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Biomarker Insights

Prognostic Value of DNA and mRNA E6/E7 of Human Papillomavirus in the Evolution of Cervical Intraepithelial Neoplasia Grade 2

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ABSTRACT

OBJECTIVE: This study aimed at evaluating whether human papillomavirus (HPV) groups and E6/E7 mRNA of HPV 16, 18, 31, 33, and 45 are prognostic of cervical intraepithelial neoplasia (CIN) 2 outcome in women with a cervical smear showing a low-grade squamous intraepithelial lesion (LSIL).

METHODS: This cohort study included women with biopsy-confirmed CIN 2 who were followed up for 12 months, with cervical smear and colposcopy performed every three months.

RESULTS: Women with a negative or low-risk HPV status showed 100% CIN 2 regression. The CIN 2 regression rates at the 12-month follow-up were 69.4% for women with alpha-9 HPV versus 91.7% for other HPV species or HPV-negative status ($P < 0.05$). For women with HPV 16, the CIN 2 regression rate at the 12-month follow-up was 61.4% versus 89.5% for other HPV types or HPV-negative status ($P < 0.05$). The CIN 2 regression rate was 68.3% for women who tested positive for HPV E6/E7 mRNA versus 82.0% for the negative results, but this difference was not statistically significant.

CONCLUSIONS: The expectant management for women with biopsy-confirmed CIN 2 and previous cytological tests showing LSIL exhibited a very high rate of spontaneous regression. HPV 16 is associated with a higher CIN 2 progression rate than other HPV infections. HPV E6/E7 mRNA is not a prognostic marker of the CIN 2 clinical outcome, although this analysis cannot be considered conclusive. Given the small sample size, this study could be considered a pilot for future larger studies on the role of predictive markers of CIN 2 evolution.

KEYWORDS: viral oncogene proteins, papillomavirus E7 proteins, E6 protein, human papillomavirus-16, cervical intraepithelial neoplasia, neoplasm regression, spontaneous, disease progression

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Introduction

Infections caused by oncogenic human papillomavirus (HPV) are a prerequisite for cervical cancer and its precursor lesion, cervical intraepithelial neoplasia (CIN).[1,2] HPV genotypes 16, 18, 31, 33, and 45 are the most prevalent types associated with cervical cancer worldwide.[2,3] These genotypes derive from two different phylogenetic species: alpha-9 HPV (types 16, 31, 33, 35, 52, 58, and 67) and alpha-7 HPV (types 18, 39, 45, 56, 59, 66, 68, and 70).[4] There are indications that HPV types belonging to the alpha-9 species occur more often in multiple infections and are correlated with more severe lesions.[4–7]

The expression of viral genes is closely regulated as the infected basal cells migrate toward the epithelial surface.

Expression of two Early (E) genes, E6 and E7, in the lower epithelial layers is also necessary for viral genome replication and cell proliferation in a productive viral infection.[8] The oncogenic potential of the high-risk HPV (HR-HPV) genotypes depends on the unregulated expression of the E6 and E7 genes.[9,10] The E6 and E7 viral proteins bind and modulate cellular gene products (p53 and pRb) that play a key role in cell DNA repair and cycle control. The resulting genomic instability is a necessary condition for cell transformation and immortalization.[11,12] Increased expression of these transcripts has been observed in high-grade squamous intraepithelial lesions (HSIL) and in cervical carcinoma.[9,11,13]

The risk of cervical neoplasia associated with infection by individual HPV types has been examined, and there are indications that specific types lead to different risks for persistence and progression.[14] In this context, HPV E6/E7 mRNA expression might be predictive of disease progression and might constitute a useful tool for screening or patient management.[9,11,15–17] These possibilities might be particularly important for the evaluation of grade-2 CIN (CIN 2) clinical outcome. Some CIN 2 lesions, like CIN 1 lesions, should not be considered true precursor stages of cervical cancer, but rather a cytopathological effect of a productive viral infection by HR-HPV. In contrast, some CIN 2 lesions exhibit a topographical change in viral gene expression similar to that of CIN 3 lesions, including an increase in HPV E6/E7 mRNA in proliferating cells.[18]

CIN 2 lesions occur more frequently in young women and therefore, more conservative management should be considered.[19,20] From a clinical standpoint, the identification of prognostic and predictive markers of regression is very important for identifying women with CIN 2 who should receive more conservative management.

