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Expressions of E2 and E7-HPV16 proteins in pre-malignant and malignant lesions of the uterine cervix

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Abstract

Continuous production of the E7 protein from different types of high risk human papilloma virus (HPV) is required for progression of malignancy. We developed antibodies against HPV type 16 E7 and E2 proteins to evaluate their utility as markers for diagnosis during early stages of cervical cancer. Forty biopsies from uterine cervices were diagnosed as low grade intraepithelial lesion (LSIL), high grade intraepithelial lesion (HSIL), squamous carcinoma (SC), in situ adenocarcinoma (ISA) and invasive adenocarcinoma (AC), all of which were infected with HPV 16. Immunohistochemistry was used to investigate the expressions of E7 and E2 (both from HPV 16) and p16. P16 was expressed in eight of 12 LSILs, in all HSILs, in 16 of 18 SC and in all ACs. E2 was expressed in six of 12 LSILs. E7 was positive in eight of 12 LSILs and in all HSIL and carcinomas. The expressions of E2 and E7 of HPV16 related to p16 expression confirmed the value of the viral oncogenic proteins as complementary to histology and support the carcinogenic model of the uterine cervix, because HPVDNA integration into cellular DNA implies the destruction of the gene encoding E2, which suppresses the expression of the E6 and E7 oncoproteins. E2 from HPV16 could be marker for LSILs, while E7 could be a marker for progression of LSILs to HSILs in patients infected by HPV16, because viral typing has little positive predictive value.

Key words: adenocarcinoma, biomarkers, E2, E7, high grade intraepithelial lesion, HPV16, immunohistochemistry, invasive adenocarcinoma, low grade intraepithelial lesion, p16, p53, squamous carcinoma, uterus

Cervical infection by papillomaviruses (HPV) is one of the most common sexually transmitted diseases worldwide. Most women are infected soon after they initiate sexual activity; the peak prevalence is in women younger than 25 years. Infections by HPV at this age often are transitory, but there is a second peak of prevalence in women at 40 years, which coincides with the age of increased incidence of cervical cancer (Castle et al. 2005, Franceschi et al. 2006).

HPV virions are produced in the differentiated layers of squamous epithelium. In the majority of cases, the virus remains episomal and the virions are released when the external layers desquamate. In a small number of cases, the high-risk types of HPV (HR-HPV) integrate into the cellular DNA and produce a persistent infection. When the HPV

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is in its episomal state, the E2 protein supresses the expression of the oncoproteins, E6 and E7. E2 could be demonstrated in normal endocervical cells in biopsies in which the genome of HPV 16 subsequently was detected. These findings are consistent with reports that suggest that E2 is important during the early stages of carcinogenesis of the cervix and that its disappearance corresponds to the appearance of E7 (Xue et al. 2010).

The protein, E7, binds the host protein, pRb, which normally is a down-regulator of the cell cycle; in its phosphorylated state, it releases the transcription factor, E2F, which translocates into the nucleus to activate the enzymes required for DNA replication. E7 also binds transcription factor AP-1 and the inhibitors of the cyclin-dependent kinases (CD). The involvement of E7 in cell cycle regulation is reinforced by the activity of the protein, E6, which binds to p53, a key factor in pro-apoptotic control (zur Hausen 2002, Doorbar 2006, Duensing et al. 2004, Sherman et al. 1997, Funk et al. 1997, Thomas and Banks 1998). The key action of E6 proteins in high risk HPV is inhibition of the function of p53, a tumor suppressor protein, by enhancing its degradation through the ubiquitin pathway. Thus the cell cannot develop the mechanisms of programmed cell death.

