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Epstein-Barr Virus (EBV) Serological Screening Combined with EBV DNA Load in Nasopharyngeal Brushings for Identification of Nasopharyngeal Carcinoma in Individuals with Chronic Complaints in Head and Neck

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Abstract

Nasopharyngeal carcinoma (NPC) is the most prevalent head and neck cancer in Indonesia, and closely linked to active Epstein-Barr virus (EBV) infection. EBV-based markers are proposed for identifying early stage of NPC. We performed a clinical, serological and viral load study in subjects presenting with chronic symptoms in head and neck to define the serologic response to EBV antigens and EBV DNA load and find early onset NPC. A total of 217 individuals were recruited and underwent clinical examination. Blood samples were taken from all subjects and nasopharyngeal brushing was collected from the majority of individuals. Initial serology analysis was done by peptide-based EBV IgA ELISA and confirmed with IgG immunoblot. As many as 75 (34.6%) subjects demonstrated positive EBV IgA ELISA with 23 (10.6%) showing high response. Sixteen individuals (7.4%) had high IgG immunoblot score. EBV DNA load was determined by real-time PCR in 65 seropositive patients and 28 (43.1%) of them showed a high level. Seropositive individuals with high viral load were sent for diagnostic work-up. Seven cases revealed a nasopharyngeal mass by CT-scan and three of them (1.4%) were histologically confirmed as NPC, two of which were early stage. Another case showed hyperplasia positive for EBV-encoded small RNA (EBER). These results indicated that elevated EBV antibody combined with high nasopharyngeal viral load can identify early NPC cases among individuals presenting with chronic complaints in head and neck in NPC endemic regions. Periodic follow-up is suggested for individuals revealing EBV seropositivity and high viral DNA load, but having normal CT-scan.

Introduction

Nasopharyngeal carcinoma (NPC) is a cancer with a geographically well-defined distribution worldwide. Its incidence is relatively low in Japan, the United States, Europe and other western countries, but high in Southern China and Southeast Asia including Indonesia. In Indonesia, in particular the local region of Yogyakarta, NPC accounts for 6.2 new cases per 100,000 persons annually, making NPC the most prevalent head and neck cancer and the fourth prevalent cancer overall. Histopathologically, undifferentiated carcinoma represents the majority of local NPC accounting for 90% of patients visiting Dr Sardjito Hospital in the years 2006 to 2010. (Hariwiyanto et al, 2011, unpublished data)

The prognosis of NPC patients is strongly influenced by tumor stage at diagnosis with 80-90% five-year survival at stage I and 50-70% at stage III-IV. Late stage NPC requires combined chemo-radiotherapy, which is associated with significant side effects and frequent recurrence. Early stage NPC may reach complete remission when treated with radiotherapy only. In our local hospital 86% of NPC patients present with late stage and progressive disease which causes a high burden of morbidity and mortality. Therefore early detection is crucial, especially in high risk populations, in order to facilitate early treatment and ensure better overall survival. Moreover, selection of suitable groups continuously exposed
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to risk factors for NPC or presenting with suspicious symptoms is considered an optimal approach for a case finding program.

Among many risk factors, Epstein-Barr virus (EBV) infection shows the strongest association to NPC. In all cases of WHO classification type III (undifferentiated) NPC, EBV gene expression is found regardless of geographical and ethnic origin.\textsuperscript{15-17} In non-endemic areas other NPC types (type II and I) showed less than 50\% of EBV detection,\textsuperscript{18} whereas in endemic areas all the type I NPC tumors were EBV-related.\textsuperscript{18,19} The close link between EBV and NPC is marked by the presence of viral DNA, RNA and protein in all tumor cells, viral lytic reactivation in the nasopharyngeal mucosa and aberrant antibodies against multiple EBV antigens in patient sera.\textsuperscript{20,21} General elevation of antibody level against EBV antigens such as viral capsid antigen (VCA), DNase, early antigen (EA) and Epstein-Barr virus nuclear antigen 1 (EBNA1) have become outstanding features for use in NPC diagnosis.\textsuperscript{22-25} The aberrant antibody levels in NPC are detectable prior to onset of the disease, showing a feasibility in predicting disease development.\textsuperscript{26-31} This made EBV serology suitable for screening purposes. Furthermore, because NPC patients frequently have elevated levels of EBV DNA either in plasma\textsuperscript{32} or in nasopharyngeal brushings,\textsuperscript{33,34} it has been suggested that DNA-based testing may be useful for NPC diagnosis\textsuperscript{34-36} and provide a prognostic marker for NPC treatment.\textsuperscript{37-39} EBV DNA load may also have a complementary role in an EBV IgA-based screening protocol to improve diagnosis accuracy.\textsuperscript{36}

