Article/Artigo

S100, CD68, and MHC class II molecule expression in cervical high- and low-grade HPV-induced lesions

Expressão de S100, CD68 e moléculas de MHC classe II em lesões cervicais de alto e baixo grau induzidas por HPV

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ABSTRACT

Introduction: Some human papillomavirus (HPV) types are involved in malignant processes in the cervical epithelium, with 99% of cases attributed to oncogenic HPV infection. This study aimed to detect S100, CD68, and major histocompatibility complex class II (MHC-II) molecules in cervical uterine epithelial samples in patients with high- and low-grade lesions induced by HPV. **Methods:** Fifty-eight samples from patients who were confirmed positive or negative for high-risk oncogenic HPV DNA, had histopathological diagnosis of cervical intraepithelial neoplasia (CIN) of grades I, II, or III, or were negative for intraepithelial lesion or malignancy were subjected to immunohistochemistry reaction to S100 protein, CD68, and MHC-II (HLA-DR alpha chain). **Results:** The presence of MHC-II predominated in samples exhibiting histopathological alterations ($p < 0.05$). S100 detection was more numerous in carcinoma samples (CIN III) (75%). Presence of this protein correlated significantly ($p < 0.05$) with histopathological findings and viral load. **Conclusions:** A small expression of CD68 was observed, which may be explained by the observation in our study having been made on random microscopic fields and not on specific areas. The findings, such as the presence of S100 protein and MHC-II expression in samples with histological alterations, could suggest that the immune system fails to control HPV replication at the early stages of infection. Further studies with larger prospective data are necessary to confirm this result.

Keywords: Human papillomavirus. Immune response. Immunohistochemistry.

RESUMO

Introdução: Alguns tipos de papilomavirus humano (HPV) estão envolvidos em processos malignos no epitélio cervical, com 99% dos casos atribuídos à infecção por HPV oncogênico. O objetivo deste estudo foi detectar a proteína S100, CD68 e moléculas de MHC-II (complexo principal de histocompatibilidade classe II) em amostras de epitélio cervical uterino, de pacientes com lesões de alto e baixo grau induzidas pelo HPV. **Métodos:** Cinquenta e oito amostras de pacientes positivos ou negativos, confirmados, para DNA de HPV de alto ou baixo risco oncogênico, e que tiveram diagnóstico histopatológico de neoplasia intraepithelial cervical (NIC) de graus I, II ou III ou foram negativas para lesão intraepithelial e malignidade (NILM), foram submetidas à reação de imunohistoquímica (IHQ) para proteína S100, CD68 e MHC-II (HLA-DR cadeia alfa). **Resultados:** A presença da molécula MHC-II predominou em amostras exibindo alterações histopatológicas ($p < 0,05$). A detecção de S100+ foi mais numerosa em amostras com carcinoma (NIC III) (75%). A presença dessa proteína correlacionou-se significantemente ($p < 0,05$) com achados histopatológicos e a carga viral. **Conclusões:** Pequena expressão CD68+ foi observada, uma possível explicação seria que em nosso estudo as observações foram feitas em campo microscópicos aleatórios e não em áreas específicas. Os achados como a presença de S100 e a expressão de MHC-II, em amostras com alterações histológicas, podem sugerir que o sistema imune falha em controlar a replicação do HPV nas fases iniciais da infecção. Maiores estudos, com mais dados prospectivos, são necessários para confirmar esses resultados.

Palavras-chaves: Papilomavírus humano. Resposta immune. Imunohistoquímica.

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INTRODUCTION

Cervical cancer is the second most frequent cause of death from cancer among women. An estimated half a million cases occur each year, almost 80% of these in developing countries[1]. Human papillomavirus (HPV) infection is sexually transmitted, with effects ranging from benign verrucae to invasive cancer. Some HPV types are involved in malignant processes in the cervical epithelium, with 99% of cases attributed to oncogenic HPV infection[2]. In the anogenital tract, HPV infects basal and parabasal squamous epithelial cells. In cases of latent infection, no cytopathic effects are observed and the epithelium is normal. Cases of productive infection may involve clinical (*Condyloma acuminatum*, invasive carcinoma) or subclinical manifestations (high- or low-grade cervical intraepithelial neoplasia)[3].

