PAPER

Are women with lupus at higher risk of HPV infection?

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Human papillomavirus (HPV) is the etiological agent of cervical cancer, the second most prevalent neoplasia among women. Although it has been proven that systemic lupus erythematosus (SLE) patients have higher frequency of cervical dysplasia, few studies have focused on HPV prevalence among them. This study aimed to investigate HPV prevalence among SLE patients and to evaluate associated risk factors, including the use of immunosuppressors (IM). Total DNA extracted from cervical samples of 173 SLE patients and 217 women (control group) submitted to routine cervical cytopathology was used as template in polymerase chain reaction (PCR)-based assays for detection of HPV DNA. HPV genotyping was performed by type-specific PCR, PCR-RFLP and/or DNA sequencing. Statistical methods included univariate analysis and logistic regression. Despite presenting significantly fewer HPV risk factors, SLE patients were found to have a threefold increase in HPV infection, mostly genotypes 53, 58, 45, 66, 6, 84, 83, 61, as compared with controls, who presented types 6, 18 and 61 more frequently. The higher rate of HPV infection was associated with immunosuppressive therapy. This study provides evidence that SLE patients have a high prevalence of HPV infection, which is even higher with the use of IM, a condition that might necessitate a more frequent cervical cancer screening program for these women. *Lupus* (2010) **19,** 1485–1491.

Introduction

Despite the previously observed higher incidence of cervical dysplasia and, possibly, cervical cancer (CC) in systemic lupus erythematosus (SLE) patients, few studies have focused on the CC etiological agent human papillomavirus (HPV).[1–5] HPV infection is considered the most prevalent sexually transmitted disease (STD) worldwide, and it has been proven to be necessary for cervical carcinogenesis by causing apoptosis resistance of DNA-damaged cervical cells, which consequently obtain a proliferative advantage by escaping p53 and p105Rb growth control.[6] Although other risk factors for CC have been described, most of them have been found to be dependent upon HPV infection and do not hold up as independent variables.[7]

In addition, CC is still the second most common cancer in women, and also a leading cause of death among women in developing countries.[8,9] Screening programs based on cervical cytology present the disadvantage of false-negative results, which may occur in up to 30% of exams performed.[7]

HPV prevalence and type distribution vary enormously depending on the characteristics of the studied population, including age, socioeconomic condition, sexual behavior, and immunocompetence status.[9] Resolution of cervical viral infection appears to be related, at least in part, to HPV antibody formation, and recruitment of macrophage, natural killer, and activated CD4+ T-lymphocytes,[10] which are immune functions found to be limited in many women with SLE.[11] Recent studies with HIV and transplant patients suggest that their higher levels of HPV infection could be due to pre-existing virus reactivation rather than new virus acquisition.[9]

Although HPV infection is a necessary condition for CC development, it must be understood as part of a multistep model for pathogenesis that includes

10.1177/0961203310372952

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the association of cofactors such as immune status (related to virus clearance), genetic predisposition, and, probably, also socioeconomic inequalities.[6]

Taking these considerations into account, we designed this study to determine whether patients with SLE are at enhanced risk of HPV cervical infection in comparison with non-SLE women matched for age and socioeconomic standing.

Methods

We conducted a cross-sectional study that included sequential SLE patients (American College of Rheumatology [ACR] criteria)[12] followed in the UERJ Rheumatology Department and a group of women without SLE who had appointments for routine cervical cancer screening at the same university. Patients were included if they did not have a known HPV infection, were not undergoing dialysis, were HIV-negative, and signed an informed consent form approved by the University Hospital Ethical Committee. Data referring to SLE manifestations, cumulative damage (by SLICC index), and use of immunosuppressors (IM) were obtained by retrospective analysis and patient interviews. Continuous use of IM for at least 12 months before inclusion was classified as intense, while use for less than 12 months was classified as mild. Data referring to sociodemographic characteristics and sexual behavior risk factors were based on a structured questionnaire and an accepted Brazilian socioeconomic scale (classes A to E). Endo-ecto cervical cells of each volunteer were collected by gynecologists using two swabs, which were immediately placed into tubes containing 1.5 ml of TE buffer (Tris-HCl 10 mM, pH 8.0; EDTA 1 mM) and transported to the laboratory on ice. Cells removed from the swabs were used for DNA extraction by proteinase K digestion. Briefly, after the cells were removed from the swabs, the material was centrifuged for 10 min at 10000 rpm, the supernatant was discarded, and the sediment containing the cells was resuspended in 500 μl of cold TE buffer. To the cell suspension was then added 5 μl of proteinase K (10 mg/ml) and 10 μl Twenn-20 (10%). The mixture was incubated for 3 h at 37°C, followed by proteinase K inactivation at 100°C for 10 min. The DNA solution was incubated at 37°C overnight and then stored at −20°C. HPV detection was performed by polymerase chain reaction (PCR)-based assays using DNA extracted from cervical cells as template. Firstly, all samples were submitted to an amplification reaction with the primers set PC03/PC04 (PC-PCR),[13] which amplifies a 110 bp fragment