The success of a screening program partly depends on reliable testing method. EBV-based serology, in particular VCA-IgA using immunofluorescence or immunoperoxidase assay (IFA/IPA) technique, has been widely used for screening.\textsuperscript{28,30,40-42} Unfortunately, IFA/IPA is not well suited for mass screening. New methods, including IgA/[EBNA1+VCA-p18] ELISA, IgG immunoblot and EBV DNA load measurement were recently developed\textsuperscript{24,34,43} and proved to provide good diagnostic value in discriminating NPC from non cancer individuals. Despite their diagnostic performance in established NPC cases, the utility for early screening is yet to be investigated. In particular EBV DNA quantification has been little explored for screening approaches.

Another key factor for a successful screening program is a well-defined population at risk. People presenting with aberrant EBV IgA in a similar pattern to NPC\textsuperscript{28,30,40} and family members of multiplex NPC cases\textsuperscript{31,42} are at higher risk for NPC. Within multiplex families elevated EBV IgA pattern was observed more frequently compared to the general population.\textsuperscript{44} Besides the above-mentioned populations, patients with a history of chronic ear and nose disease were also found to have risk for NPC.\textsuperscript{45-47} Patients with NPC often present with symptoms related to the development of the malignancy but which cannot be considered tumor specific. The first clinical symptom may present as epistaxis, nasal obstruction and discharge due to the presence of tumor mass in the nasopharynx; tinnitus and deafness as a consequence of the extension of tumor mass to paranasopharyngeal space causing the dysfunction of Eustachian tube; headache, diplopia, facial pain and numbness
associated with the superior extension of tumor; and swollen lymph node in the neck representing metastatic tumor.\textsuperscript{48} Furthermore, similar nasal and otological symptoms are defined at presentation of Indonesian NPC in early stages.\textsuperscript{(Adham et al., Chinese J Cancer 2011, accepted)} However, a seroepidemiology study of combined EBV IgA and EBV DNA detection has not been done before among subjects presenting with chronic symptoms in head and neck. Therefore, we aimed at determining the serologic responses and viral quantification among these individuals and assess possible presence of early stage NPC. A 3-step EBV-based screening approach was designed and applied to a selected group of prospective patients with defined head and neck complaints.

**Methods**

**Subject recruitment**

A prospective case finding and follow-up study was conducted by clinical examination, EBV specific serological analysis and nasopharyngeal EBV DNA load measurement in patients visiting the outpatient-clinic at the Department of Ear, Nose and Throat (ENT), Dr. Sardjito Hospital, Yogyakarta, Indonesia. The study was carried out from July 2006 to December 2010. Recruited subjects came to the department with chief complaints of chronic problems in the head and neck area which were not responsive to standard treatment (antibiotic, anti-allergic). The clinical symptoms included rhinitis, nasal obstruction, epistaxis, tinnitus, hearing impairment, discomfort in ear (sensation of ear fullness or pain), headache, facial pain, dizziness and dysphagia. All subjects were recruited with informed consent. Each individual was given a clinical examination including detailed inspection of the nasopharynx using a mirror or by nasendoscopy. The examinations were carried out by ENT specialist doctors or by residents undergoing clinical rotation in this department. Human subject review committee in Yogyakarta approved this project and informed consent was obtained from all participants.

