

ROLE OF p16INK4a, hTERT AND K-ras IN EARLY DETECTION OF GYNECOLOGICAL MALIGNANCIES

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The fundamental problems set by cancer are those related to the specificity of diagnosis markers. Due to cancer's several faces, it is hard to identify a single marker aimed to discover correctly early stage transformation, and this is why we were trying to correlate the expression profiles of some human genes that seem to become affected in cervical cancers. In this context, we studied the p16INK4a, hTERT and K-ras clinical utility as diagnosis markers, as well as their role in early detection of disease in preclinical stages.

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 while semi-quantitative expression levels of p16INK4a, hTERT, K-ras were estimated in RT-PCR.

Results: In single high-risk genotype infections, p16:ras, p16:hTERT correlations are very increased ($r=0.66$ respectively $r=0.63$) in comparison to ras:hTERT ($r=0.12$). In co-infections, an inversion of the correlations occurs, increasing ras:hTERT correlations ($r=0.61$) in comparison to p16:ras, p16:hTERT ($r=0.15$ respective $r=0.02$). Positive correlations were observed in the HSIL/cancer group for all possible association of the analyzed markers: p16:ras, p16:hTERT, ras:hTERT. In conclusion, we consider that the analyzed molecules (p16INK4a, k-ras and hTERT) are of real interest as tumoral markers used in gynecological oncology, playing a role in the improvement of the diagnosis of high degree lesions and that of low degree lesions which could progress towards cancer

Key words: Early detection of gynecological malignancies; p16INK4a; k-ras; hTERT.

INTRODUCTION

Human Papilloma Virus (HPV) is recognized as a major factor of genetic alterations associated with pre-invasive/invasive cancer. These observations have stimulated the development of strategies for the prevention of tumor appearance and led to the design of prophylactic vaccines. A number of very important limitations of HPV vaccination must be considered: the short duration of protection achieved by these vaccines; the prophylactic vaccines are effective pre-exposure to the virus so that the target population for vaccination is

represented by girls from 9–10 years; the existence of different vaccine variants with variable effectiveness. To eradicate the disease, screening routine should continue to detect and treat women infected before vaccination or HPV types that are not covered by the vaccine. Pap test detects cell transformation induced by HPV but with certain limitations. In addition, HPV detection and genotyping cannot distinguish between forms, which require therapeutic treatment. Therefore, it is necessary to develop new markers with role in diagnostic of lesions that can progress towards cancer.

p16INK4a is a tumour suppression protein that inhibits cyclin dependent kinase CDK4/6. Normally p16INK4a protein acts as a negative regulator of cell proliferation, but this inhibitory action is ineffective in proliferative cells infected with high risk HPV¹. p16INK4a expression targets negative feedback control of the Rb protein, enhancing the p16INK4a growth levels. Increasing expression of p16INK4a is the result of pRb inactivation by the E7 HPV protein². Increased levels of p16INK4a were observed in transformed cell lines, cervical carcinoma, high-grade intraepithelial lesions (HSIL)^{3,4}, but also in the normal squamous epithelium of cervix^{5,6}. Some reports indicate that p16INK4a is not expressed in cervicitis and in the metaplastic epithelium in the absence of CIN⁷. However, other authors argue that a low level of p16INK4a was found in the glandular epithelium and cervical metaplasia⁸. On the other hand, strong expression of p16INK4a was strictly associated with the presence of high risk HPV rather than low-risk HPV^{5,9}.

Telomerase is a RNA-dependent DNA-polymerase that synthesizes telomeres' DNA and provides molecular bases for unlimited proliferative potential. 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. Generally, hTERT is repressed in normal cells but over regulated in immortal cells suggesting that hTERT represents a 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 represent a central mechanism by which HPV infections can lead to malignant transformation of cervical mucosa^{10,11}.

Ras is a GTP-ase involved in human carcinogenesis through apoptosis inhibition and cell cycle promotion. Wild-type Ras gene expression possesses antioncogenic properties. Frequent alterations of ras gene which constitutively activates ras were reported. Oncogenic ras mutations were found in 30% in human cancers indicating the importance of this gene in cell proliferation. Ras gene activation in HPV infections has been described in particular in cervical carcinomas¹², but the information on involvement in premalignant lesions is limited.

The purpose of the present study was to investigate the utility of p16INK4a, hTERT and K-ras as diagnosis markers (differentiation of the malignant disease from the benignant one or that of a certain pathological state from another), as well as their role in early detection (in order to evaluate the risk or the liability of healthy suspects, or in detecting the disease in preclinical stages).