In addition to the changes caused by E6 and E7, the viral protein, E5, promotes proliferation of the infected cells by altering protein trafficking in the endoplasmic reticulum and by recycling the epidermal growth factor receptor and the β-receptor of platelet derived growth factor. E5 also reduces the levels of HLA class I molecules on the cell surface (Bouvard et al. 1994, Valle et al. 1995, Maufort et al. 2007) to prevent antigen presentation. When the virus becomes integrated into cellular DNA, the information encoded by E5 is lost; therefore, it is not a determining factor in the development of a malignant lesion (Schwarz et al. 1985**).**

Viral typing to identify the oncogenic HPV has not shown predictive value for the development of low grade intraepithelial lesions (Arbyn et al. 2005). It is thought that 12 − 14% of low squamous intraepithelial lesions (LSILs) progress to high squamous intraepithelial lesions (HSIL) (Nasiell et al. 1986), and without treatment, a few of those lesions progress to invasive cancer (Holowaty et al. 1999). Consequently, investigators have searched for other parameters with predictive value, such as the protein, p16INK4a (p16), which accumulates in cells infected by HR-HPV when the oncoprotein, E7, is expressed (Sano et al. 1998, D'Amico et al.2004).

P16 is coded by the gene CDKN2A, which interacts with the protein, pRB. P16 blocks the activity of the kinase, CDK4/6, that phosphorylates pRB to release the factor, E2F. When the cell is infected by HR-HPV, E7 binds to pRB irreversibly. In this case, p16 has no effect on inhibition of the cell cycle and accumulates in the nucleus and cytoplasm. For this reason, p16 can be detected by immunochemistry (Wentzense et al. 2007).

We investigated the expressions and utility of proliferation markers, E7, E2 of HPV 16, and p16 in LSILs, HSILs in squamous cervical carcinoma and in in situ and invasive adenocarcinoma of the uterine cervix.

Material and methods

Tissue samples

All tissue samples for histologic examination and diagnostic purposes were anonymized for our study; therefore, no informed consent was required. Our study was approved by the Institutional Review Board at the Clinical Hospital-University of Buenos Aires and the latest version of the Helsinki Declaration (World Medical Association Declaration of Helsinki 2013) (World Medical Association 2013) was followed.

We used 40 archived paraffin blocks of samples of LSILs (n = 12), HSIL (n = 4), squamous carcinoma (SC, n = 18), in situ adenocarcinoma (ISA, n = 3) and invasive adenocarcinoima (AC, n = 3) for our study. All specimens had been infected with HPV 16.

HPV16 typing

The biopsies were deparaffinized in xylene in two steps: immersion for 30 min at 56° C and for 20 min at room temperature. DNA was extracted from five 7 μm sections for each biopsy using the commercial kit, ADN HP PCR Template Preparation (Roche Diagnostics, Indianapolis, IN). To monitor DNA extraction and the lack of PCR inhibition, qPCR amplification was performed in a final volume of 20 μl using Eva Green as intercalating fluorescent dye (KAPA HRM FAST; Biosystems, Woburn, MA) with primers: beta actin fw 5´-TGCGTGACATTAAGGAGAAG-3´and beta actin rv 5´-GCTCGTAGCTCTTCTCCA-3´ that amplify a 99 bp fragment of human beta actin. Detection of HPV-16 was performed using a Rotor Gene Thermocycler (Qiagen, Hilden, Germany) with type-specific PCR primers (Fontaine et al. 2007).

Synthesis of monoclonal antibodies

Ascites from five clones of hybridomas that produced IgG anti-HPV16 E7, three clones that pro-

duced IgG anti-HPV16 E2, and one clone that produced anti-p16INK4a were developed using Balb/c and incomplete Freund's adjuvant using standard methodology (Shepherd et al. 2000).

Monoclonal antibodies (IgGs) were characterized using purified immunochemical techniques and the recombinant proteins as antigens. Briefly, equal amounts of the recombinant proteins used as antigen to generate the antibodies were resolved using SDS-PAGE, blotted and subjected to western blot analysis using the monoclonal antibodies. The affinity of the antibodies was verified by enzyme-linked immunoassay (ELISA). The specificity was tested by measuring the cross reactivity of the antibody with the same proteins from other HPV strains. Reactivity was verified against endogenously produced antigen in the tumor cell lines CaSki (HPV16-transformed) by western blotting. The osteosarcoma cell line, U2OS, which is not HPV-related, was used as a negative control (Dantur et al. 2009).

