Improved Accuracy of Detection of Nasopharyngeal Carcinoma by Combined Application of Circulating Epstein–Barr Virus DNA and Anti-Epstein–Barr Viral Capsid Antigen IgA Antibody

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Background: Circulating Epstein–Barr viral (EBV) DNA and anti-EBV capsid antigen IgA (IgA VCA) represent two of the most sensitive peripheral blood markers of nasopharyngeal carcinoma (NPC), but direct comparative studies of these two markers are lacking. Methods: The sensitivities and specificities of IgA-VCA and EBV DNA for diagnosis of NPC were determined in 139 new cases of NPC and 178 healthy individuals, respectively. EBV DNA was also assessed in 36 healthy family members identified as having false-positive IgA-VCA results at a screening clinic. EBV DNA was measured by a real-time quantitative PCR assay with a detection limit of 60 copies/mL. IgA-VCA was measured by semiquantitative indirect immunofluorescent method; a titer ≥1/10 was taken as positive. Results: The sensitivities of EBV DNA and IgA-VCA for diagnosis of NPC were 95% (95% confidence interval, 91–98%) and 81% (73–87%), respectively. The combined marker panel had an overall sensitivity (positive result by either marker) of 99%. The concentrations of both markers showed dependence on cancer stage. The specificities of EBV DNA and IgA-VCA were 98% (96–99%) and 96% (91–98%), respectively. Among 36 healthy family members with false-positive IgA-VCA results, three-fourths had undetectable EBV DNA, whereas the others had increased EBV DNA concentrations that were significantly lower than in NPC patients. Conclusions: For diagnosis of NPC, EBV DNA identifies almost all false-negative IgA-VCA cases and gives a 99% diagnostic sensitivity when combined with IgA-VCA. In the screening setting, EBV DNA identifies three-fourths of false-positive IgA-VCA cases. The selective application of EBV DNA in an IgA-VCA-based screening protocol could improve screening accuracy with only moderate increases in cost.

The association between specific serologic responses to Epstein–Barr virus (EBV) and nasopharyngeal carcinoma (NPC) has been exploited to develop serologic tumor markers for this cancer (1). The anti-viral capsid antigen IgA antibody (IgA-VCA), measured by indirect immunofluorescence (1) or ELISA (2), is one of the most widely used antibody markers used for assisting in diagnosis (1, 3) and for screening (4–9). Its sensitivity in the diagnosis of WHO type II and III NPC in areas endemic (1) and nonendemic (3) for the disease has been generally reported to be ~85–90% (1, 3, 4, 10–14), although a broader range had been reported in some studies. A false-positive rate of 2.36–6.71% had been found in screening studies in areas with endemic NPC (5), whereas in case–control studies a false-positive rate of 9–18% has been reported (3).

In attempts to improve the sensitivity and specificity of...
a peripheral blood-based marker for NPC, several alternative markers had been developed. These were based on antibodies to different antigenic components of the EBV (9, 15–29). Attempts to use panels of antibody-based markers have generally been useful in improving the accuracy of NPC detection (13, 15, 20, 21, 29–32). Plasma/serum cell-free EBV DNA is a more recently developed peripheral blood-based molecular marker for NPC. It is conceptually distinct from other peripheral blood markers in that it directly measures tumor-derived EBV genomic material rather than an antibody response to genomic or peptidic components of the EBV. Although initial studies based on qualitative measurement systems showed that plasma EBV DNA has a sensitivity of only 59–75% for diagnosis of NPC (33–35), its sensitivity in quantitative assays was as high as 90% (36). Such a diagnostic sensitivity appears to be at least as good as that of IgA-VCA, although direct comparative studies of the two markers have yet to be reported. Of further interest is the observation that plasma EBV DNA was found to be rarely detectable in patients who had complete eradication of cancer (37) and in individuals without cancer (34, 36, 37). These observations suggest that the marker may have a role in the cancer screening setting. In the present study, we aimed to directly compare the sensitivities and specificities of the two markers in NPC patients and individuals without cancer and to explore whether the markers play complementary roles in the diagnosis of and screening for NPC.

Materials and Methods

SAMPLE SIZE

The sample size of the present study was based on estimation of sensitivity and specificity rates postulated at ~80–90%. The estimate is considered accurate within a variation of 10%. To have 80% power to test the postulated rates at 5% significance with a one-sided test, we need to enter ~90 patients. The same number of patients is adequate to draw a 95% confidence interval around the sensitivity or the specificity with a range of 10%.

