HPV16 E2 Is an Immediate Early Marker of Viral Infection, Preceding E7 Expression in Precursor Structures of Cervical Carcinoma

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Abstract

The viral E2 gene product plays a crucial role in the human papillomavirus (HPV) vegetative cycle by regulating both transcription and replication of the viral genome. E2 is a transcriptional repressor of the E6 and E7 viral oncogenes for HPV types 16 and 18, which are involved in cervical cancers. Using new polyclonal antibodies against the HPV16 E2 protein, we showed that E2 is expressed at various precursor stages of cervical carcinoma by immunohistochemistry on paraffin-embedded clinical samples. E2 was found to be highly expressed in the nuclei and cytoplasm of cells forming the intermediate and upper layers of cervical intraepithelial neoplasia (CIN). We could show that the expressions of E2 and p16INK4a (surrogate marker for oncogenic E7 expression) were exclusive in most of the cases, thus implying that E2 is not expressed together with high levels of E7. Moreover, we found that E2 is expressed in a subset of columnar cells adjacent to the CIN. We could show that expression of E2 is topologically distinct from the proliferation markers p63 and Ki67, whereas it coincides with the expression of cytokeratin K13, a marker of squamous cell differentiation. Expression of E2 also topologically coincides with episomal amplification of viral genomes in the upper layers of CIN1. These in vivo data thus validate previous assumptions of the crucial role of E2 in the early steps of HPV infection and of its negative link with expression of the viral E6 and E7 oncogenes. Cancer Res 70(13): 5316–25. ©2010 AACR.

Introduction

Cervical carcinoma is the second leading cause of cancer-related death in women worldwide and has been shown to be linked to infection by high-risk human papillomaviruses (HPV), with a prominent role for types 16 and 18 present in 70% of these cancers. Cervical cancer develops several years after primary viral infection and follows a progression from low-grade to high-grade cervical lesions before full carcinogenic transformation. Some of the viral proteins involved in this progression are known, although its precise mechanisms are not fully understood. Stages preceding the invasive cervical squamous carcinoma are called cervical intraepithelial neoplasia (CIN). They correspond to various stages of invasion of the epithelium by undifferentiated basal cells going from CIN1, which is close to normal fully differentiated epithelium, to CIN3, containing mostly undifferentiated cells. The definition and description of these stages are mainly pathologic and only a few markers are used to help and sustain the diagnosis. In addition, the viral vegetative cycle has not yet been clearly described in these precancerous lesions and a better knowledge of the expression of the viral genes is needed to help understand the progression of the disease.

The HPV E2 protein has been extensively studied for more than 20 years following its first description as a potent transcriptional transactivator in the bovine papillomavirus model (1). However, and in contrast with the bovine model, E2 is a transcriptional repressor of the viral oncogenes E6 and E7 for human genital papillomaviruses (2). In cancer, transcriptional repression of E6 and E7 is relieved by integration of the viral genome into the cellular genome, which is accompanied by the disruption of the E2 open reading frame (3). This event is thus considered critical for the progression from CIN to cancer. At the same time, viral DNA replication is abolished because E2, together with the viral helicase E1, is required for viral DNA replication to occur (4). Altogether, these data suggest a crucial role for E2 early in HPV infection and its gradual disappearance following HPV DNA integration. Therefore, most cervical carcinoma cell lines do not express E2, except for one cell line established from a low-grade HPV16 lesion which contains HPV16 episomes and has been shown to express E2 (5, 6). Expression of the viral oncogenes E6 and E7 has been more thoroughly studied in cervical carcinoma cell lines as well as in vitro in organotypic cell cultures. However, they were not usually detected by direct antibody staining but rather by using surrogate markers for...
E7, such as mini-chromosome maintenance, proliferating cell nuclear antigen, and bromodeoxyuridine incorporation, as markers of DNA replication, or p16INK4a, which has been found activated in cervical cancers as a result of functional inactivation of pRb by E7 (7). Indeed, this last marker has been widely used for staining of clinical biopsies to help in the diagnosis of CIN2 or CIN3, and it is now admitted that its detection perfectly matches the expression of E7 itself in the CIN lesions (8–10).

