

Prevalence of Epstein-Barr virus infection in palatine tonsils hyperplasia through the immunoreexpression of the LMP-1 marker

Avaliação da prevalência de infecção por Epstein-Barr vírus na hiperplasia de tonsilas palatinas através da imunoreexpressão do marcador LMP-1.

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ABSTRACT

Introduction: LMP-1 (latent membrane protein 1) is a membrane protein found in EBV and can be identified through immunohistochemistry.

Objectives: To evaluate the prevalence of EBV virus in tonsillar tissue samples correlating with tonsil size, age and gender.

Methods: An LMP-1 immunohistochemical study was performed in slides from patients with tonsil hyperplasia.

Results: The sample consisted of 120 slides, 66 were from male. The average tonsillar size was 6.0 cm² (1.5-14.0) and the average age was 6.5 years (2-18). Overall, 72 patients were positive for the virus, the majority being male (51%) and preschoolers (51.4%). For comparative analysis, the sample was divided: group 1 (positive immunohistochemistry) and group 2 (negative immunohistochemistry). The average age was 6.74 years (± 4.14) and 6.19 years (± 3.82) for group 1 and 2, respectively. Group 1 had 37 preschoolers, 23 school-age children and 12 adolescents. Group 2 had 28 preschoolers, 14 school-age children and 6 adolescents ($p=0.71$). To evaluate the presence of virus, the slides were divided according to area: G ($\geq 6\text{cm}^2$) and P ($< 6\text{cm}^2$). In G group, 54 slides were positive, while in P group, 18 were positive. Group 1 presented 54 of G group slides and 18 of P group slides, with no statistical difference, but with a tendency to positivity ($p=0.09$).

Conclusion: Most of the sample was positive for the presence of Epstein-Barr virus. There was no difference in the virus presence or absence when correlated with the tonsillar size, age or sex.

KEYWORDS: Herpesvirus 4. Epstein-Barr virus infections. Hyperplasia. Palatine. Tonsil. Tonsillectomy.

Central Message

Pharyngeal and palatine tonsils are fundamental structures at the immune response by being present at the ingested or aspirated antigens path. These structures may undergo morphological modifications, such as total volume increase, due to lymphoid follicles or histopathological changes resulting from recurrent infections. These modifications are examples of indications for tonsillectomy. It is possible that EBV directly affects the survival of tonsillar cell, interfering with the apoptosis mechanism.

Perspective

Most of the sample was positive for EBV in the tonsillar tissue. There was also no significant difference in the positivity or not for EBV when correlated with tonsillar size, age and sex of the patients.

RESUMO

Introdução: LMP-1 (proteína latente de membrana-1) é uma proteína de membrana encontrada no Epstein-Barr vírus (EBV) e que pode ser identificada através da imunistoquímica.

Objetivo: Avaliar a prevalência do EBV em amostras de tecido tonsilar correlacionando com o tamanho da tonsila, idade e sexo. **Método:** Realizou-se um estudo imunistoquímico de LMP-1 para EBV em

lâminas de pacientes com hiperplasia de tonsilas.

Resultados: A amostra foi composta por 120 lâminas, sendo 66 (55%) de pacientes do sexo masculino. O tamanho médio tonsilar foi 6,0 cm² (1,5-14,0) e a idade média 6,5 anos (2-18). Ao todo, 72 pacientes (60%) tiveram positividade para o vírus, a maioria meninos (51%) e pré-escolares (51,4%). A amostra foi dividida em: grupo 1 (imunistoquímica positiva) e grupo 2 (imunistoquímica negativa). A idade média foi de 6,74 anos ($\pm 4,14$) e 6,19 anos ($\pm 3,82$), para o grupo 1 e 2, respectivamente. O grupo 1 apresentou 37 pré-escolares, 23 escolares e 12 adolescentes. O grupo 2 foi composto por 28 pré-escolares, 14 escolares e 6 adolescentes, sem diferença estatística ($p=0,71$). Para avaliar a presença do EBV, as lâminas foram divididas de acordo com a área: G ($\geq 6\text{cm}^2$) e P ($< 6\text{cm}^2$). No grupo G, 54 lâminas foram positivas, enquanto no grupo P, 18, sem diferença estatística, mas com tendência a positividade ($p=0,09$).