CANCER CASES FOR DETERMINATION OF SENSITIVITY

We recruited 139 patients with newly diagnosed NPC at our institution (group A) and collected data prospectively. Consecutive consenting patients were recruited at the oncology clinic in the period May 1998 to December 2000. Histologic details were available in all cases and were confirmed to be WHO type II/III carcinoma in 138 cases and WHO type I in 1 case. Tumor staging was according to the International Union Against Cancer 1997 stage classifications (38). Staging work up included in all cases clinical examination; computed tomography scans of the nasopharynx, skull base, and neck; chest radiography; and serum alkaline phosphatase measurements. Table 1 details the gender, age, and tumor stage distribution of the patients. Serum IgA-VCA and plasma EBV DNA were assessed before commencement of oncologic therapy.

Table 1. Characteristics of study participants.

<table>
<thead>
<tr>
<th></th>
<th>Cancer patients (group A)</th>
<th>Healthy controls (group B)</th>
<th>Family members with positive IgA-VCA (group C)</th>
</tr>
</thead>
<tbody>
<tr>
<td>n</td>
<td>139</td>
<td>178</td>
<td>36</td>
</tr>
<tr>
<td>Gender, % male</td>
<td>74</td>
<td>26</td>
<td>39</td>
</tr>
<tr>
<td>Age, years</td>
<td>Median 48</td>
<td>48</td>
<td>39</td>
</tr>
<tr>
<td></td>
<td>Mean (SD) 49 (12)</td>
<td>52 (12)</td>
<td>39 (10)</td>
</tr>
</tbody>
</table>

NON-CANCER CASES (CONTROLS) FOR DETERMINATION OF SPECIFICITY

We recruited 178 healthy individuals ≥30 years at a community center (group B). The age and gender distribution of the cohort are detailed in Table 1. The age selection served to enhance the comparability with the cancer patient group, in consideration of the uncommon occurrence of NPC below the age of 30. To further address the clinical question of whether selective analysis for EBV DNA in IgA-VCA-positive individuals could reduce false positives in the screening setting, another cohort of 36 family members of NPC patients with known positive IgA-VCA results (titer ≥1/10) were recruited at a NPC screening clinic during a follow-up visit (group C). After blood sampling, the participants were followed up at 6-month to 1-year intervals and had a minimum follow-up time of 1 year. Their noncancer status was defined by the absence of clinical evidence of disease at 1 year or more after the EBV DNA assessment time point. The age and gender distributions of the non-cancer cohorts are detailed in Table 1. The higher proportion of females reflects the gender distribution of individuals attending the screening clinic.

TUMOR MARKER ASSAY

Plasma EBV DNA was measured by real-time quantitative PCR as described previously (36). DNA was extracted from plasma samples with a QIAamp Blood Kit (QIagen) according to the “blood and body fluid protocol” as recommended by the manufacturer. A total of 400–800 µL of the plasma samples was used for DNA extraction per column. The exact amount was documented for calculation of the target DNA concentration. A final volume of 50 µL was used to elute the DNA from the extraction column, and 5 µL of the eluted DNA was used per PCR. Circulating EBV DNA concentrations were measured by a real-time quantitative PCR system that amplified a DNA segment in the BamHI-W fragment region of the EBV genome. The principles of real-time quantitative PCR and the reaction set-up procedures were as described previously (36). Data were collected with an ABI Prism 7700 Sequence Detector and analyzed with the Sequence Detection System software (Ver. 1.6.3) devel-
oped by PE Biosystems. Results are expressed as EBV genome copies/mL of plasma. Endogenous β-globin was used as an “amplification control”, and all plasma DNA samples were also subjected to real-time PCR analysis for the β-globin gene, which gave a positive signal on all tested samples, thus demonstrating the quality of the extracted DNA. Multiple negative water blanks were included in every analysis. Results were expressed as DNA copies/mL of plasma. The lower limit of reliable quantification was 5 copies/assay, although occasionally 1 copy/assay was also detectable (the latter corresponding to 12 copies of EBV DNA/mL of plasma).

The precision of the EBV DNA assay near the lower limit of detection was determined by 20 replicate extractions of pooled NPC plasma samples with a mean EBV DNA concentration of 100 copies/mL. Because no recovery experiments had been performed, the results, expressed in copies/mL and assuming 100% recovery, represent the minimum estimates of the concentrations. Each of these replicate extractions was then subjected to real-time EBV DNA PCR. The CV at this low concentration of circulating EBV DNA was 58%.

EBV IgA-VCA was measured by a semiquantitative immunofluorescence method (1). Briefly, B95.8 cell preparations were air-dried and fixed in cold acetone in wells on glass slides, and dilutions of patient sera were applied. After incubation the slides were washed with phosphate-buffered saline, and fluorescein isothiocyanate-conjugated anti-human IgA was applied to each well at appropriate concentrations. After incubation and washing, the slides were mounted with buffered glycerol and read with a fluorescence microscope. The antibody titer of the test serum was read as the reciprocal of the highest dilution showing definite apple-green fluorescence in 20% of the cells. A titer ≥1/10 was taken as positive. This cutoff titer was commonly adopted in previous studies on the marker (4).