As for the earlier stages of HPV infection and the origin of CIN, there are no specific markers except for the presence of viral DNA which could be detected by in situ hybridization (11). We reasoned that E2, as a replication factor, could represent an early marker and that detection of this viral protein could help in characterizing the evolution of HPV infections and their malignant progression. Recent studies have shown a decrease of the presence of the E2 gene in CIN2–3 compared with earlier stages (12). As for the detection of the E2 protein, two previous studies from the same group have shown E2 expression in sections of biopsies of HPV16-associated CIN1 (13, 14). Here, we show that CIN1 sections of formalin-fixed, paraffin-embedded cervical biopsies are stained with E2 antibody in the intermediate and upper layers of formalin-fixed, paraffin-embedded cervical biopsies are associated CIN1 (13, 14). Here, we show that CIN1 sections of formalin-fixed, paraffin-embedded cervical biopsies are stained with E2 antibody in the intermediate and upper layers, but not in the basal layers, and that E2 is expressed both in the nuclei and cytoplasm of these cells. In CIN2 and CIN3 sections, staining of E2 moves up to the upper layers and does not coincide with p16INK4a, p63, or Ki67 staining. In CIN1, E2 staining overlaps with the differentiation marker K13 and with DNA replication. E2 therefore seems to be an excellent marker of precursor stages of cervical cancer.

Materials and Methods

Tissue specimens

A total of 99 formalin-fixed and paraffin-embedded (FFPE) cervical specimens from cervical punch biopsies, loop electrosurgical excision procedure, cold knife cervical conization, and hysterectomy samples were selected from the archives (during March 2009 to September 2009) of the Department of Pathology of two hospitals: Changning Maternity and Infant Health Hospital, Shanghai, China, and the National University Hospital affiliated with National University of Singapore, Singapore. The specimens included 5 normal cervical tissues, 35 CIN1, 31 CIN2, and 28 CIN3 lesions. Tissues were fixed in neutral-buffered formalin immediately after being taken from patients and processed into paraffin-waxed blocks. Diagnosis of specimens was validated by two pathologists according to the classification criteria of the WHO. The study was approved by the Institutional Review Boards of the National University of Singapore (NUS-IRB 09-218) and the Medical Ethical Committee of Changning Maternity and Infant Health Hospital.

Preparation and specificity of the anti-HPV16 E2 antibodies

A fragment encompassing the COOH-terminal part of E2 containing half of the hinge and the DNA-binding domain (E2C: from amino acids 209 to 365) was cloned in-frame with glutathione S-transferase (GST), expressed in bacteria and purified before injection into two rabbits. Affinity-purified and depleted antibodies were prepared by purification on GST beads containing the GST-E2C fragment. HaCaT cells were grown in DMEM supplemented with 10% fetal bovine serum, infected with recombinant adeno-viruses expressing GFP-E2 HPV16 or GFP alone at a multiplicity of infection of 200. The cells were harvested 24 hours after infection for Western blot as previously described (15) with the anti-GFP antibody (Torrey Pines), E2 serum (1:1,000), E2 affinity-purified antibodies (1:50), and depleted antibodies (1:500). Infected HaCaT or HeLa cells were grown on coverslips in DMEM supplemented with 10% fetal bovine serum, fixed in 4% formaldehyde, permeabilized by 0.1% Triton X, and blocked with 10% goat serum. They were then incubated with HPV16 E2 serum or affinity-purified antibodies, followed by a goat anti-rabbit antibody conjugated with Alexa 568 (Invitrogen) and counterstained with 4′,6-diamidino-2-phenylindole (Sigma-Aldrich).

