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Evaluation of Commercial EBV RecombLine Assay for Diagnosis of Nasopharyngeal carcinoma

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ABSTRACT

Background: In recent years a number of Epstein-Barr virus (EBV) proteins were defined as being immunodominant for either IgM, IgG or IgA immunoresponses, yielding promising markers for diagnostic serology. Specific reactivity patterns to these proteins have been described for infectious mononucleosis (IM), nasopharyngeal carcinoma (NPC), various types of lymphoma, and healthy EBV carriers.

Objectives: To compare the NPC-related diagnostic value of EBV RecombLine test (Mikrogen, Germany) with a standardized immunoblot assay [15] and to define the diagnostic value of individual EBV marker proteins in a population with high incidence of NPC.

Result: Sera from Indonesian NPC patients taken at primary diagnosis (n=108) were analyzed for IgG and IgA reactivity and compared with regional healthy blood donors (n=62), non-NPC patient controls (n=10) and IM patients (n=10). Most NPC patients and controls showed strong IgG reactivity to VCA-p18, -p23, and EBNA1, limiting their diagnostic use. Few (<20%) healthy donors and patient controls showed IgG reactivity to EA proteins p47/p54 and p138, yielding combined sensitivity/specificity and PPV/NPV values of 92.6%/98.3% and 99.0%/98.1%, for diagnosing NPC. NPC sera showed significantly more EBV reactive IgA antibody (>80% positive) than controls (<10% positive), although being less broadly reactive and significantly less strong compared to IgG. For IgA best results were observed for RecombLine EBNA1 with sensitivity/specificity and PPV/ NPV values of 92%/89% and 93.4%/85.9%, respectively.

Conclusion: In high incidence NPC regions with low incidence IM yet high prevalence of EBV infection, both RecombLine IgG and IgA tests provide a useful alternative to the more complex cell-extract based immunoblot assay as confirmation test for NPC diagnosis in particular when using EA and EBNA1 as discriminators in IgG and IgA testing, respectively.

INTRODUCTION

The Epstein-Barr virus (EBV) is a human oncogenic herpesvirus associated with a spectrum of diseases, including infectious mononucleosis (IM) [21], Burkitt’s lymphoma (BL) [13], Hodgkin disease [23, 44], extranodal T/NK cell lymphoma [7, 39], immunoblastic B-cell lymphomas in immunocompromised individuals [33], gastric carcinoma [38] and nasopharyngeal carcinoma (NPC) [46]. Transmitted in saliva, EBV usually infects infants without any symptoms and is highly prevalent in developed countries. After primary infection EBV persists for life in B-lymphocytes and mucosal epithelia in the oro- and nasopharynx [37, 45]. Increased hygiene may delay primary EBV infection to adolescence, which then in 50% of cases may associate with a symptomatic disease, named IM or kissing disease, as seen in developed countries, but which is rare in the developing world. Worldwide over 90% of adults are EBV carrier as reflected by persistent IgG antibody responses to a limited number of EBV proteins [27, 40]. EBV reactivation is implicated in a number of chronic and malignant diseases and associates with aberrant anti-EBV antibody responses that may be used diagnostically [1, 8, 17, 26].

EBV has been implicated as causal factor in the development of NPC, although environmental cofactors also play a role [31]. Poorly and non-differentiated NPC (WHO type-II/III), which are 100% EBV associated, are highly prevalent in South-China, most of South-East Asia and in some parts of Africa and Arctic regions as well. In Indonesia, especially in Southern part of Central Java, NPC ranks in the top 5 of most prevalent cancers [34]. Sero-epidemiological studies have indicated a close relation between EBV infection and NPC as revealed by elevated IgG and especially IgA to VCA, EA and EBNA complexes [19, 20]. NPC patients particularly have elevated titers of IgA anti-EBV antibodies, reflecting the tumor’s origin in the mucosa of the nasopharynx [10]. Aberrant EBV serology is commonly used to support NPC diagnosis and provides an affordable approach for population screening to identify individuals with high NPC risk [28]. Indirect immunofluorescent assay (IFA) methods are still used as golden standard for EBV serodiagnosis in NPC. IFA allows the separate analysis of antibody responses to viral capsid antigen (VCA), early antigen (EA) and nuclear antigens (EBNA), each comprising multiple proteins and requiring different cell lines for specific analysis [17, 19]. However, this method is time consuming, subjective and not suitable for large-scale automatic handling [2, 18, 40]. Enzyme-linked immunosorbent assay (ELISA) techniques are increasingly used and showed a better sensitivity and specificity compared to IFA [6, 17, 24, 28, 29]. Development of ELISA requires the availability of high quality EBV antigens, either derived from native antigen [11, 12, 30, 43] or purified recombinant protein [1, 6, 18]. Commercial ELISA kits for the detection of EBV-specific antibodies have been available since late 1980s, but show considerable variation in diagnostic performance providing limited standardization [9, 18, 41, 42].