Immunodeficiency is associated with low numbers of Langerhans cells and the presence of HPV-induced lesions[4,5]. The presence of antigen-presenting cells (APC), like Langerhans cells and macrophages, and the activity of such cells by major histocompatibility complex class II (MHC-II) molecule expression at the early stages of infection are therefore important factors for disease resolution. We know that Langerhans cells are the most efficient APCs to initiate primary response by naïve T-lymphocyte activation[6] and that macrophages are the APCs that differentiate T-helper lymphocytes in the effector phase of cellular immunity and humoral response[7].

Evidence suggests that E5, a small, highly hydrophobic protein observed in cell cultures and located in internal compartments of the endoplasmic reticulum and Golgi complex, inhibits the transport of proteins, including class I and class II MHC molecules, to the cell surface[8,9].

Although oncogenesis has been strongly associated with HPV-related neoplastic progression

and inhibition of immune response[10], the exact mechanisms responsible for activating effective immune response to HPV remain unknown. Systemic immune abnormalities have been demonstrated in cases of uterine cervical cancer[11,12]. Moreover, the tumor microenvironment may be capable of controlling local immune response, thereby interfering with disease progression[13,14].

In local immune response, the initial mechanisms of presentation of antigens and their components, like MHC-II expression, are essential for activating and maintaining effective immune responses capable of promoting virus elimination or limiting replication to low levels. The purpose of the present study was to describe the presence of S100 protein, CD68 marker, and MHC-II molecules (HLA-DR+) in epithelial lesions of the uterine cervix of patients with high- and low-grade HPV-related lesions. As these markers can be found in Langerhans cells, macrophages, and others cells, characterizing the antigen presentation events is important for efficient immune response.

METHODS

Subjects

The study included samples of uterine cervical lesions collected from women aged 18 to 65 years, assisted for routine analysis or for clinical suspicion of HPV infection from 2000 to 2002 at the Centro de Prevenção ao Câncer, a cancer prevention center located in Campo Grande, the capital city in the State of Mato Grosso do Sul, Brazil.

Fifty-eight samples of paraffin-embedded cervical stroma were used. Of these, 44 were positive for high-risk oncogenic HPV DNA, as detected using the hybrid capture II (HCII) test (Digene), and had a negative histopathological diagnosis for intraepithelial lesion or malignancy (NILM) or cervical intraepithelial neoplasia (CIN) grade I, II, or III. In this last group we normally have the samples classified as *in situ* or invasive carcinoma, but in this work the samples considered carcinoma *in situ* or invasive were considered separately, as with other authors[15], because the immune response to the neoplastic process can be different from that to the pre-neoplastic process. In this study we included yet another 14 samples obtained from patients who were negative for HPV DNA, as revealed by HCII, and with NILM results. Viral load, quantified by HCII, was classified into four groups, according to Lorincz et al.[16]: from 0.1 to less than 10, from 10 to less than 100, from 100 to less than 1,000, and from 1,000 or more relative light units/positive control to group B (RLU/PCB). The data for histopathological diagnosis and viral load **(Table 1)** were provided by a previous study[17].

Immunohistochemistry

The immunohistochemistry (IHC) reaction was performed as proposed by Santos and colleagues[18]. Antigen retrieval was performed by heating sections in 10mM citrate buffer at pH 6.0. To detect IHC markers, the following monoclonal antibodies were used: mouse anti-human S100 protein (Zymed, ref. 18-0046); mouse anti-human CD68, clone KP1 (Dako, ref. M0814); and mouse anti-human HLA-DR, alpha chain, clone TAL.1B5 (Dako, ref. M0746). The detection system was a Universal LSAB + kit/-HRP (Dako, ref. K0690), and diaminobenzidine (Dako, ref. K3468) was used as chromogen. All sections were examined under a 10× objective with confirmation at 40×, and ten randomly chosen microscopy fields/per region were observed. Only epithelium was considered for S100 protein, only stroma for CD68, and both epithelium and stroma for MHC-II. The percentage of cells stained was categorized as follows: 0 for the absence of cells (negative), 1 for a small number of cells, and 2 for a large number. Two previously calibrated independent observers ($\kappa = 0.091$) carried out the observation; conflicting results were resolved by consensus.

TABLE 1 - Histological classification of uterine cervical epithelial lesion and viral load of HPV DNA of the samples.

Histopathological findings	Number	Percentage
NILM	24	41.3
CIN I	10	17.2
CIN II	9	15.5
CIN III	7	12.2
CS	8	13.8
Total	**58**	**100.0**
Viral load		
Negative	14	24.1
0.1 to <10 RLU/PCB	8	13.8
10 to <100 RLU/PCB	7	12.1
100 to <1000 RLU/PCB	17	29.3
≥1,000 RLU/PCB	12	20.7
Total	**58**	**100.0**

HPV: human papillomavirus; **DNA:** deoxyribonucleic acid; **NILM**: negative for intraepithelial lesions and malignancy; **CIN**: cervical intraepithelial neoplasia; **CS**: carcinoma samples; **RLU/PCB**: relative light units/positive control to group B.

