

The Detection of Complexed Proteins E6/P53 and E7/Prb In Relation to Carcinogenesis of the Uterine Cervix

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Abstract

Objective: The aim of our work was to identify by Western Blot technique (WB) the oncoproteins generated by E6 and E7 genes of high risk Human Papillomavirus (HR-HPV), resulting from integration of viral genomes into cervical cell DNA and their interactions with tumor suppressor human proteins p53 and pRb in premalignant or malignant cervical lesions.

Methods: The study was performed on 2,500 women from Caserta Local Health Authority who underwent cervical cytology from June 2010 to September 2011. Informed consent of participants was obtained. The cell samples taken by brushing were stored in liquid based cytology (LBC) and in physiological solution for WB analysis. Cytology diagnoses was based on 2001 Bethesda classification. Proteomic research was performed according to size after extraction and SDS (Sodium Dodecyl Sulfate) electrophoresis. Proteins of interest were detected by primary monoclonal antibodies and WB analysis.

Results: Cytology results of 2,500 women were positive for abnormalities of epithelial cells in 3.1%. Atypical squamous cells of undetermined significance (ASC-US) were 1.3%, Low Squamous Intraepithelial Lesion (L-SIL) 1.7% and High Squamous Intraepithelial Lesion (H-SIL) or worse 0.2%. The integration of E6 and E7 genes with their oncoproteins expression were found in 3.1% of ASC-US and in all cases of SIL or worse. On the other hand positive interactions of E6/p53 proteins and E7/pRb were found in 4.8% of L-SIL, in 75.0% of H-SIL or worse and in no ASC-US.

Conclusions: Results show that the integration of viral genomes (E6 and E7) is present in all H-SIL or worse and in several L-SIL, but only the most advanced lesions, histologically confirmed, have shown the E6/p53 interaction and E7/pRb. Our protocol allows a selection of women with advanced lesions toward cancer to obtain appropriate management.

Keywords: E6/p53 and E7/pRb; Biomarkers cervix carcinoma; Proteomic; Western Blotting; Lsil and Hsil; Cervical vaginal screening

Background

Human Papillomavirus (HPV) are the primary cause of cervix cancer, involved in 90% of all cases [1,2]. HPV can be divided into two groups: low risk HPV (such as type 6 and 11) and high risk HPV (such as type 16 and 18).

There is sufficient morphological and epidemiological consensus for the assumption that SIL is a dynamic disease in which more cases regress spontaneously [3], whereas others progress to the invasive state [3]. Invasive Cervical Cancer (ICC) alters pathways involved in cell cycle control, interacting with and neutralizing the regulatory functions of two important tumor suppressor proteins, p53 and pRb [4].

Two early HPV genes, E6 and E7, are known to play a crucial role in tumorigenesis. Studies both in vitro and in vivo show that the function of E6 proteins and E7, particularly of the "high risk HPV types", are

essential for neoplastic cellular transformation. In summary, when low grade lesion occurs HPV DNA penetrates into host nuclei and is present in episomal state [4,5] and E6 expression and E7 is regulated by viral gene and by host cell.

The mechanisms by which HPV integrates its DNA into human genome are not fully understood although there are a number of hypotheses. The integration typically takes place in correspondence of sequence of E2 gene, with destruction of genes E2, E4, E5 and part of L2, while the sequences of E6 and E7 genes are joined to cellular sequences thereby generating more stable transcripts. Consequently, the expression of E6 and E7 is increased and uncontrolled, also due to the loss of brake inhibitory represented by E2 [4,5]. These oncogenes from the 'high risk' viruses have been shown to alter pathways involved in cell cycle control, interacting and neutralizing the regulatory functions of two important tumor suppressor proteins, p53 and pRb, and deregulating key signal transduction pathways.

The expression of two viral oncoproteins is the event necessary for the initiation of a wide range of complex events that develop cancer and activation between E6 and E7 proteins respectively with p53 and pRb proteins. It becomes quite imperative that their interaction is

targeted in order to court a cure against cervical cancer. Western Blotting (WB) could be used to do it [6]. WB can detect on women with positive cytology testing only the proteins of interest extracted with co-immunoprecipitation that is considered to be the gold standard assay especially for protein-protein interactions [7,8].