Conclusão: A maior parte da amostra foi positiva para o Epstein-Barr vírus. Não houve diferença significativa na positividade ou não do vírus quando correlacionada com o tamanho tonsilar, a idade e o sexo dos pacientes.

PALAVRAS-CHAVE: Herpes humano 4. Infecções por vírus Epstein-Barr. Hiperplasia. Tonsila palatina. Tonsilectomia.

INTRODUCTION

Pharyngeal and palatine tonsils are fundamental structures at the immune response by being present at the ingested or aspirated antigens path. The palatine tonsils are located at the lateral portions of Waldeyer's ring and the pharyngeal tonsil (adenoid) is located at the posterosuperior nasopharynx. These structures may undergo morphological modifications, such as total volume increase, due to lymphoid follicles or histopathological changes resulting from recurrent infections. These modifications are examples of indications for tonsillectomy.¹

Recurrent tonsillitis are very common in children and may lead to chronic activation of the immune system, culminating in pharyngeal and palatine tonsils lymphoid tissues hypertrophy.² It may be related to complications like superior airway obstruction, otologic dysfunctions, snore and sleep apnea. The etiology of the hypertrophy is unknown, being often associated to bacterial infection.³ Some studies show a close relation between viral infections and tonsillitis, being the Epstein-Barr virus (EBV) one of the most involved in acute and recurrent cases.⁴ With the emergence of new technologies for molecular research, viruses have been increasingly detected in tonsil's chronic infection and, among them, EBV.⁵

EBV is a DNA virus which belongs to Herpesviridae family, also known as Human herpesvirus type 4, and which main transmission occurs through saliva. First, oropharynx and nasopharynx epithelial cells are infected and then the B cells.⁶ Primary infection in children is often asymptomatic and tends to remain latent in memory B cells.⁷

During EBV's latent infections, the expression of viral genes that encode nuclear antigens (EBN A-1, 2, 3A, 3B, 3C and LP), latent membrane proteins (LMP-1, LMP-2A and LMP 2B), RNA molecules (EBER-1 and 2), and others can occur.⁸ From that state, EBV may be associated with malignant conditions, such as Hodgkin's or Non-Hodgkin's lymphoma, gastric and oropharyngeal carcinomas.^{9,10}

LMP-1 is a latent membrane protein found in EBV that is closely related to epithelium transformation and tumoral development, and can be identified through immunohistochemistry (IHC).¹¹ It reduces the cell response to differentiation signals, increases the ability to invade collagen matrix and turns human fibroblasts into keratinocytes. LMP-1 is also capable to induce apoptosis, activating anti-apoptotic proteins.¹²

Therefore, this study aims to evaluate the prevalence of EBV infection in pediatric patients by LMP's immunoexpression and to verify associations between the profile of patients affected by hyperplasia and EBV infection.

METHODS

This study was approved by the Institutional Ethics Committee under number 2.722.571 in 06/17/2018. To characterize the pediatric population, the following age range has been adopted: infant (28 days to 2 years),

preschool child (2 years to 6 years), school-age child (6 years to 12 years) and adolescent (12 years to 19 years).

An evaluation of slides from surgical pieces taken from patients with tonsillar hyperplasia, submitted to tonsillectomy from August to December 2018, was carried out. Patients with age upper than 18 years or whom anatomopathological examination did not reveal tonsillar hyperplasia were excluded. Palatine tonsils obtained from tonsillectomy were sectioned on their major axis, and, from there, the area was measured.

At first, H&E stained slides were reviewed in order to confirm the histological type of lesion. Tissue microarray (TMA) blocks were produced by extracting tissue cores from different paraffin donor blocks. From the paraffin blocks, tissue microarray blocks were produced in order to generate tissue microarray slides for immunohistochemical analysis through LMP-1 protein. This process was divided into two different phases: first, a IHC staining technique was applied on the material with anti-EBV monoclonal antibodies, through DAKO autostainer (DAKO Corp. Carpinteria, CA, USA), after that, the stained areas were examined. The EBV positivity criteria was nuclear and/or cytoplasmatic LMP-1 immunopositivity in keratinocytes through all epithelial layers.

Statistical analysis

All results and information obtained were tabulated according to data protocol and then expressed by graphics and tables. Statistical analysis was performed by parametric and non-parametric methods, with significance of $p < 0.05$.