Data analysis

The data were organized in spreadsheets for statistical analysis (chi-square test), carried out using SPSS 10.0 software. For statistical analysis, the histopathology results were grouped as CIN I, CIN II/III, and carcinoma samples (CS). The last CS was considered a separate group because immune responses to neoplastic processes can differ from those to pre-neoplastic conditions. The data were analyzed for possible correlations between histological or viral load groups and the presence of IHC markers.

Ethical considerations

The investigation was approved by the Research Ethics Committee of the Universidade Federal de Mato Grosso do Sul (UFMS) (protocol 1355).

RESULTS

Relationship between the presence of IHC markers and histopathological findings

Table 2 shows data on MHC-II expression and the presence of CD68 and S100 for each type of histopathological finding. Low amounts of MHC-II molecules were expressed in 79.2% of NILM samples. Greater expression of this molecule was found in CIN I, reaching maximum values in CS (57.1%). Histopathological findings correlated significantly ($p < 0.05$) with the presence of MHC-II molecules.

Low CD68 presence predominated in about 70% to 90% of NILM, CIN I, and CIN II/III samples. In CS, however, low and high numbers of these cells were equally distributed. No correlation was found between CD68 presence and histopathological findings in any of the groups.

S100 protein, on the other hand, was present in small numbers in 70.8% of NILM samples, but in high quantities in CIN I and CIN II/III samples (50-60%), as well as in CS samples (75%). Presence of S100 correlated significantly with histopathological findings ($p < 0.05$). **Figure 1** shows S100 and CD68, in addition to MHC-II molecules, detected using the IHC reaction. **Figure 2** shows NILM samples with marker absence.

Relationship between the presence of IHC markers and viral load

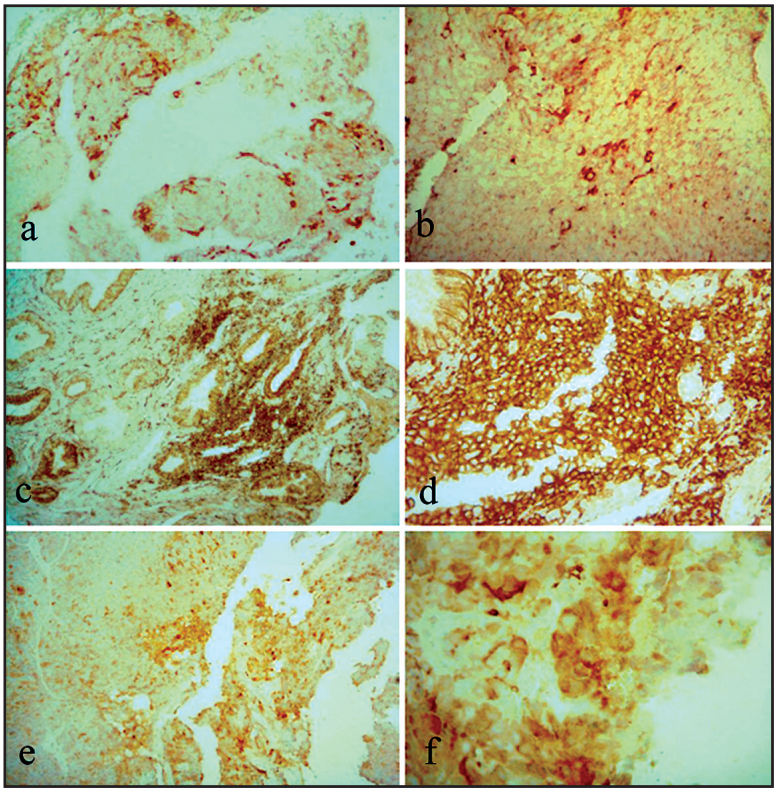
No significant correlation was found between MHC-II expression, CD68 presence, and viral load. As shown in **Table 3**, most samples revealed low levels of MHC-II molecules and CD68 (60-80%), regardless of viral load, with a slight predominance of MHC-II expression (42.9%) and CD68 presence at high levels (28%) among samples having a viral load of 10 to less than 100 RLU/PCB.

