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The impact of glucocorticoids and anti-cd20 therapy on cervical human papillomavirus infection risk in women with systemic lupus erythematosus

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OBJECTIVE: To identify the prevalence and factors associated with cervical human papillomavirus infection in women with systemic lupus erythematosus

METHODS: This cross-sectional study collected traditional and systemic lupus erythematosus-related disease risk factors, including conventional and biologic therapies. A gynecological evaluation and cervical cytology screen were performed. Human papillomavirus detection and genotyping were undertaken by PCR and linear array assay.

RESULTS: A total of 148 patients were included, with a mean age and disease duration of 42.5 ± 11.8 years and 9.7 ± 5.3 years, respectively. The prevalence of squamous intraepithelial lesions was 6.8%. The prevalence of human papillomavirus infection was 29%, with human papillomavirus subtype 59 being the most frequent. Patients with human papillomavirus were younger than those without the infection (38.2 ± 11.2 *vs.* 44.2 ± 11.5 years, respectively; $p = 0.05$), and patients with the virus had higher daily prednisone doses (12.8 ± 6.8 *vs.* 9.7 ± 6.7 mg, respectively; $p = 0.01$) and cumulative glucocorticoid doses (14.2 ± 9.8 *vs.* 9.7 ± 7.3 g, respectively; $p = 0.005$) compared with patients without. Patients with human papillomavirus infection more frequently received rituximab than those without (20.9% *vs.* 8.5%, respectively; $p = 0.03$). In the multivariate analysis, only the cumulative glucocorticoid dose was associated with human papillomavirus infection.

CONCLUSIONS: The cumulative glucocorticoid dose may increase the risk of human papillomavirus infection. Although rituximab administration was more frequent in patients with human papillomavirus infection, no association was found. Screening for human papillomavirus infection is recommended in women with systemic lupus erythematosus.

KEYWORDS: Cervical Human Papillomavirus Infection; Systemic Lupus Erythematosus; Risk Factors; Rituximab.

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INTRODUCTION

Systemic lupus erythematosus (SLE) is a multisystemic disease that mainly affects young women and is caused by autoantibodies to a variety of autoantigens. SLE has been associated with cervical dysplasia (1-3), for which some risk factors have also been identified, including a history of sexually transmitted disease, early onset of sexual activity, the number of sexual partners, and low educational levels (3). Immunosuppressive therapy can increase the risk of

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viral infections, such as cervical human papillomavirus (HPV) infection, which is strongly associated with cervical dysplasia (4,5). Furthermore, a high risk of vulvar cancers has been found in patients with SLE, and one important factor is the possibility of altered clearance of viruses, particularly HPV, which is linked to this malignancy and cervical cancer (6). A recent analysis of a multicenter SLE cohort demonstrated that the standardized incidence ratio (SIR) for cervical cancer is consistent with increased risk (SIR 1.65, 95% CI 1.09-2.41) (7).

There is also a relationship between immunosuppressive therapy and cervical abnormalities (8). However, some studies have found no association between cervical HPV infection and immunosuppressive therapy (3,9). In recent years, new, targeted therapies have been administered to SLE patients, and there has been some evidence for the efficacy and safety of B cell depletion by anti-CD20 therapy with rituximab. Although there was a case report of rituximab administration and JC papovavirus infection in a patient with non-Hodgkin lymphoma (10), the effect of this type of therapy on cervical HPV infection is unknown.

In Mexico, the prevalence of HPV in cervical samples is estimated at 9.3% (11), and the prevalence of cervical cancer in women in the general population (aged ≥35 years old) ranges between 0.5% and 0.9% (12). Mexican patients with SLE have an elevated risk of major organ involvement (13). This type of patient usually requires immunosuppressive drugs and even biologic therapy, which may increase the risk of squamous intraepithelial lesions in patients with cervical HPV infection. The prevalence of cervical HPV infections in Mexican women with SLE was recently evaluated (5).

The objective of this study was to identify the prevalence and factors associated with cervical HPV infection in women with SLE.

