The Role of Epstein-Barr Virus DNA Load and Serology as Screening Tools for Nasopharyngeal Carcinoma

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Sponsorships or competing interests that may be relevant to content are disclosed at the end of this article.

Abstract

Objective. Screening for nasopharyngeal carcinoma (NPC) among family members has been advocated in endemic populations in view of significantly increased risks. We aimed to compare the role of Epstein-Barr virus (EBV) DNA load and EBV IgA serology as tools for screening patients with a first-degree family history of NPC.

Study Design. Case-control study.

Setting. Tertiary referral center.

Subjects and Methods. Serum samples were compared from 293 newly diagnosed NPC patients and 475 individuals with a first-degree family history of NPC. EBV DNA load was measured by real-time quantitative polymerase chain reaction, while EBV viral capsid antigen (VCA) IgA and EBV early antigen (EA) IgA titers were measured by immunofluorescence assays.

Results. NPC patients had a significantly higher median EBV DNA load as compared with unaffected family members (835.4 vs 18.8 copies/mL; \(P \leq .001\)). At 100 copies/mL, EBV DNA load demonstrated a sensitivity of 76.8% and a specificity of 85.6%. A positive EBV-EA IgA titer (\(\geq 1:10\)) gave a sensitivity of 85.0% and a specificity of 96.4%. There was good correlation between EBV DNA load and EBV serology titers (Spearman’s \(\rho = .536\) and \(.594\) for EBV-VCA IgA and EBV-EA IgA, respectively; \(P < .001\)). Receiver operating characteristic analysis demonstrated that EBV-VCA IgA and EBV-EA IgA were better classifiers than EBV DNA load (areas under the curve: 0.942, 0.926, and 0.880, respectively) in distinguishing NPC patients and unaffected family members.

Conclusion. EBV DNA load and EBV IgA serology demonstrate good sensitivity and specificity as screening tools. EBV-EA IgA gave the best sensitivity and specificity profile as a screening tool for NPC among high-risk family members.

Keywords

nasopharyngeal carcinoma, Epstein-Barr virus, DNA load, serology, family screen

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Among high-risk family members, however, there have been limited studies evaluating the effectiveness of EBV DNA load and EBV serology as screening tools. Previous studies involving small numbers of NPC patients and high-risk family members showed EBV DNA detection rates of 53% to 74% for NPC patients and 0% to 15% for high-risk family members. However, EBV DNA load and serology have not been compared together as screening techniques for high-risk family members. As screening for NPC among family members has been recommended in endemic areas, evaluating the specificity and sensitivity of EBV tests in such a setting will be useful to establish their role in a screening program.

In this study, we aimed to compare the role of EBV DNA load and EBV IgA serology as tools for screening patients with a first-degree family history of NPC.

Materials and Methods

Study Population

Between 2006 and 2012, serum samples were collected from NPC patients as well as healthy family members with a first-degree family history of NPC. Informed consent was taken from all participants, and the study was approved by the Institutional Review Board (National Healthcare Group Domain Specific Review Board 2006/00409).

All NPC patients were biopsy proven, and blood samples were taken before treatment was initiated. All family members were evaluated closely at 6-month intervals, with a thorough head and neck examination, including nasopharyngeal endoscopy. Nasopharynx biopsies were taken if a mass or mucosal irregularity was observed, if there was any positivity of EBV-EA IgA, or if EBV-VCA IgA was raised at 1:160 and beyond, following national cancer screening guidelines.

As part of the standard workup for our patients, EBV-VCA IgA and EBV-EA IgA titers were measured with immunofluorescence assays. In addition, EBV DNA load was measured with real-time quantitative polymerase chain reaction analysis. All the above laboratory tests were performed at the World Health Organisation Immunology and Training Research Centre, Singapore.