Immunohistochemistry staining

Consecutive sections from paraffin-embedded tissues were processed for immunohistochemical staining with purified anti–HPV16 E2C (1:50), anti-p16INK4a (1:100, clone JC8; Santa Cruz Biotechnology), anti-p63 (1:100, clone 4A4; BD PharMingen), anti-Ki67 (1:50, rabbit polyclonal antibody; Novus), anti-keratin 13 (1:100, clone KS-1A3; Novoceastra), and keratin 14 (supernatant; clone LO002), a gift from Birgit Lane, Institute of Medical Biology (Immunos, Singapore). Sections were dewaxed in xylene and rehydrated through descending ethanol concentrations. Endogenous peroxidase activity was blocked by incubation in 0.3% hydrogen peroxide in methanol for 30 minutes. Epitope retrieval was performed by heating the sections at 121°C for 10 minutes in 10 mmol/L of citrate buffer (pH 6.0) then blocked by preincubation in 10% goat serum for 20 minutes. Primary antibodies were added for 1 hour at room temperature, whereas secondary antibodies were conjugated to peroxidase-labeled dextran polymer for 30 minutes (Dako EnVision+ Peroxidase system). After washing in tap water, 3,3′-diaminobenzidine substrate-chromogen system (Dako) was added for color development. Sections were counterstained with hematoxylin, dehydrated, and mounted in DPX. As a positive control, infected HaCaT cells expressing GFP-E2 or GFP alone were pelleted, fixed with formalin, and embedded in paraffin.

DNA extraction, HPV genotyping, and detection in microdissected samples

DNA extraction was carried out by using a column-based extraction protocol with QIAamp DNA FFPE Tissue Kit (Qiagen), according to the instructions of the manufacturer. Genotyping was performed by using the commercial HPV GenoArray test kit (HybrBio Limited, Hong Kong). DNA extracted from microdissected E2-positive and -negative columnar cells were subjected to reverse transcription-PCR with primers corresponding to the NH2- and COOH-terminal...
domains of the E2 protein: NH2-domain: 5′-ACCTACGTGAC-CATATGACTATTGGAA, 5′-TCCCATTTCTCTGGCCTTGT; COOH-domain: 5′-AGTGTCGTCTACATGGCATTGG, 5′-CACGTTGCCATTCACTATATG.

In situ hybridization

The in situ hybridizations were performed with the wide spectrum HPV biotinylated DNA probe sets able to detect 11 types of anogenital HPV (Dako), according to the guidelines of the manufacturer. Two different in situ hybridization detection kits were used. One was for the detection of episomal HPV DNA (Dako) and the other for the detection of integrated copies, GenPoint Tyramide Signal Amplification System (Dako).

Results

Specificity of the HPV16 E2 antibodies

Polyclonal antibodies were produced by injection in rabbits of the purified GST fusion proteins prepared with the carboxyl-terminal domain (amino acids 209–365) of the HPV16 E2 protein. The best serum was then tested in Western blots against extracts of HaCaT cells infected with recombinant adenoviruses expressing HPV16 or heterologous HPV18 GFP-E2 proteins. As shown in Fig. 1A, the serum efficiently detected HPV16 E2 with some cross-reaction with HPV18 E2, although the level of detection of the heterologous E2 protein was low compared to its detection with the GFP antibodies (Fig. 1A, right). The high background

![Figure 1](https://example.com/image1)