**Protocol and method of laboratory investigation**

At enrollment a serum sample from venous blood was taken from each subject (n= 217) and epithelial brushings of the nasopharyngeal region were collected from 166 individuals. Brush sampling was performed using a cytobrush (Cytobrush-plus, Medscand, Malmo, Sweden) applied to the nasopharynx via a protective tubing to avoid nasal fluid contamination.\textsuperscript{34} The laboratory investigation protocol was designed with a 3-step approach. Firstly, serum from each individual was tested in ELISA for IgA reactivity to the combined VCA-p18 and EBNA1 antigens. This assay, hereupon referred as EBV IgA ELISA, was performed as described previously and a positive seroreactivity result was defined with a cutoff value (CoV) of OD\textsubscript{450} > 0.354. This single-assay combination provided good sensitivity and specificity (85.4% and 90.1%, respectively) in distinguishing NPC cases from non-cancer individuals.\textsuperscript{24}
Additionally, a cutoff point distinguishing strong from weak responders was determined at OD$_{450} > 1.000$ (approximately three times of CoV). (Hutajulu et al., J Med Virol 2011, accepted) For individuals refusing venous blood collection, EBV IgA ELISA was done on sera taken by finger-prick sampling.\textsuperscript{49}

A serology confirmation test was subsequently performed using IgG diversity analysis by immunoblot as described before.\textsuperscript{43} The score was based on the intensity and diversity of IgG reactive bands on the immunoblot ranging between 0-4. Scores of 0-2 represent negative to normal reactive immunoblot patterns, as encountered in healthy EBV carriers, whereas scores of 3-4 are referred to as high score and possible NPC pattern, as defined before.\textsuperscript{25,43,50} As a confirmation test to EBV IgA ELISA, IgG immunoblot increased the sensitivity and specificity of discriminating NPC from unaffected EBV-carriers to 98.0\% and 99.2\%.\textsuperscript{24} Samples with an EBV IgA ELISA result higher than the CoV and/or high score of IgG immunoblot at the time of enrollment were designed as belonging to the seropositive group.

EBV DNA quantification in the nasopharyngeal brushing was done for individuals with a seropositive score for both EBV IgA ELISA and IgG immunoblot. EBV DNA quantity was analysed with LightCycler\textsuperscript{TM} real-time PCR targeting the 213bp region of EBNA1.\textsuperscript{34} The CoV was set on 2,300 copies/brushing presenting the mean +3*SD of a cohort of controls.

Individuals with elevated IgA seroreactivity or high score of IgG immunoblot and/or elevated viral load were sent for clinical work-up including CT-scan and pathological biopsy from the nasopharynx. Patients confirmed having NPC were sent to oncology department for cancer treatment. Individuals with seropositivity and high viral load including cases without nasopharyngeal mass on CT scan or revealed no malignancy in histopathology confirmation were invited to undergo clinical monitoring and laboratory re-testing every 6-12 months. Serum and brushing samples collected at enrollment and on subsequent monitoring were tested in different batches. Schematic flow of subject recruitment, laboratory investigation and follow up is summarized in Figure 1.

**Statistical analysis**

The proportion of EBV IgA seroreactivity as the main marker in relation to other parameters was analysed using Chi-square or Fischer’s exact test. The median of seroreactivity among the groups of immunoblot score was compared using one way ANOVA. The correlation between EBV IgA and EBV DNA load as well as age were performed using correlation coefficient testings (Pearson’s correlation and Spearman’s rank correlation). Computations were done using the SPSS 15 software and significance was determined with p < 0.05.
Figure 1. Protocol of subject recruitment, laboratory investigation and follow-up.
Results

Clinical and laboratory testing in individuals with chronic complaints in head and neck