The aim of our work is to identify the protein products of E6 genes and E7 that are detected from integrated phase HPV, and activation between E6 proteins and E7 respectively with the p53 proteins and pRb on cytological diagnoses of ASC-US, LSIL and HSIL or worse, using LBC and WB technique.

Therefore, the early detection of interaction between E6/p53 and E7/pRb could be useful to modify the management of cervical cancer treatment.

Material and Methods

The study was performed from June 2010 to September 2011 on 2,500 women from Local Health Authority of Caserta who underwent spontaneous cervical cytology. Informed consent of participants was obtained for extra tests that we carried out on positive pap test only (ASC-US or worse). Negative samples were not tested because our goal was to develop a method and then perform further analysis to evaluate it. The cell samples taken by brushing for cytology were stored in ThinPrep PapTest vials (Hologic™ - Massachusetts, USA). Samples for WB analysis (Semi-Dry Electrobloetter AICOS – Denmark) were stored at 4°C in extra vials with 1 ml of physiological solution, no alcohol was used to avoid the possible precipitation of the protein. The histological diagnosis was performed in 64/78 cases: 18 ASC-US, 42 L-SIL and 4 H-SIL.

Cytology

For cytological diagnosis was used the classification of Bethesda 2001 including:

- Negative for intraepithelial lesion or malignancy.
- Atypical squamous cells of undetermined significance (ASC-US).
- Atypical squamous cells cannot exclude high grade SIL (ASC-H).
- Low grade squamous intraepithelial lesion (L-SIL).
- High grade squamous intraepithelial lesion (H-SIL)
- Squamous cell carcinoma
- Abnormal glandular cells (AGC) (specify: endocervical, endometrial or NOS)
- Abnormal glandular cells (AGC), vs. neoplastic (specify: endocervical, endometrial or NOS)
- Adenocarcinoma (specify: endometrial, endocervical, extrauterine or NOS)
- Other malignancies (specify)

Protein extraction

In the group of patients with ASC-US or worse diagnoses we performed the extraction of proteins from the second samples by centrifugation of the solution and recovery of cellular material. Cells were lysated in a specific buffer which dissociates the proteins and inhibits the cellular proteases. Then the extracted proteins were treated with SDS (Sodium Dodecyl Sulfate) anionic detergent. SDS solubilizes

and denatures the proteins and adds negative charges that can migrate by electrophoresis.

Western blot

The Semi-Dry Electrobloetter-of-Aicos Denmark was used with a constant current of 360 mA for about 2 hours. E6 and E7 proteins were labelled with monoclonal antibodies (Santa Cruz Biotechnology). The proteins of interest were separated according to their size. When the electrophoresis identified extra bands at molecular weight much higher than those of E6 and E7 it induced us to hypothesize that there was also interaction between E6/p53 and E7/pRb. In order to confirm this hypothesis we performed on E6 positive samples and E7, an additional WB analysis with primary monoclonal antibodies respectively anti-p53 and anti-pRb (Santa Cruz Biotechnology).

Results

Cytology results of 2,500 women were positive for abnormalities of epithelial cells in 78 women (3.1%). ASC-US were 32 (1.3%); L-SIL 42 (1.7%) and H-SIL or worse 4 (0.2%). The integration of E6 genes and E7 with their oncoproteins expression were found in 2/32 ASC-US (3.1%); in 42/42 L-SIL (100%) and in all 4 cases of H-SIL or worse (100%). Figure 1 shows the positive result of WB analysis using primary monoclonal antibodies anti-E6, anti-E7, anti-p53, anti-pRb, displayed in single band and/or in extra bands. Positive interactions of E6/p53 proteins and E7/pRb were found in 2 L-SIL (4.8%) and in 3 H-SIL or worse (75.0%) and in no ASC-US (Table 1). The histological diagnosis of cases in which the biopsy was performed has given the following results: 2 CIN 1-2 (11.1%) in ASC-US; 37 CIN 1 (88.1%), 2 CIN 2 (4.8%) and 4 CIN 3 (9.5%) in L-SIL; 3 CIN3 (75.0%) and 1 Invasive Squamous Cell Carcinoma (25.0%) in H-SIL or worse (Figures 2 and 3).