S100 protein was found in large numbers in 62.5% of the samples having 1 to less than 10 RLU/PCB and in 83.3% of samples with 1,000 or more RLU/PCB. Presence of S100 protein correlated significantly with viral load ($p < 0.05$).

TABLE 2 - Classification of uterine cervical epithelial lesion samples by presence of MHC-II molecules, CD68, and S100+ cells.

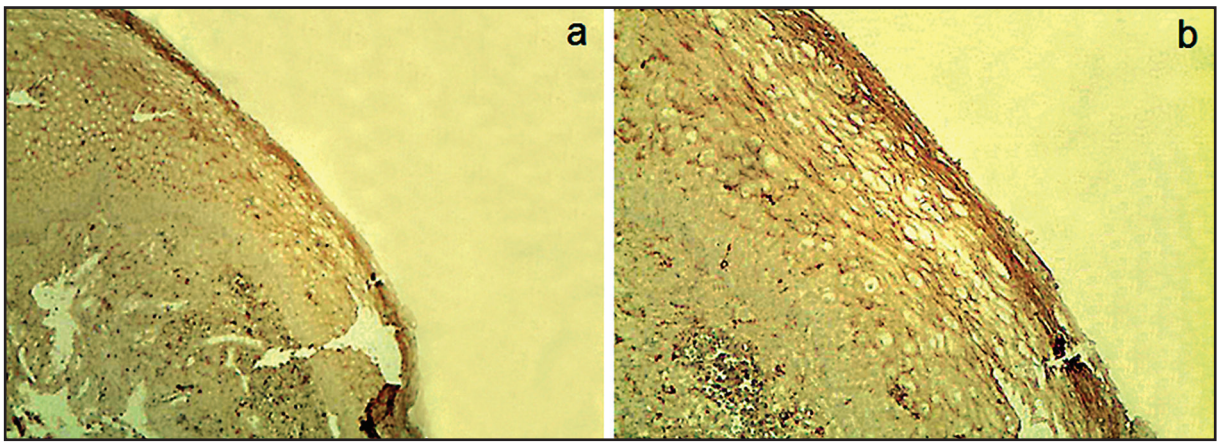
	Immunohistochemistry							
	MHC-II							
	0		1		2		Total	
Histopathological findings	n	%	n	%	n	%	n	%
NILM	4	16.7	19	79.2	1	4.2	24	100.0
CIN I	0	0.0	6	60.0	4	40.0	10	100.0
CIN II/III	3	18.8	8	50.0	5	31.3	13	100.0*
CS	0	0.0	3	42.9	4	57.1	7	100.0*
Total	**7**	**12.3**	**36**	**62.2**	**14**	**24.7**	**58**	**100.0**
	CD68							
	0		1		2		Total	
Histopathological findings	n	%	n	%	n	%	n	%
NILM	4	16.7	18	75.0	2	8.3	24	100.0
CIN I	1	10.0	8	80.0	1	10.0	10	100.0
CIN II/III	1	6.3	14	87.5	1	6.3	16	100.0
CS	0	0.0	4	50.0	4	50.0	8	100.0
Total	**6**	**10.3**	**44**	**75.9**	**8**	**13.8**	**58**	**100.0**
	S100							
	0		1		2		Total	
Histopathological findings	n	%	n	%	n	%	n	%
NILM	4	16.7	17	70.8	3	12.5	24	100.0
CIN I	0	0.0	5	50.0	5	50.0	10	100.0*
CIN II/III	0	0.0	6	37.5	10	62.5	16	100.0
CS	0	0.0	2	25.0	6	75.0	8	100.0
Total	**4**	**6.9**	**30**	**51.7**	**24**	**41.4**	**58**	**100.0**

MHC-II: major histocompatibility complex; **CD68:** cluster of differentiation 68; **S100:** S100 protein; **NILM**: negative for intraepithelial lesions and malignancy; **CIN**: cervical intraepithelial neoplasia; **CS**: carcinoma samples. Statistical analysis: χ^2 test. * $p < 0.05$.



a, b, e and **f:** carcinoma; **c** and **d:** cervical intraepithelial neoplasia III.

FIGURE 1 - Macrophages (a: 100x; b: 400x), major histocompatibility complex-II molecules (c: 100x; d: 400x), and Langerhans cells (e: 100x; f: 400x) detected by immunohistochemistry reaction in chorium cells from cervical mucosa.



a and **b:** negative for intraepithelial lesion or malignancy.

FIGURE 2 - Negative immunohistochemistry reaction in chorium cells from cervical mucosa. (a: 100x; b: 400x).