MATERIALS AND METHODS

Patient selection and assessment

In this cross-sectional study, consecutive female patients who presented at the Systemic Autoimmune Disease Research Unit of General Regional Hospital No. 36, Instituto Mexicano del Seguro Social, Puebla, Mexico, and fulfilled the 1997 American College of Rheumatology revised criteria for the classification of SLE (14) were recruited. Patients were eligible for the study if they were married or sexually active. Patients were excluded if they were pregnant or had had a hysterectomy, cervical cancer, or a previous diagnosis of papillomavirus infection. None of the patients had been immunized against any HPV subtypes. The local institutional ethics committee approved the study, and written informed consent was obtained from all participants. All women with abnormal Pap smears were referred for gynecologic follow-up.

The study visit for each participant included a structured interview detailing demographic information and medical history, including sexual, gynecological, and obstetric histories, and a gynecological examination, including the collection of samples for a Pap test and HPV test. The Systemic Lupus Erythematosus Disease Activity Index, validated for the Mexican population (mexSLEDAI) (15), and the Systemic Lupus International Collaborating Clinics/American College of Rheumatology damage index (SLICC/ACR DI) for SLE (16) were used to assess disease activity and damage, respectively. The drug history was determined by chart review. The daily prednisone dose at the study visit and the cumulative glucocorticoid (GC) dose were measured. The continuous use of immunosuppressive therapy (azathioprine, leflunomide, methotrexate, mycophenolic acid, and cyclophosphamide) and rituximab in the three years before study recruitment was recorded. Rituximab administration (2×1 g) was added to immunosuppressive treatment (azathioprine, leflunomide, methotrexate or mycophenolic acid) in patients with refractory disease.

The genitalia were clinically examined by a single gynecologist, who examined the vulva, vagina, and cervix of each woman. Pap smears were collected using conventional techniques. Briefly, the cervix was visualized after insertion of a speculum into the vagina with rotation using gentle pressure. A Cytobrush was inserted two-thirds of the way into the endocervical canal and external cervix and was rotated 360°; the material obtained was fixed on a microscope slide. A second Cytobrush was inserted into the endocervical canal, and the material obtained was deposited in a 15 ml tube containing 3 ml of phosphate-buffered saline (PBS) (137mM NaCl, 1.8mM $KH2PO_4$, 2.7mM KCl, 10mM Na_2HPO_4).

All Pap smears were evaluated by a cytopathologist who was blinded to the results of the gynecological examinations. Cervical cytology results were classified according to the 2001 Bethesda system as negative for intraepithelial lesion or malignancy; epithelial cell abnormalities that include atypical squamous cells; low-grade squamous intraepithelial lesion (LGSIL); high-grade squamous intraepithelial lesion (HGSIL); squamous cell carcinoma; atypical glandular cells; endocervical adenocarcinoma in situ; adenocarcinoma; or other (17).

Determination of HPV in cervical smears

All samples were labeled and stored at -20℃ until DNA extraction. Cervical smears were shaken vigorously and centrifuged for 10 min at 4000 rpm. The pellets were resuspended in 1 ml PBS, and a 0.5 ml aliquot was used for DNA extraction using the QIAamp DNA Mini (Qiagen, Hilden, Germany) kit, according to the manufacturer's recommendations. Purified DNA was suspended in 100 µl of water and stored at -20℃ until use. DNA integrity was verified by 1% agarose gel electrophoresis.

HPV detection by PCR assay

HPV was detected using PGMY09/11 primers (18) which allow for the detection of more than 30 HPV genotypes.

The final volume of the PCR reaction was 25 µl, including 10.5 µl of the DNA sample, 10 pmol of each PGMY09/11 primer, and the Taq PCR Master Mix kit (Promega, Madison, WI, USA). Amplification was performed in a thermal cycler (PTC 200, MJ Research, Watertown, MA, USA) using 40 cycles of 95℃ for 1 min and 55℃ for 1 min to allow for alignment and 72℃ for 1 min to allow for elongation. These cycles were followed by a final extension at 72℃ for 5 min. A fragment of the cyclophilin gene was amplified as a control for the samples. The amplification products were analyzed by 1.5% agarose gel electrophoresis. The gels were stained with ethidium bromide (1 mg/ml), observed in an ultraviolet transilluminator, and photographed using a digital camera Canon (Melville, NY, USA) (18-20).