The objective of this study was to evaluate whether HPV groups and the expression of E6/E7 mRNA are prognostic of the clinical outcome of CIN 2.

Methods

Study design, participants, and ethical aspects. A cohort study was carried out between January 2005 and December 2008 at the Women's Hospital of the State University of Campinas (UNICAMP), Brazil. The selection of women followed strict criteria to ensure that none of them suffered adverse effects because of a delay in the treatment of their CIN 2. This study derived from a study previously conducted to evaluate CIN 1 management; the previous study was approved by the Institutional Review Board of the School of Medical Sciences of UNICAMP. The Institutional Review Board approved this CIN 2 study with the recommendation to include women with previous low-grade squamous intraepithelial lesion (LSIL) cytology, but not HSIL, leading us to include cases from the CIN 1study. All 50 women included in the study read and signed the consent form.

Subject selection and follow-up. A total of 4732 women with a cervical smear showing LSIL were invited to participate by letters sent with the cervical smear reports; 1584 responded to this invitation and were admitted to the colposcopy clinic (Fig. 1). All of these women underwent colposcopic examination and biopsies were carried out whenever a suspicious image was found. Prior to the colposcopic examination, a cervical sample was taken for a second cervical smear, and residual material was rinsed and maintained in 1.0 mL of Universal Collection Medium (QIAGEN Inc.) for HPV-DNA detection and genotyping. A cervical sample for HPV E6/E7 mRNA analyses also was taken and rinsed in RNAlater (Ambion, Inc.).

Women were considered eligible for this study if they satisfied the following criteria: (1) the second cervical smear showed LSIL; (2) histological diagnosis of CIN 2; (3) lesion and squamocolumnar junction completely visualized by colposcopy; (4) not pregnant; (5) no evidence of any immunodeficiency; (6) no history of therapy for neoplasms; and (7) having a fixed address and able to at least provide a fixed telephone number. Of the 1584 women who responded to the initial invitation letter, 1534 were not included in this study because of biopsy revealing no neoplasia, CIN 1, CIN 3, or worse; a negative colposcopy and negative second cervical smear at admission; a second cervical smear showed CIN 3 or worse; unsatisfactory colposcopy; personal reasons (Fig. 1).

Fifty women satisfied the inclusion criteria, agreed to participate in the study, and signed the informed consent. These women were followed over a 12-month period and a cervical smear and colposcopy were performed every three months. The women who showed a worsening of the suspect image during the colposcopy relative to the previous controls were subjected to biopsy. When the biopsy revealed CIN 2 or less, the woman was kept in follow-up every three months; when the biopsy revealed CIN 3, immediate treatment by excision of the lesion was performed. On completing one year of follow-up, all the women who still showed any cytological or colposcopy abnormalities underwent complete diagnostic evaluations and biopsies of suspicious areas and were offered treatment by excision.

Cytopathologic and histopathologic diagnosis. Second cervical smears were analyzed by the same cytopathologist in accordance with the 2001 Bethesda System recommendations.[21] In cases in which the diagnosis was HSIL, a differentiation between CIN 2 and CIN 3 was carried out.[22] The biopsies were evaluated according to the criteria of the World Health Organization and classified as no neoplasia, CIN 1, CIN 2, or CIN 3.[23] All histopathological analyses were carried out at the same laboratory, and diagnoses were performed by a single pathologist, who was unaware of the HPV results.