We recently revealed the underlying diversity of anti-EBV antibody responses in NPC and showed that IgG and IgA responses are frequently directed against different EBV antigens [15], which may explain in part the inconsistent results of prior studies. Analysis of the spectrum of IgG and IgA responses to different EBV proteins was shown to be relevant for NPC diagnosis [15, 24]. Multiple studies have focused on the use of individual recombinant EBV proteins, but no single protein test was sufficient to reliably diagnose NPC and a combination of test is required [25]. The complementary use of EBNA1 with EAd was reported achieve the sensitivity more than 90% [5, 22]. In addition, combination of serology and EBV DNA load testing has been proposed [3, 35]. Most recently a synthetic peptide-based ELISA combining VCA and EBNA1 in a single test was described advanced EBV serology for more standardization [14].

In this study we present the results of the evaluation of Mikrogen EBV RecombLine assay, which uses a selected set of purified recombinant EBV proteins...
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on a polymer strip to detect human IgM, IgG or IgA antibodies. Special emphasis is given on the detection of IgG and IgA antibodies in sera from defined NPC patients from Yogyakarta, Indonesia, in comparison with IgG and IgA antibodies from healthy regional controls and IM patients. As reference, the results were compared with a well-standardized in-house immunoblot assay using recombinant protein or natural antigens HH514 cell line as described recently (8, 15, 30).

MATERIALS AND METHODS

Sera and antibodies. Sera were obtained from the archives of the Gadjah Mada University (GMU), Yogyakarta, Indonesia and the VU medical center (VUmc) in Amsterdam, the Netherlands. Healthy donor sera were from volunteers recruited among laboratory personnel at VUmc (n=13), used only for initial studies, or collected at regional blood banks in Yogyakarta, Indonesia (n=62). Sera from IM patients (n=10) were sampled at regional health centers in the Netherlands. Sera from NPC patients (n=108) and non-NPC cancer patients (n=10) were collected at ENT Department, GMU Sardjito Hospital, Yogyakarta, Indonesia. The NPC sera were taken on the first visit of patients during 2001-2003 and comprise patients presenting with different stages of disease as outlined in Table 1. All sera were examined for EBV IgG and IgA reactivity in a recently described one-step synthetic peptide-based ELISA system (14). NPC was confirmed by biopsy, and paraffin slides were stained for EBV presence by standard EBER-RISH using PNA kit (DAKO, Glostrup, Denmark) and by EBNA1 detection using monoclonal antibody OT1X (4). NPC staging was done by ENT examination and CT-scan and classified according to the 1996 UICC criteria. Monoclonal antibodies to individual EBV proteins were produced at VUmc and tested as described before comprising antibodies to EBNA1 (OT1x), VCA-p18 (OT15E), EAd-p47/54 (OT14E), and EAd-p138 (OT13B) (15, 30).

Table 1. Disease stage of NPC patients

<table>
<thead>
<tr>
<th>Stage</th>
<th>No of patients</th>
</tr>
</thead>
<tbody>
<tr>
<td>I</td>
<td>3</td>
</tr>
<tr>
<td>II</td>
<td>8</td>
</tr>
<tr>
<td>IIA</td>
<td>18</td>
</tr>
<tr>
<td>IIIB</td>
<td>6</td>
</tr>
<tr>
<td>IIIA</td>
<td>17</td>
</tr>
<tr>
<td>IIIB</td>
<td>44</td>
</tr>
</tbody>
</table>

Figure 1(A). RecombLine reactivity of healthy blood donor sera from Yogyakarta region in Indonesia. IgG reactivity patterns observed in Indonesian blood donors are limited to either VCA-p18 or -p23 paralleled by a positive IgG EBNA1 response in most (95%) samples. VCA-p23 showed strongest staining compared to VCA-p18 and EBNA1. A marginal IgG response was found against EAd-p138 and EAd-p47/54. Almost no IgA reactivity was detected against any EBV marker except some marginal reactivity against VCA-p18.