Identification of viral genotypes

HPV DNA genotyping was performed with the Linear Array (LA) HPV assay (Roche Diagnostic) in samples with a positive HPV PCR test. The LA assay uses the PGMY09/PGMY11 primer set, which amplifies a 450-bp fragment of the L1 gene, and it can detect 37 HPV genotypes (6, 11, 16, 18, 26, 31, 33, 35, 39, 40, 42, 45, 51, 52, 53, 54, 56, 57, 58, 59, 61, 62, 64, 66, 67, 68, 69, 70, 71, 72, 73, 81, 82, 83, 84, IS39, and CP6108). On each strip, two different concentrations of β-globin probes are present, which serve as internal controls to assure adequate amplifiable DNA in each specimen. Beta-globin negative samples were considered inadequate and genotyping test was repeated. Genital HPV has traditionally been classified into low-risk (LR) and high-risk (HR) types. HR HVP types are associated with HGSIL and cervical cancer. The HPV types classified in the HR group include 16, 18, 31, 33, 35, 39, 45, 51, 52, 56, 58, 59, 68, 73, and 82 (21).

The assay was performed according to the manufacturer's instructions. Briefly, DNA was amplified in a total volume of 100 µl, containing 50 µl of sample DNA and 50 µl of the master mixture provided by the manufacturer. The amplification protocol was as follows: 9 min of denaturation at 95℃; 40 cycles of 30 s of denaturation at 95℃, 1 min of annealing at 55℃, and 1 min of elongation at 72℃; and a final extension for 5 min at 72℃. After amplification, the whole PCR product was denatured and hybridized with oligonucleotide probes immobilized on strips. After a stringent wash, the hybrids were detected by the addition of streptavidin-horseradish peroxidase conjugate, which binds to the biotinylated PCR primers, and a substrate (hydrogen peroxide and 3, 3′, 5, 5′-tetramethylbenzidine) that generates a purple precipitate at the probe line.

Statistical analysis

Quantitative variables are expressed as means ± standard deviations (SDs), and qualitative variables are expressed as frequencies (%). The means of two groups were compared using Student's unpaired t-test. Proportions between groups were compared using the chi-square test or Fisher's exact test. Odds ratios (ORs) with 95% confidence intervals (CIs) were used to estimate the association between each possible risk factor and cervical HPV infection.

Logistic regression was used to determine the independent risk factors for cervical HPV infection, and variables with a significance of $p<0.05$ in the univariate analysis were included in the multivariate analyses. The final models were assessed for interaction effects. All analyses were two-tailed, and p-values ≤0.05 were considered significant. The statistical analysis was performed using SPSS for Windows, version 18.0 (SPSS, Chicago, IL, USA).

RESULTS

The sociodemographic, clinical, and treatment variables of the 148 SLE patients included are shown in Table 1. Sixty-three patients (42.5%) were postmenopausal. At the study visit, the mean mexSLEDAI and SLICC scores were 2.3±2.1 and 1.3±0.4, respectively. The mean mexSLEDAI score since disease onset was 3.54±1.96. Eighteen patients had received rituximab, of whom three were non-responders (16.6%). The median daily doses of each immunosuppressive agent were azathioprine 100 mg per day, methotrexate 15 mg per week, leflunomide 20 mg per day, and mycophenolic acid 2 g per day. Most patients who had received intravenous cyclophosphamide had been administered the NHI protocol.

Table 1 - Sociodemographic, clinical, and treatment characteristics of patients with systemic lupus erythematosus.