Immunohistochemistry for viral oncoproteins and p16

The biopsies were fixed with 5% formalin, dehydrated through graded alcohols, cleared in xylene, embedded in paraffin, sectioned at 2 − 4 μm and mounted on slides with adhesive (Superfrost Plus-ESCO; Thermo Scientific-Erie Scientific Co., Portsmouth, NH). After deparaffinization, the sections were re-hydrated through graded ethanols, then placed in pH 6.0 citrate buffer for 30 min at 96 − 98° C for antigen retrieval. The slides were cooled in the same buffer for 20 min, then placed in phosphate buffer, pH 7.2 (PBS). Each sample was incubated for 90 min at room temperature with one of the following primary antibodies: anti-E2-HPV16, anti-E7-HPV16 or anti-p16 (Leloir Institute, Buenos Aires, Argentina). After washing with PBS, the slides were incubated with HRP Streptavidin Label (CytoscanTM HRP detection System; Cell Marque, Rocklin, CA) for 20 min, rinsed with PBS, incubated with Polyvalent Biotinylated Link (CytoscanTM HRP detection System; Cell Marque) for 20 min, rinsed again, then incubated for 10 min with the developing solution of diaminobenzidine and hydrogen peroxide (Negri et al. 2004). Negative controls consisted of samples of normal glandular squamous and stromal cervical tissues from paraffin embedded sections of cervices from uteri removed for benign disease such as myoma or prolapse. Biopsies from HSIL, AC and SC, all infected by HPV 16, were used as positive controls.

The expressions of p16INK4a, E2 and E7 in SILs were scored as follows: (0) negative, (1) focal and weak cytoplasmic staining with or without nuclear staining in superficial or intermediate layers, and (2) diffuse and strong nuclear or cytoplasmic staining in the lower 2/3 or in all layers. The expressions of p16, E2 and E7 in ISA, AC and SC were considered negative if the cells did not express markers; slightly positive if fewer than 20% of the cells expressed the markers; positive if more than 20%, but fewer than 80%, of cells expressed the markers; and strongly positive if more than 80% of cells expressed the markers. For ISA, the presence of the marker in the cells was considered positive.

Table 1. P16 expression in biopsies of uterine cervix infected by HPV16.

Samples	Total	Positive	Negative
SC	18	16 (strong)	2
ISA + AC	6	16 (strong)	0
LSIL	12	8 (score 2)	4 (score 0)
HSIL	4	4 (score 2)	0

SC, squamous carcinoma; ISA, in situ adenocarcinoma; AC, adenocarcinoma; LSIL, low-grade squamous intraepithelial lesion; HSIL, high-grade squamous intraepithelial lesion.

Table 2. E7 expression in biopsies of uterine cervix infected by HPV 16.

Samples	Total	Positive	Negative
SC	18	18 (strong)	0
ISA + AC	6	6 (strong)	0
LSIL	12	8* (score 2)	4
HSIL	4	4 (score 2)	0

*Two biopsies co-expressed E2-HPV 16.

Results

P16 was positive in eight of 12 LSILs (score = 2); these eight LSILs progressed to HSIL. P16 was

Table 3. E2 expression in biopsies of uterine cervix infected by HPV16.

Samples	Total	Positive	Negative
SC	18	0	18
ISA + AC	6	0	6
LSIL	12	6* (score 1 − 2)	6
HSIL 40	4		

*Two biopsies co-expressed E7-HPV16.

