ASSESSING THE RISK OF NASOPHARYNGEAL CARCINOMA ON THE BASIS OF EBV ANTIBODY SPECTRUM

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We have evaluated the performance of 3 new EBV ELISA for the diagnosis of nasopharyngeal carcinoma (NPC). The tests were specific for EBNA 1 IgA, EBNA 1 IgG and zta IgG, respectively. Their distinct antigenic specificity permits these assays to be used in concert in an approach that differentiates patients and apparently healthy subjects on the basis of their antibody spectrum. By so exploiting a distinguishing feature of NPC first described by the late Werner Henle that the patients sustain high levels of a broad spectrum of serum EBV antibodies, this approach achieved a sensitivity of 92% and a specificity of 93%, surpassing the performance of each of these assays individually. The enhanced performance is especially useful in population screening. It was shown that relative risk of NPC sustained by apparently healthy subjects residing in a high incidence area for NPC in the Pearl River estuary in Southern China may vary according to EBV antibody spectrum. The risk of the cancer was markedly reduced with odds ratios of 0.009 for 59% of those who had low level of all 3 antibodies. The risk was increased as antibody spectrum broadens and the risk was the highest with an odds ratio of 138 for 0.4% of those who had high levels of all 3 antibodies. Thus, EBV antibody spectrum may serve to guide follow-up measures for early detection of the cancer and/or risk counseling according to level of the risk of the cancer sustained by the screened individuals.

Key words: EBV serology; NPC risk; NPC diagnosis

Nasopharyngeal carcinoma (NPC) patients sustain higher levels of serum EBV-specific antibodies, especially antibodies of the IgA isotypes, than healthy carriers and patients with other head and neck cancers.1,2 First described by the late Werner Henle and his colleagues,1 this characteristic feature had been widely applied since in serologic diagnosis of suspected NPC. In this approach, a positive finding supports clinical suspicion of the disease and provides an additional indication for further investigation by endoscopy and, if so indicated, histopathology to confirm diagnosis of the cancer. Especially for patients with early disease, when symptoms are often nonspecific and innocuous, this approach can facilitate early treatment and thereby better ensure survival.3 Previous population studies also associate high level of EBV-specific antibody in an otherwise healthy subject with an increased incidence of NPC.4–7 and on the basis of which EBV serology is included as part of cancer screening programs in some areas with high incidence of the cancer.

EBV-specific antibodies are traditionally determined by immunofluorescence or immunoenzymatic methods against EBV-transformed lymphoblastoid cell lines.8,9 Objective enzyme immunoassays (ELISA) specific for individual EBV-specified peptides have become increasingly available.9–14 Most of these assays afford comparable specificity and sensitivity for serologic diagnosis of NPC as do the traditional methods, and they have the additional advantage of avoiding inherent assay errors relating to subjectivity of the traditional methods. Moreover, increasing availability of these assays with distinct antigenic specificity makes it possible to use the assays in combination in a new approach to effect diagnosis of NPC based on EBV antibody spectrum rather than the level of individual antibodies. The new approach more fully exploits the advantage of the outstanding serologic feature of NPC than the conventional approach based on antibody level determined by individual assay. Consequently, the performance of different combined assays was enhanced compared to individual assays. The enhanced performance may find an especially useful application in population screening for the early detection of NPC.

MATERIAL AND METHODS

Serum samples were obtained from 121 newly diagnosed NPC patients before treatment and 332 apparently healthy subjects (HS). Residing in Zhongshan and nearby areas, the HS were those who voluntarily subscribed to an NPC screening program organized by Zhongshan Peoples’ Hospital. They included 186 males and 146 females with age ranging from 19 to 81 (median = 47). The patients were referred to this hospital or Queen Mary Hospital, Hong Kong SAR, for treatment. They included 89 males and 32 females with age ranging from 18 to 78 (median = 34). Both cities are situated in the Pearl River estuary and have an incidence of NPC of 14 and 19 per 105, which are among the highest in the world.15–17 The sera were stored in aliquots at −20°C. After thawing, the samples were kept at 4°C and used within 2 weeks.