MATERIALS AND METHODS

Patients: The study was conducted on the following groups [mean age: normal / inflammatory aspect (31.9 ± 8.1) (range 19–46); ASCUS (37 ± 9.2) (range 25–55); LSIL (35.8 ± 8.2) (range 24–51); HSIL / cancer (41.5 ± 8.3) (range 30–42)]. Informed consent was obtained from each participant. The project had the approval of the Ethics Committee of Stefan S. Nicolau Institute of Virology. 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 preparation has been colored with Harris hematoxylin (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 system.

DNA extraction: DNA extraction from swabs was done with Quiagen kit according to the manufacturer procedures. For handling biochemical processes in molecular diagnostics, DNA was resuspended in 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 INNOLIPA kit (Innogenetics), according to manufacturer instructions. Briefly, a fragment of L1 HPV region was amplified in a PCR reaction using biotinylated nucleotides; denatured amplicons were hybridized with specific oligonucleotide probes that are fixed on the nitrocellulose membrane. After hybridization and stringent washing, streptavidin conjugated with alkaline phosphates and chromogen incubation revealed the positive sample as a purple precipitate. Interpretation of the results was done with the specific read card of the INNO-LIPA 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 and RT: A 0.5 ml aliquot of the sample was mixed with 0.5 mL phosphate buffered saline (PBS) and centrifuged at $60\,000 \times g$ 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 aliquot of this sample was stored at -20°C as a control to test for DNA contamination.

Gene expressions: The mRNA expression was semi-quantitatively evaluated after RT-PCR amplification. Briefly, 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. 2µl aliquots of the reverse-transcribed cDNA were amplified by PCR using specific primers^{9,10,12}. The PCR products were resolved by electrophoresis in 2% agarose gels and analysis of the bands intensity was performed using ImageJ 1.33u.

Statistical analysis: Correlation between variables was assessed by calculating the nonparametric Spearman's correlation coefficient (GraphPad Prism, 1994). $P < 0.05$ was considered significant.

RESULTS

HPV detection

We used Innolipa for HPV detection and genotyping, a sensitive method that has frequently detected multiple infections among women with normal cytological Pap test and intraepithelial cervical lesions. Our study group included 42 HPV positive cases; from these, 4 cases were non-genotyping using this test and from 38 cases, HPV was detected as single infection in 20 cases and

multiple HPV infection was observed in 18 cases. The frequency of different genotypes (single and multiple infections) is presented in the Figure 1A. Stratification by cytological grade is presented in Figure 1B.

Correlation between the expression profiles of the three analyzed genes in pre-invasive lesions and cancer (integration of results)

The correlations between the expression levels of the three genes in NILM, pre-invasive lesions and in cancers are presented in Figure 2.

Positive correlations were observed in the HSIL/cancer group for all possible association of the analyzed markers: p16:ras, p16:hTERT, ras:hTERT. Some of these positive correlations, like ras:hTERT or negative between p16:ras and p16:hTERT in the ASCUS and LSIL groups could be a result of lavages contamination with lymphocytes (HPV infections in the LSIL and ASCUS cytodiagnosis groups are generally productive infections that generate inflammatory reactions). Despite their clinical utility as markers for potentially invasive lesions and for the early cancer detection, however, a disadvantage of these non-invasive or minimally-invasive methods limiting their use is that they are incapable of detecting the production source of each mRNA (keratinocytes or activated lymphocytes). Our results prove that all of the investigated markers correlate positively in cases of hrHPV infections and negatively in lrHPV infections (Fig. 3).