Figure 1(B). RecombLine reactivity of IM sera

Five of ten IM sera showed IgM, IgG and IgA antibodies to one or more EBV marker protein(s). IgG was relatively weak and most clearly directed against EAd-p138 and p47/54 with less reactivity to VCA-p18 and -p23 markers. No IgM anti-EBNA1 was detected. IgA reactivity showed a similar reactivity distribution as IgG, IgA reactivity to EAd-p138 and p47/54 was predominant over VCA-p18/p23 in most cases without EBNA1 reactivity, except in one sample (not shown).
RESULTS

Reference antibody staining. RecombLine strips containing five recombinant proteins (EBNA1, VCA-p18, VCA-p23, EAd-p138, and EAd-p47/54) were analyzed with monoclonal antibodies (MoAbs) to verify the position, identity and reactivity of the individual antigen bands on the strips. The reactivity of the MoAbs used confirmed the specific localization and reactivity of the individual EBV antigen bands. VCA-p23 was not analyzed, due to unavailable monospecific antibody.

EBV reactivity in healthy donor sera. The IgG and IgA reactivity of internal control sera were tested in each run. The EBV negative donor (SJ) did not show any reactivity, confirming specificity. Two healthy EBV carriers (JM, MV) showed the expected IgG reactivity against EBNA1 plus VCA-p18 and/or-p23 and a faint (below cut-off) reactivity against EAd-p47/54 but not against EAd-p138. No IgA reactivity was detected. The chronic EBV patient (RR) showed the expected IgG reactivity with VCA and EA proteins, without IgG to EBNA1. A weak IgA response was detected against VCA-p23 and the EAd markers. The EBV-specific IgG and IgA reactivity of an additional set of nine blinded sera from healthy Caucasians were analyzed. Two donors were non-reactive, subsequently confirmed as EBV negative by reference ELISA and in-house immunoblot testing. The remaining seven donors all were EBV seropositive showing strong IgG reactivity to both VCA-p18 and VCA-p23 markers and one of them showed strong IgG-EBNA1 reactivity, whereas two of them showed weak IgG-EBNA1. Three donors showed some IgG reactivity to the EAd protein bands, which remained at or below cut-off. No IgA antibodies were detected in these healthy donors, except in 2, with IgA reactivity at the cut-off level for VCA-p18 in one donor and for VCA-p23 in the other (data not shown).

As Indonesian control population, 62 samples were randomly selected from 254 healthy blood donors from Blood Bank Yogyakarta, which gave IgG reactivity either to EBNA1 or VCA-p18, or to both of the proteins, except 1 rare EBV negative sample (14). The EBV-specific IgG and IgA reactivity in those sera were analyzed (Fig. 1A). Details are summarized in Table 2. Only one Indonesian donor proved to be EBV negative, not showing any reactive band in the EBV RecombLine assay, which was confirmed by a negative reaction in all other serological tests (data not shown). Otherwise the IgG reactivity patterns in Indonesian blood donors closely resemble those of healthy Caucasian EBV carriers. Predominant IgG reactivity was found for VCA-p18 and somewhat less to VCA-p23 and EBNA1, whereas 100% of EBV seropositive donors had responses to either VCA or EBNA1 or both. An occasional marginal IgG response was found against EAd-p138 and EAd-p47/54. Almost no IgA reactivity was detected against any EBV marker except some marginal reactivity against VCA-p18, which parallels our recent findings in EBV IgA ELISA (14).

EBV IgM, IgG and IgA reactivities in sera from IM patients. In a third series of experiments, 10 heterophile antibody positive sera from patients with serologically confirmed acute IM (positive VCA-IgM ELISA) were analyzed. The age of IM...
Patients ranged from 18 to 25 with an average of 22. Some examples of IgM, IgG and IgA responses are shown in Fig. 1B and the overall results are summarized in Table 2.