The serum samples were tested by the traditional immunoenzymatic tests for IgA VCA antibodies according to Yi et al.6 and by ELISA’s specific for EBNA-1 IgG (ENG), EBNA-1 IgA (ENA) and zta IgG (ZG), respectively. These 3 assays were produced by coating microtiter plates with purified glutathione transferase (GST) fusion proteins of EBNA-1 encoded by a cloned BKRF 1 sequence11 or zta encoded by another cloned BZLF1 sequence,18 respectively. The ELISAs were supplied by Sinoclone Hong Kong Ltd. (Hong Kong). Serum samples were diluted 1 in 1,000 for ENG and 1 in 100 for the other tests. The assays were performed according to manufacturer’s instructions. Briefly, 100 (l of diluted patient’s serum in blocking buffer was added in each well and incubated for 1 hr at 37°C. The wells were then washed 4 times in washing buffer (0.05% tween-20 in PBS), filled with 100 (l of diluted horseradish peroxidase-conjugated goat antiserum) and incubated at 37°C for another hour. The wells were washed as

Received 22 February 2001; Revised 15 June 2001; Accepted 30 July 2001

DOI 10.1002/jic.1641

Grant sponsor: Croucher Foundation; Grant sponsor: Industry Support Funds Scheme of Hong Kong SAR.

In memory of the late Werner Henle.

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before and then 100 l of TMB was added. The colour development was stopped after 30 min by the addition of 100 l of 0.3 M H2SO4. Optical absorbance was measured at 450 nm against 620 nm.

RESULTS

Serologic diagnosis of NPC by ELISA and immunofluorescent assays

Serum samples were obtained from 322 apparently healthy adults (HS) and 121 NPC patients residing in the Pearl River estuary, which has one of the highest incidences of NPC in the world. The patient samples were taken before the patients received treatment, and both groups of study subjects were EBV-seropositive having VCA IgG and EBNA antibody titer of >1:10 as determined by the immunofluorescence assays. Another 7 serum samples were from EBV-seronegative donors, which were not reactive for VCA IgG or EBNA antibody. The samples were tested by 3 new ELISAs specific for EBNA 1 IgA (ENA), EBNA 1 IgG (ENG) and zta IgG (ZG) antibodies, respectively. Interassay variations were minimized by expressing antibody levels in relative OD (rOD) units calculated as ratios of mean OD values of test samples to that concurrently determined for a positive reference serum. Table I shows that median and range of the antibody levels were higher for patients than HS and that the results obtained for the EBV-seropositive and EBV-seronegative HS correlated with their respective EBV serologic status determined by the traditional immunofluorescence assays.

Antibody levels of patients and EBV-seropositive HS exhibited a bimodal distribution and the capacity of ELISA to differentiate the 2 groups of individuals, i.e., the capacity of the tests to diagnose NPC depends on the separation of the 2 curves. To optimize assay performance, we plotted sensitivity and specificity calculated for different cutoff values (Fig. 1), sensitivity being percentage of patients having rOD exceeding the given cutoff rOD, and specificity being percentage of HS having rOD, which is equal to or below the cutoff rOD. The optimum cutoff value corresponds to the intercept of the 2 curves, where percent of patients having rOD values exceeding the optimum rOD (i.e., sensitivity) equals the percent of HS having rOD equal to or less than the optimum rOD (i.e., specificity). Comparison of results obtained by different ELISAs (Table II) showed that ENA assay is more diagnostic of NPC than ENG assay and that, in turn, is more diagnostic than the ZG assay. Reproducibility of the assays was assessed by repeated testing of a panel of 10 sera containing different levels of the antibodies. Coefficients of variation thus determined for the 3 assays were from 10–16%. The same samples were tested by immunofluorescence assay specific for VCA IgA antibody. The results showed that the traditional assay had similar specificity as, but higher sensitivity than, ENA and ENG.