RESULTS

The sample consisted of 120 slides, of which 66 (55%) were from male patients and 54 (45%) from female. The average tonsils size was 6.0 cm² (1.5-14.0). The mean age was 6.51 (2-18) years. In all, 72 (60%) patients were positive for EBV at IHC. For comparative analysis, the sample was divided in two groups, by group 1 consisting of samples with positive IHC and group 2 by samples with negative IHC (Table 1).

TABLE 1 - Correlation between variables (age, sex, age range and tonsillar size) with or without the presence of EBV by IHC

Variable	Group 1 Positive IHC	Group 2 Negative IHC	P
Age			
Avarege	6.7	6.2	0.23
Median	5	5	
Sex			
Female	35 (64.8%)	19 (35.2%)	0.33
Male	37 (56.1%)	29 (43.9%)	
Age range			
Preschool child	37 (51.4%)	28 (58.3%)	0.71
School-age child	23 (31.9%)	14 (29.2%)	
Adolescents	12 (16.7%)	6 (12.5%)	
Tonsillar size			
G (area ≥6cm ²)	54 (65.1%)	29 (34.9%)	0.09
P (area <6cm ²)	18 (48.6%)	19 (51.4%)	

Regarding gender, group 1 had slightly more boys, 37 of the 72 (51%) patients. Group 2 presented 60% of male patients (29 of 48 patients). 37 (56.1%) of the analyzed slides from the 66 boys were positive for EBV at the IHC and 29 (43.9%) were negative. As for the slides of the 54 girls, 35 (64.8%) were positive for EBV at the IHC and 19 (35.2%) were negative.

The mean age was 6.74 (± 4.14) years and 6.19 (± 3.82) years, to group 1 and 2, respectively. Group 1 had 37 (51.4%) preschoolers, 23 (31.9%) school-age children and 12 (16.7%) adolescents. Group 2 was composed by 28 (58.3%) preschoolers, 14 (29.2%) school-age children and 6 (12.5%) adolescents, with no statistically significant difference between the two groups ($p = 0.71$). No patient was younger than 2 years old. Regarding the slides of the 65 preschoolers analyzed, 57% were positive for EBV at the IHC and 43% were negative. Of the school-age children, 62.2% were positive and 37.8% were negative. Considering adolescents, 66.7% presented positivity and 33.3% were negative for EBV.

Regarding the tonsillar size, group 1 had a mean of 6.21 cm² (± 1.67), while group 2 presented a mean of 5.81 cm² (± 1.49), with no statistically significant difference, but showing tendency toward positivity ($p = 0.09$). To evaluate the presence of EBV, the slides were also divided into two groups, according to the area: G (greater than or equal to 6 cm²) and P (less than 6 cm²). In G group, 54 (65.1%) slides were positive at the IHC and 29 (34.9%) were negative. In P group, 18 (48.6%) slides were positive at the IHC and 19 (51.4%) were negative. Group 1 presented 54 (75%) of slides in the G group and 18 (25%) of slides in the P group. Whereas group 2 had 29 (60.4%) 60% of the slides in the G group and 19 (39.6%) 40% in the P group, with no statistically significant difference ($p = 0.09$).

DISCUSSION

Most of the time, EBV infection has a benign course at childhood, however, when the first infection affects adolescents, it leads to infectious mononucleosis (IM) 30 to 70% of the time, when up to 20% of B lymphocytes are infected by the virus.⁶ Despite most cases are asymptomatic¹³, the diagnosis is made by the classic triad (fever, lymphadenopathy and pharyngitis), added to laboratory tests, which include atypical lymphocytosis and heterophile antibodies. IM is usually self-limiting, but may be associated with some early or late complications, including manifestations of the respiratory, cardiovascular, genitourinary, gastrointestinal and nervous systems. The mechanisms can be by direct viral invasion, chronic immune response due to reactivation of latent virus or even to several EBV DNA mutations.⁶ In tonsils of patients with IM, lymphoid cells infected with EBV are normally found in extrafollicular areas.¹⁴

The in situ hybridization (ISH) and the polymerase chain reaction (PCR) are methods used to identify the EBV virus. PCR grants the detection of minimal amounts of DNA, in addition to allow the use of paraffin-embedded

biopsies, permitting retrospective studies.⁹ Beyond being used in frozen and paraffin-embedded tissues, ISH grants the location of positive EBV cells in the tissue structure.⁴ IHC permits the identification of viral proteins found at the latent infection in replication.⁹

The EBV prevalence in tonsils varies depending on the used method to detect the virus presence. Two studies with ISH identified 29% and 60.5% of EBV association with tonsillitis, one of them compatible with the one found in our sample (60%).^{4,13} Alternatively, Dias et al.⁹, when using the PCR method, found a prevalence of 54.1% in tonsillar tissue.