of the human *β-globin* gene, in order to verify the integrity and quality of the DNA extracted. The reaction mixture consisted of 1× PCR buffer (75 mM Tris-HCl pH 9.0; 50 mM KCl; 20 mM $(NH_4)SO_4$); 1.5 mM $MgCl_2$; 40 μM dNTPs (mixture of the four deoxyribonucleotides dATP, dCTP, dGTP, dTTP); 0.12 μM of each primer; 0.2 U DNA polymerase (BIOTOOLS); and 2.5 μl of the extracted DNA in a final volume of 25 μl. Two molecular primer sets designed to the L1 viral gene, MY09/MY11[14] and GP05+/06+,[13] which amplify fragments of approximately 450 bp and 150 bp, respectively, were used for estimating HPV prevalence. For MY-PCR (amplification reaction with the primers set MY09/MY11) a reaction mixture of 25 μl was prepared, containing 1× PCR buffer; 2 mM $MgCl_2$; 100 mM dNPTs; 0.2 μM of each primer; 0.3 U DNA polymerase (BIOTOOLS); and 5.0 μl of the extracted DNA. HPV DNA detection using a GP system (GP-PCR - amplification reaction with the primers set GP05+/GP06+) was performed in a reaction mixture composed of 1× PCR buffer; 1.5 mM $MgCl_2$; 100 μM dNTPs; 0.4 μM of each primer; 0.6 U DNA polymerase (BIOTOOLS); and 5.0 μl of the extracted DNA. PCR conditions for PC- and MY-PCR comprised an initial denaturation for 5 min at 95°C, 40 cycles of denaturation for 1 min at 95°C, annealing for 1 min at 55°C, and extension for 1 min at 72°C, and a final extension for 10 min at 72°C. PCR conditions for GP-PCR consisted of an initial denaturation for 5 min at 94°C, 40 cycles of denaturation for 30 s at 95°C, annealing for 1 min at 44°C, and extension for 30 s at 72°C, and a final extension for 10 min at 72°C. Amplicons were submitted to 6% polyacrylamide gel electrophoresis (PAGE) and stained with ethidium bromide. All positive samples for MY and/or GP system were genotyped for HPV 6, 11, 16 and 18 by type-specific PCR, based on the methods previously reported.[15] We have used the type-specific primers: TAGTGGGCCTATGGCTCGTC and TCCATTAGCCTCCACGGGTG, for HPV 6; GGAATACATGCGCCATGTGG and CGAGCAGACGTCCGTCCTCG, for HPV 11; GCCTGTGTAGGTGTTGAGG and TGGATTTACTGCAACATTGG, for HPV 16; and GTGGACCAGCAAATACAGGA and TCCAACACGTGGTCGTTGCA, for HPV 18, to amplify 280 bp, 360 bp, 246 bp, and 162 bp fragments, respectively. The individual reaction mixtures using the four pairs of type-specific primers were composed of 1× PCR buffer (20 mM Tris-HCl, pH 8.4; 50 mM KCl); 2 mM $MgCl_2$; 100 mM of dNTPs; 0.2 mM each primer; 0.5 U Platinum®Taq DNA polymerase (INVITROGEN); and 1 μl of extracted DNA in a final volume of 25 μl. Amplification conditions