**EBV antibody spectrum for diagnosis of NPC and risk assessment**

The distinct antigenic specificity of ELISAs had given rise to 8 antibody profiles shown in Table III. The majority of patients had high level (H) of all 3 antibodies (profile 8), with rODs exceeding the optimum cutoff values determined for the respective assays. Other patients’ sera had high levels of 2 antibodies (profiles 5–7), and few had high level of 1 of the antibodies only (profiles 2–4). Consequently, results obtained with patients’ samples by the 3

![Image](image-url)

**FIGURE 1**—Optimizing the performance of EBV ELISAs for diagnosis of NPC. EBV antibody contents in sera from 121 NPC patients and 332 apparently healthy subjects were determined by ELISA specific for EBNA 1 IgG, EBNA 1 IgA and zta IgG, respectively. Antibody levels were calculated as ratios of OD unit obtained with the test samples to that obtained concurrently with a positive control serum and were expressed as relative OD (rOD) units. The results were plotted as percent of the patients’ samples having rOD exceeding the different cutoff rOD (sensitivity, filled box) and percent of HS having rOD, which is low or equal to the different cutoff rOD (specificity, open diamond). The optimum cutoff that best differentiates the patients and HS is at the intercept of the 2 curves. Median antibody level corresponds to rOD at 50% of the respective groups of study subjects.

**TABLE I**—SERUM EBV ANTIBODY LEVELS OF EBV-SEROPOSITIVE NPC PATIENTS AND SERONEGATIVE HEALTHY SUBJECTS

<table>
<thead>
<tr>
<th>Test subjects</th>
<th>EBNA 1 IgA</th>
<th>EBNA 1 IgG</th>
<th>zta IgG</th>
</tr>
</thead>
<tbody>
<tr>
<td>NPO (n = 121)</td>
<td>2.55</td>
<td>0.37–7.5</td>
<td>1.69</td>
</tr>
<tr>
<td>HS EBV + (n = 332)</td>
<td>0.54</td>
<td>0.23–3.9</td>
<td>0.79</td>
</tr>
<tr>
<td>HS EBV − (n = 7)</td>
<td>0.17</td>
<td>0.14–0.26</td>
<td>0.22</td>
</tr>
</tbody>
</table>

Serum samples of HS subjects were tested at 1:10 dilution by immunofluorescent assays for VCA IgG; 1:100 by EBNA 1 IgA ELISA; 1:1,000 by EBNA 1 IgG ELISA; and 1:100 by zta IgG ELISA. Antibody levels determined by ELISAs expressed as rOD, calculated as ratios of mean OD of test samples to that concurrently determined for the positive reference serum. The latter was a pooled serum from NPC patients diluted for use in the ELISAs. It has a VCA IgG and IgA titer of 1:5,120 and 1:2,800, respectively, determined by immunofluorescent assay and a EBNA titer of 1:640 determined by anticomplement immunofluorescent assay. VCA IgG titer of 1:10 or greater indicates EBV seropositive (EBV+) and titer of <1:10 indicates EBV seronegative (EBV−).
ELISAs combined was determined as the percent of patients having antibody levels exceeding the optimum cutoff level determined in Figure 1. Specificity was defined as the percent of healthy subjects having antibody levels lower or equal to the optimum cutoff level. The cutoff level used for the traditional VCA IgA test was 1.10 serum dilution. Sensitivity of 3 ELISAs combined was defined as the percent of NPC patients having antibody profiles 5–8, which were associated with an increased NPC risk, and specificity was defined as the percent of HS having antibody profiles 1–4, which were associated with reduced NPC risk (see Table III and text for detail).

ELISAs exhibited a high degree of positive concordances but a low negative concordance. Namely, patients’ samples tested to be positive by 1 of the ELISAs were likely to be tested positive by the other tests also, but samples tested to be negative by 1 test is likely to be positive when tested by another test. Conversely, the majority of HS have low level (L) of all 3 antibodies (profile 1), having rODs equal to, or lower than, optimal cutoff values of the respective tests. Other HS had high level of 1 of the antibodies and few had high levels of 2 or all 3 antibodies. Consequently, results obtained with HS samples exhibited a high degree of negative concordance and a low degree of positive concordance.

