The presence of human papillomavirus and Epstein–Barr virus in male Chinese lichen sclerosus patients: a single center study

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This study aimed to investigate the presence of human papillomavirus and Epstein–Barr virus in male lichen sclerosus patients. We extracted DNA from formalin-fixed paraffin-embedded foreskin tissue blocks of 47 male LS patients and 30 healthy men and performed real-time PCR test to detect HPV and EBV. None of the 47 LS patients and 30 healthy men had detectable HPV infection. EBV was detected in 18 LS patients (38.3%) and four healthy men (13.3%), the difference is significant (P < 0.05). Tissue blocks with significant inflammatory reaction tend to have higher EBV load. HPV has no significant relationship with LS. Male LS patients have higher EBV infection rate, but the role of EBV in the pathogenesis of LS needs further investigate.

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INTRODUCTION

Lichen sclerosus (LS) is a chronic inflammatory disease that commonly affects urogenital skin. This disease affects male and female at the same time. In male it’s also referred to as balanitis xerotica obliterans (BXO), because it frequently causes skin lesion of glans. The exact reason of this disease is currently unclear. A variety of factors are believed to have relations with this disease, such as chronic infection, poor hygiene, and autoimmune factors.¹ Human papillomavirus (HPV) has known oncogenic effect, and is thought to have possible relationship with LS.² Many studies have been carried out on this topic, and came out with largely negative results, that is, HPV prevalence among LS patients are similar or even lower than normal population.²⁻⁵ Epstein–Barr virus (EBV) infection is common in developing countries and has possible connection with autoimmune diseases like multiple sclerosis and Sjögren’s syndrome.⁶⁻⁸ It’s generally believed that LS patients might have underlying autoimmune factors,⁶⁻¹⁰ so EBV infection could possibly play some roles in the pathogenesis of LS. One study reported that female LS patients might have higher prevalence, which indicated that EBV might be related to the development of LS.¹ The main aim of this study is to investigate the presence of HPV and EBV in male LS patients, and further clarify the possible link between virus infection and LS. To our knowledge, it’s the first study to investigate EBV presence in male LS patients.

MATERIALS AND METHODS

Patients

This study was approved by the Ethics Committee of Shanghai 6th Hospital. We searched the medical records of our hospital from 2009 to 2014 for male patients with the diagnosis of LS. Inclusion criteria are: (1) have pathologic evidence of LS; (2) Have typical clinical manifestation of LS (secondary phimosis, white plaques in foreskin or glans, urethral stricture); (3) Have accessible paraffin embedded tissue; (4) Have conclusive medical record covering history of present and previous diseases, clinical manifestations, laboratory tests and imaginary exams. Patients who did not receive any surgery in our hospital were excluded. 47 patients matched all standards and were included in this study. They were divided into two groups according to the stage of their disease. The first group (n = 36, mean age 49.1) consists of late stage patients who had developed long urethral strictures. All of them had prolonged dysuria (from 4 to over 30 years), and urethrogram showed urethral strictures ranged from 3 to 15 cm. Thirty-six patients all received lingual mucosa urethroplasty, and their skin lesions on the glans were biopsied. The other group (n = 11, mean age 30.8) consists of early stage patients without urethral stricture or only with meatal stenosis. They came to our hospital with the complaint of phimosis, dysuria or white plaque on foreskin. All of them had complete phimosis. Seven of them had typical clinical manifestation of LS, and four of the seven patients were found to have meatal stenosis. These 11 patients all received circumcision, and four patients with meatal stenosis received meatomomy. The pathologic examination of excised foreskin revealed LS characteristics in all 11 patients. None of the 47 patients had any systemic diseases like RA, lymphoma, SLE or AS. None of these patients had visible genital papilloma. Control group consists of 30 healthy adults (mean age 24.5) who received circumcision for cosmetic or other personal reasons. They all have normal appearing, retractable foreskin except excessive length, and pathologic exam showed nothing special. 11 men from control group were college students who were sexually inactive, the rest 19 reported only one sexual partner. Three of the 47
LS patients reported to be sexually inactive, two reported two partners, and the rest of them all claimed to have only one sexual partner.

**DNA extraction**

DNA samples were extracted from formalin-fixed paraffin-embedded (FFPE) tissue blocks. To ensure high DNA yield, we used commercialized FFPE extraction kits and followed its standard extraction protocol. Each paraffin block was sliced off three 2-μm-thick slices. The paraffin of these slices was dissolved with xylene, and we washed off xylene with absolute alcohol. The remaining tissue was digested with protease CK. DNA was extracted from digested tissue using extraction buffer provided by the kit. The extracting buffer was then passed through the DNA-binding columns to bind DNA. Finally, we washed the columns twice to collect DNA samples.