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comprised an initial denaturation at 94°C for 5 min, 40 cycles of denaturation for 1 min at 94°C, annealing for 2 min at 60°C, and extension for 1 min at 72°C, and a final extension for 10 min at 72°C. PCR products, along with molecular weight standards, were submitted to 6% or 8% PAGE and stained with ethidium bromide. In each PCR run, the negative control consisted of a reaction mixture with all PCR reagents and water replacing the DNA template, and an HPV-positive sample previously confirmed by sequencing was used as a positive control. All PCR reactions were performed using a thermocycler, *ThermoHybaid – PCR Express*. HPV-positive samples for the MY system were identified by restriction fragment length polymorphism (RFLP), individually digesting 5–10 μl of amplicons with the restriction endonucleases *Bam*HI, *Dde*I, *Hae*III, *Hinf*I, *Pst*I, *Rsa*I, and *Sau*3AI (New England BioLabs), according to manufacturer's instructions. The digestion products were submitted to 8% PAGE followed by ethidium bromide staining. The obtained digestion profiles were compared with the patterns of different HPV types previously described by Bernard *et al.*[16] All cases who presented doubtful results had their DNA sequence determined using the ABI 3130 sequencing platform (Applied Biosystems).

With the assumption of a 14% difference in HPV prevalence between the groups and 0.8 power to detect the differences between them, the number of cases was calculated as 110 for each group. Data were expressed as the mean ± SD for normally distributed variables and the median for non-normally distributed variables. Adjusted odds ratio (OR) tests for trend with 95% confidence interval (CI) were obtained by multivariate (MV) logistic regression, and comparisons among groups were based on χ^2, Mann–Whitney or Student's *t*-test as appropriate. The same MV model was employed to analyze the association of high-risk HPV types and the variables of risk studied.

Results

A total of 173 SLE patients, all fulfilling the ACR revised criteria,[12] and 217 women without SLE with cervical material adequate for PCR analysis (*β globin* gene as internal control) were studied. Most of the women included were Afro-descendents of low social status (classes C/D), and half of them were married. Mean (SD) age was 39.7 (11.2) years for SLE patients and 37.3 (10.3) years for controls. Patients with lupus were more likely to belong to social classes A/B, to have a higher

median monthly per capita income (U$168 versus U$124), and to have had a higher age at sexual debut, and were less likely to have had first sexual intercourse before the age of 17. They also had a lower number of lifetime sexual partners and less frequent use of hormonal contraception, and were also less frequently current/former smokers. There was no difference between the groups in frequency of previous STD, but, among those with previous STD, women with SLE had proportionally more previous HPV infections (Table 1). Current sexual activity was not different between groups (71.1% versus 77.9%), but lupus patients were more likely to be at menopause than control (32.7% versus 15.2%, $p = 0.0001$). The use of IM was classified as intense in 85 patients and mild in 37. Fifty-one patients had never received any IM. Among SLE patients, MY09/MY11 primers allowed detection of 82.3% and GP05+/06+ detection of 53% of HPV-positive cases. For the control group, the HPV DNA exclusive detection rates for MY09/MY11 and GP05+/06+ primers were three (30%) and six (60%), respectively.

Prevalence of HPV was significantly higher in SLE patients than in controls (20.2% versus

Table 1 Sociodemographic characteristics, and sexual and behavior risk factors

	SLE group (n = 173)	Control group (n = 217)	p *Value*
Age (mean ± SD) (years)	39.7 ± 11.2	37.3 ± 10.3	0.023[a]
Range (years)	20 – 77	17 – 66	
Ethnic group			
Caucasoid	59 (34.1%)	67 (31.9%)	
Afro-Brazilian	62 (35.8%)	75 (35.7%)	
Black	50 (28.9%)	68 (32.4%)	0.845[b]
Social Class			
A/B	44/165 (26.6%)	29 (13.4%)	
C/D	121/165 (73.3%)	187 (86.6%)	0.0001[b]
Age at sexual debut (mean ± SD)	19.3 ± 3.89	17.6 ± 3.5	0.0001[a]
≤17 years	54 (31.2%)	105 (48.4%)	0.0002[b]
Lifetime sexual partners (mean ± SD)	3.3 (± 3.0)	4.7 (± 6.3)	0.006[b]
≥4	58 (33.5%)	83 (38.3%)	0.200[b]
Previous STD	33/173 (19.1%)	45 (21%)	0.706[b]
Condiloma–HPV/STD	25/36 (69.4%)	12/55 (21.8%)	0.0001[b]
Previous/current use of hormonal contraception	114 (65.9%)	175 (80.6%)	0.0003[b]
Ever or current smoking	48 (27.7%)	119 (55.1%)	0.0001[b]