The expected distribution of 2 groups of test subjects having different antibody profiles was calculated on the basis of the intrinsic sensitivity or specificity determined for each test individually (Table III). This approach assumes that each test provides an independent determination of the EBV antibody status of the test subjects. This assumption was justified by low negative concordance between patients’ test results and low positive concordance between test results obtained for HS. The finding that the expected distribution of HS and patients closely correlate with the observed distribution of the respective groups of test subjects (correlation coefficients = 0.990 and 0.999) further supported this assumption.

Odds ratios were calculated as ratios of the expected distribution of NPC to HS having the different antibody profiles (Table III). Compared to the general population, the results suggested that the relative risk of NPC is increased for HS, who had the EBV serologic profiles 5 to 8. Odds ratios vary from 4 for those having serologic profile 5 to as high as 138 for those having serologic profile 8. Conversely, the relative risk of NPC was reduced for those having the serologic profiles 1 to 4. Odds ratios vary from as low as 0.009 for those having serologic profile 1 to 0.3 for those having serologic profile 4. The results showed that 93% of HS have low levels of 2 or all 3 antibodies (profile 1–4) with a reduced odds ratio of 0.3 or less, while 82% of NPC patients have high levels of at least 2 of the antibodies (profile 5–8) with increased odds ratios of 4–138. Thus, for patients suspected of having NPC, an increased odds ratio may be considered to be compatible with a diagnosis of the cancer. The sensitivity and specificity achieved by utilizing the 3 tests in concert surpasses that achievable by each of these tests alone as well as that afforded by the traditional immunofluorescent test (see Table II). Moreover, 6.4% of the 6.8% of HS having an increased odds ratio may be considered as having a moderate risk of NPC with odds ratios of 4 to 10. The remaining 93.6% are considered as having a high risk of NPC with an odds ratio of 138. It is thus possible to assess relative risk sustained by individual test subjects on the basis of their EBV antibody spectrum.

### Table II - Sensitivity and Specificity EBV Antibody Assays for the Diagnosis of NPC

<table>
<thead>
<tr>
<th>Assay¹</th>
<th>% Sensitivity (n = 121)</th>
<th>% Specificity (n = 332)</th>
</tr>
</thead>
<tbody>
<tr>
<td>EBNA1 IgA</td>
<td>85</td>
<td>86</td>
</tr>
<tr>
<td>EBNA1 IgG</td>
<td>83</td>
<td>86</td>
</tr>
<tr>
<td>zta IgG</td>
<td>79</td>
<td>80</td>
</tr>
<tr>
<td>3 ELISAs combined²</td>
<td>92</td>
<td>93</td>
</tr>
<tr>
<td>VCA IgA</td>
<td>93</td>
<td>87</td>
</tr>
</tbody>
</table>

¹EBV antibodies were determined by 3 ELISAs respectively specific for EBNA 1 IgG, EBNA 1 IgA and zta IgG and by immunoezymatic method specific for VCA IgA. Sensitivity of the ELISA was defined as the percent of patients having antibody levels exceeding the optimum cutoff level determined in Figure 1. Specificity was defined as the percent of healthy subjects having antibody levels lower or equal to the optimum cutoff level. The cutoff level used for the traditional VCA IgA test was 1.10 serum dilution. Sensitivity of 3 ELISAs combined was defined as the percent of NPC patients having antibody profiles 5–8, which were associated with an increased NPC risk, and specificity was defined as the percent of HS having antibody profiles 1–4, which were associated with reduced NPC risk (see Table III and text for detail).