TABLE 3 - Classification of samples by viral load by presence of MHC-II molecules, CD68, and S100+ cells.

	Immunohistochemistry							
	MHC-II							
	0		1		2		Total	
Viral load	n	%	n	%	n	%	n	%
0 (negative)	3	21.4	11	78.6	0	0.0	14	100.0
1 (1 to <10 RLU/PCB)	1	12.5	4	50.0	3	37.5	8	100.0
2 (10 to <100 RLU/PCB)	0	0.0	4	57.1	3	42.9	7	100.0
3 (100 to <1,000 RLU/PCB)	2	11.8	10	58.8	5	29.4	17	100.0
4 (≥1,000 RLU/PCB)	1	8.3	7	58.3	4	33.3	12	100.0
Total	**7**	**12.1**	**36**	**62.1**	**15**	**25.9**	**58**	**100.0**
	CD68							
	0		1		2		Total	
Viral load	n	%	n	%	n	%	n	%
0 (negative)	3	21.4	11	78.6	0	0.0	14	100.0
1 (1 to <10 RLU/PCB)	1	12.5	6	75.0	1	12.5	8	100.0
2 (10 to <100 RLU/PCB)	0	0.0	5	71.4	2	28.0	7	100.0
3 (100 to <1,000 RLU/PCB)	1	5.9	14	82.4	2	11.8	17	100.0
4 (≥1,000 RLU/PCB)	1	8.3	8	66.7	3	25.0	12	100.0
Total	**6**	**10.3**	**44**	**75.9**	**8**	**13.8**	**58**	**100.0**
	S100							
	0		1		2		Total	
Viral load	n	%	n	%	n	%	n	%
0 (negative)	3	21.4	11	78.6	0	0.0	14	100.0
1 (1 to <10 RLU/PCB)	0	0.0	3	37.5	5	62.5	8	100.0
2 (10 to <100 RLU/PCB)	0	0.0	4	57.1	3	42.9	7	100.0
3 (100 to <1,000 RLU/PCB)	1	5.9	10	58.8	6	35.3	17	100.0
4 (≥1,000 RLU/PCB)	0	0.0	2	16.7	10	83.3*	12	100.0
Total	**4**	**6.9**	**30**	**51.7**	**24**	**41.4**	**58**	100.0

MHC-II: major histocompatibility complex; **CD68:** cluster of differentiation 68; **S100:** S100 protein; **RLU/PCB**: relative light units/positive control to group B. Statistical analysis: χ^2 test. *: $p < 0.05$.

DISCUSSION

Host defense mechanisms, even if limited, require TCD4 lymphocytes to recognize the antigen associated with MHC-II molecules presented by Langerhans cells or macrophages[6,7].

Several markers, including OKT6, ATPase, and HLA-DR, have been identified in Langerhans cells. In HPV infection, as well as in CIN, reductions of as much as 60% have been found in the number of these cells, while those with the S100 marker have been reported as entirely absent. These findings suggest that selective reduction of S100+ cells could be the cause of localized immunodeficiency[6].

Local immunity mediated by Langerhans cells is thought to be a key defensive mechanism against HPV infection and the development of cervical cancer. The present investigation revealed a significant correlation ($p = 0.001$) between the presence of S100+

cells and low-grade lesions. In fact, this marker was present in large numbers in high- and low-grade lesions, but in low numbers in NILM samples. The latter finding might stem from the absence of adequate stimulus for antigen presentation[19,20].

Comparisons of histological findings and frequency of S100+ cells in HPV-negative cervix samples versus samples from women infected with oncogenic high-risk HPV[21] have shown that the presence of these cells in small numbers in HPV-positive patients may represent a mechanism of viral escape from immune response, thus reducing antigen presentation. A reduced presence of S100+ cells was also observed in the present study, revealing that these marked cells are prompted to migrate to affected areas only when histopathological alterations become more pronounced. The occurrence of Langerhans cells and other APC infiltration represent better prognosis in cases of intraepithelial neoplasia and invasive and squamous carcinoma of the uterine cervix[22], thyroid, lungs, and stomach[23-25].

HLA-DR, a member of the MHC-II family of transmembrane receptors, is primarily expressed on B cells, onto which it presents antigenic peptides for recognition by CD4+ T-cell receptors. This interaction is central to antigen specificity in adaptive immune responses. There is a consensus that HLA-DR expression represents an intense local action of cytokine inductors[26].