CIN 2 clinical outcome. The final CIN 2 outcome was classified as regression, persistence, or progression according to the following criteria:

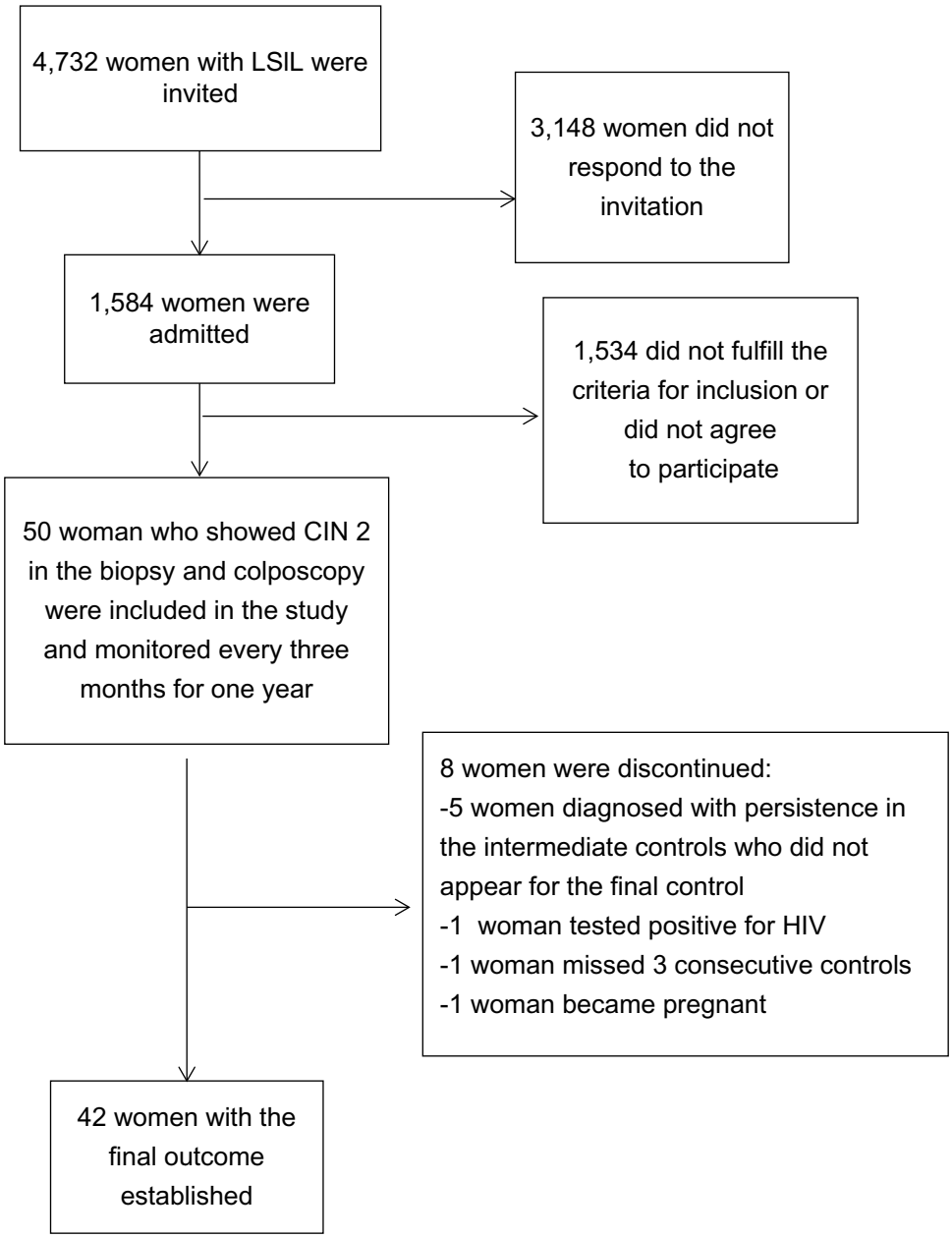

Figure 1. Selection and inclusion of study subjects.