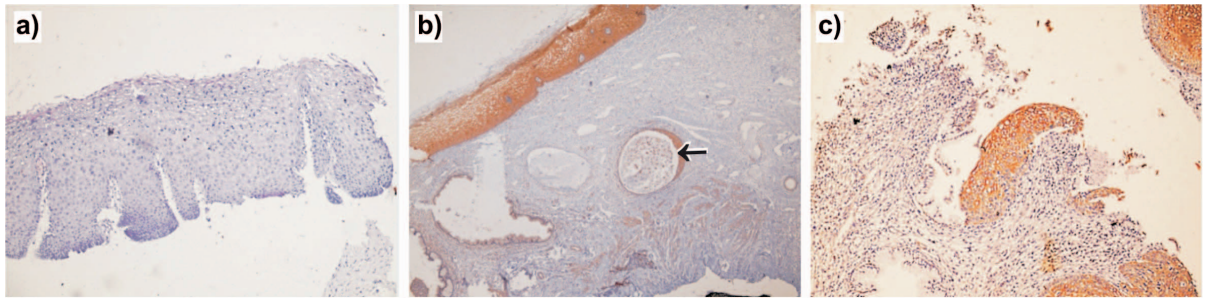

Fig. 1. a) E2-HPV16 in LSIL. b) E7-HPV16 in LSIL. The marker is expressed also in an intraglandular metaplasia (arrow). c) P16 in LSIL. Immunohistochemistry. Hematoxylin counterstain. 100 × for all panels.

expressed in all four HSILs (score = 2), in 16 of 18 SC and in all six AC.

E2 was positive in only six of 12 LSILs. Two of the six samples, which co-expressed E7, corresponded to lesions that progressed to HSIL, while the remaining four returned to normal epithelium. E2 was not expressed by HSILs or AC.

E7 was positive in eight of 12 LSILs. The eight LSILs that expressed E7 progressed to HSIL and were positive for p16. E7 was positive in all of the HSILs and AC (Tables 1 – 3; Figs. 1 – 5).

Discussion

Integration of HR-HPV into the host genome is required for malignancy to develop, because it increases the expression of viral oncogenes (E6/E7). The HR-HPV E7 oncoprotein inactivates the protein, pRb, which is required to maintain the malignant phenotype. Consequently, p16 is overexpressed; therefore, it can be used as a marker for progression of the lesion (Volgareva et al. 2004).

E7 is an oncoprotein in HR-HPV, because it binds to pRB and triggers cell transformation. E7 is produced also in LR-HPV, but in very low amounts and the bond to pRB is weak. We used a novel antibody that we produced in the Leloir Institute, Buenos Aires, Argentina, that binds to an epitope of the E7 of HR-HPV, but also has some reactivity with the stroma.

All of the SC and AC expressed E7 and were detected by our novel antibody. E7 also was detected in all AC, although the transformation steps from glandular epithelium to AC infected by HR-HPV are unknown.

SC with HPV 16 were positive for p16 in 88% of cases and they were positive for E7 in all cases (Tables 1, 2); these results show a high correlation between the two markers.

The use of p16 immunohistochemistry for diagnosis is limited by the lack of uniformity in scoring systems, although its use would be beneficial for decreasing inter-observer variations for diagnosis of LSILs (van Bogaert 2012). P16 has the advantage that its expression depends only on the presence of E7 from HR-HPV and is independent of the type of high-risk HPV.

E7 in high risk HPV was expressed in all cases of SC, while E2 was negative. E2 open reading frame (ORF) during viral integration allows the maximum expression of E6 and E7, because E2 is an inhibitor of viral oncoproteins (Desaintes et al. 1997). This mechanism explains the high levels of E7 expression in SC. E7 detection in the cytoplasm of cells was explained by Dantur et al. (2009) (see below).