Two hundred and seventeen patients were examined during years 2006-2010. Table 1 shows the basic features of demography, clinical and laboratory findings at enrollment. There were 87 males (40.0%) and 130 females (60.0%), aged between 13 and 80 years (median=35.94 years). The patients initially visited the ENT clinic having various chief chronic complaints as detailed in Table 1, with rhinitis (51%) and nasal obstruction (22%) being most common. Thirty patients had a history of chronic allergy to some substances such as dust, house dust mite, corn pollen, smoke or human epithelial skin. Seventy-five out of 217 individuals (34.6%) showed elevated EBV IgA seroreactivity above the CoV level in their sera on the first visit. Strong EBV IgA responses (level seroreactivity of >1.000) were demonstrated in 23 out of these 75 seropositive individuals (30.6%) or 10.6% from the total population (data not shown). The seroprevalence was non-significantly higher among females (47/130, 36.2%) compared to males (28/87, 32.2%) (p=0.547). The relative frequency of EBV IgA ELISA positive responders increased somewhat with age, but no significance was reached (p=0.229). However, correlation analysis on the individual age revealed a weak but significant correlation between seropositivity and age (Pearson's correlation r=0.272 p<0.01) (Figure 2). When compared to the particular chief complaint, no symptom showed a significant association to EBV IgA seroreactivity (p=0.380). There was no difference in EBV IgA seroreactivity in patients with or without a history of chronic allergy (p=0.449) (Table 1).

Serology confirmation using IgG immunoblot diversity showed that 16 out of all individuals (7.4%) had a score 3-4 (referred to as abnormal score or suggestive NPC pattern) (Table 1) and no difference was found between seroreactivity and the score (p=0.180). Furthermore, no significant variation of median of ELISA reactivity among groups of immunoblot reactivity was demonstrated (p=0.155) (Figure 3). From 75 patients determined positive by EBV IgA ELISA and/or IgG immunoblot, 65 nasopharyngeal brushings were available for EBV DNA load measurement. Viral DNA load quantification demonstrated high level in 28 out of 65 patients (43.1%) and low level or undetectable in 37 out of 65 patients (56.9%) (Table 1). No correlation was found between EBV IgA serology and EBV DNA load (p=0.653) (Figure 4). As many as 19 patients with elevated EBV DNA load underwent CT-scan investigation. Some patients were also sent to the imaging department because of extreme elevated EBV IgA or other clinical reason such as nasopharyngeal defect seen by nasendoscopy. Seven individuals showed nasopharyngeal mass and underwent nasopharyngeal biopsy.
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Table 1. Characteristics and laboratory testing of study participants

<table>
<thead>
<tr>
<th>Characteristic</th>
<th>Frequency</th>
<th>% EBV IgA Seropositivity</th>
<th>p value</th>
</tr>
</thead>
<tbody>
<tr>
<td>EBV IgA ELISA at baseline</td>
<td>217</td>
<td>75(34.6)</td>
<td>0.195</td>
</tr>
<tr>
<td>Gender, n= 217</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Male</td>
<td>87</td>
<td>28(32.2)</td>
<td>0.547</td>
</tr>
<tr>
<td>Female</td>
<td>130</td>
<td>47(36.2)</td>
<td></td>
</tr>
<tr>
<td>Ages (years), n= 207</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>&lt;20</td>
<td>33</td>
<td>9(27.3)</td>
<td>0.229</td>
</tr>
<tr>
<td>21-30</td>
<td>60</td>
<td>16(26.7)</td>
<td></td>
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<tr>
<td>31-40</td>
<td>35</td>
<td>11(31.4)</td>
<td></td>
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<tr>
<td>41-50</td>
<td>38</td>
<td>15(39.5)</td>
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<tr>
<td>51-60</td>
<td>30</td>
<td>14(46.7)</td>
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</tr>
<tr>
<td>&gt;60</td>
<td>11</td>
<td>6(54.5)</td>
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<tr>
<td>Chief complaints, n= 217</td>
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<td></td>
<td></td>
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<tr>
<td>Rhinitis</td>
<td>112</td>
<td>40(35.7)</td>
<td>0.380</td>
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<td>Nasal obstruction</td>
<td>48</td>
<td>17(35.4)</td>
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<td>Epistaxis</td>
<td>11</td>
<td>5(45.5)</td>
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<td>Tinitus</td>
<td>7</td>
<td>2(28.6)</td>
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<tr>
<td>Ear dyscomforrtess</td>
<td>7</td>
<td>0(0.0%)</td>
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<tr>
<td>Headache</td>
<td>11</td>
<td>4(36.4)</td>
<td></td>
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<tr>
<td>Facial pain</td>
<td>11</td>
<td>4(36.4)</td>
<td></td>
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<tr>
<td>Dizziness</td>
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<td>0(0.0)</td>
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<tr>
<td>Dysphagia</td>
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<td>3(60.0)</td>
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<tr>
<td>Allergic test, n= 34</td>
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</tr>
<tr>
<td>Negative</td>
<td>4</td>
<td>1(25.0)</td>
<td>0.627</td>
</tr>
<tr>
<td>Positive</td>
<td>30</td>
<td>13(43.3)</td>
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<td>Immunoblot score at baseline, n= 217</td>
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<tr>
<td>0-2</td>
<td>211</td>
<td>67(31.8)</td>
<td>0.177</td>
</tr>
<tr>
<td>3-4</td>
<td>16</td>
<td>6(50.0)</td>
<td></td>
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<tr>
<td>EBV DNA load at baseline, n= 65</td>
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<tr>
<td>Low level</td>
<td>37</td>
<td>33(89.2)</td>
<td>0.405</td>
</tr>
<tr>
<td>High level</td>
<td>28</td>
<td>24(85.7)</td>
<td></td>
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</tbody>
</table>