- Progression: biopsy showing CIN 3 at any time during follow-up.
- Persistence: biopsy showing CIN 1 or CIN 2 at the 12-month follow-up.
- Complete regression: cervical smear, colposcopies, and biopsies without neoplasia at any time during follow-up and confirmed at the 12-month follow-up.

At the three-, six-, and nine-month follow-ups, persistence was determined when no changes were detected in the colposcopy image and/or the cervical smear revealing ASC-US, LSIL, or HSIL (CIN 2). Women were subjected to biopsy when the HSIL was classified as CIN 3. A colposcopy-directed biopsy that showed CIN 1 or CIN 2 was characterized as persistence, while detection of a CIN 3 led to a designation of progression. The analysis included data on all of the follow-up visits.

The women who showed progression to CIN 3 were submitted to large loop excision of the transformation zone (LLETZ). Women who exhibited regression of the lesion at the intermediate visits completed the scheduled follow-up to ensure that the lesion actually regressed.

HPV genotyping. HPV-DNA genotyping was carried out using a reverse line-blot hybridization assay that involved the hybridization of a 450 nt PCR amplicon generated by PGMY primer set to a nylon strip containing immobilized probes. The strip contained two levels of β-globin control probes, 18 HR-HPV probes (16,18, 26, 31, 33, 35,

39, 45, 51, 52, 55, 56, 58, 59, 68, 73, 82, and 83) and 9 low-risk HPV (LR-HPV probes 6, 11, 40, 42, 53, 54, 57, 66, and 84). PCR reagents, probe strips, and developing reagents were kindly supplied by Roche Molecular Systems Inc. (Pleasanton, CA).

Detection of HPV E6/E7 mRNA. Total nucleic acid was extracted from the sample stored in 1 mL of RNAlater® with the NucliSENS® easyMAG® (bioMérieux) system according to the manufacturer's instructions.

The E6/E7 mRNA of HR-HPV types 16, 18, 31, 33, and 45 was identified using the NucliSENS® EasyQ® HPV kit v1 (bioMérieux), which is based on real-time nucleic acid sequence-based amplification, according to the manufacturer's recommendations. In order to prevent false-negative results because of insufficient cellular material, degradation of the mRNA, or inhibition of amplification, the quality of the extracted nucleic acid was monitored by testing for an internal control mRNA, human U1 small nuclear ribonucleoprotein-specific protein A (U1-A).

Statistical analysis. The odds ratios (OR) for CIN 2 progression or persistence, with the associated 95% confidence intervals (95% CI), were calculated for every three-month follow-up period. The CIN 2 regression rates over time according to HPV types, viral groups, and HPV E6/E7 mRNA expression were evaluated using the Kaplan–Meier method and compared with the log-rank test, assuming a significance level of 5%. Analyses were carried out using Epi-Info version 7.0 and SAS version 9.1.3.

Results

This study included 50 women, with a mean age of 26.5 ± 7.3 years and a median age of 25 years (range 17–47 years); one woman was excluded from the HPV analysis because beta-globin, an internal reaction control, was not detected. The overall prevalence of HPV was 93% (46/49), of which 90% (44/46) of cases were HR-HPV. The prevalence of a single infection was 46% (21/46), and multiple infections represented 54% of all cases (25/46). Of the HPV-positive women, 48% (22/46) were infected with HPV 16, either as a single infection or as multiple infections. The second most prevalent type was type 33 (19%), followed by types 52 (15%), 58 (10%), and 31 (9%). HPV types 18 and 45 were identified in only one case each. Among the women, 59% (27/46) of women were predominantly infected by alpha-9 species, 8.7% (4/46) of cases harbored alpha-7 species, and 19.6% (9/46) of cases were infected by both alpha-9 HPV and alpha-7 HPV. Six women (13%) had infections with HPV types belonging to other HPV species (Table 1).