All IM sera had detectable IgM, IgG and IgA antibodies to one or more EBV marker protein(s). IgM was relatively weak, but was most clearly directed against the EAd-p138 and -p47/54 markers and to a lesser extend to the VCA-p18 and -p23 markers, confirming previous data (1, 27, 40). No IgM anti-EBNA1 was detected. IgA reactivity showed a similar reactivity distribution as IgM, without EBNA1 reactivity. IgG reactivity was most clearly detected and showed variation between individual IM patients. Like IgM and IgA, the IgG reactivity against EAd-p138 and -p47/54 was dominant over VCA-p18/-p23 in most cases. IgG anti-EBNA1 was not detected except for a weak response in one sample, which is in line with the early IM status of these sera.

EBV IgG and IgA reactivity in NPC and non-NPC cancer patients. Indonesian non-NPC cancer controls (n=10) showed a similar pattern of reactivity as regional healthy controls, except for one patient with weak IgG and IgA to EAd-p47/54 and three patients with weak IgG to VCA-p18/-p23.

All NPC samples (n=108) were collected at primary diagnosis prior to any therapeutic intervention. Most of the patients (87.6%) presented with advanced disease in stages III and IV (Table 1). The male to female ratio of NPC patients was 2.35 (73/31) with age ranging from 18 to 80 with an average 47.4 years. Examples of the IgG and IgA analysis are shown in Fig. 2 and the data are summarized in Table 2. Non-NPC cancer controls consisted of head and neck cancer cases collected at a similar ENT-unit with ages ranging from 11 to 70 (mean 41) years.

Comparison of EBV banding reactivity of NPC sera using the RecombLine system and in-house immunoblot. Immunoblot assay was carried out with in-house recombinant and "native" EA-VCA HH514 cell antigens as described before (15, 30). HH514 blot strips revealed the reactivity to many EBV lytic proteins and in-house immunoblot. In this experiment we focused on the reactivity to EBNA1, VCA-p18, EAd-p47/54 and -p138, with a combined overall sensitivity and specificity for NPC diagnosis are 92.6% and 98.3%, yielding a PPV and NPV of 99% and 88%, respectively (Table 3). For IgA antibody, all EBV markers can be used for discriminating NPC cases from normal donors, with combination sensitivity and specificity for each protein shown in Table 3. EBNA1-IgA gave the best sensitivity, followed by EAd-p47/54 and -p138, with a combined overall sensitivity and specificity for NPC diagnosis compared to EBNAs and VCA IgG in comparison with similar analysis in IM patients. Only two NPC patients had a negative IgG response to either p138 or p47/54. Nearly 60% of NPC cases showed IgG to EBNA1, 37-50% had IgG to VCA markers and 58-68% had IgG responses to EA markers. Although visible as weak staining, IgG responses to EBNA1 and VCA markers remained below the cut-off in about 37-47% of NPC cases and were by definition considered negative in the analysis. Maximum 10% of healthy donors and non-NPC controls show IgA reactivity with low intensity (scored as weak) to single EBV marker, except reactivity of non-NPC to VCA-p18, which reached 30%. In NPC patients IgA reactivity to EAd-p47/54 and -p138 was stronger compared to EBNA1 and VCA, but in a limited number of NPC cases EBV-IgA antibodies were weak or undetectable. Overall 12.6% (n=12) NPC patients failed to make detectable EBV-IgA antibodies as detected by RecombLine assay. However, these sera were EBV-IgA positive in VCA+EBNA1 peptide and EAg-extract ELISAs and showed an aberrant reactivity pattern in immunoblot (14, 15, 30). For all individual EBV markers NPC patients had significantly stronger IgG and IgA responses compared to IM patients and healthy EBV carriers. IgG and IgA reactivity patterns did not show a clear correlation. IgA being less broadly reactive and significantly less strong than IgG yet clearly more reactive than in IM patients and regional controls. Excluding IM patients, the serological responses observed in NPC patients for IgG to EAd-p47/54 and EAd-p138 and for IgA to all EBV markers (Table 2) were significantly different from regional healthy EBV carriers and non-NPC patients (P<0.05). Using IgG analysis, the best markers to discriminate NPC cases from healthy donors are EAd-p47/54 and -p138, with a combined overall sensitivity and specificity for NPC diagnosis are 92.6% and 98.3%, yielding a PPV and NPV of 99% and 88%, respectively.