Immunofluorescence Assay

EBV-VCA IgA and EBV-EA IgA titers were measured with the immunofluorescence assay previously described by Fones-Tan et al. Slides coated with P3HR1 cells were used for EBV-VCA tests, and slides coated with Raji cells were used for EBV-EA tests. Aliquots (20 μL) of serially diluted sera (1:10, 1:40, 1:160, and 1:640) were added to the individual wells of the EBV-VCA and EBV-EA slides. The slides were incubated at room temperature in a moist chamber for 30 minutes, then rinsed in phosphate-buffered saline (PBS), and washed in PBS by immersing for 2 × 5 minutes with gentle agitation. The slides were drained, and fluorescein-conjugated anti-human IgA rabbit immunoglobulin (SPD Scientific, Singapore) diluted 1:10 in PBS was added to individual wells of the slides. The slides were incubated at room temperature in a moist chamber for a further 30 minutes, washed in PBS, and drained. Finally, the slides were read with a fluorescent microscope at 16× objective (Zeiss) or 20× objective (Olympus).

DNA Extraction and Measurement

DNA was extracted from 250 μL of serum via the QIAamp Blood Kit (Qiagen, Hilden, Germany), per the blood and body fluid protocol as recommended by the manufacturer, and eluted with 30 μL of AE buffer. Real-time quantitative polymerase chain reaction (PCR) targeting the BamHI-W region was performed. Amplification primers W-44F (5′-CCCAACACTCCACCACCC-3′) and W-119R (5′-TCT TAGGAGCTGTCCGAGGG-3′; Sigma-Aldrich, St Louis, Missouri) and the dual-labeled Taqman fluorescent probe 450025 (5′-(6FAM)CACACACTACACACACCCCGTCT(TAMRA)-3′; Life Technologies, California) were used. Real-time PCR reactions were set up in a reaction volume of 25 μL with components supplied in a TaqMan PCR Reagent Kit (Life Technologies). Each reaction comprised 12.5 μL of 2× TaqMan Uni Mix, 300 nM of each amplification primer, 200 nM of fluorescent probe, and 10 μL of DNA sample. Thermal cycling was performed with an initial denaturation step of 20 minutes at 95°C, followed by 50 cycles of 1 minute at 95°C and 20 minutes at 60°C. A standard curve was run in parallel with each reaction, using DNA extracted from the EBV-positive cell line Namalwa.

Statistical Analysis

Data were entered into a database, reviewed for errors, and statistically analyzed with SPSS 16.0. Mann-Whitney U test was used to compare the medians of continuous variables. To compare the median EBV viral load for each stage of disease, Kruskal-Wallis test was used. Probability values ≤ 0.05 were regarded as statistically significant. All probability values were based on 2-sided tests.

The Kruskal-Wallis test was used to compare the median EBV viral load at different titer levels for both EBV-VCA IgA and EBV-EA IgA. Bivariate correlation analysis with Spearman’s correlation was also performed to determine the relationship between EBV DNA load and geometric mean titers for both serology tests. To allow for a comparison between the screening tests, the sensitivity and specificity of each test was calculated at different cutoff values. Receiver operating characteristic (ROC) curves were also performed for EBV DNA load, EBV-VCA IgA, and EBV-EA IgA to give a reflection of the overall sensitivity and specificity of each test. Area under the ROC curve was used to compare the 3 test methods.

Results

Study Population

A total of 293 NPC patients and 475 family members with a first-degree family history of NPC were recruited. Family
members were reviewed at 6-month intervals with a thorough head and neck examination, including nasopharyngeal endoscopy. Nasopharynx biopsies were taken if a mass or mucosal irregularity was observed, if there was any positive EBV-EA IgA titer, or if EBV-VCA IgA was raised at 1:160 and beyond. During the course of screening, 4 family members were identified to have NPC and were excluded from the analysis.

The median duration of follow-up for the remaining 471 unaffected family members was 60.0 months (range: 0-120 months, mean: 52.5 months). Of 471 unaffected family members, 95 (20.1%) had biopsies of the nasopharynx performed, all of whom were negative for malignancy. Of 471, 56 (11.9%) unaffected family members had a clinic visit, none of whom demonstrated any symptoms or signs of NPC. The demographic characteristics of NPC patients and family members are included in Table 1. As the demographic characteristics of both groups were different, a matched analysis was also performed to validate the study’s findings, as detailed later.