HPV, human papillomavirus; IM, immunosuppressor; SD, standard deviation; SLE, systemic lupus erythematosus; STD, sexually transmitted disease. The sum of episodes of HPV/STD infections exceeds the number of cases of STD because some patients presented more than one type of STD in life.
[a]Student's *t*-test.
[b]χ^2.

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7.3%; $p = 0.0001$). Among SLE women, univariate analysis (UV) showed that the variables associated with HPV were four or more lifetime sexual partners, previous HPV infection, previous STD, and intense use of IM. HPV high-risk types according to IARC classification[17] were detected in 12 (42.9%) SLE patients (58, 45, 66, 33, 16 and 68) and in four (40%) controls (18, 16 and 58) ($p = 0.82$) among all cases in which HPV genotyping was possible. HPV genotypes 26, 53 and 66, which are considered to be of probable high risk by Muñoz et al,[18] despite the latter being classified as high-risk by IARC,[17] were detected exclusively in patients with SLE (five cases – 17.9%). Low-risk types were detected in six (21.4%) SLE patients (6, 40, 61 and 70) and in five (50%) controls (6 and 61) ($p = 0.19$). Multiple infections were found in five SLE patients (21.7%) and in one (11.1%) control ($p = 0.75$). HPV 53 and 58 (five cases each) and 45, 66, 6, 84, 83, 61 (two cases each) were the most prevalent in the SLE group. In controls the most frequent were HPV genotypes 6 (three cases), 18 (two cases) and 61 (two cases). Compared with HPV-negative SLE patients, HPV-positive patients have continuously used IM for longer periods, but this difference was not statistically significant (median time of 84.9 versus 38.8 months, $p = 0.08$, Mann–Whitney); however, they had higher median cyclophosphamide cumulative dose (10.1 versus 7.7 g, $p = 0.049$) and higher median prednisone cumulative dose (38.1 versus 20.2 g, $p = 0.02$, Mann–Whitney).

Although women with mild or no IM use presented higher prevalence of HPV than controls (12.5% versus 7.3%), this difference was not statistically significant ($p = 0.12$). We could not detect any sociodemographic characteristic or sexual behavior associated with HPV in controls by intra-group univariate analysis (Table 2). When a backward stepwise multivariate logistic regression model including all of the risk factors previously identified by univariate analysis ($p < 0.15$, Wald χ^2)

Table 2 Unadjusted intra-group ORs for potential risk factors associated with HPV infection by univariate analysis for SLE and control groups

Variable	SLE group (n = 173)			Control group (n = 217)		
	number of patients	Prevalence % of HPV+	Unadjusted OR (95% CI)	No. of women	Prevalence % of HPV+	Unadjusted OR (95% CI)
Age at first intercourse						
≤17 years	55	10	0.38	104	7.7%	0.96
>17 years	118	24	(0.15–0.97)	113	7.9%	(0.36–2.60)
Lifetime sexual partners						
≥4	58	32.8%	3.01	81	6.2%	0.75
<4	115	13.9%	(1.4–6.45)	136	8.0%	(0.25–2.24)
STD						
Yes	33	33.3%	4.50	46	6.5%	0.84
No	140	17.1%	(1.95–10.40)	170	7.6%	(0.23–3.10)
Previous HPV infection						
Yes	25	40%	2.62	11	9.1%	1.16
No	136	17.6%	(1.37–6.64)	206	7.3%	(0.14–9.57)
Hormonal contraceptive ever or current use						
Yes	114	19.3%	0.69	175	7.4%	1.02
No	59	22.0%	(0.39–1.83)	42	7.1%	(0.27–3.75)
Marital Status						
Not Married	89	21.3%	1.54	110	8.2%	1.27
Married	84	19.0%	(0.54–2.42)	107	6.5%	(0.46–3.55)
Ever or current smoking						
Yes	48	16.6%	0.72	105	10.5%	2.50
No	125	21.6%	(0.31–1.73)	112	4.4%	(0.84–7.47)
SLICC						
<1	78	23.1%	1.38	NA	NA	NA
≥1	95	17.9%	(0.65–2.89)			
Intense use of IM						
Yes	85	28.3%	2.75			
No	88	12.5%	(1.25–6.06)	NA	NA	NA

