Human papillomavirus and Epstein-Barr virus infection in benign thyroid lesions

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Abstract
Introduction: The objective was to investigate the correlation between Epstein-Barr virus (EBV) and human papillomavirus (HPV) infection in the development of benign thyroid lesions.
Material and methods: 29 cases of Hashimoto’s thyroiditis (HT), 133 cases of thyroid adenoma, and 34 cases of HT with thyroid adenoma paraffin embedded tissue samples were used for EBV and HPV quantitative detection.
Results: None of the tissue samples carried HPV DNA. In HT tissue samples, the positive rate of EBV was 55.2% (16/29). In thyroid adenoma tissue samples, the positive rate was 37.6% (50/133). In HT combined with thyroid adenoma tissue samples, the positive rate of EBV was 67.6% (23/34). There was no correlation between EBV infection and clinical features such as age and gender.
Conclusion: The occurrence and development of benign thyroid lesions are closely related to EBV infection. HT combined with thyroid adenoma may be more susceptible to EBV infection than simple HT and thyroid adenoma, which provides a new idea for the diagnosis and treatment of benign thyroid lesions.

Keywords: Hashimoto’s thyroiditis; thyroid adenoma; EB virus; human papilloma virus

Introduction
Benign thyroid lesions are common endocrine diseases, which have a significant negative impact on the quality of life of patients [1]. Hashimoto’s thyroiditis (HT) and thyroid adenoma are common benign thyroid lesions. HT is a chronic autoimmune thyroid disease with autoimmune thyroid tissue as an antigen. It is characterised by increased thyroid volume, substantial lymphocyte infiltration, and the presence of specific antibodies against thyroid antigens [2]. HT is considered to be the main cause of hypothyroidism [3]. Studies have found that the increase in the incidence of HT is related to environmental factors, including changes in health conditions, increased iodine intake, new treatment methods and chemical agents [4]. Thyroid adenoma is the most common benign tumour in the thyroid gland. It originates from thyroid follicles and is a nodular structure confined to the thyroid gland. It does not cause penetration or penetration of its capsule [5]. Study has shown that thyroid adenoma may be used as a precancerous lesion [6]. At present, there is no reliable immunohistochemical or molecular detection method that can clearly distinguish follicular adenoma and follicular thyroid carcinoma [7]. The specific pathogenesis of the above 2 benign thyroid lesions is still unclear.

Studies have shown that viral infection is a possible pathogenic factor of autoimmune thyroid diseases (AITD). Viruses can activate innate and acquired immunity and trigger the occurrence of HT, such as Epstein-Barr virus (EBV) [8]. In addition, in a study on human papillomavirus (HPV) vaccination and autoimmune diseases, it was found that the risk of HT was significantly increased in women vaccinated with HPV vaccine [9]. Our research group has conducted a number of studies since 1998, confirming that HPV and EBV play an important role in the pathogenesis of head and neck diseases. Teng [10] found that the occurrence and development of parotid gland tumours are related to HPV, and preliminarily divided the HPV infection subtypes of Chinese people into high-risk subtypes. Xue [11] found that adenoidal hypertrophy in children is closely related to EBV infection by real-time fluorescence-based quantitative polymerase chain reaction (PCR).

Despite these findings, studies on the expression of HPV and EBV genes in HT and thyroid adenoma...
patients have been rare. In this study, real-time fluorescence quantitative polymerase chain reaction (PCR) and flow-through hybridisation were used to detect the expression of HPV and EBV DNA in patients with HT and thyroid adenoma, and the correlation between clinical characteristics such as age and gender and viral expression was analysed. The results of this study provide novel insight into the role of HPV and EBV in the pathogenesis of benign thyroid lesions.

Material and methods

Case source
Twenty-nine cases of simple HT tissue samples, 133 cases of simple thyroid adenoma tissue samples, and 34 cases of HT combined with thyroid adenoma tissue samples were all obtained from the archived wax blocks of the Department of Pathology, Gongli Hospital, Pudong New Area, Shanghai, from 2013 to 2022. This study was approved by the Ethics Committee of Shanghai Pudong New Area. In HT, there were 28 females and one male, aged 29–79 years, with an average age of 53.62 ± 12.592 years. In thyroid adenoma, there were 95 females and 38 males, aged 19–83 years, with an average age of 52.53 ± 13.124 years. There were 33 females and one male in HT with thyroid adenoma, aged 17–72 years, with an average age of 51.44 ± 13.274 years.

