected appreciable hTERT levels in HPV 16/18 co-infections and in additional samples infected with another high risk genotype other than HPV16/18, respectively HPV53. Our results show that low risk lesions, meet hTERT expression only in the presence of HPV (especially high-risk HPV). A LSIL case with HPV: 11 introduced hTERT expression, but at low level. In a careful reassessment of cytological diagnostic it was found that in this case markers of inflammation and a rich bacillary flora could influence the result. Taking into account these observations, hTERT expression in patients with normal smear without inflammatory infiltrate might be associated with the initiation and progression of cervical lesions, telomerase activation being a relatively early event in cervical carcinogenesis. Analysis of hTERT expression can be used to decrease the rates of false-negative diagnosis in cytology tests, but only in association with morphological observation cell on cytological smear.

The 5p15 region amplification (where hTERT gene is located) was observed in various tumours [18]. These data suggest that the hTERT locus may be a potential target for amplification by HPV during the oncogenic process in cervical cancers [19, 20].

It is not clear whether HPV16 infection activates directly telomerase *in vivo*. Most studies examining cervical tissue or cervical lavage indicate telomerase activation in CIN and carcinoma, only after progression at intraepithelial lesion [22, 23, 24]. Taken together, *in vitro* and *in vivo* studies suggest that infection with HPV16 and the concomitant expression of E6 is associated with telomerase activation. Cervical lesions containing hrHPV in early stages does not present telomerase activity or present it at low levels [23, 25]. Some results show that telomerase activity appears only when E6 is expressed at elevated levels, these results supporting our hypothesis, i.e. detection mRNA hTERT only in a group of hrHPV positive patients.

Interaction between telomerase and hrHPV can explain our observations. E6 hrHPV oncoprotein (but not lrHPV) activates telomerase by induction gene transcripts [21, 22]. In order to induce hTERT transcripts E6 is dependent on Myc activity and E6/E6-AP interaction [26, 27]. hTERT expression increased with CIN grade, in agreement with idea that telomerase activation and telomere maintaining is essential for immortalization and represents an important step in cancer genesis. The hTERT expression is in agreement with E6 hrHPV role in telomerase activation, but this association lost its significance due to strong association between hTERT and stage of intraepithelial lesion. One feasible explanation may be done by the recent experiments concerning the monitoring the E6/ E7 dynamics and telomerase expression along with the progressive grades [28]. The initial increased levels of E6 decrease dramatically, while hTERT mRNA expression and telomerase activity increase by 10 and respectively 4 fold. This means that the telomerase activation by E6 HPV is an early event and selection of clones with increased telomerase activity will lead to tumour progression and this intimate association with gradual lesions hides hTERT association with E6 hrHPV. In our study, in the low grade cytological group (normal / inflammatory and ASCUS patients) only hrHPV infected subjects present hTERT expression claiming that hTERT mRNA expression

analysis can be used as an succour of cytology in order to decrease the rates of false-negative cytology diagnosis of samples.

hTERT mRNA expression level detection, even in the presence of HPV, must be interpreted with suspicion due to lymphocytes contamination of the biological samples. Thus, quantification of hTERT expression loses its clinical usefulness in the management of patients with ASCUS or LSIL. In addition, we detected hTERT only in 70% of HSIL /cancer cases, considering that is not very specific. Another group found a significant correlation between hTERT mRNA levels and hrHPV genotypes, suggesting that HPV infections target instability either by viral DNA integration in the host genome, or by repealing the control paths leading to telomerase activation. In addition, they noticed a significant correlation between viral load and mRNA hTERT expression, indicating that the viral load is involved in activation of hTERT and the presence of cervical neoplasia.

In conclusion, the association between the expression of hTERT, the presence of hrHPV, as well as dysplasia grade suggests that the hTERT activation may be a central mechanism by which HPV infections lead to malignant transformation. Analysis of hTERT expression can be used in diagnosis to decrease the false-negative cytology tests but only as an adjuvant requiring correlation with the results of morphological features.

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