HPV, human papillomavirus; IM, immunosuppressor; NA, not applicable; OR, odds ratio; SLE, systemic lupus erythematosus; STD, sexually transmitted disease.

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was used for the SLE group, the three independent risk factors for cervical HPV infection were intense use of IM (OR 3.45, 95% CI 1.41–8.39, $p < 0.006$), history of four or more lifetime sexual partners (OR 3.26, 95% CI 1.39–7.61, $p < 0.006$), and history of previous HPV infection (OR 3.55, 95% CI 1.20–10.43, $p < 0.02$). When the end-point was set to the presence of high-risk HPV infection, we could not detect any independent associated risk factor.

Objective disease activity evaluation was not obtained systematically for all patients, but Systemic Lupus Erythematosus Disease Activity Index (SLEDAI)[19] was available in 31 of the 35 HPV-positive patients. Among these cases, 21 were in remission (SLEDAI < 2), five had only laboratory abnormalities, and five had clinical and laboratory signs of disease activity. Most of the HPV-negative patients were also in remission or had mild active disease.

Discussion

The present study demonstrates a threefold increase in HPV prevalence among SLE patients as compared with women without SLE, which has been associated with IM use. Interestingly, patients with SLE, and with mild or no use of IM, presented higher rates of HPV infection than controls, despite presenting a significantly lower number of classic HPV risk factors. The absence of statistical significance for this difference may have been related to the small sample of non-immunosuppressed patients included.

Few studies have focused on HPV prevalence among SLE women,[3–5] despite their higher frequency of cervical dysplasia.[2] As HPV prevalence varies widely among different populations, results obtained in France, the United Kingdom, and China probably do not reflect HPV prevalence in other areas.[3–5]

The French and English studies that found higher HPV prevalence among SLE patients included few cases and/or used a purposely biased population for comparison.[3,4] Neither study could confirm any relationship with IM use, but it was claimed that this lack of association was related to the small number of cases analyzed. In the Chinese study, which included 85 SLE patients and a large number of control women, only HPV high-risk types were found to be more prevalent in SLE patients (11.8% versus 7.3%), and the authors could not detect any association with IM use, but

they argued that this may have been related to the small sample size studied.[5] In the present study, we used the same pair of primers used by Tam et al.,[5] but, in order to enhance HPV detection sensibility, we added a second pair, GP05+/06+, which was able to detect six (18.2%) previous SLE-negative HPV cases with MY09/11 primers. It is possible that we have included more severe SLE patients than the Chinese study, as the median SLICC value found was 1, versus 0 by Tam et al.[5] On the other hand, we have included more patients who were continuously taking IM, our patients have used significantly higher cumulative doses of azathioprine and steroids, and 70.5% overall had used IM for some time. Besides higher disease severity and more frequent intense use of IM, differences in sexual behavior also probably interfered with the higher HPV prevalence observed in the present study than in Tam's.[5]

SLE women with long-term use of IM (and higher HPV prevalence) also had a higher mean SLICC index than patients with mild or no use of IM (mean 1.5 ± 1.5 versus 0.5 ± 0.5, $p = 0.01$). These results suggest that IM use is associated with higher HPV prevalence, but it is not possible to exclude the influence of SLE immune disturbances per se,[2,3,12] as inefficient activation of innate immunity and ineffective priming of the adaptive immune response facilitate viral persistence, a key feature of HPV-associated cervical lesions.[9] Regarding the importance of HPV as a causative agent for abnormal pap smears, we found a higher prevalence of low and high grade squamous intraepithelial lesions (L/HSIL) among SLE patients who presented HPV (12/35 (34.3%) versus 4/138 (2.9%) respectively, χ^2, $p = 0.0001$, data not shown). Notwithstanding these findings, we could not safely establish in this study that virus persistence was the reason for those cytological abnormalities, as data were obtained in a cross-sectional design. At the same time, among controls we found only one case of LSIL, who curiously did not present HPV in cervix by PCR methods.