**Detection**

HPV and EBV DNA were detected with real-time PCR. We used commercialized test kits. Primer sequences used in our reactions are provided in Table 1. The sensitivity of HPV test kit is 10^3 copies per sample. HPV amplification target is protein L1 gene, covering 18 high-risk HPV types (16, 18, 31, 33, 35, 39, 45, 51, 52, 56, 58, 59, 68, 73, 82, 26, 53, and 66). Beta-actin gene is used as internal positive control (IPC). The sensitivity of EBV test kit is 5 × 10^2 copies per sample. EBV amplification target is EBNA1 gene. The EBV test kit also provided dilution series of target DNA from 10^4 to 10^7 as quantification reference. The amplification and detection were carried out on an Applied Biosystems StepOne real-time PCR system. The results of real-time PCR were collected and analyzed with software provided by ABI. Each sample had three Ct (Cycle threshold) values for three target: beta-actin gene (internal control), protein L1 (HPV) and EBNA1 (EBV). According to quality control standards of the kits, samples with the Ct of beta-actin <37 were considered valid. Ct of HPV target < 35 indicated HPV infection (positive). With the provided diluted series of EBNA1 DNA, we can calculate the EBV load of each sample using standard curve method. Samples with EBV target Ct <30 and calculated virus load higher than 5 × 10^3 is considered EBV positive.

**Data collection and analysis**

All patients’ slides were reviewed by one pathologist. By his request, we provided him with the patients’ clinical information like the appearing of skin lesion, age and urethrogram, but he is blind to patients’ personal information. The pathologist reviewed the inflammatory reaction of each slide and classified them into three grades: severe, moderate, slight. DNA extraction and PCR procedures were performed by an experienced technician who was not given any information about these patients.

Data were collected and analyzed with IBM SPSS 22 (IBM). We performed descriptive and analytical statistic methods like Chi-square test and signed-rank test.

**RESULTS**

The mean age of control group is younger than LS group (24.5 vs 44.8, P < 0.01). Since healthy young adults are our only source of normal foreskin samples, the age of control group is naturally younger. And LS is a chronic disease commonly seen in the senior population. It’s very difficult to find healthy foreskin samples from healthy people that matches the age of LS patients, so we have to use these control samples from younger people.

All samples’ beta-actin target saw amplification (Ct all lower than 30), indicating no problem in the extraction and amplification process. All samples’ HPV DNA target had no amplification in 40 cycles. The HPV positive control sample amplified normally. That means all 77 specimens, LS patients and normal controls alike, have no detectable infection of high-risk HPV virus.

Results of EBV detection were shown in Table 2. Fifteen of 36 late stage LS patients are EBV-positive, 11 early LS patients has 3 positive, and control group has 4. The EBV infection rate of LS patients is higher than control group (38.3% vs 13.3%, P < 0.05). Signed-rank test showed that the virus load of EBV-positive LS patients is higher than positive samples from health control group (P < 0.05). The infection rate of late stage patients is higher than early stage patients, but not statistically significant (41.7% vs 27.3%, P = 0.49). Signed-rank test showed that late stage patients have higher virus load than early stage patients (P < 0.05).

The mean age of EBV-positive patients is older than negative patients (44.5 vs 33.8, P < 0.01). But as the mean age of control group is significantly younger, this result has no clinical value. The mean sexual partner of EBV-positive patients is only slightly higher than negative (0.95 vs 0.80, P = 0.16). EBV itself is not a sexually transmitted virus, and some studies reported that LS patients usually have reduced sexuality. The result of our experiment is concordant with this result.

We reviewed the inflammatory reaction of each specimen, and the result is listed in Table 3. About 51% of LS patients (24 of 47) had severe inflammatory reaction, significantly higher than normal control (3 of 30, P < 0.01). These patients had typical inflammatory cell infiltration in dermis (Figure 1). 31 of 36 late stage LS patients had severe or moderate inflammatory reaction, but five patients had significant homogenous base beneath epidermis and only a small amount of inflammatory cells (Figure 2). Specimens with calculated EBV DNA copies over 1 × 10^7 all had severe or moderate inflammation. Five late stage LS patients had homogenous band and only slight inflammation. Two of them are EBV negative, the other three had EBV load of 5.2 × 10^7, 9.1 × 10^6 and 6.4 × 10^6, just slightly above the detection limit.

**DISCUSSION**

The pathogenesis of LS is still not clear yet. Various factors are believed to have relationship with LS, but none of them has solid proof. Chronic

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**Table 1: The primer sequences used in the PCR amplification**

| Target | Forward primer | Reverse primer |
|--------|----------------|----------------|
| EBV    | GTAGAAGGCAATTTCAC | TTTCTACGTACCTCAGCC |
| HPV    | TCTCTCATGACACATGTC | CACAGTTGTATGTATTGTTGT |

**Table 2: EBV presence and virus load in LS patients and controls**

| EBV positive (%) | EBV load range (copies) | Geometric mean EBV load of positive samples |
|------------------|-------------------------|------------------------------------------|
| Lat stage LS     | 15/36 (41.7)           | 5.2×10^5–4.1×10^6                  | 1.5×10^5                           |
| Early stage LS   | 3/11 (27.3)            | 5.7×10^4–7.4×10^4                  | 6.7×10^4                           |
| Healthy control  | 4/30 (13.3)            | 7.2×10^6–9.6×10^6                  | 2.7×10^6                           |