**Figure 1.** Control experiments for the rabbit anti-HPV16 E2 antibodies. A, Western blots were done with serum, purified and depleted serum, from a rabbit immunized with the HPV16 COOH-terminal domain of E2 (amino acids 209–365) on extracts of HaCaT cells infected by recombinant adenoviruses expressing HPV18 and HPV16 E2 proteins as indicated. Detection by the anti-GFP antibodies is shown as a control. B, detection by immunofluorescence of the HPV16 GFP-E2 protein in infected HaCaT cells showing colocalization of the GFP and Alexa 568 fluorescence as well as nuclear and cytoplasmic localization. C, immunofluorescence in infected HeLa cells as in B. D, immunohistochemistry staining on paraffin-embedded pellets of HaCaT cells infected with the HPV16 E2 or the GFP recombinant adenoviruses with the serum (dilution 1:1,000) and affinity-purified antibodies (dilution 1:50) as indicated.
seen in the Western blots is due to E2 being a very unstable protein which is degraded through the proteasome after ubiquitination (16), in addition, major caspase 3 degradation products (as shown by asterisks in Fig. 1A) have previously been described (15). The sera prepared against the carboxyl-terminal domain of E2 were depleted by an excess of purified E2 proteins on GST beads and affinity-purified antibodies were recovered from the beads. Both depleted and affinity-purified HPV16 E2 antibodies were assayed in Western blots. Although the affinity-purified antibodies could detect HPV16 E2 with minimal background and cross-reacted with HPV18 E2, the depleted serum only revealed background (Fig. 1A).

HaCaT cells were either infected with GFP-HPV16 E2 or with the GFP expressing recombinant adenoviruses to test for the specificity of the E2 antibody in immunofluorescence. We have previously shown that E2 proteins from high-risk HPV are expressed both in the nucleus and cytoplasm of infected cells due to active nucleocytoplasmic shuttling (15). As expected, we found GFP fluorescence in the nuclei and cytoplasm of E2-infected HaCaT cells and we could show that this perfectly colocalized with the signal found with purified anti-HPV16 E2 revealed by Alexa 568 (Fig. 1B). In addition, E2 could form cytoplasmic speckles as previously described (17). We also found a

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Figure 2. Detection of E2 expression by immunohistochemistry with the affinity-purified HPV16 E2 antibodies in sections of clinical samples (magnification, ×400). A, normal cervical epithelium and junction between noninfected and HPV16-infected epithelium. B, two different clinical samples with CIN1 sections. C, two different clinical samples with CIN2 sections. D, two different clinical samples with CIN3 sections.
similar colocalization of GFP-E2 fluorescence with the Alexa immunofluorescence in these speckles in infected HaCaT cells (Fig. 1B, bottom). Noninfected cells (data not shown), or cells infected with the negative control GFP adenovirus, were not stained by Alexa with a low background with the E2 serum and an even lower background with the affinity-purified antibody (Fig. 1B; data not shown). We tested the E2 antibodies in infected HeLa cells, in which E2 is also expressed both in the nucleus and cytoplasm of infected cells, and a perfect colocalization of the GFP fluorescence and Alexa staining was seen, specifically with the purified antibodies (Fig. 1C). Cells not infected or infected with the GFP adenovirus gave a low background, as shown in Fig. 1C (top).

We then proceeded to stain HPV16 GFP-E2 or GFP-infected HaCaT cell pellets embedded in paraffin to check for the efficiency and specificity of the serum and affinity-purified antibodies for E2 detection by immunohistochemistry as positive controls for clinical samples (Fig. 1D). Both could detect E2 expression efficiently but the background in the negative control GFP-infected cells was undetectable only with the purified antibodies (Fig. 1D, compare right and left). We therefore decided to use the E2 affinity-purified antibodies at 1:50 dilution for subsequent immunohistochemistry staining.

Characterization of the clinical samples and staining by the affinity-purified E2 antibodies

Clinical samples containing biopsies of CIN1 to CIN3, embedded in paraffin blocks, were sectioned for subsequent genotyping and immunohistochemistry staining. From a total of 99 specimens, we obtained 20 HPV16-positive samples that were further characterized for E2 staining. We first checked that the purified E2 antibodies did not stain a section of normal cervical epithelium and that we could find the junction between noninfected and infected tissues in the same HPV16-positive section (Fig. 2A), thus establishing the specificity of the purified antibodies in clinical samples. In all HPV16-positive samples, E2 staining could be found specifically in CIN lesions in the intermediate or upper layers but never in the basal layers (Fig. 2). In CIN1 and some of the CIN2, both the nuclei and cytoplasms of the cells of the intermediate layers were stained with a particularly strong signal in the nuclei of koilocytes (Fig. 2B and C). In CIN2, E2 staining was also detected, although it could be restricted to the cells of the upper layers (Fig. 2C, right). Examination of different CIN3 samples showed different E2 staining patterns, E2 staining could be either maintained in upper layers or almost completely lost (compare left and right, Fig. 2D).