**EBV Serology**

EBV antibody titers were higher in NPC patients than unaffected family members. All 293 patients (100%) with NPC had detectable EBV-VCA IgA titers, compared with 323 of 471 unaffected family members (68.6%). The proportion of detectable EBV-EA IgA titers was lower, with 249 of 293 (85.0%) of NPC patients having detectable titers but only 17 of 471 (3.6%) of unaffected family members. Overall, the distribution of antibody titers for both EBV-VCA IgA and EBV-EA IgA was significantly different between both groups ($P < .001$ for both, Pearson $\chi^2$), as shown in Figure 1.

Regarding NPC patients alone, the proportions of EBV-VCA IgA titers was similar at each stage of disease ($P = .185$, Pearson $\chi^2$), while EBV-EA IgA titers increased with more advanced disease ($P = .004$, Pearson $\chi^2$), as shown in Figure 2.

**EBV DNA Load**

Patients with NPC had higher median EBV DNA load levels when compared with unaffected family members (835.4 vs 18.8 copies/mL; $P < .001$, Mann-Whitney $U$ test). Median EBV DNA load increased with overall stage of disease, extent

<table>
<thead>
<tr>
<th>Sample size</th>
<th>Family Members</th>
<th>Nasopharyngeal Carcinoma Patients</th>
<th>$P$ Value</th>
</tr>
</thead>
<tbody>
<tr>
<td>Sex</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Male</td>
<td>240 (51.0)</td>
<td>222 (75.8)</td>
<td>&lt;.001</td>
</tr>
<tr>
<td>Female</td>
<td>231 (49.0)</td>
<td>71 (24.2)</td>
<td></td>
</tr>
<tr>
<td>Age, y, mean ± SD (range)</td>
<td>44.6 ± 11.7 (17-76)</td>
<td>51.7 ± 10.6 (19-90)</td>
<td>&lt;.001</td>
</tr>
<tr>
<td>Ethnicity</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Chinese</td>
<td>448 (95.1)</td>
<td>232 (79.2)</td>
<td>&lt;.001</td>
</tr>
<tr>
<td>Malay</td>
<td>9 (9.0)</td>
<td>36 (12.3)</td>
<td></td>
</tr>
<tr>
<td>Indian</td>
<td>2 (0.4)</td>
<td>4 (1.4)</td>
<td></td>
</tr>
<tr>
<td>Others</td>
<td>12 (2.5)</td>
<td>21 (7.2)</td>
<td></td>
</tr>
</tbody>
</table>

*Values presented as n (%) except where noted otherwise. Bold format indicates $P < .05$. 

**Figure 1.** Distribution of EBV-VCA IgA and EBV-EA IgA titers among NPC patients ($n = 293$) and family members ($n = 471$). EA, early antigen; EBV, Epstein-Barr virus; NPC, nasopharyngeal carcinoma; VCA, viral capsid antigen.
of primary tumor, nodal status, and the presence of distant metastasis ($P < .001$).

EBV DNA load demonstrated an increasing trend with increasing serology titers for both EBV-VCA IgA ($P < .001$, Kruskal-Wallis test) and EBV-EA IgA ($P < .001$, Kruskal-Wallis test; Figure 3). On bivariate analysis, EBV DNA load also showed good correlation with geometric mean titers for both EBV-VCA IgA (Spearman’s $\rho = .536$, $P < .001$) and EBV-EA IgA (Spearman’s $\rho = .594$, $P < .001$), with a stronger correlation observed for EBV-EA IgA.

Comparison of Screening Tests

The sensitivities and specificities for the 3 screening tools at different cutoff values are detailed in Table 2. A comparison between NPC patients ($n = 293$) and unaffected family members was performed ($n = 471$). Sensitivity and specificity were calculated at each serology or DNA load level based on whether NPC patients could be accurately identified. At 100 copies/mL, EBV DNA load demonstrated a sensitivity of 76.8% and a specificity of 85.4%. Overall, EBV-VCA IgA gave the best sensitivity, while EBV-EA IgA was the most specific. A positive EBV-EA IgA titer ($\geq 1:10$) gave a sensitivity of 85.0% and a specificity of 96.4%.