The study was approved by our institution, and written informed consent was obtained from all study participants.

Results

CANCER CASES (GROUP A): SENSITIVITY OF MARKERS

When we used detectable (≥60 copies/mL of plasma) circulating EBV DNA concentrations and an IgA-VCA titer ≥1/10 to define positive results, the sensitivity of EBV DNA [95% confidence interval (CI), 91–98%] was higher than that of IgA-VCA (81%; 95% CI, 73–87%; Table 2). We observed a difference in sensitivities among both early-stage and advanced-stage disease (Table 2). The 7 cases that were false negative for EBV DNA (stage I, 2 cases; stage II, 3 cases; stage III, 0 cases; stage IV, 2 cases) did not overlap with the 27 cases that were false negative for IgA-VCA (stage I, 5 cases; stage II, 9 cases; stage III, 8 cases; stage IV, 5 cases) except for 2 cases. When EBV DNA and IgA-VCA were combined as a marker panel and the sensitivity of the panel was defined as positive results by either marker, the panel had a sensitivity of 99%.

CANCER CASES (GROUP A): RELATIONSHIP BETWEEN MARKER CONCENTRATION AND CANCER STAGE

The distributions of EBV DNA concentrations and IgA-VCA titers according to individual cancer stage are shown in Figs. 1 and 2, respectively. To assess the relationship between EBV DNA concentration and IgA-VCA titer and cancer stage, we tested the variance by first transforming EBV DNA values to a log scale and IgA-VCA titers to a linear scale. We found a significant relationship for each of the two markers with cancer stage (P < .01 for EBV DNA; P = 0.04 for IgA-VCA). Multiple group comparisons were also performed for marker concentrations among different cancer stages. We found that EBV DNA concentrations were significantly different between stage I and each of the other stages and between stages II and

<table>
<thead>
<tr>
<th>NPC stage</th>
<th>n</th>
<th>Sensitivity of IgA-VCA</th>
<th>Sensitivity of EBV DNA</th>
</tr>
</thead>
<tbody>
<tr>
<td>All stages</td>
<td>139</td>
<td>81% (112/139)</td>
<td>95% (132/139)</td>
</tr>
<tr>
<td>Stages I + II</td>
<td>50</td>
<td>72% (36/50)</td>
<td>90% (45/50)</td>
</tr>
<tr>
<td>Stages III + IV</td>
<td>89</td>
<td>85% (76/89)</td>
<td>98% (87/89)</td>
</tr>
<tr>
<td>95% CI</td>
<td>78–92%</td>
<td></td>
<td>95–100%</td>
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</tbody>
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**Table 2. Comparison of sensitivities of markers by stage (group A).**

**Fig. 1.** Scattergram of distribution of EBV DNA according to cancer stage. x axis, cancer stage; y axis, EBV DNA concentrations in log scale.
IV. The IgA-VCA titers were significantly different only between stages I and IV. In other words, the concentrations of both markers exhibited a certain degree of cancer stage dependence, which was more significant for EBV DNA.

CONTROLS (GROUP B): SPECIFICITY OF MARKERS
The specificity for EBV DNA was 98% (174 of 178) with a 95% CI of 96–99%, and the specificity for IgA-VCA was 96% (170 of 178) with a 95% CI of 91–98%. There were four false-positive cases for EBV DNA and eight false-positive cases for IgA-VCA, but these cases did not overlap except for one case. Among the eight individuals with false-positive IgA-VCA results, seven had a titer of 1/20 and one had a titer of 1/40. The EBV DNA concentrations in the four individuals with false-positive EBV DNA results were 72, 164, 239, and 3287 copies/mL, respectively, which were significantly lower than the concentrations in NPC (group A) patients ($P < 0.01$, Wilcoxon rank-sum test). For the only individual with false-positive results for both markers, the EBV DNA concentration was 164 copies/mL and the IgA-VCA titer was 1/10.

HEALTHY FAMILY MEMBERS OF CANCER CASES WITH INCREASED IgA-VCA TITERS (GROUP C)
Among the 36 healthy family members who had increased IgA-VCA titers ($\geq 1/10$) identified at a screening clinic and who did not have evidence of cancer at follow-up, 27 had undetectable EBV DNA. For the other nine individuals, all had EBV DNA concentrations $\leq 344$ copies/mL, apart from one individual with a concentration of 4840 copies/mL. For these nine individuals with false-positive results for both markers, EBV DNA concentrations were significantly lower than those of true-positive cases (i.e., group A NPC patients; $P < 0.01$, Wilcoxon rank-sum test).