Figure 3. Staining of E2 does not coincide with staining of p16 and proliferation markers p63 and Ki67. A, a section showing adjacent CIN1 (right of the arrow) and CIN2–3 (left of the arrow) stained by the E2C antibody on the left and the p16 antibody on the right (magnification, ×100). B, higher magnification of the junction of the same sample as in A, serial adjacent sections were stained with E2, p16, p63, and Ki67 antibodies as indicated (magnification, >200).
Altogether, E2 staining of clinical samples indicated a strong expression of the viral protein, inversely correlating with the grade of the lesion, as expected from previously published data.

**E2 is highly expressed in epidermal compartments that express low or undetectable levels of E7 as judged by p16$^{INK4A}$, Ki67, and p63 surrogate markers**

We then proceeded in using the well-established p16$^{INK4A}$ staining as a surrogate marker for E7 expression and compared it with the staining of E2. Indeed, a recent article described a complete overlap between p16$^{INK4A}$ and anti-p63 in the CIN2–3 neoplasia and not in the columnar cells (magnification, ×200). C, microdissected positive columnar cells area for DNA extraction. D, reverse transcription-PCR amplification of the DNA of E2-positive and E2-negative columnar cells. Values were obtained using the formula $2^{\Delta CT}$ after normalization with cellular 18S DNA amplification.

Higher magnification of the p16 staining showed a clear demarcation between CIN2 (left) and CIN1 (right of the arrow in Fig. 3B). In contrast, E2 staining was high in the intermediate and upper layers of CIN1, completely devoid of p16 staining (Fig. 3B, top).

The transcription factor p63 has been shown to stain specifically proliferative keratinocytes as well as being a marker of proliferation and transformation in cervical neoplasia (18). We examined the proliferative state of the cells expressing E2 by using p63 and Ki67, a well-established proliferative marker, to stain consecutive sections and found that, as for p16, the CIN2–3 lesions were stained in all the layers by Ki67 and p63, whereas the adjacent CIN1, stained by E2, expressed Ki67 and p63 only in the basal and parabasal layers (Fig. 3B, bottom). We deduce from these experiments that E2 staining is not detectable in proliferative cells, either because it is not expressed or expressed at low levels, whereas it is highly expressed in differentiated cells, and that furthermore, high levels of E7 expression do not occur concomitantly with E2.

Figure 4. Columnar cells were stained by E2 but not by proliferation markers and contain HPV DNA. A, staining of the columnar cells, as indicated by arrows, of an HPV16 sample proximal to the transformation zone with the E2C antibody (left) and staining of a negative sample from an uninfected patient (right; magnification, ×200). B, serial sections of the same sample as A, stained with anti-p16$^{INK4A}$ and anti-p63 in the CIN2–3 neoplasia and not in the columnar cells (magnification, ×200). C, microdissected positive columnar cells area for DNA extraction. D, reverse transcription-PCR amplification of the DNA of E2-positive and E2-negative columnar cells. Values were obtained using the formula $2^{\Delta CT}$ after normalization with cellular 18S DNA amplification.
Columnar cells of the endocervix at the transformation zone express E2