DNA extraction

0.01–0.05-g paraffin specimens were weighed and put into a 1.5-mL centrifugal tube. 1 mL of xylene was added, and the wax was removed after incubating at room temperature for 10 minutes and the xylene poured out. 1 mL 100% anhydrous ethanol was added, incubated for 10 minutes, then poured out anhydrous ethanol. 1 mL 95% ethanol was added, incubated for 10 minutes, and poured out. Ethanol was allowed to volatilise completely at room temperature for 15 minutes. 400 μL solution I and 1 μL 50 mg/mL proteinase K, mixed. The samples were incubated at 56°C for 3 hours and boiled in water for 15 minutes. 400 μL solution II was added after cooling, fully mixed, placed at room temperature for 2 minutes, centrifuged at 14,000 rpm for 5 minutes, and the supernatant removed. 60 μL solution III was added and fully dissolved. 1 μL was used as a PCR template for amplification or stored at −20°C. Solutions I, II, and III were all from the reagents in Cape’s DNA extraction kit (cell lysate).

HPV genotyping

In this study, 37 HPV genotyping kits (PCR + flow-through hybridisation) were used to detect and genotype HPV. The kit was purchased from Chaozhou Hybrilbio Biochemical Co., Ltd. HPV genotyping quantitative detection included HPV6, 11, 42, 43, 44, 44, 16, 18, 31, 33, 35, 39, 45, 51, 52, 53, 56, 58, 59, 66, 68, 81, 26, 34, 40, 54, 55, 57, 61, 67, 69, 70, 71, 72, 73, 82, 83, and 84 types. According to GenBank HPV DNA amplification was designed: MY09 primer sequence, 5'-CCAGGTAAGTGAGACTTTTA-3'; and downstream primer, 5'-CCAGGTAAGTGAGACTTTTA-3'. The expression of HPV and EBV DNA was not detected in 29 cases of HT, 133 cases of thyroid adenoma, and 34 cases of HT with thyroid adenoma. EBV DNA was positive in 55.2% (16/29) of HT tissues, 37.6% (50/133) of thyroid adenoma tissues, and 67.6% (23/34) of HT with thyroid adenoma tissues. We further found that there was a significant difference in EBV infection rate between HT with thyroid adenoma and simple thyroid adenoma (p < 0.05), while there was no difference with simple HT (p > 0.05, Tab. 1).

EBV DNA detection

It was detected by EB virus nucleic acid detection kit (PCR-fluorescence probe method), which was purchased from Shengxiang Biotechnology Co., Ltd. According to GenBank EBV DNA segment, the following primer sequences for EBV DNA amplification were designed: upstream primer, 5'-CCAGGTAAGTGAGACTTTTA-3'; and downstream primer, 5'-CCCTCTAGGACCTCTGCC-3'. EBV DNA was rapidly lysed and released by nucleic acid release agent. A pair of specific primers and a specific fluorescent probe were designed for the conserved region of EBV nucleic acid. With PCR reaction solution and other components, real-time fluorescence quantitative PCR detection technology was used to achieve rapid detection of viral DNA. For the samples with cycle threshold (Ct) value ≤ 39, EBV DNA positive was reported, for Ct > 39 samples, the internal standard test was positive, and EB virus DNA was reported to be lower than the lowest limit of the detection kit, and when Ct value > 40, the test results were invalid.

Statistical analysis

The chi-square test was used to compare the relationship between the patient group and the positive rate of the virus and clinical characteristics. The data were analysed using SPSS version 26.0, and p < 0.05 was considered statistically significant.

Results

The expression of HPV and EBV

HPV DNA was not detected in 29 cases of HT, 133 cases of thyroid adenoma, and 34 cases of HT with thyroid adenoma. EBV DNA was positive in 55.2% (16/29) of HT tissues, 37.6% (50/133) of thyroid adenoma tissues, and 67.6% (23/34) of HT with thyroid adenoma tissues. Although a greater number of EBV-positive patients were positive — the positive rate was 46.2%, there was no difference with simple HT (p > 0.05, Tab. 1).

The relationship between EBV infection and clinical characteristics of samples

There was no significant correlation between the prevalence of EBV and the patient’s gender and age (p > 0.05, Tab. 2). There were 15 male patients who were EBV positive — the positive rate was 37.5%, and 72 female patients were positive — the positive rate was 46.2%. Although a greater number of EBV-positive patients were female, this difference was not significant. Forty-two cases of EBV-positive patients were seen in patients with average age of 53 years or less, with a positive rate of 46.2%. There were 44 EBV-positive cases in patients older than 53 years, with a positive rate of 43.6%. The difference was not statistically significant.

Discussion

EBV was found under an electron microscope by culturing cells in Burkitt’s lymphoma tissue. As a member of
the herpesvirus family, EBV is a B-lymphotropic virus. Before entering B cells, the envelope glycoprotein gp350 binds to the viral receptor CD21 molecule on the surface of B cells. Although the main target cells of EBV are B lymphocytes, a wider range of cell types of infection occurs in immunocompromised hosts, especially epithelial cell lines. EBV infects epithelial cells in vitro, produces viruses, and lyses cells. EBV infection usually occurs through contact with oral secretions, and B cells in the oropharynx may be the main site of infection in humans [12]. In a study on the expression of EBV inAITD, the positive rate of EBV was 11.8%, and EBER was highly expressed in lymphoid tissues (>30%). EBV infection may become a link between AITD disease and thyroid tumours [13]. We should note that immunocompromised hosts may also be infected even without clinical features. EBV infection factors must be considered when finding unknown causes of immunocompromised patients with fever [14].