**Table 3: Inflammatory reaction of LS patients and controls**

| Inflammatory reaction | Total |
|-----------------------|-------|
| Severe                | 4     | 11   |
| Moderate              | 20    | 36   |
| Slight                | 3     | 30   |

Notes: LS: lichen sclerosus
infection, including virus infection, is once believed to be a risk factor of LS.\textsuperscript{11} Human papillomavirus, due to its high prevalence and oncogenic effect, is suspected to be a risk factor of LS, but many studies denied this hypothesis. These studies covered European, North America and middle-east populations, but Asia is left out. From a recent study carried out in China, the prevalence of high-risk HPV among rural Chinese males is 6.4%.\textsuperscript{12} But in our study, none of the 77 samples has detectable high-risk HPV DNA. This result is a little surprising and naturally brings us to consider the extraction and detection process. We performed 2 runs for these 77 samples. In both runs, we detected all samples’ IPC target below 30 cycles, and the positive control and negative control were all normal. Thus, we can assume that our extraction and amplification worked alright, and these 77 samples were indeed all HPV negative. We believe that the low prevalence of HPV infection among the control group may be related to, since the control group mainly consists of urban school-age adolescents who are well-educated and largely sexual inactive. On the other hand, all LS patients have no sign of genital HPV infection. This result is concordant with the majority of other such studies that the presence of HPV in genital area has no relationship with LS. Furthermore, the 11 early stage LS patients have no HPV infection too. That further indicated that HPV may not play any important role in the pathogenesis of LS.

EBV infection can be common among children or adolescents. It’s estimated that in China, over 90% of children under 5 years have detectable EBV antibodies. EBV is a known risk factor of nasopharyngeal carcinoma, and has possible relationship with autoimmune diseases. EBV infection is usually self-limited, but after infection, most patients remain in latent infection state, during which the virus remain in a small portion of B cells in circulation and lymphatic organs. In this state, the virus has no significant cytopathic effects, and the infected B cells don’t release infectious virus.\textsuperscript{13} But the persistent existence of virus DNA in lymphocytes may induce immortalization and trigger malignancy, and has possible relationship with many autoimmune diseases.\textsuperscript{14–16} One article has reported higher infection rate of EBV in vulvar LS patients.\textsuperscript{4} There is also a report about the presence of EBV in oral lichen planus, a similar atrophic disease that involves oral mucosa.\textsuperscript{17}

In our study, 13.3% of healthy foreskin samples have detectable EBV. Because male foreskin normally does not contain lymphoid tissue, we believe that the EBV in healthy foreskin samples mainly came from the B cells in the residual blood of the skin tissue. Small amount of bleeding is inevitable during circumcision, pinching and stretching during the surgery may also contribute to local congestion. The amount of residual blood in the excised tissue can be greatly variable. Considering the high prevalence of EBV infection in developing countries,\textsuperscript{18} the EBV test could possibly be positive when there is enough amount of residual blood in the excised tissue. We should notice that the virus load of control group never exceeds 1 × 10\textsuperscript{5}.

Our study shows that LS patients have higher EBV infection rate and higher virus load. Samples with EBV load higher than 1 × 10\textsuperscript{5} all had significant inflammatory reaction. Considering the highest virus load from control group does not exceed 1 × 10\textsuperscript{3}, and that virus load mainly comes from circulating lymphocytes, we can assume that the EBV in LS affected tissue mainly comes from inflammatory cells. The pathological change of early stage LS is characterized by hyperkeratosis and inflammatory cell infiltration of dermis, but previous studies about penile and vulvar LS indicate that these inflammatory cells are wholly or mainly T cells which could not host EBV.\textsuperscript{19–21} We believe that although the percentage of B cells is small, the large amount of inflammatory cells in the tissue could still contribute to high absolute number of B cells that could host EBV. There is also a study that reported high portion of B cells in two LS cases.\textsuperscript{22} Also, five late stage LS samples with reduced inflammatory reaction had low or no virus load. All these facts suggest that the inflammatory cells are the main source of EBV in LS affected tissue, rather than circulatory B cells.

The high prevalence of EBV in LS affected tissue and the high EBV load in late stage LS patients suggest that local EBV infection may be related to LS, but it’s unclear whether the EBV infection initially triggered the inflammation changes, or the presence of EBV caused unspecific inflammation to eventually transform into LS. Though many late stage LS patients have high EBV load, some patients with very long history of LS and panurethra stricture have diminished inflammation and low EBV load. That may suggest that in late stage of the disease, the advance of LS is independent from local EBV infection, but it still lacks evidence. After all, the exact role of EBV in the pathogenesis of LS is still unclear.

CONCLUSION

HPV has no relationship with the pathogenesis of male LS. The affected tissue of male LS patients contains higher load of EB virus than healthy tissue, indicating possible association of EBV infection with the pathogenesis of LS.

AUTHOR’S CONTRIBUTIONS

YMZ collected samples, carried out experiments, analyzed data and wrote the manuscript. QF organized this project, supervised the entire experiment and helped editing the manuscript. XZ helped organizing this project.

COMPETING FINANCIAL INTERESTS

The authors declared no competing financial interests.

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