Table 2. Antibody responses of some sets of sera to EBV proteins

<table>
<thead>
<tr>
<th>Sample</th>
<th>IgG positive (%)</th>
<th>IgA positive (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>NPC</td>
<td>EAd-p138</td>
<td>81.1</td>
</tr>
<tr>
<td></td>
<td>EAd-p47/54</td>
<td>82.4</td>
</tr>
<tr>
<td>Non-NPC</td>
<td>EAd-p138</td>
<td>15.0</td>
</tr>
<tr>
<td></td>
<td>EAd-p47/54</td>
<td>20.3</td>
</tr>
<tr>
<td>IM</td>
<td>EAd-p138</td>
<td>50.0</td>
</tr>
<tr>
<td></td>
<td>EAd-p47/54</td>
<td>25.0</td>
</tr>
<tr>
<td>Normal</td>
<td>EAd-p138</td>
<td>82.5</td>
</tr>
<tr>
<td></td>
<td>EAd-p47/54</td>
<td>10.6</td>
</tr>
</tbody>
</table>

In NPC patients IgA reactivity with low intensity (scored as weak) to single EBV marker, except reactivity of non-NPC to VCA-p18, which reached 30%. In NPC patients IgA reactivity to EAd-p47/54 and -p138 was stronger compared to EBNA1 and VCA, but in a limited number of NPC cases EBV-IgA antibodies were weak or undetectable. Overall 12.6% (n=12) NPC patients failed to make detectable EBV-IgA antibodies as detected by RecombLine assay. However, these sera were EBV-IgA positive in VCA+EBNA1 peptide and EAg-extract ELISAs and showed an aberrant reactivity pattern in immunoblot (14, 15, 30). For all individual EBV markers NPC patients had significantly stronger IgG and IgA responses compared to IM patients and healthy EBV carriers. IgG and IgA reactivity patterns did not show a clear correlation. IgA being less broadly reactive and significantly less strong than IgG yet clearly more reactive than in IM patients and regional controls. Excluding IM patients, the serological responses observed in NPC patients for IgG to EAd-p47/54 and EAd-p138 and for IgA to all EBV markers (Table 2) were significantly different from regional healthy EBV carriers and non-NPC patients (P<0.05). Using IgG analysis, the best markers to discriminate NPC cases from healthy donors are EAd-p47/54 and -p138, with a combined overall sensitivity and specificity for NPC diagnosis are 92.6% and 98.3%, yielding a PPV and NPV of 99% and 88%, respectively (Table 3). For IgA antibody, all EBV markers can be used for discriminating NPC cases from normal donors, with combination sensitivity and specificity for each protein shown in Table 3. EBNA1-IgA gave the best sensitivity, followed by EAd-p47/54 and VCA markers. Both IgA-EAd-p47 and -p138 gave the best specificity.
Serology profiles and disease stage, age and gender of NPC patients. To further evaluate the value of Mikrogen RecombLine commercial test for NPC diagnosis, we analyze the IgG and IgA reactivity in NPC patients in relation with stage, age and sex. The male/female ratio was 2.35 with an age distribution 18-80 (mean 47.4). No correlation was observed between the serology profile (i.e. number of reactive bands and intensity of staining) with age, sex and stage (data not shown).

**DISCUSSION**

EBV-associated diseases are characterized by distinct antibody pattern to various EBV encoded antigen specificities, as defined by IFA (19) and immunoblot analysis (8, 15, 27, 40). Our previous studies revealed considerable heterogeneity in antibody responses to individual proteins in different EBV linked diseases, which may be exploited for diagnostic use. A broad range of commercial methods is available for serological diagnosis of EBV infection, but these show considerable differences (9, 16, 36, 41, 42).