*Seropositivity is defined as EBV IgA ELISA OD$_{450}$ > 0.354
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Figure 2. Scatter diagram of EBV IgA ELISA seroreactivity versus age (years) among individuals presented with chronic symptoms in head and neck (n = 207). Pearson correlation analysis revealed a weak correlation between the two parameters (r = 0.272 p< 0.01).

Figure 3. Boxplot of EBV IgA ELISA seroreactivity (OD_{450} value) versus IgG immunoblot score among individuals presented with chronic symptoms in head and neck (n = 217). Groups of score 3 and 4 were fused to enhance the statistic power since score 4 was only demonstrated by 2 subjects. There is no significant variation of means of seroreactivity among the groups (one way ANOVA p= 0.155). The symbol of “” represented mild outlier and “*” represented extreme outlier.
Nasopharyngeal carcinoma cases detected at enrollment

From 217 subjects at enrollment, three individuals, two males and one female, were identified as having a nasopharyngeal tumor confirmed as NPC by histopathology assessment. This number represents 1.4% of the total patients and 4% of the seropositive group. Their age was 61, 42 and 25 years and all three patients showed classic signs and symptoms of underlying NPC such as chronic rhinitis, nasal obstruction and epistaxis for some years prior to examination. All three showed irregularity on nasendoscopy, but none had obvious enlarged lymph nodes in the neck. Two out of them were diagnosed as early stage (1 and 2) and 1 was stage 3 NPC. All three newly diagnosed cases showed strong seroreactivity ELISA (> 1.000) and high immunoblot score (3-4). From only one of them a nasopharyngeal brushing was available which showed a high level of EBV DNA load similar to established NPC cases (symbolized with open circle in Figure 4).

Figure 4. Scatter diagram of EBV IgA ELISA seroreactivity versus (log) EBV DNA load (copies/brushings) among individuals presented with chronic symptoms in head and neck (n= 65). There was no correlation between the two parameters (Spearman’s rank correlation r= -0.057 p= 0.653) suggesting both EBV parameters are independent markers. Open circle symbolized one of the newly diagnosed NPC cases.
Of the seven cases showing a nasopharyngeal mass in the CT-scan but revealing a negative result of nasopharyngeal biopsy, two individuals demonstrated a defect of nasopharyngeal mucosa under nasendoscopy. One of them also showed severe hyperplasia and a positive result of Epstein-Barr-encoded small RNA (EBER) which is widely used as golden standard for EBV detection. Unfortunately this patient refused to undergo further monitoring. The pathological examination of the biopsy of the other five patients revealed chronic inflammation.