The above figures relating to the results of the Western Blot with primary monoclonal antibody anti E6 and E7 show, for each sample, a dual-band response. The presence of extra band at molecular weight far superior to those of E6 and E7 induced us to hypothesize that there was also the interaction between E6/p53 and E7/pRb.

Discussion

Cervical cancer screening programmes must be performed to detect and treat precancerous lesions before invasive disease develops. However, cytological testing may lack sensitivity and an increasing number of women develop invasive cancer following a negative result. HPV testing detects high grade lesions but also detects transient infections that are not associated with the development of high grade lesions; thus HPV testing has a poor positive predictive value. HR-HPV DNA integration into the human genome is one of the key stages in the onset of malignant progression and so it makes a very plausible positive predictive biomarker of invasive disease [9-11]. Thus understanding the biology and mechanisms behind HR-HPV integration will aid the prevention and/or detection of many HPV related cancers.

E6 and E7 HR-HPV early genes are known to play a crucial role in tumor formation. The expression of the two viral oncoproteins represents the event necessary for the initiation of a wide range of complex events leading to cancer development. The over expression of E6 proteins and E7 could induce their binding to p53 and pRb tumor

suppressor proteins. The interaction between E6/p53 and E7/pRb leads to cervical cancer. Physical state of HPV can be estimated by calculating HPV E2: E6/E7 ratio by real-time PCR amplification of HPV [12-14] but HPV E2: E6/E7 ratio may not reliably distinguish integrated DNA in a background of episomal DNA [12].

The main aim of our study was to distinguish clearly the episomal phase from integration status, so we decided to study SIL by WB technique. The advantage of the WB technique is that it is a very sensitive and highly specific for detecting the proteins of our interest [12]. The other advantage is that the blotting is the only technique able to determine if the HPV infection is at episomal state or integrated into host cell DNA [12].

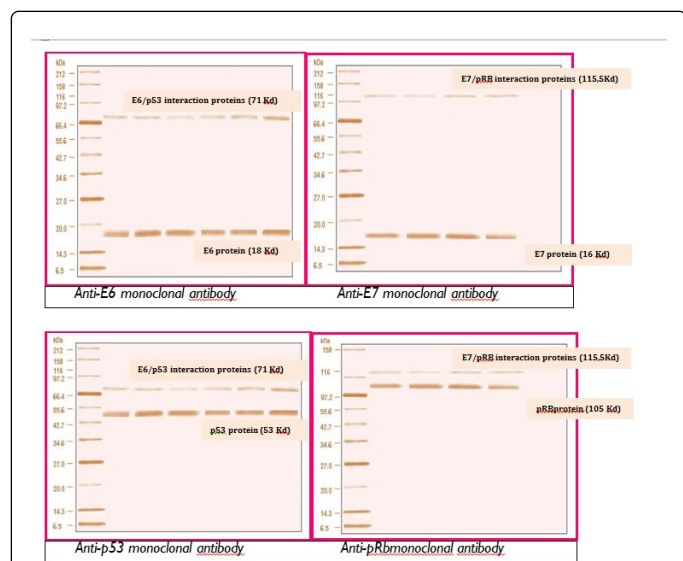


Figure 1: Western blot obtained from some of the samples, where, in addition to E6 and E7 was also found to their interaction with p53 and pRb respectively.