Surprisingly, we also detected E2 expression in cells that are not squamous cells of the ectocervix structures but rather columnar cells of the uterine endocervix (arrows in Fig. 4A, left). We could detect a strong specific staining of columnar cell nuclei only at the border of the endocervix and close to E2-positive CIN lesions as shown in Fig. 4A. This staining seemed highly specific because it was not found in a total of nine samples from uninfected women, an example of which is shown in Fig. 4A (right). In addition, in the same positive sample, negative areas could be found further away from the junction (data not shown). Specific expression of E2 in columnar cells thus occurs proximal to E2-positive squamous cell lesions, close to the transformation zone. It is in this zone, where the columnar cells meet the stratified epithelium of the ectocervix, that HPV infection is considered to be initiated (reviewed in ref. 19). Columnar cells positive for E2 staining were negative for p16\(^{INK4a}\) and p63 staining as expected from previous data (8, 18), whereas the adjacent CIN2–3 were stained by the two markers (Fig. 4B).

We sought to determine whether these cells were able to replicate the viral DNA but could not detect proper DNA replication by \textit{in situ} hybridization. However, the use of laser capture and reverse transcription-PCR on a positive sample (Fig. 4C) allowed us to detect HPV DNA, encompassing the complete E2 gene, in E2-positive columnar cells but not in negative cells from uninfected patients (Fig. 4D).

E2 is expressed in differentiated lesions negative for p16 expression but positive for expression of cytokeratin K13

CIN express different cytokeratins depending on their differentiated status (20). To be able to better characterize...
the lesions expressing E2, we decided to stain serial sections for the presence of E2 and the p16 marker and for two cytokeratins, K13 and K14. Keratin 13 is a marker of parabasal and upper layers in normal cervix epidermis, whereas in contrast, K14 is a marker of the basal cells (20, 21). E2 antibody stained all the layers, except the basal layers, of a CIN1 section, with an almost perfect coincidence with K13 staining (Fig. 5A), whereas in adjacent serial sections, p16 staining was completely absent and keratin 14 was found only in the basal layers (Fig. 5A). In contrast, in a CIN2–3 lesion originating from the same sample, E2 staining clearly decreased together with the staining of K13, whereas in contrast, staining of p16 and K14 increased (Fig. 5B). We deduced from these experiments that, indeed, E2 staining coincided with keratinocyte differentiation. Altogether, these results confirmed high expression of E2 mostly in differentiated keratinocytes.

**HPV DNA replication coincides with E2 expression**

The next obvious question was whether the cells expressing E2 were replicating the viral genomes. We studied HPV replication by *in situ* hybridization methods that allowed the detection of episomal and/or integrated HPV DNA as previously described (8). Episomal DNA replication, as a diffuse dark blue nuclear signal, was readily detectable in cells scattered in the E2-positive intermediate and upper layers of CIN1 lesions (Fig. 6A, left, E2 staining; right, DNA hybridization). Interestingly, high levels of DNA amplification (dark blue nuclei) were often detected in areas that were in close proximity to high E2 expression (brown nuclei), but often not completely overlapping. We could also detect integrated viral DNA as punctate black signals in most nuclei of cells from more advanced CIN3 lesions (Fig. 6B and C, right), in which E2 was found expressed at low levels in similar compartments of a serial adjacent section (Fig. 6B and C, left). A higher magnification of this CIN3 section shows that there might be coincidence between E2 expression and DNA integration, thus inducing genomic instability *in vivo* as suggested recently (22, 23). More work is needed to accurately correlate DNA amplification with the expression of viral proteins in clinical samples but the data presented here is in good agreement with the role of E2 in DNA replication and in early stages of the HPV vegetative cycle.