Nine cases were not included for mRNA analysis because the internal control mRNA (U1-A) was negative. E6/E7 mRNA of HPV 16, 18, 31, 33, and 45, were detected in 49% (20/41) of the cases; 60% (12/20) were HPV 16, followed by genotypes 33 (30%), 31 (15%), 45 (5%), and 18 (5%) (Table 1).

CIN 2 final outcome data up to 12-months of follow-up were available for 42 women. Five women did not have confirmation of CIN persistence at the 12-month follow-up, one woman had an HIV diagnosis, one woman missed three consecutive follow-up visits, and one woman got pregnant (Fig. 1).

The frequency of CIN 2 regression during the 12-month follow-up was 74% (31/42); progression to CIN 3 occurred in 24% of cases (10/42), and persistence was observed in 2% of cases (1/42). No case of progression to invasive carcinoma was detected during follow-up. We observed 26/31 (84%) spontaneous regression cases at the six-month follow-up (data not shown).

We observed that the presence of alpha-9 HPV was significantly associated with CIN 2 progression at the three-month follow-up, with an OR (95% CI) of 7.0 (1.41–34.68). During the remaining follow-up visits, the association between alpha-9 HPV and CIN 2 progression showed an OR ranging from 2.94 to 5.79, but with a wide 95% CI. The association of HPV 16 with the progression of CIN 2 revealed an OR ranging from 2.95 (at three months) to 4.0 (at 12 months), but with a wide 95% CI. HR-HPV and alpha-7 HPV were not associated with CIN 2 progression (Table 2). At the three-month follow-up, the association between E6 and E7 mRNA expression of HPV 16, 18, 31, 33, and 45, and CIN 2 progression had an OR of 3.37 (0.55–16.65); E6/E7 mRNA of HPV 16 was associated with an OR of 5.14 (0.55–48.37) (Table 2).

The rate of CIN 2 regression up to the six-month follow-up was 75% for low-risk HPV/negative HPV women, and it was 100% up to the 12-month follow-up. For women infected with HR-HPV, these rates were 60.6 and 74.5%, respectively, but the difference between HR-HPV and low-risk HPV/negative HPV was not significant ($P = 0.16$; Figure 2 A). For women with alpha-9 HPV, the CIN 2 regression rate up to 12-months of follow-up was 69.4%; this rate for women harboring other HPV species or a HPV-negative status was 91.7%, a statistically significant difference ($P < 0.05$; Figure 2B). For women with HPV 16, the CIN 2 regression rate up to the 12-month follow-up was 61.4%, while for other HPV types or an HPV-negative status, this rate was 89.5%, a statistically significant difference ($P < 0.05$; Figure 2C). The CIN 2 regression rate up to the 12-month follow-up for women who were positive for E6/E7 mRNA of HPV 16, 18, 31, 33, and 45, was 68.3 and 82.6% for mRNA-negative women, but this difference was not statistically significant ($P = 0.08$; Figure 2D).

Discussion

According to the present study, women infected with alpha-9 HPV, including HPV 16, are less likely to have CIN 2 regression up to 12 months of follow-up. The log-rank test revealed a regression rate of 24.1% at the three-month follow-up for women infected with alpha-9 HPV, and a rate of 58.3% for women with non-alpha-9 HPV and/or low-risk HPV or negative HPV. From the three-month follow-up to the 12-month

Table 1. Distribution of DNA-HPV genotyping and expression of E6/E7 mRNA for HPV 16, 18, 31, 33, and 45, in women with CIN 2.