Variable	N = 148
Age, mean ± SD, years	42.5±11.8
Formal education, mean ± SD, years	11.0±4.4
Current smoker, n (%)	16 (10.6)
Age at first intercourse, mean ± SD, years	20.4±3.7
Pregnancies, n (%)	133 (88.1)
0	18 (12.1)
1-2	62 (41.8)
3 or more	68 (45.9)
Number of sexual partners, n (%)	
1	105 (70.9)
2	24 (16.3)
3 or more	19 (12.8)
Oral contraceptive use, n (%)	1 (0.7)
Disease duration, mean ± SD, years	9.7±5.3
Previous medication	
Antimalarials, n (%)	144 (95.4)
Daily prednisone (mg/d), mean ± SD	10.5±6.8
Cumulative GC dose (g), mean ± SD	11.0±8.4
Azathioprine, n (%)	47 (31.3)
Methotrexate, n (%)	29 (19.2)
Leflunomide, n (%)	9 (5.9)
Mycophenolic acid, n (%)	5 (3.3)
Cyclophosphamide, n (%)	4 (2.7)
Cumulative cyclophosphamide (g), mean ± SD	5.8±3.2
Rituximab, n (%)	18 (12.1)

SD: Standard deviation; GC: glucocorticoid.

Forty-three patients (29%) had HPV infections, of whom 31 (72%) had HR HPV infections, and 20 (13.5%) had ≥2 HPV infections. The most prevalent HR HVP types were 59 (34.8%), 18 (18.6%), 62 (16.2%), and 16 (13.9%).

Cervical HPV (+) patients were younger than cervical HPV (-) patients (38.2±11.2 years *vs.* 44.2±11.5 years, respectively; $p=0.05$) and had higher prednisone doses (12.8±6.8 mg *vs.* 9.7±6.7 mg, respectively; $p=0.01$) and higher cumulative GC doses (14.2±9.8 g *vs.* 9.7±7.3 g, respectively; $p=0.005$). The mexSLEDAI score and SLICC score did not significantly differ between the two groups. Although cervical HPV (+) patients were more likely to have received immunosuppressive therapy than cervical HPV (-) patients, the difference was not significant (58.1% *vs.* 54.1%, respectively; $p=0.7$). However, mycophenolic acid therapy (9.3% *vs.* 0.9%, respectively, $p=0.02$; OR: 10.6, 95% CI: 1.15-98.4) and prior rituximab therapy (20.9% *vs.* 8.5%, respectively, $p=0.03$; OR: 2.8, 95% CI: 1.03-7.7) were more frequent in cervical HPV (+) patients than in HPV (-) patients. The cumulative cyclophosphamide dose was higher in HPV (+) patients than in HPV (-) patients (6.3±3.4 g *vs.* 5.2±3.0 g, respectively; $p=0.05$). Table 2 shows the differences between patients with and without cervical HPV infections. The prevalence of cervical HVP infection did not differ significantly according to the response to rituximab therapy.

In the logistic analysis, only the cumulative GC dose was associated with cervical HPV infection (OR: 1.03; 95% CI 1.01-1.11), with no association found with other traditional risk factors or immunosuppressive treatments. Similarly, there was no association with rituximab therapy.

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Table 2 - Sociodemographic, clinical, and treatment characteristics of patients with systemic lupus erythematosus with and without cervical HPV infection.*

Variable	Patients with HPV (N = 43)	Patients without HPV (N = 105)	*p*-value
Age, mean ± SD, years	38.2 ± 11.2	44.2 ± 11.5	0.05
Current smoker, n (%)	4 (9.3)	12 (11.4)	0.40
Formal education, mean ± SD, years	10.7 ± 4	11.2 ± 4.6	0.30
Pregnancies, n (%)			
0	8 (18.6)	10 (9.5)	0.07
1-2	21 (48.8)	43 (41)	0.08
3 or more	14 (32.6)	52 (49.5)	0.06
Age at first intercourse, mean ± SD, years	20.4 ± 3.4	20.5 ± 3.8	0.80
Number of sexual partners, n (%)			
1	27 (62.7)	78 (74.2)	0.30
2	8 (18.6)	16 (15.2)	0.40
3 or more	8 (18.6)	11 (10.4)	0.20
Oral contraceptive use, n (%)	1 (2.3)	0 (0)	0.29
Disease duration, mean ± SD years	9.5 ± 6.2	9.8 ± 6.1	0.77
mexSLEDAI at study visit, mean ± SD, score	2.3 ± 2.1	2.1 ± 2.0	0.70
mexSLEAI since diagnosis, mean ± SD, score	4.0 ± 2.0	3.3 ± 1.9	0.08
SLICC/ACR DI, mean ± SD, score	1.2 ± 0.5	1.3 ± 0.4	0.80
Previous medication			
Antimalarials, n (%)	36 (83.7)	81 (77.1)	0.50
Daily prednisone dose, mean ± SD, mg	12.8 ± 6.8	9.7 ± 6.7	0.01
Cumulative GC dose, mean ± SD, mg	14.2 ± 9.8	9.7 ± 7.3	0.005
Azathioprine, n (%)	16 (37.2)	30 (28.5)	0.33
Methotrexate, n (%)	5 (11.6)	25 (23.8)	0.17
Leflunomide, n (%)	5 (11.6)	6 (4.7)	0.28
Mycophenolic acid, n (%)	4 (9.3)	1 (0.9)	0.02
Cyclophosphamide, n (%)	3 (6.9)	1 (0.9)	0.07
Cumulative CYC (g), mean ± SD	6.3 ± 3.4	5.2 ± 3.0	0.50
Rituximab, n (%)	9 (20.9)	9 (8.5)	0.03