Discussion
The anti-VCA IgA antibody was chosen as the reference marker for this study because of its high sensitivity for diagnosis of NPC and the relative abundance of data related to this marker with its long track record in both case-control studies and population screening studies. Plasma/serum cell-free EBV DNA represents a direct measure of EBV genomic material and is conceptually distinct from other antibody-based markers for NPC. There is much clinical interest in comparing EBV DNA and IgA-VCA, first because the sensitivities of both markers for diagnosis of NPC are among the highest reported for peripheral blood markers of NPC, and second because their different mechanisms of production may allow minimization of false-positive cases when used in conjunction, which is an important issue in the cancer screening setting.

The present study shows that the two markers have complementary roles in the diagnosis of and screening for NPC. There is almost no overlap of false-negative cases for these two markers and only limited overlap of the false-positive cases. This allows a potential diagnostic sensitivity of 99% when the two markers are used in a panel. The clinical interpretation is that if a patient is strongly suspected to have NPC, a negative result for both markers would make the diagnosis of NPC extremely improbable and should lead to consideration of alternative diagnoses. Such a high sensitivity has been reported for only a very limited number of marker panels (20).

Although the reasons for false negativity of the markers is not certain, we observed that 5 of the 7 cases that were
false negative for EBV DNA had stage I-II tumors, whereas the distribution of the 27 false-negative IgA-VCA cases was relatively even between early- and advanced-stage tumors. In other words, the two markers appear to exhibit different degrees of cancer stage dependency.

Although improvement in sensitivity is commonly paralleled by a reduction in specificity, the present study on healthy family members of NPC patients showed that use of EBV DNA can eliminate approximately three-fourths of the false-positive IgA-VCA results. Although the cost of the EBV DNA test (approximately US $75.00 per test) is appreciably higher than that of the IgA-VCA test (less than US $10 per test), the latter can be used as the first step in a screening protocol, followed by the EBV DNA test in cases with positive IgA-VCA results. Such selective application of the EBV DNA test in an IgA-VCA-based screening protocol may lead to a significant improvement in accuracy of screening with only a limited increase in cost. Although the widely used anti-early antigen IgA antibody marker may serve a similar purpose of eliminating an appreciable proportion of false-positive IgA-VCA results, its sensitivity in detecting NPC is only ~70% (1, 10, 12). Thus, its combination with IgA-VCA in the screening setting may lead to missing an appreciable proportion of true cancers. This limitation is overcome by EBV DNA, which has a 90% sensitivity in detecting early-stage cancer and 95% overall detection rate for NPC.

The reason for the presence of detectable EBV DNA and IgA-VCA in 2–4% of healthy individuals without cancer is not clear, although the EBV DNA concentrations in false-positive cases were much lower than in NPC patients. Previous studies had shown that EBV DNA and EBER (small EBV-encoded RNA) are detectable only in tumor cells and not in nasopharyngeal tissue of healthy individuals (39). However, there have also been reports documenting EBV DNA in nonmalignant tissue (40–45). Some individuals with increased IgA-VCA had been shown to have nasopharyngeal lymphoid hyperplasia, in which EBV DNA and EBER were detectable (46). The alternative explanation for the higher rate of EBV DNA positivity in apparently healthy individuals with increased IgA-VCA titers is that they actually harbor occult NPC that was not clinically apparent (44, 47). An early study on healthy family members of NPC patients had also reported an increased incidence of IgA-VCA seropositivity in family members, and it was postulated that an autosomal recessive gene might be involved in the IgA-VCA response (48). Continued follow-up surveillance of healthy individuals with increased IgA-VCA titers, especially in the family member group, is worthwhile to exclude emergence of cancer (4). These individuals may also serve as a target group for tissue sampling by noninvasive methods, such as nasopharyngeal brushing for EBV DNA (49–51), which has been reported to have a high accuracy in diagnosis of NPC.

In conclusion, the strategy of combined marker panels has been increasingly explored in NPC with encouraging results (13, 30, 31). In the cancer screening setting, the sensitivity, specificity, quality control, and costs are relevant considerations. To date, only a few markers or marker panels have been reported to have both a sensitivity and specificity >90% for detection of NPC (15, 17, 18, 36). The sensitivity of 99% of the combined IgA-VCA/EBV DNA marker panel and the specificity of 96–98% of the respective markers in the present study warrant further studies in the screening setting. Because plasma/serum EBV DNA concentrations are based on direct measurement of genomic material in the peripheral blood, this marker is conceptually advantageous in terms of quality control and has potential to serve as a reference marker for comparison of results obtained with different EBV antibody-based markers, which are linked to different EBV source antigens.

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