Antibodies against HPV 16E7 in the uterine cervix have been characterized extensively by immunohistochemistry. E7 is the pivotal viral oncoprotein, because its expression is required to maintain the transformed phenotype of the infected cell. We

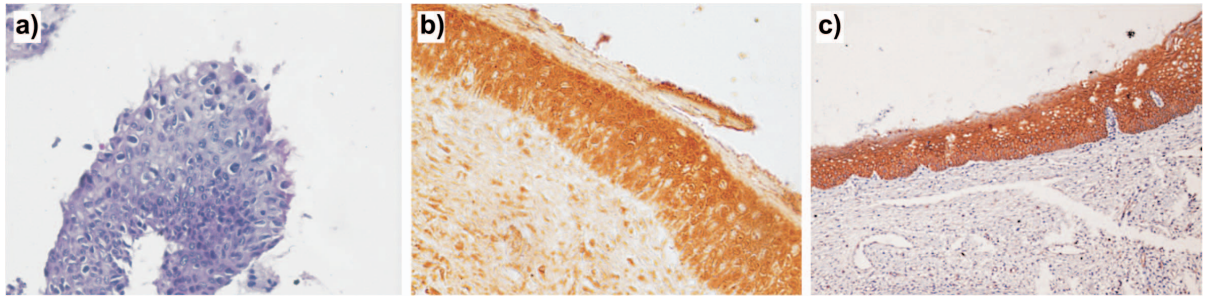

Fig. 2. a) E2-HPV16 in HSIL. b) E7-HPV16 in HSIL. c) P16 in HSIL. Immunohistochemistry. Hematoxylin counterstain. 100 × for all panels.