Another study has shown the presence of EBV in children with recurrent tonsillitis (53.8% of cases), tonsils hypertrophy (32% of cases) and adenoids hypertrophy (41.2%), through PCR. It also found the presence of EBV in both palatine and pharyngeal tonsillar tissue. This research indicates that this virus tends to be present in different locations in the lymphoid epithelial tissues of the upper respiratory tract. The persistence of the virus can be a pathogenic potential for developing lymphoid hypertrophy and recurrently stimulate inflammation.²

Endo et al.⁴ researched the existence of EBV in the tonsillar tissue of 42 patients with recurrent tonsillitis and 43 patients without infections, but with tonsillar hyperplasia, and detected 29.4% of positive cases through ISH. There was no statistical difference between the groups.

A further study included tonsillar pharyngeal tissue of 71 children (21 with 12-24 months old and 50 with 25 months to 13 years) and, using ISH, found the presence of EBV in 60.5% of adenoids, similar to our results with IHC. In 21 samples of adenoid tissue from younger children (1-2 years), 7 (33%) were positive for EBV and almost all of them were positive for CD20, confirming the preference of EBV for the B lymphocyte. They concluded that children older than 25 months are more likely (twice) to be infected by EBV when compared to younger ones.¹³ Our study did not embrace children under 24 months.

It is known that EBV is associated with malignant neoplasms, especially in immunosuppressed patients. In immunocompetent individuals, there is the hypothesis that tonsils are a site of viral replication.⁹ In a study by Babcock (1998), episomal and linear forms of EBV DNA were detected in tonsillar lymphocytes, concluding that viral DNA replicates in healthy and persistently infected individuals.¹⁵

It is possible that EBV directly affects the survival of tonsillar cell, interfering with the apoptosis mechanism. This mechanism is accepted as one of the main in the pathogenesis of cellular hypertrophy. It is also possible that EBV activates lymphoid proliferation and thus cause hyperplasia.¹²

Regarding the patient's profile found in our study, there was a prevalence of preschooler (51.4%), 55% of them male. However, another study found a higher frequency in females (62.5%), between 6 and 12 years (58.8%).⁹

Another research looked over the presence of EBV and human papillomavirus in 104 Chinese children with hypertrophy of the palatine and pharyngeal tonsils and correlated with the clinic and possible complications. The

prevalence of EBV infection was 45.5% in palatine and 69.2% in pharyngeal tonsils. The EBV DNA was found at 64.2% of preschoolers and 73.6% of school-age children, a value higher than the one found by us (57% and 62.2%, respectively).⁵ As in the present study, the positivity to EBV was not related to age, gender or disease duration.^{5,16} Yet, another study found a statistically significant difference between EBV positivity in children among 3 and 6 years and 11 months (23+/61) and patients above 7 years (2+/24/ p=0.02).⁴

At the research carried out by Dias et al, children with average age of 7.4 years were selected, slightly higher than ours (6.5 years), and diagnosis of chronic and hypertrophic tonsillitis. Of the 24 tonsils, 16 had a positive beta-globin gene amplification, proving the constitutional DNA presence in the material. Of these 16, 10 had EBV genome amplification. Around 54% of the tonsils were infected by the EBV. They also identified the LMP-1 in 37.5% of the studied tonsils, a lower value than the one found in our research, demonstrating that IHC may be an initial investigation method for latent EBV infection.⁹

CONCLUSION

Most of the sample was positive for EBV in the tonsillar tissue. There was also no significant difference in the positivity or not for EBV when correlated with tonsillar size, age and sex of the patients.

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Conflict of interest: none

Financial source: None

This study was approved by the Institutional Ethics Committee under number 2.722.571 in 06/17/2018

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