HT is also known as chronic lymphocytic or autoimmune thyroiditis. The incidence of women is higher than that of men, and the incidence of white people is higher than that of black people, and the prevalence increases with age. Although the specific pathogenesis is unknown, it is related to genetic effects, environmental triggers, and epigenetic effects [2]. As one of the risk factors, the effect of the virus on AITD has been studied, but the results are controversial [15]. Thyroid adenoma is a monoclonal benign capsular tumour, which is composed of the offspring of a mutant cell. Its growth, iodine metabolism, and thyroid hormone secretion are independent of the control of normal thyroid stimulating hormone (TSH) [16].

In this study, there was no HPV infection in HT, thyroid adenoma, and HT combined with thyroid adenoma samples. We found that the positive rates of EBV were, respectively, 55.2%, 37.6%, and 67.6% in simple HT, simple thyroid adenoma, and HT combined with thyroid adenoma. Compared with simple HT and thyroid adenoma, the positive rate of EBV in HT combined with thyroid adenoma was higher, and the difference in positive rate between HT combined with thyroid adenoma and simple thyroid adenoma was statistically significant. There was no correlation between the rate of EBV positivity and clinical characteristics such as age and gender in benign thyroid lesions. The difference in the EBV-positive rate in different studies may be caused by the difference in the number of detection samples and detection methods. As a B-lymphotropic virus, EBV appears in chronic lymphocytic thyroiditis in line with the characteristics of the virus, and it may also lead to the production of thyroid adenoma as a mutation factor.

**Conclusion**

This study reveals that EBV exists in benign thyroid lesions, but with no HPV, and HT combined with thyroid adenoma tissues are more susceptible to EBV infection than HT and adenoma tissues alone. The specific pathogenesis after infection remains to be further explored.

### Table 1. The correlation between Epstein-Barr virus (EBV) positive rate and disease type in benign thyroid lesions

<table>
<thead>
<tr>
<th>Benign thyroid lesions</th>
<th>Cases</th>
<th>Expression of EBV</th>
<th>p-value</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>Positive</td>
<td>Negative</td>
</tr>
<tr>
<td>HT</td>
<td>29</td>
<td>16 (55.2%)</td>
<td>13 (44.8%)</td>
</tr>
<tr>
<td>HT with thyroid adenoma</td>
<td>34</td>
<td>23 (67.6%)</td>
<td>11 (32.4%)</td>
</tr>
<tr>
<td>Thyroid adenoma</td>
<td>133</td>
<td>50 (37.6%)</td>
<td>83 (62.4%)</td>
</tr>
<tr>
<td>HT with thyroid adenoma</td>
<td>34</td>
<td>23 (67.6%)</td>
<td>11 (32.4%)</td>
</tr>
</tbody>
</table>

HT — Hashimoto’s thyroiditis

### Table 2. The relationship between Epstein-Barr virus (EBV) positive rate and clinical features in benign thyroid lesions

<table>
<thead>
<tr>
<th>Characteristic</th>
<th>Cases</th>
<th>Expression of EBV</th>
<th>p-value</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>Positive</td>
<td>Negative</td>
</tr>
<tr>
<td>Sex</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Male</td>
<td>40</td>
<td>15 (37.5%)</td>
<td>25 (62.5%)</td>
</tr>
<tr>
<td>Female</td>
<td>156</td>
<td>72 (46.2%)</td>
<td>84 (53.8%)</td>
</tr>
<tr>
<td>Age (years)</td>
<td></td>
<td></td>
<td></td>
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<tr>
<td>≤ 53</td>
<td>95</td>
<td>42 (44.2%)</td>
<td>53 (55.8%)</td>
</tr>
<tr>
<td>&gt; 53</td>
<td>101</td>
<td>44 (43.6%)</td>
<td>57 (56.4%)</td>
</tr>
</tbody>
</table>
Data availability statement
Original data generated and analysed during this study are included in this published article or in the data repositories listed in References.

Ethics statement
This study was performed with the approval of the Ethics Committee of Gongli Hospital, Pudong New Area, Shanghai (Approval No. 2020 Research Review No. 09) and was conducted in accordance with the Declaration of Helsinki.

Author contributions
Y.L. and R.Z.: conceptualisation and writing — original draft preparation and data duration; Y.Z.: writing — review and editing; X.C. and S.H.: project administration and funding acquisition. All authors have read and agreed to the published version of the manuscript.

Conflict of interests
The authors have declared that no competing interest exists.

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