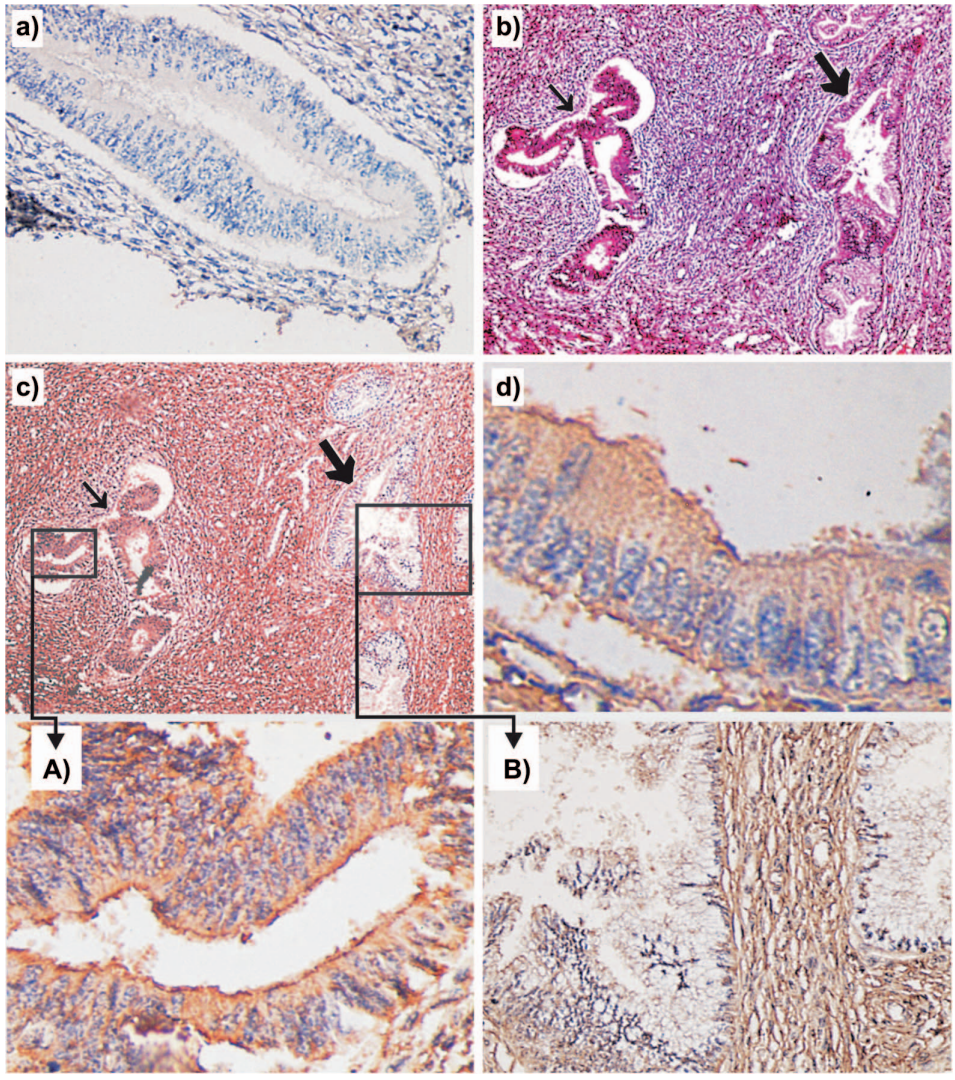

Fig. 3. a) E2-HPV16 in AC in situ of endocervix. Immunohistochemistry with hematoxylin counterstain. ×400. b) AC in situ (thin arrow) and normal gland (thick arrow) in endocervix. H & E. ×100 . c) E7-HPV16 in the same case as in (b). Note strong expression of E7-HPV16 in the neoplastic cells (thin arrow) and the lack of staining in the normal gland (thick arrow). Immunohistochemistry with hematoxylin counterstain. ×100. A) Detail of E7 immunostaining for ISA. B) Detail of E7 immunostaining for normal endocervix. d) P16 in ISA. Immunohistochemistry with hematoxylin counterstain. ×800.

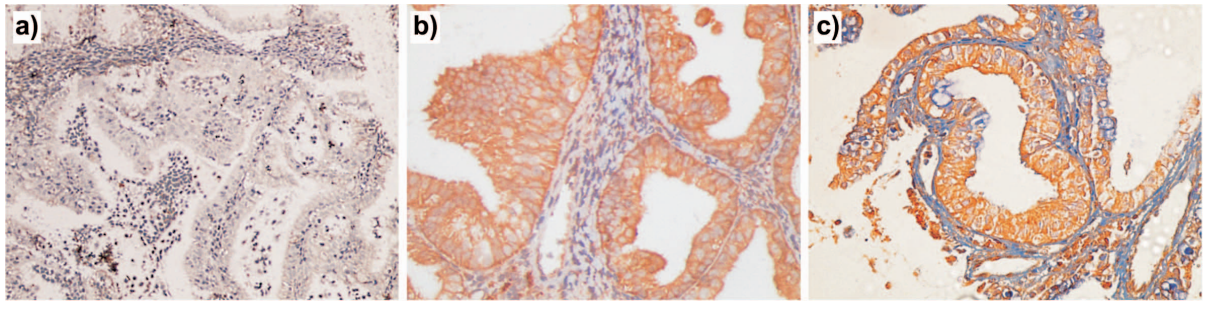

Fig. 4. a) E2-HPV16 in AC. ×100. b) E7-HPV16 in AC. ×400. c) P16 in AC. Immunohistochemistry with hematoxylin counterstain. 400 × for all panels.