HPV SPECIES	N	HPV DNA	E6/E7 mRNA
Alpha-9	5	16	Positive for HPV 16
	2	16	Negative
	2	16; 58	Negative
	1	16; 58	Positive for HPV 16
	1	16; 33**	Positive for HPV 33
	1	16; 33; 6	Positive for HPV 33
	1	16; 31	Positive for HPV 16 and 31
	1	16; 51; 52; 53	Negative
	1	16; 67	Positive for HPV 16
	1	16; 33; 52**	Positive for HPV 16 and 33
	1	16; 33; 52	Invalid test*
	1	31	Invalid test
	1	31	Positive for HPV 31
	1	31; 33**	Positive for HPV 33
	1	33	Positive for HPV 33
	1	33	Invalid test
	1	35	Negative
	1	35; 58; 73	Invalid test
	1	58	Negative
	1	52; 53	Negative
	1	52; 82	Invalid test
Alpha-7	1	39	Negative
	1	39; 51; 53; 56	Negative
	1	56; 62; 84	Negative
	1	45**	Positive for HPV 45
Alpha-9 + Alpha-7	1	16; 56	Positive for HPV 16
	1	16; 33; 68	Positive for HPV 33
	1	16; 18; 56	Positive for HPV 16 and 18
	1	16; 56; 66	Negative
	1	16; 68; 84	Positive for HPV 16
	1	18; 31; 33**	Positive for HPV 18 and 31
	1	52; 53; 66; 82	Invalid test
	1	39; 52; 54; 56; 68	Negative
	1	35; 56; 58; 62; 73; 82	Negative
Other HR-HPV species	1	26	Negative
	1	51	Invalid test
	1	82	Invalid test
	1	71	Invalid test
Other low-risk species	1	6	Negative
	1	81	Negative
Negative	3	Negative	Negative
Beta-globin negative**	1	No data	Negative

Notes: *Invalid test: negative internal control (U1-A). **These tests were considered positive for HPV detected by DNA and RNA tests.

follow-up, the Kaplan–Meier curves remained approximately parallel, indicating that the difference between the regression rates was established during the first three months of follow-up (Fig. 2). These data are in accordance with the OR of 7.00 (1.41–34.68) for persistence and/or progression for women with alpha-9 HPV infection at the three-month follow-up (Table 2). Alpha-9 HPV, mainly types 16, 31, and 33, are associated with a very high risk for cervical cancer and these data suggest that CIN 2 progression is strongly dependent on these HPV genotypes.[24] Trottier et al (2006) suggested that the alpha-9 HPV may have faster and more extensive effect on infected cervical epithelia.[4] Similar findings were observed when HPV 16 was analyzed alone (Fig. 2B and C).

We failed to detect a statistically significant difference between HR-HPV and a low-risk HPV/HPV-negative status (Fig. 2A). We expected an association between HR-HPV infection and CIN 2 progression, but maybe this effect is not powerful enough to be detected with the sample size studied. Conversely, these results suggest that the risk level of HPV 16 for CIN 2 progression is high enough to be demonstrated with a sample of low statistical power. Similar findings were also observed for alpha-9 HPV, but it could be an effect of the HPV 16 because of its high prevalence.

Data on incident HPV associated with CIN in HPV-naïve women included in the control group of the Future I/II Studies showed that HPV 18 and non-HPV 18 alpha-7 species were more prevalent in CIN 2 cases than in CIN 3 cases, which may indicate that this HPV does not have an important role in CIN2 progression. Similar data were also obtained for HPV 51 and 56.[25] Pitta *et al* (2009) reported that women infected with alpha-9 HPV were more likely to have CIN 3 than those infected with alpha-7 HPV or other genotypes.[6] Cross-sectional population-based data from the POBASCAN study revealed that the risk of CIN 3 is increased for HPV 16 and 33 (alpha-9 HPV), but not for HPV 18 and 45 (alpha-7 HPV).[26]

No association has been demonstrated between HPV E6/E7 mRNA and CIN 2 clinical outcome, but it is relevant to point out that the OR for persistence and/or progression at the three-month follow-up were 5.14 for HPV 16 mRNA and 3.37 for HPV 16, 18, 31, 33, and 45 (Table 2). Based on these data, although they are not statistically significant, we

Table 2. Risk for progression/persistence versus regression of CIN 2 according to HPV and E6/E7 mRNA status.