The Mikrogen EBV RecombLine assay provides a versatile, well-standardized approach for simultaneous assessment of IgM, IgG and IgA antibody responses to the individual immunodominant EBNA1, VCA, and EAd markers in sera of patients with different EBV syndromes and healthy individuals. The identity of individual markers could be verified by specific monoclonal antibodies. The aim of this study was to evaluate the Mikrogen EBV RecombLine assay, with special emphasis to detect IgG and IgA antibodies in NPC sera from Indonesia, compared to IgG and IgA responses in non-NPC cancer patients and healthy regional controls as
well as IM patients. Furthermore, the results were compared with a standardized in-house immunoblot using natural EBV antigen from EA+VCA induced HH514 cells (8, 15).

Overall, especially IgG reactivity to EBNA1 and EA markers (p47/54 and p138) antigens showed good specificity and sensitivity for discriminating NPC patients from healthy EBV carriers, non-NPC cancer patients and IM patients. The reactivity of VCA-p23 IgG was lower than VCA-p18 in most cases, except in IM. It was initially reported that sera from most IM patients contained detectable levels of IgG and IgM to p23, but only few studies addressed this topic (1, 18, 32).

A negative EBV RecombLine IgG reaction correctly reflects EBV-seronegative immune status and can reliably be determined in a population of high EBV prevalence. The sensitivity/specificity for defining virus carrier status by virtue of detecting IgG to the combination of VCA-p18/p23 plus EBNA1 is 100%, whereas IgG reactivity for the individual markers has lower values. IgG to VCA-p18 was somewhat more prevalent over IgG to VCA-p23 in the healthy EBV carrier population. A positive IgM reactivity for any of the VCA and/or EA markers is indicative for acute IM, which is confirmed by absent IgG-EBNA1 in 90% of cases analyzed (1). EA-IgG antibodies were found in all IM patients, whereas VCA-IgG was detected in fewer individuals, with a predominance of IgG to VCA-p23. Weak IgA responses to various EBV markers are frequently observed in IM patients, but also prevail in NPC patients for EBNA1 and VCA markers. These weak responses are not observed in other populations and may represent real antibody reactivity. However, the manufacturer’s instructions consider these to be negative.

The specificity of using RecombLine IgG and IgA reactivity to EBV-EA markers for NPC diagnosis confirmed in Indonesia with a population at high risk NPC prevalence, where IM is not prevalent. When compared to regional controls, of which 99% were EBV VCA/EBNA1 IgG seropositive, EAd-p47/54+EAd-p138 IgG emerged as the most distinctive diagnostic parameter showing a PPV and NPV of 99% and 88%, respectively (Table 3). IgA to EAd-p47/54+EAd-p138 was less frequently detected and was sometimes absent for both EAd-p47/54 and p138 markers, thus yielding inferior diagnostic values. IgG EBNA1 provided the best overall diagnostic values, which is in line with previous findings (14, 22). Overall IgG and IgA signal to the individual markers were significantly stronger in NPC patients compared to IM patients and healthy EBV carriers, reflecting a higher affinity and concentration. The antibody-recognition pattern in NPC patients differs from that in patients with other EBV syndromes, which reflects the distinct underlying viral activity in NPC (15).

CONCLUSION

The EBV RecombLine approach provides a useful addition to the currently available panel of EBV serological tests for NPC diagnosis and may be used to reliably detect abnormal anti-EBV antibody patterns. This test can be applied as a confirmation test in screening and diagnosis of NPC in populations with high EBV prevalence such as Indonesia and other parts of SE-Asia.

Acknowledgements

We thank the NPC team of Dr. Sardjito Hospital, Faculty of Medicine, Gadjah Mada University, Indonesia for support in collecting patient samples and Dr. Bambang Hariwijanto (ENT specialist) and Dr. Harijadi (pathologist) for providing clinical and pathological data. We also thank the EBV team in Dept. Pathology, Vrije Universitairer Medical Centre, Amsterdam, the Netherlands, for providing facilities and assistance. Mikrogen GmbH is acknowledged for providing EBV RecombLine test kits. This research was funded by the Netherlands Cancer Foundation (grant KWF-IN 2000-02 and 2004-17) and by the European Union (grant Asia-link, Contract no:ASI/B7-301/98/679-034).
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Reference


Commercial EBV Recombine for NPC diagnosis