Despite the lack of objective disease activity evaluation for all patients in this study, we employed the SLEDAI for most HPV-positive SLE patients (88.6%) and found that 70% of them were in remission, possibly associated with intense immunosuppressive treatment at the time of inclusion. On the other hand, even patients without highly active disease may present impaired immune functions that could facilitate HPV acquisition and/or reduced virus clearance, as shown by Tam in a recent report.[20]

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In the present study, in which the main objective was to establish HPV prevalence and associated factors among SLE patients with different levels of immunosuppression, and which included the largest sample of patients submitted for prospective HPV detection by highly sensitive PCR methods, it was possible to establish a clear association of HPV infection with the use of IM. Although it has not been previously demonstrated, this finding is not completely unexpected, as these agents act mainly by inhibition of adaptive immune responses, which are involved in blocking papillomavirus infection.[21] This suggests that antibodies play a role in neutralizing infection, providing the rationale for a preventive vaccine approach.

The recent development of two highly immunogenic HPV vaccines has raised important issues about their use in SLE and other high-risk groups of patients, as vaccination traditionally represents the most cost-effective approach to combat infectious diseases. Because both bivalent and quadrivalent vaccines are composed of non-infectious virus-like particles (VLP) that resemble native virions,[22,23] their use in immunosuppressed patients should not be contraindicated.[24] These HPV-VLP vaccines, delivered by intramuscular injection, circumvent viral epithelial evasion strategies, and produce an effective T-cell-dependent B-cell response, which generates high levels of L1-specific serum neutralizing antibodies and immune memory.[21] On the other hand, the effectiveness of vaccines in SLE patients remains controversial, particularly for those taking steroid and immunosuppressive agents.[25] Another aspect that must be taken into account is that the most prevalent high-risk genotypes detected in SLE patients in the present study (58 and 53) are not included in the two commercially available vaccines. Studies that included women transplant recipients or women with HIV, both in conditions of long-term immunosuppression, also observed high prevalence of HPV genotypes not usually detected in non-selected women,[9,26,27] an aspect that might influence the effective protection to be obtained with the current available HPV vaccines for these patients.

Furthermore, at present, there are no data regarding HPV vaccination of women with lupus. Most important, however, is the fact that evidence is currently insufficient to recommend for or against vaccination of women aged 19 through 26 years, and there is no evidence for women over the age of 26 years,[28] similar to the median age in our patients when the first symptoms of SLE appeared (27 years – data not shown). The extent to which the use of these highly effective HPV vaccines might help to prevent cervical cancer in adult patients already diagnosed with SLE remains to be further studied.

This study did not have sufficient statistical power to determine whether there was an increase in the prevalence of HPV infection among patients without IM in comparison to women without SLE. However, immune disturbances related to the disease and use of steroids might explain, at least in part, the higher HPV prevalence among patients with low or no use of IM versus controls. Although this result did not reach statistical significance, it was nevertheless unexpected because the patients presented significantly fewer risk factors than controls.

Our findings extend previous observations and therefore provide evidence in support of the general recommendation to institute a more stringent program for cervical cancer screening for SLE patients, particularly when immunosuppression is needed. This finding is particularly important, given the frequent observation of a lack of routine cancer screening for SLE women, possibly because rheumatologists are usually more worried about specific and/or urgent matters such as active skin or renal disease.

In conclusion, this study provides solid evidence that SLE patients have a higher risk of presenting HPV infection, which is associated with the use of IM. Longitudinal studies including SLE patients with and without IM treatment are required to determine whether the higher levels of infection are related to increased acquisition or reduced virus clearance.

Funding

Conflict of interest statement

None declared.

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