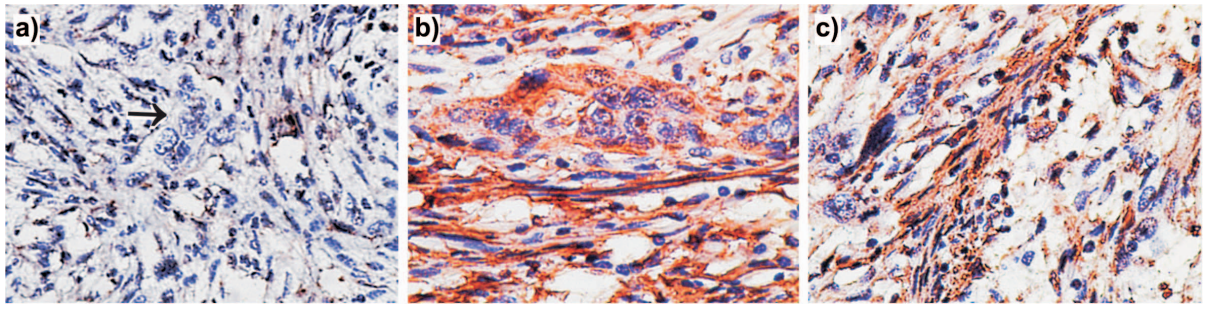

Fig. 5. a) E2-HPV16 in SC. Note that neoplastic cells at the center are negative for E2. b) E7-HPV16 in SC. c) P16 in SC. All panels, ×400. Immunohistochemistry with hematoxylin counterstain.

investigated this protein for the first time in both in situ and invasive cervical AC using a monoclonal antibody that recognizes an epitope in the C-terminus of this oncoprotein. Our findings corroborate the role of E7 in the development of these highly malignant neoplasms (Dreier et al. 2011).

The conformational, structural and chemical behavior of E7 has been characterized extensively (Alonso et al. 2002) and several conformational E7 species have been identified. Although E7 historically has been considered a nuclear dimer, it can form high molecular weight spherical oligomers, especially in the cytoplasm of transformed cells (Dantur et al. 2009). These oligomers, which are the predominant species in transformed cells, contain epitopes that are not exposed in the dimer and can be detected with new antibodies.

E2 and E7 have opposite expressions. Deregulated expression of E7 occurs when the HPV genome becomes integrated into the host genome, which disrupts the E2 ORF and therefore the transcriptional repression of E2 is lost. This was verified in four of 12 LSILs where E2 was positive, while E7 and p16 were not expressed; in this sub-group, no cervical pathology was detected during follow-up six months later. Both E2 and E7 markers were expressed in two of 12 LSILs (Fig. 6), which indicates that the virus was partially integrated (Shulzhenko et al. 2014). These two cases, together with six cases of LSIL with E7 expression (negative for E2) progressed to HSIL; in all cases, p16 confirmed the malignant potential of these lesions. HPV 16 E7 is a marker of progression of LSILs in biopsies that contain this viral type. P16 is a progression marker for any high risk virus. The model for cervical carcinogenesis requires viral DNA integration into cellular DNA; this mechanism leads to increased expression of viral oncoproteins. We confirmed the model, because the disappearance of E2 produced during integration coincided with the expression of E7. Therefore, E2 and E7 can be useful as complementary markers for histopathological studies. E2 and E7 of HPV 16 could be markers for precursor stages of cervical cancer, E2 for LSILs and E7 for progression of LSILs to HSILs in patients infected by HPV16, because viral typing alone has low predictive value.

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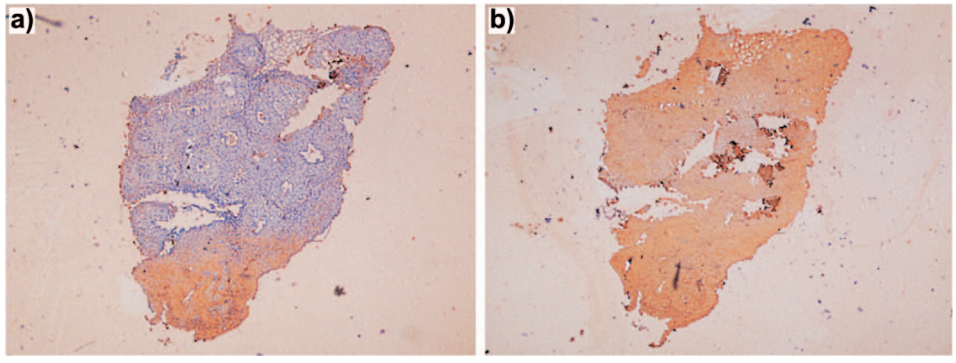

Fig. 6. a) E2-HPV16 in LSIL. b) E7-HPV16 in LSIL from same biopsy shown in (a). Immunohistochemistry. ×40.

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