*HPV: human papillomavirus; SD: standard deviation; GC: glucocorticoid; CYC: cyclophosphamide.

Six percent of patients had low-grade squamous intraepithelial lesions, and 0.6% had high-grade squamous intraepithelial lesions, according to the 2001 Bethesda system classification. None of the patients had squamous cell carcinoma or adenocarcinoma.

DISCUSSION

Several studies have investigated the prevalence of and risk factors for cervical HPV infection in SLE patients. Although immunosuppressive therapy has been studied as a risk factor for HPV infection in these patients with contradictory results (3-5,8), biologic therapy has not previously been analyzed as a possible risk factor.

We found a prevalence of cervical HVP infection of 29%, which was higher than in some studies. Tam et al. (22) found that 12.5% of SLE patients had a persistently high risk of HPV, which increased to 25% three years after diagnosis. Rojo-Contreras et al. (5) recently found a prevalence of 14.7% in Mexican SLE patients. In contrast, a Brazilian study found a prevalence of 80.7% (9). These differences may be due to environmental, cultural, or genetic risk factors or geographical variations (23). Our study assessed a greater number of HPV types than previous studies (24). HR HPV type 59 was the most prevalent type in our cohort (34.8%). Tam et al. (3) found subtype 59 in only 1.2% of patients with SLE, in whom the most prevalent HR HPV subtype was 16 (4.7%). In addition, 13.5% of our patients had ≥2 HPV subtypes, which is higher than in some studies (3,5) but lower than the 21.7% found by Klumb et al. (24).

The prevalence of cervical HPV infection decreases sharply in women after the age of 30 years (25). We found a higher prevalence of cervical HPV in younger patients with SLE, possibly related to sexual activity. However, we found no associations between the variables measuring sexual activity and infection, possibly due to the cross-sectional nature of the study.

Disease activity may be associated with abnormal cervical cytology in juvenile-onset SLE (26). However, we found no association between cervical HPV infection and lupus activity measured by the mexSLEDAI at the study visit or the mean activity since disease onset.

The cumulative GC dose was the only risk factor associated with cervical HPV infection in our SLE patients. The cumulative GC dose was recently associated with Toll-like-receptor (TLR) downregulation and the risk of HPV infection (27). Prednisolone suppressed the functions of TLR-stimulated human plasmacytoid dendritic cells, thereby reducing the ability to clear HPV infection (28).