The traditional immunofluorescent method first developed by Henle⁴ is routinely used in most centers to assist diagnosis of NPC. In high-incidence areas for the cancer, the method is also widely used in population screening for the early detection of the cancer. The approach is based on the finding by Henle⁴ that NPC patients sustain high levels of a broad spectrum of EBV antibody, with high levels of antibodies of the IgA subtype being an especially outstanding feature. For patients suspected of having NPC, an increased serum level of VCA IgA antibody is considered to be an indication for further clinical investigations. If the clinical findings so indicate, then histopathology is performed to confirm the diagnosis of NPC. Since early symptoms of NPC are innocuous, serologic diagnosis has become an integral part in the diagnostic workup for suspected cases of NPC. For apparently healthy subjects, elevated antibody levels were associated with an increased risk of the cancer. Previous population studies carried out in high-incidence areas for NPC estimated the occurrence of the cancer among apparently healthy subjects having an elevated serum level of VCA IgA to be about 1% per year.⁵,⁶,⁷ In practice, this serves to alert the individuals and clinicians to early symptoms of the cancer.

Despite its usefulness, the traditional VCA IgA assay suffers from the disadvantage that it is a subjective test produced with cell lines, which are latently infected with EBV. The antigen specificity of the assay, however, is directed against those antigens expressed by a small percent of the cells, which are undergoing EBV replication. The percent of these permissive cells varies and spectrum of viral antigens expressed by these cells is not known. Consequently, the antigenic specificity of the assay is not defined, and it is difficult to ensure the uniformity of different batches of the cells used to produce different batches of the test. Moreover, reading of test results may vary between operators. These difficulties have been overcome by the new ELISA in that the recombinant antigens used to produce the assays have been purified to at least 85% homogeneity⁸ and that operator errors are avoided by objectivity of the assays.

In our present study, we have described 2 approaches to further enhance the performance of the assays. The first aims to minimize intertest variation by expressing OD values of test samples as ratios to the OD value concurrently determined for the positive control sample. Expressed as relative OD values, coefficient of variation of the assays was found to be 10% for ENA, 12% for EAG and 16% for ZG. The second approach aims to optimize assay performance by selecting the cutoff rOD, which best separates HS and patients. This was achieved by plotting the sensitivity specificity calculated for different cutoff rOd values. The optimum cutoff value corresponds to the intercept of the 2 curves, where sensitivity and specificity of the assay are equal.

An additional, and probably the most important, advantage of the new assays is that, when used in concert, these combined assays allow us to exploit more fully the outstanding serologic feature of NPC first described by Henle in a new approach to effect diagnosis and early detection of NPC. This approach assumes that each assay affords an independent determination of a different aspect of the EBV serologic status of test subjects. The assumption is based on the prior knowledge that each test has a distinct and defined antigenic specificity, and this is evidenced by the discrepancy between the test results obtained for individual test subjects by the 3 tests that have given rise to 8 serologic profiles. The contention is supported by the close correlation between the observed and the expected distribution of test subjects having different serologic profiles, the expected values being calculated on the basis of the intrinsic performance of each test individually on the assumption that each test provides an independent determination of the EBV serologic status of test subjects.
Increased odds ratios of 4 to 138, and these profiles are, therefore, considered to be diagnostic of NPC. Based on these criteria, the sensitivity of diagnosis of NPC was expected to be 92% and the specificity 93%, the latter being calculated as the percent of HS having the other 4 profiles, which are associated with reduced odds ratios. Thus, the 3 tests combined afford similar sensitivity to, and higher specificity than, the traditional VCA IgA.

The new approach finds an especially useful application in population screening for the early detection of NPC. The combined test results served to separate HS into 3 groups according to level of NPC risk sustained by the individual. The first group comprising 93% of HS has a reduced risk of NPC with odds ratios ranging from 0.009 to 0.3. Another 6.5% has a moderate level of risk with odds ratios of 4 to 10. This is similar to the risk level sustained by those who have elevated levels of VCA IgA antibody determined by the traditional test, and the knowledge of the risk serves to alert these individuals and their physicians to early symptoms of NPC. In addition, the combined test results serve to identify a third group comprising an estimated 0.4% of HS, who are at high risk of NPC with an odds ratio of 138. Even though these individuals may not show symptoms of NPC, the high level of risk would seem to be sufficient indication for clinical workup to effect diagnosis of the cancer. Thus, the new approach may make it possible to serologically stratify individuals according to the level of risk of NPC they sustain and that, in turn, will serve to follow-up measures for early detection of the cancer.

REFERENCES