To compare the effectiveness of the screening tools in correctly classifying NPC patients and unaffected family members, ROC curve analysis was performed. The areas under the ROC curve for EBV-VCA IgA and EBV-EA IgA were 0.942 (95% confidence interval [95% CI]: 0.926-0.958) and 0.926 (95% CI: 0.903-.950), respectively. The area under the ROC curve for EBV DNA load was 0.880 (95% CI: 0.866-0.894; Figure 4). ROC analysis suggests that EBV-VCA IgA and EBV-EA IgA were better classifiers than EBV DNA load in distinguishing NPC patients and unaffected family members.

Matched Analysis

As NPC patients and family members were different in sex, age, and ethnicity, we also performed a matched analysis of
the data. We compared 200 NPC patients and 200 unaffected family members, matched for age, sex, and ethnicity. Similar findings were demonstrated. The areas under the ROC curve for EBV-VCA IgA, EBV-EA IgA, and EBV DNA load were 0.953 (95% CI: 0.935-0.972), 0.927 (95% CI: 0.898-0.956), and 0.878 (95% CI: 0.843-0.912), respectively. The best test statistic was for a positive EBV-EA IgA titer (≥1:10), with a sensitivity of 88.5% and a specificity of 96.5%.

**Discussion**

Almost all NPC patients in this study had detectable EBV DNA levels, similar to the high detection rates among Hong Kong and Taiwanese patients.14,18 However, in countries with lower prevalence of NPC, the detection rates for EBV DNA among patients were lower.10 Consistent with current literature, EBV DNA load in our study correlated well with stage of disease, stage of primary tumor, nodal status, and the presence of distant metastasis. EBV-EA IgA titers also increased with more advanced stage of disease, which is not surprising, as the early antigen complex consists of a variety of virus-encoded enzymes essential for viral replication. This trend did not reach statistical significance for EBV-VCA IgA, probably because a large proportion of NPC patients already had high EBV-VCA IgA titers (Figure 2).

As screening for NPC among family members has been recommended in endemic areas, it is important to compare the EBV serology titers and EBV DNA load levels between NPC patients and unaffected family members to determine their sensitivity and specificity in the context of family screening.

The family members in our study were subject to close follow-up, with an average duration of 52.5 months. Apart from the 4 family members who were identified to have NPC, the remaining 471 were disease-free and healthy at the date of last follow-up. Due to frequency and duration of follow-up and our low threshold of performing a nasopharynx biopsy—when a mass or mucosal irregularity was observed, if there was any positive EBV-EA IgA titer, or if EBV-VCA IgA was raised at 1:160 and beyond (per national cancer screening guidelines5)—we are confident that the 471 family members compared in this study were disease-free and healthy.

Interestingly, a large proportion (71%) of our family members had positive EBV-VCA IgA titers—higher than healthy controls in a previous local study (34%) and also significantly higher than families from Taiwan (28.4%), Greenland (25%), and Guangzhou (32%-34%).20-23 The high percentage of EBV-VCA IgA-positive family members reduces the specificity of EBV-VCA IgA as a screening test and may limit the role of EBV-VCA IgA as a screening tool among high-risk family members in our population.

EBV DNA load correlated well with EBV VCA and EA IgA serology titers, similar to previous observations that EBV-VCA IgA-positive individuals had significantly higher EBV viral loads.20 These findings are consistent with previous