The recent addition of biologic therapies, such as rituximab, to the treatment of SLE led us to include this factor in our analysis to identify any possible association with the risk of cervical HVP infection. The possible mechanism by which anti-CD20 therapy might increase susceptibility to HPV infection is unclear. Rituximab administration results in profound depletion of normal B cells for several months, but immunoglobulin levels remain unaltered in most patients. These effects may occur because long-lived plasma cells do not express CD20. The lack of an effect on immunoglobulin levels suggests that rituximab administration could have a minimal effect on the occurrence of infections. Depletion of B cells would be expected to result in poor antibody responses to new antigens. Several studies have reported that patients receiving rituximab exhibit decreased to absent humoral responses to new

antigens compared with recall antigens (29-31). Our results showed that patients treated with rituximab had a higher prevalence of cervical HPV infection than those treated with conventional immunosuppressive therapy. However, this possible association was lost after adjusting for others factors, such as GC. Similarly, Abud-Mendoza et al. (32) found no associations between HPV infection and rituximab in patients with SLE and rheumatoid arthritis. One possible reason why rituximab was not associated with cervical HPV infection might have been that other routes, such as the innate immune response, could have been affected in these patients. Innate immune abnormalities have been reported in patients with SLE and HPV (27).

In the univariate analysis, mycophenolic acid was associated with cervical HPV infection, although the association disappeared in the multivariate analysis, possibly because few patients were analyzed. A very recent Mexican study detected low levels of B and NK cells and an enhanced risk of HPV infection in SLE patients receiving mycophenolate mofetil (32). We also found no association between HPV infection and other immunosuppressive therapies, such as azathioprine and methotrexate. Rojo-Contreras et al. (5), who also studied Mexican SLE patients, found no association between cervical HPV and azathioprine but did find an association with methotrexate and a longer duration of prednisone therapy (5). These differences may be explained by the different methods used to assess the impact of these therapies and to geographical variations in the prevalence of HPV in Mexican women (23). A positive association has been found between cervical dysplasia and cyclophosphamide (33,34), and Klumb et al. (24) found higher cumulative cyclophosphamide doses in SLE patients with HPV infections. In our study, cyclophosphamide was not associated with cervical HPV infection.

The importance of vaccination, including HPV immunization, in preventing and reducing infectious morbidity and mortality in SLE patients was recently suggested (35,36). However, the onset of some autoimmune diseases, particularly SLE, following HPV vaccination has also been reported (37). Mok et al. (38) evaluated the immunogenicity and safety of GARDASIL, a quadrivalent HPV vaccine, in patients with SLE, and they found that the vaccine was well tolerated and reasonably effective in patients with stable SLE and that it did not induce an increase in lupus activity or flares.

Our study is not without limitations. First, there were the inherent difficulties of cross-sectional studies and the effect of sample size in establishing causal relationships and identifying incidence. Longitudinal studies with more patients who have received biologic therapy are necessary to establish associations. All the patients receiving rituximab had been refractory to conventional treatment, which was still continued during rituximab therapy, and the combination of therapies could have resulted in a greater risk of HPV infection than the administration of biologic therapy alone. Furthermore, although we measured the cumulative GC and cyclophosphamide doses, we did not measure the cumulative doses of other immunosuppressive agents, identifying only those drugs that had been administered and the median daily doses. In addition, we did not measure B cell counts to reflect depletion due to rituximab treatment or the role of other elements of the innate immune system.

In conclusion, women with SLE, and particularly younger patients, had an increased prevalence of cervical HPV infection. Our results suggest that the cumulative GC dose may increase the risk of HPV. Although rituximab administration was more frequent in patients with HPV infections, no associations were found. Screening for HPV infection is recommended in women with SLE, particularly those receiving high GC doses. Longitudinal studies are necessary to demonstrate these possible associations and to evaluate the natural history of cervical HPV infection in women with SLE who are receiving biologic therapies.

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■ AUTHOR CONTRIBUTIONS

Mendoza-Pinto C substantially contributed to conception and design, and drafted the manuscript. Garcia-Carrasco M substantially contributed to conception and design, obtained funding and administrative, technical, and material support. Vallejo-Ruiz V acquired and interpreted data, drafted the manuscript. Taboada-Cole A substantially contributed to conception and design. Muñoz-Guarneros M, Lara LV and Reyes-Leyva J analyzed and interpreted data. Solis-Poblano JC provided study supervision. Pezzat-Said E obtained funding and administrative, technical, and material support. Aguilar-Lemarroy A and Jave-Suarez LF acquired data. Ramos-Alvarez G provided administrative, technical, and material support. Lopez-Colombo A contributed to study planning, and provided administrative and technical support. All authors approved the final drafting of the manuscript.

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