VARIABLE	ODDS RATIOS (95% CI)			
	3 MONTHS (n = 39)	6 MONTHS (n = 41)	9 MONTHS (n = 40)	12 MONTHS (n = 42)
HR-HPV	1.83 (0.23–14.71)	1.91 (0.18–20.23)	1.91 (0.16–20.74)	Undefined
HPV 16	2.95 (0.72–12.11)	1.91 (0.52–7.01)	2.91 (0.58–12.09)	4.0 (0.88–18.19)
HPV alpha-9	7.00 (1.41–34.68)	3.66 (0.67–19.97)	2.94 (0.45–16.22)	5.79 (0.65–51.51)
HPV alpha-7	1.22 (0.25–5.91)	2.0 (0.47–8.56)	3.0 (0.59–15.29)	1.88 (0.33–8.34)
E6/E7 mRNA for HPV 16, 18, 31, 33, 45	3.37 (0.55–16.65)	1.56 (0.39–6.25)	1.30 (0.29–5.76)	1.63 (0.37–7.2)
E6/E7 mRNA for HPV 16	5.14 (0.55–48.37)	1.11 (0.25–5.04)	1.21 (0.17–6.27)	1.71 (0.37–7.97)

consider the hypothesis that the HPV E6/E7 mRNA might be a short-term prognostic factor for CIN 2, which is in accordance with the role of the HPV E6/E7 mRNA, but not a long-term prognostic factor because a case DNA-HPV positive and E6/E7 mRNA negative can become DNA-HPV positive and E6/E7 mRNA positive, changing the clinical behavior of the lesion.[8] For this reason, DNA-HPV positive probably is a much better clinical finding than an E6/E7 mRNA negative expression for clinical management of HPV-induced lesions.

HPV E6/E7 mRNA was detected in 49% of the 41 women whose mRNA test was valid. Although the E6/E7 mRNA was detected in CIN 2 cases that persisted or progressed, these transcripts were also detected in 68.3% of CIN 2 cases that regressed, irrespective of HPV type. These findings are in accordance with the fact that HR-HPV expresses E6/E7 mRNA even in productive CIN 2 lesions.[8] The high regression rates observed in this study suggest that the selected CIN 2 cases more closely resemble CIN 1 than CIN 3, and CIN 1 also can express E6/E7 mRNA.[8,16]

According to the model proposed by Snijders et al (2006), CIN 1 and some CIN 2 lesions that harbor HR-HPV types may display viral expression patterns suggestive of productive viral infections.[18] In these infections, active viral replication and virion production are strongly coupled with the differentiation program of the infected epithelium, which is characterized by low levels of viral activity in the infected basal cells. Conversely, some CIN 2 lesions, as well as CIN 3 lesions, exhibit a dramatic topographical change in viral gene expression, including deregulation of E6/E7 expression. Once a CIN lesion has developed, altered transcriptional regulation