<table>
<thead>
<tr>
<th>EBV</th>
<th>Sensitivity</th>
<th>Specificity</th>
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<tbody>
<tr>
<td>Viral capsid antigen IgA</td>
<td></td>
<td></td>
</tr>
<tr>
<td>≥1:10</td>
<td>0.997</td>
<td>0.314</td>
</tr>
<tr>
<td>≥1:40</td>
<td>0.976</td>
<td>0.546</td>
</tr>
<tr>
<td>≥1:160</td>
<td>0.908</td>
<td>0.854</td>
</tr>
<tr>
<td>1:640</td>
<td>0.666</td>
<td>0.987</td>
</tr>
<tr>
<td>Early antigen IgA</td>
<td></td>
<td></td>
</tr>
<tr>
<td>≥1:10</td>
<td>0.850</td>
<td>0.964</td>
</tr>
<tr>
<td>≥1:40</td>
<td>0.765</td>
<td>0.985</td>
</tr>
<tr>
<td>≥1:160</td>
<td>0.553</td>
<td>0.998</td>
</tr>
<tr>
<td>1:640</td>
<td>0.328</td>
<td>1.000</td>
</tr>
<tr>
<td>DNA load</td>
<td></td>
<td></td>
</tr>
<tr>
<td>≥25</td>
<td>0.904</td>
<td>0.575</td>
</tr>
<tr>
<td>≥50</td>
<td>0.823</td>
<td>0.720</td>
</tr>
<tr>
<td>≥100</td>
<td>0.768</td>
<td>0.854</td>
</tr>
<tr>
<td>≥150</td>
<td>0.710</td>
<td>0.917</td>
</tr>
<tr>
<td>≥200</td>
<td>0.689</td>
<td>0.934</td>
</tr>
<tr>
<td>≥300</td>
<td>0.642</td>
<td>0.962</td>
</tr>
<tr>
<td>≥400</td>
<td>0.597</td>
<td>0.977</td>
</tr>
<tr>
<td>≥500</td>
<td>0.580</td>
<td>0.981</td>
</tr>
<tr>
<td>≥1000</td>
<td>0.464</td>
<td>0.989</td>
</tr>
<tr>
<td>≥5000</td>
<td>0.222</td>
<td>0.998</td>
</tr>
<tr>
<td>≥10,000</td>
<td>0.154</td>
<td>1.000</td>
</tr>
</tbody>
</table>

Abbreviation: EBV, Epstein-Barr virus.
research that patients with EBV IgA antibodies had depressed virus-specific T-cell immunity.\textsuperscript{24}

The median EBV DNA load for high-risk family members in our study was significantly lower than that of NPC patients. If a cutoff had to be suggested, a level of 100 copies/mL for EBV DNA load appeared to give the best balance between sensitivity and specificity. Although lower cutoff levels will give a better sensitivity, selecting low levels may result in poor test reliability, as PCR tests are prone to stochastic effects with small quantities of DNA, due to inconsistent hybridization of primers during amplification.\textsuperscript{25,26} In addition, the specificity of the test decreased significantly with lower copy numbers.

At a level $\geq 100$ copies/mL, serum EBV DNA was detectable in 14.6% high-risk family members, comparable to high-risk family members in Taiwan (15%) and Greenland (5%).\textsuperscript{18-20} However, in a study performed in Tunisia, where NPC has an intermediate incidence, EBV DNA was not detectable in any high-risk family members.\textsuperscript{10} In a separate study examining the role of EBV DNA in recurrent NPC, we also found a level $\geq 100$ copies/mL to be useful in detecting recurrent NPC.\textsuperscript{27}

Any successful screening program for NPC will require a test that is both sensitive and specific to accurately stratify patients for further investigation and to optimize the cost-effectiveness of the screening process. Overall, we felt that EBV-EA IgA gave the best sensitivity and specificity profile. Although additional tests for EBV-VCA IgA or EBV DNA load may improve sensitivity, the significantly lower specificity of these tests will result in a large group of patients with positive titers or raised DNA load being subject to further investigation and an extended period of follow-up. In contrast, the EBV-EA IgA is highly specific and avoids the above problems. Patients with a positive EBV-EA IgA titer should be treated seriously, warranting further investigation with either a nasopharyngeal biopsy or magnetic resonance imaging.

**Conclusion**

Both EBV DNA load and EBV IgA serology demonstrated good sensitivity and specificity as screening tools. Overall, EBV-EA IgA gave the best sensitivity and specificity profile as a screening tool for NPC among high-risk family members.

**Author Contributions**

Joshua K. Tay, conceived the study, collected the data, performed the analysis, wrote and approved the manuscript; Soh Ha Chan, conceived the study, performed the laboratory experiments, and wrote and approved the manuscript; Chwee Ming Lim, conceived the study, collected the data, revised and approved the manuscript; Chor Hiang Siow, collected the data, revised and approved the manuscript; Han Lee Goh, collected the data, revised and approved the manuscript; Kwok Seng Loh, directed the work, recruited the patients, conceived the study, performed the analysis, wrote and approved the manuscript.

**Disclosures**

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