**Discussion**

Although the involvement of HPV infection in cervical cancer has been established for more than 20 years, the actual progression of the disease from HPV infection to productive
infection and to cancer is not completely understood. The functions of the viral proteins and regulation of transcription and replication of the viral genome have been extensively studied in cell cultures and differentiated keratinocytes in raft cultures in vitro (19, 24). These studies allowed for an extensive knowledge of the functions of the viral oncogenes E6 and E7 (reviewed in refs. 25, 26). The E2 gene encodes for a fascinating multifunctional protein which is involved in regulating transcription as well as replication, but which is also able to interact with several key cellular factors leading to modulation of the cell cycle and apoptosis (15, 17, 27). E2 is not expressed in cervical carcinoma cell lines and we and others have shown that its reintroduction into HPV-positive cells induced a repression of the E6 and E7 transcription, thus inducing an arrest of cell proliferation (28–30). As for the absence of E2 in cervical cancer in vivo, it has not yet been conclusively demonstrated and assumptions from previous data are that E2 should not be expressed in cancers and that its expression should be reduced in high-grade cervical neoplasia.

On this point of view, the data presented here are in complete agreement with these assumptions. We could also show that E2 and E7 are expressed at high levels in an exclusive fashion, E2 expression being high in low-grade CIN1 lesions which express low levels of the transforming proteins, whereas it is decreased in CIN2–3 lesions in which high levels of the transforming proteins are expressed. Furthermore, when we examined the viral DNA by in situ hybridization, we could show that E2 is expressed in cells of the intermediate and upper layers of CIN1 where viral DNA replication occurs. In addition, viral genome integration takes place in cells in which E2 expression is decreased. Altogether, our staining of clinical samples confirmed previous assumptions that E2 acts as a repressor of the E6 and E7 transcription and as an activator of the viral DNA replication in vivo. Two previous reports describe the expression of HPV16 E2 in CIN and its relationship with viral DNA replication, which are in complete agreement with our data, although they did not correlate the expression of E2 with the viral oncogenes (13, 14). Interestingly, the BPV1 E2 protein has also been found to be expressed at high levels in the intermediate layers of bovine papillomas, perfectly matching viral DNA amplification (31, 32). As for the relationship between E2 expression and viral genome integration in the cellular genome for the HPV model, our data only indicates that E2 is highly expressed during viral DNA replication. We cannot deduce from these data when and how the viral DNA are integrated and whether there is a complete overlapping between this event and loss of E2 expression.

We have also previously shown that the high-risk E2 proteins are shuttling from the nucleus to the cytoplasm of expressing cells (15), and found that indeed E2 staining is located in both cell compartments in vivo. E2 is also an unstable protein which is degraded at the G1–S transition of the cell cycle through the SCF^Muv^ ubiquitin ligase in cycling cells (33), which implies that E2 should be stabilized in G0-arrested differentiating cells. We could therefore hypothesize that the high levels of E2 detected in CIN1, compared with CIN2–3, might be due to cell cycle arrest of the differentiated cells in CIN1 where E2 would be stabilized in contrast with the proliferative cells of the CIN2–3 lesions. This assumption was supported by the expression pattern of the proliferative markers p63 and Ki67 and differentiation marker cytokeratin K13. Direct staining of E2 thus seems to be a powerful tool to recognize infected low-grade lesions that are differentiated and could otherwise be mistaken with normal epithelia.

In this respect, we found that E2 is also highly expressed in the nuclei of an unexpected new cell type, the columnar cells of the endocervix. E2 expression is detected in these cells only when they are proximal to the CIN lesions, and therefore, could represent a precursor state for HPV infection that has not been previously documented. We were intrigued by these results and wanted to check whether viral DNA replication could be detected in these cells, which are not squamous cells and are therefore theoretically not suitable for a complete viral cycle. We could detect the presence of HPV16 DNA in these cells indicating that they could probably replicate HPV DNA. More work is needed, however, to determine the extent of the viral cycle in these cells and whether they are able to produce virus to propagate the infection. Nevertheless, staining of E2 in these cells indicate that they might be primary targets of viral infection in the transformation zone where HPV infection starts. Altogether, our data describe E2 as a new marker that can identify early stages of HPV infection before morphologic changes occur.

**Disclosure of Potential Conflicts of Interest**

No potential conflicts of interest were disclosed.

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