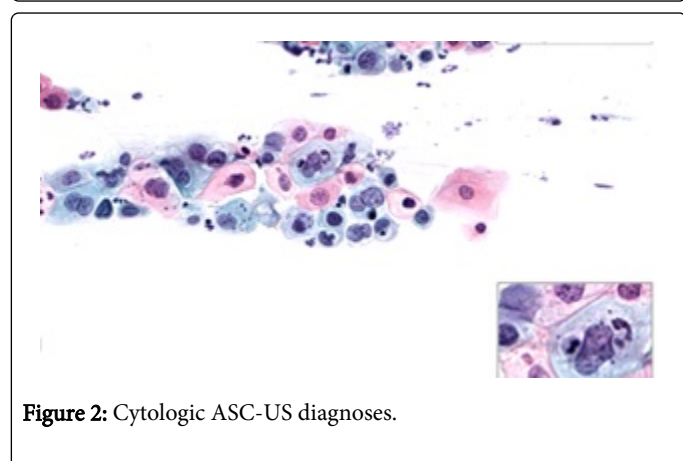


Figure 2: Cytologic ASC-US diagnoses.

Our study identified the E6 proteins and E7 for diagnostic categories of ASC-US, L-SIL and H-SIL or worse using WB technique. The second objective was to search lesions that were in carcinogenetic progression by testing the E6/p53 interaction and E7/pRb. These three information items (episomal phase, integration and/or interaction)

indicate a different risk in progression of SIL that may be use especially in management of H-SIL lesions or worse.

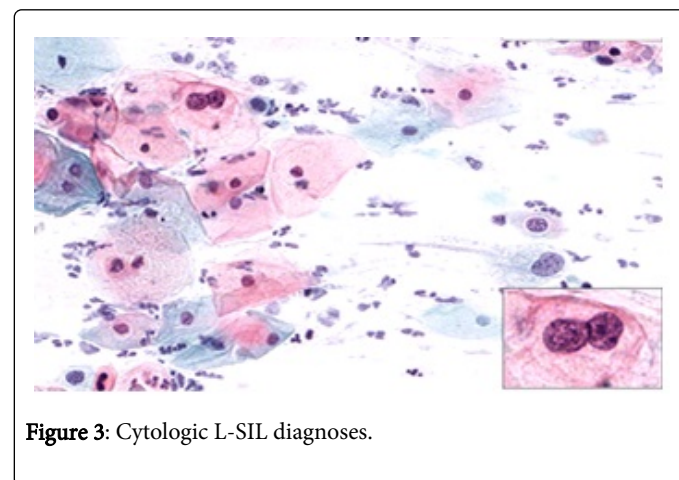


Figure 3: Cytologic L-SIL diagnoses.

Cytology	No. of cases	E6 & E7 detected	E6/p53 & E7/pRb interaction	
			absent	present
			Histology	Histology
Negative	2,422 (96.9%)	Not tested	Not tested	Not tested
Positive	78 (3.1%)	48 (61.5%)	43 (89.6%)	5 (10.4%)
ASC-US	32 (1.3%)	2 (3.1%)	2 (100.0%)	0)
L-SIL	42 (1.7%)	42 (100.0%)	40 (95.2%)	N.37 CIN1+N.3 CIN2 2 (4.8%)
H-SIL+	4 (0.2%)	4 (100.0%)	1 (25.0%)	N.1 CIN3 3 (75.0%) N.2 CIN3+N.1 CA.SQ.INV.

Table 1: Cytology, Histology and Western Blot relation of cases studies for integration/interaction of E6/p53 proteins and E7/pRb.

Using WB technique we have identified the integration of E6 proteins and E7 on ASC-US or worse lesions and their E6/p53 interaction and E7/pRb. From our data we can see that integration is found in all H-SIL but proteins interaction is found in three cases. The integrated cases decrease in L-SIL and proteins interaction are uncommon. So in future we may be able to follow the carcinogenetic progression of SIL by identifying high-risk cases E6/p53 positive testing and E7/pRb.

Now, the most promising cytological biomarkers for cervical cancer screening are p16 (INK4a)/Ki-67 dual immunostaining testing [15-21]. Although some of the biomarkers are very promising for this purpose, no studies have evaluated how accurately these biomarkers classify or predict the outcome [22-27]. Our work can be seen as an encouraging preliminary study. About the potential role of the WB technique, we believe that in the future it is necessary to make an extensive study of a large series, which includes Pap test negative and positive for SIL. So it

will be possible to evaluate the sensitivity, specificity and accuracy of this method.

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