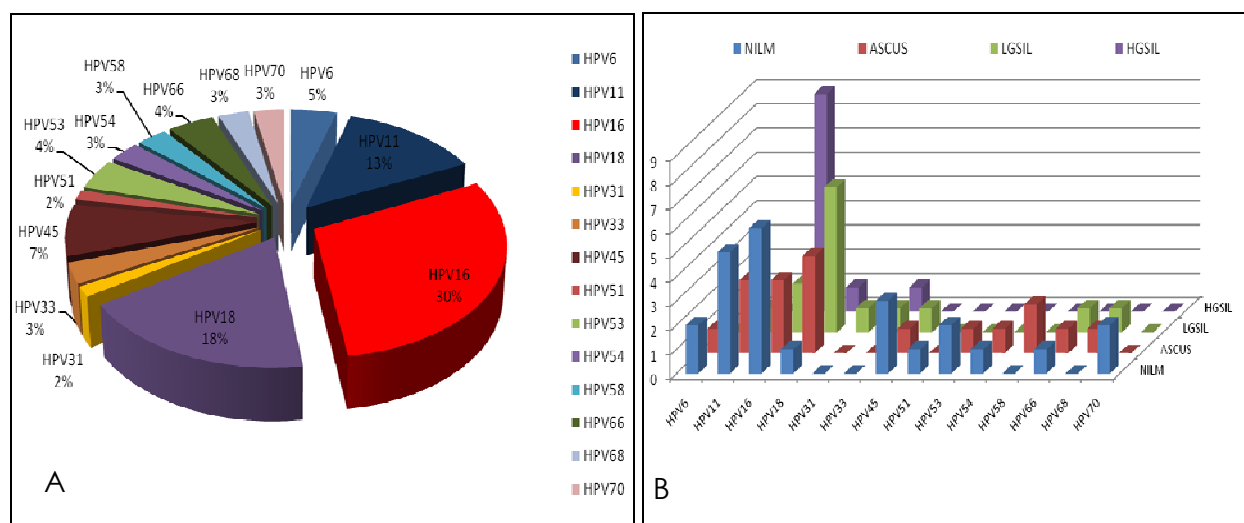


Fig. 1. Dispersion of HPV genotype (A); Analysis of HPV genotype according to cytology (B).

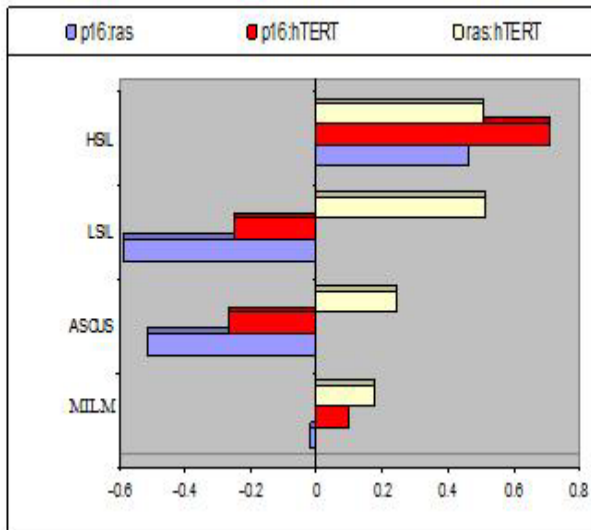


Fig. 2. Correlation between the expression profiles of the 3 analyzed genes in NILM, pre-invasive lesions and cancer.

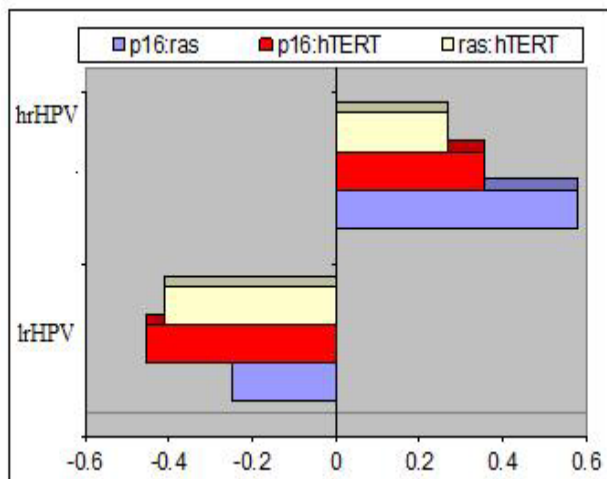


Fig. 3. Analysis of p16: ras, p16: hTERT, ras: hTERT correlations according to the presence of risk genotypes in single infections or co-infection.

Making an analysis related to the presence of co-infections or of infections with a single high-risk genotypes (Fig. 4), we have noticed that:

- In single high-risk genotype infections, p16:ras, p16:hTERT correlations are very increased ($r=0.66$ respectively $r=0.63$) in comparison to ras:hTERT ($r=0.12$)
- In co-infections, an inversion of the correlations occurs, increasing ras:hTERT correlations ($r=0.61$) in comparison to p16:ras, p16:hTERT ($r=0.15$ respective $r=0.02$).

These results and the fact that unique infections have especially been detected in high degree lesions while HPV co-infections were found in

ASCUS, LSIL or NILM cytodiagnosis samples, proves that all analyzed parameters can be used as diagnosis markers for high degree lesions. Having in view that the utility of the tumoral markers in early diagnosis of malignant tumors is strictly correlated to their sensitivity and specificity, we consider that the use of the associations between at least 2 markers, like p16 expressions correlated with hTERT presence, detects the early stages of cellular transformation with a much higher probability. Ras expression, correlated to the presence of hTERT expression does not allow systemic detection of a neoplastic pathology in early stages, but only in advanced stages of the disease. Most likely, this limitation could be resolved if the detection allows single cell quantification (for example *in situ* hybridization).