Malignant transformation of infected cells and neoplasia are effects of viral DNA integration, which at this infection stage might inhibit antigen presentation, since HPV interferes with MHC-I and MHC-II synthesis, hindering T-cell activation[27]. In inflammatory conditions and viral infections, expression of higher levels of MHC-II molecules is responsible for more pronounced T-cell activation[28].

Langerhans cells originally express MHC-II molecules, but HPV-16 and HPV-18 infections cause a decrease in these molecules in the cervical epithelium[29]. Loss of MHC-I molecules has been reported in the progression of cervical lesions and can serve as a prognostic marker for vulvar disease, as can antigen expression by MHC-II[30]. Our results showed that higher levels of MHC-II expression occurred at the stages in which viral replication was accompanied by histopathological changes, as was the case of CS.

Inflammatory infiltration by macrophages and CD4 T-lymphocytes has been observed in spontaneous regression of condyloma, and specific CD4 T-lymphoproliferative response to HPV E2 antigen was associated with virus elimination. On the other hand, CD8 T-cells specific for E6 and E7 antigens have been reported for patients with large lesions or cervical tumors. It was thus possible to observe that T-helper type 1 response is decreased when IL-2, INF-γ, and TNF-α production is low in patients with high levels of intraepithelial lesions[31].

Murine cells expressing HPV-16 E6 protein have been found to induce lysis by macrophages, but not by natural killer cells[32]. Low expression of CD68 molecules was observed in the present study, demonstrating reduced presence of this marker at all stages of infection, predominantly in CS.

In a study of 35 samples of cervical stroma following conization and with a four-year follow-up, no association was found between CD68+ cells and recurrence of CIN III, as detected by IHC reaction[33]. Some authors suggest, however, that the inflammatory response is responsible for the worst prognostic in cervical disease, and showed that it had a direct relationship between the increasing grade of the lesion and the number of macrophages in the epithelium[34]. In Hammes and colleagues the authors showed that cases of low-grade squamous epithelial lesions with moderate to intense inflammatory regions had almost two times more macrophages than did those with negative to weak reactions[34].

Other authors have found similar results with the significant increase of macrophages in carcinoma *in situ* and have suggested that this cell participates in malignant cell migration and invasion by production of angiogenic factors, once a direct relation was observed between macrophage and blood vessels[15,35,36].

A small expression of CD68+ cells was observed in our study, demonstrating the low presence of this marker in all phases of infection; these cells are predominant only in carcinoma samples. One possible explanation for this discrepancy is that in our study the observation was made on ten random microscopic fields and not on specific areas with CIN lesions and CS. Therefore, our sample size was small, and further studies with larger prospective data are necessary to confirm this result.

By relating, on the one hand, the magnitude of viral load and, on the other, the presence of the structures linked to immune response activation, a predominance of S100 protein was found in samples from patients at the viral replication stage (≥1,000 RLU/PCB). The relationship between viral load and the presence of CD68 and MHC-II molecules revealed their distribution to be virtually uniform at all levels. Studies have demonstrated that viral load in HPV infection can serve as a marker for the progression of pre-cancerous lesions[37,38]. This is corroborated by the results of the present investigation showing that, at non-replication stages of infection, the stimulus for immune response activation was not operating, allowing viral infection to persist, thus contributing to malignant transformation.

The evaluation of NILM samples, which were negative (n = 14) and positive (n = 10) for HPV DNA (data not shown), revealed few S100 or CD68+ cells and low levels of MHC-II molecules in both groups. In NILM samples that were positive for HPV DNA, S100 was present in large numbers only when the viral load was ≥1,000 RLU/PCB, suggesting that intense viral replication may be necessary for antigen presentation and immune response activation.

The presence of Langerhans cells at the initial stage of HPV infection could represent an indicator of infection control by the immune system. At more advanced stages, however, an increase in the number of these cells may be an indication that antigen presentation by these cells to T lymphocytes fails to occur at the initial stage. Periods of intense replication, when the virus causes characteristic cellular alterations and the infection is productive, are accompanied by the occurrence of immune response. The persistence of viral replication and cellular alterations become risk factors for malignancy. The findings of this article, such as the predominant presence of S100, CD68, and MHCII expression in samples with histological alterations, could suggest that the immune system fails to control HPV replication at the early stages of infection.

In this study we concluded that at the non-replication stages of infection and in the consequent absence of histological alterations, the stimulus for immune response activation was not operating, allowing viral infection to persist, thus contributing to malignant transformation. Further studies with larger prospective data are necessary to confirm this result.

CONFLICT OF INTEREST

The authors declare that there is no conflict of interest.

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