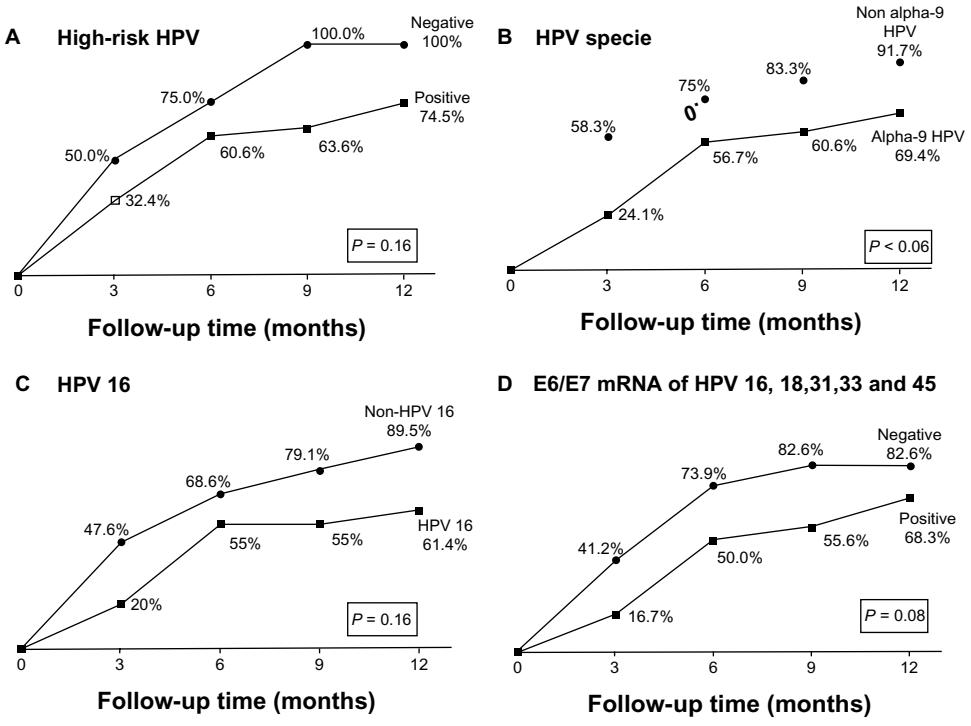

Figure 2. Cumulative spontaneous regression of CIN 2 at the 3-, 6-, 9-, and 12-month follow-ups. (**A**) HR HPV; (**B**) HPV species; (**C**) HPV 16; (**D**) E6/E7 mRNA of HPV 16, 18, 31, 33, and 45.

of the viral E6/E7 genes occurs, resulting in genomic instability and distinguishing the process of cell transformation from a productive viral infection.

The subjects of the present study were selected based on two cervical smears showing LSIL, and a biopsy that revealed CIN 2. This criterion may have selected CIN 2 lesions with characteristics of productive infection, which would be consistent with Snijders' model and the high rate of regression observed in our study. This topic was discussed in a previous publication about CIN 2 management.[27]

The greatest originality of this study was our analysis of the expectant management for women with biopsy-confirmed CIN 2 and previous cytological tests showing LSIL, which revealed a high rate of regression over 12 months. Alpha-9 HPV, especially the detection of HPV 16 at diagnosis, could be a prognostic factor of CIN 2 progression and might identify women who would not benefit from conservative CIN 2 management. If so, HPV genotyping has a higher potential for use in clinical practice than the detection of HPV E6/E7 mRNA. This study is not sufficient to conclusively support recommendations for CIN 2 expectant management. Prudently, only women with high adherence to the follow-up regimen should be considered for expectant management; otherwise, immediate lesion treatment is recommended. Given the small sample size, this study could be considered a pilot for future larger studies on the role of predictive markers of CIN 2 evolution.

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Author Contributions

Conceived and designed the experiments: LCZ, MGD. Analyzed the data: LCZ, MGD, SHRS. Wrote the first draft of the manuscript: LCZ, MGD, SHRS. Contributed to the writing of the manuscript: IDCGS, LLV. Agree with manuscript results and conclusions: IDCGS, LLV, LR, PH, MCC. Jointly developed the structure and arguments for the paper: IDCGS, LLV, MCC, LR, PH. Made critical revisions and approved final version: MGD, IDCGS, LLV, LR, PH, MCC, SHRS, LCZ. All authors reviewed and approved of the final manuscript.

DISCLOSURES AND ETHICS

As a requirement of publication the authors have provided signed confirmation of their compliance with ethical and legal obligations including but not limited to compliance with ICMJE authorship and competing interests guidelines, that the article is neither under consideration for publication nor published elsewhere, of their compliance with legal and ethical guidelines concerning human and animal research participants (if applicable), and that permission has been obtained for reproduction of any copyrighted material. This article was subject to blind, independent, expert peer review. The reviewers reported no competing interests.

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