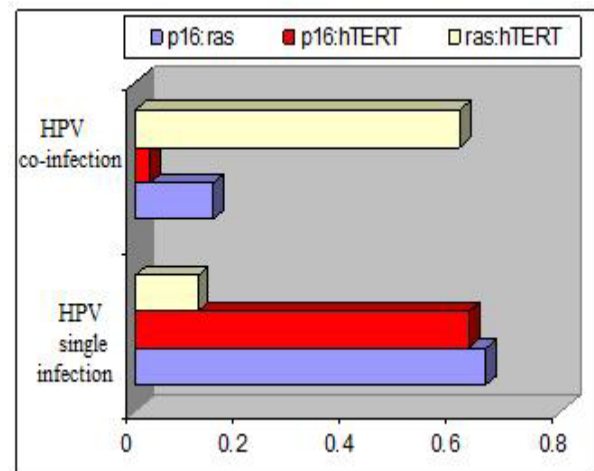


Fig. 4. The analysis of the correlations between p16:ras, p16:hTERT, ras:hTERT in high risk genotypes unique and respectively co-infections.

DISCUSSIONS

Because of their characteristics, cancers have many similarities to normal tissues of origin, which makes it impossible to differentiate between pathogen and non-pathogen correctly based on the current information (by means of at least one characteristic). Mainly through its various types, cancer ultimately mixes simplicity and differences that characterize the tissues and the normal growth processes, which become impossible to differentiate by help of a single feature. Therefore, the fundamental problems set by cancer are those related to the specificity of diagnosis markers. "Tumoral marker" terminology was introduced in the medical vocabulary, meaning substances or molecules whose detection or accumulation is

associated with the presence and development of malignant tumors. The clinical and paraclinical diagnosis possibilities at the present time do not find tumors but starting from 1 cm (10^9 cells) dimensions, which triggers the necessity of identifying them using one or several tumoral markers to serve as screening tests. Unfortunately, nowadays, except a few particular cases, no tumoral marker allows systematic detection of a neoplastic pathology; firstly, because of the sensitivity and specificity characteristics, and secondly because of the costs.

Due to cancer's several faces, it is hard to identify a single marker aimed to discover correctly early stage transformation, and this is why we were trying to correlate the expression profiles of some human genes that seem to become affected in cervical cancers. In this context, we studied the p16INK4a, hTERT and K-ras clinical utility as diagnosis markers, as well as their role in early detection of disease in preclinical stages. To highlight the clinical value of p16INK4a, H-ras, hTERT expressions as potential early markers of tumor, we considered the following four criteria: 1) tumor marker is absent in healthy subjects or with benign disease, 2) tumor marker is produced exclusively by tumor cells; 3) tumor marker is present frequently in neoplastic diseases; 4) tumor marker is also present in subclinical disease. Therefore, among the specific host cellular factors as markers for early diagnosis were analyzed: p16INK4a protein – negative regulator pRb; K-ras, a G protein, located on the surface of internal membrane involved in transducer message from outside the cell; hTERT – catalytic subunit of telomerase.

Our results claim the existence of a positive correlation in the HSIL/cancer group for all analyzed possibilities: p16:ras, p16:hTERT, ras:hTERT. However, the positive correlations between ras:hTERT and the negative correlations between p16:ras and p16:hTERT in the ASCUS and LSIL groups, were associated to lavages contamination with lymphocytes (HPV infections in LSIL and ASCUS groups of cytodiagnosis are generally productive infections which generate inflammatory reactions). A limitation of the used method is the incapacity to determine the production source for each mRNA (keratinocytes or activated lymphocytes). The aim of this study was to establish their clinical utility for the early diagnosis of potentially invasive lesions by means of non-invasive or minimally-invasive methods.

This hypothesis is also sustained by the idea that all of the investigated markers correlate positively in cases of hrHPV infections and negatively in lrHPV infections. In single high-risk genotype infections, p16:ras, p16:hTERT correlations are very increased ($r=0.66$ respectively $r=0.63$) in comparison to ras:hTERT ($r=0.12$). In co-infections, an inversion of the correlations occurs, increasing ras:hTERT correlations ($r=0.61$) in comparison to p16:ras, p16:hTERT ($r=0.15$ respective $r=0.02$). These results and the fact that unique infections have especially been detected in high degree lesions while HPV co-infections were found in ASCUS, LSIL or normal/inflammatory cytodiagnosis samples, proves that all analyzed parameters can be used as diagnosis markers for high grading lesions. The utility of the tumoral markers in early diagnosis of malignant tumors is strictly correlated to their sensitivity and specificity. We consider that the use of the associations between at least 2 markers, such as p16 expressions correlated with hTERT presence, detects the early stages of cellular transformations with a much higher probability. Ras expression that correlates to the presence of hTERT expression does not allow systematic detection of neoplastic pathology in early stages, but only in advanced stages of the disease. Most likely, this limitation could be resolved if the detection allows single cell quantification (for example *in situ* hybridization).

CONCLUSION

In conclusion, we consider that the analyzed molecules (p16INK4a, k-ras and hTERT) are of real interest as tumoral markers used in gynecological oncology, playing a role in the improvement of the diagnosis of high degree lesions and that of low degree lesions which could progress towards cancer.

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