**EBV serology profile and EBV DNA load during follow-up**

A total of 129 follow-up examinations, ranging from 6 to 36 months, were performed for seropositive subjects during subsequent hospital visits. Sixty-six of the 75 seropositive patients have undergone a first follow-up, 34 a second, 17 a third, 8 a forth and 4 a fifth monitoring. There were 33 individuals showing sustained elevated EBV IgA seroreactivity, five of which demonstrated a dynamic fluctuation above CoV. Seven individuals showed fluctuation between seronegative and seropositive. Twenty-six individuals reverted to seronegative. Figure 5 displayed a representative serologic pattern of baseline and follow up serial of EBV IgA seroreactivity. Up to now the monitoring has not yet resulted in detection additional NPC case. The subject with EBER positive hyperplasia and elevated serology plus EBV DNA load in the brush, continued to refuse further examination. Along with EBV IgA ELISA, IgG immunoblot was also performed on these individuals during follow-up. The fluctuation in immunoblot score usually paralleled EBV IgA ELISA. NPC brushing was collected inconsistently during follow-up due to the refusal of some participants at different times of clinical visits. Like the serologic pattern, there was no specific pattern of increase or decrease of EBV DNA quantification during monitoring (data for serial IgG immunoblot score and EBV DNA load not shown).

**Discussion**

Seroepidemiology studies for defining NPC risk have been done on unselected healthy subjects\textsuperscript{28,30,40} or on selected groups of high risk multiplex families.\textsuperscript{31,42} The present study is the first to determine the EBV serologic pattern in a group of individuals presenting with general chronic head and neck symptoms suggestive of NPC. With a limited number (217) of patients analyzed, three newly NPC cases (1.4%) were diagnosed at the first investigation, plus one case of EBER-positive hyperplasia. This rate of new cases at enrollment was higher than the rate detected by previous studies (0.03-0.09\%).\textsuperscript{30,31} These studies were conducted in a bigger population with longer monitoring time rendering more NPC identification at the end of screening. Our results indicate that screening in a selected population with defined chronic head and neck complaints is proper for an NPC case finding program.
Figure 5. Representative fluctuating serological pattern at baseline (1) and follow-up of EBV IgA seroreactivity among individuals with chronic complaints in head and neck. Antibody levels followed pattern of sustained elevation (n= 33) with some dynamic patterns (n= 5) as represented by HR 007 and HR 038; fluctuation between seropositive and seronegative (n= 7) as represented by HR 035; or negative-reverting pattern (n= 26) as represented by HR 124. HR= high risk (individuals presenting with chronic complaints in head and neck); CoV= cuttoff value (set at 0.354).

From the three NPC cases identified in our study population, two cases presented as early stage NPC. An additional case with EBER positive hyperplasia and positive EBV test results refused further investigation. All four cases were first identified by EBV IgA ELISA and confirmed by IgG immunoblot score. All showed elevated IgA seroreactivity at intake, not only above the standard CoV but also above the point of strong reactivity (i.e. >3x CoV). Of the three NPC subjects, only one nasopharyngeal brushing was available because the first two cases were identified when EBV DNA load testing was not yet incorporated in the routine protocol. Inspite of this, our results demonstrate the utility of EBV-based serology for initial NPC risk screening in this defined patient population.

The general seropositivity (> CoV) in EBV IgA ELISA in the group of patients with chronic head and neck complaints (34.6%) was comparable to first-degree relatives of established NPC cases and matched case-controls in our local population (41.2% and 39.5%, respectively).(Hutajulu et al., J Med Virol 2011, accepted) However, in the seropositive subgroup of patients with head and neck complaints, 30.6% of patients showed seroreactivity with strong response (with value more than 3 times of standard CoV). This is in line with 36.9% strong responders in first-
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degree relatives of NPC cases, both being higher than the 14.7% strong responders in the EBV IgA positive subgroup of healthy EBV carriers in the general community. (Hutajulu et al., J Med Virol 2011, accepted)

Prospective population-based studies conducted in endemic areas of China and Taiwan have explored the relation between EBV antibodies and NPC onset, suggesting that EBV VCA IgA is a suitable seromarker for NPC screening. Most reported studies using VCA IgA have deployed IFA as the golden standard method for NPC serodiagnosis. However, this test has technical and interpretation limitations preventing standardization for routine and mass screening. IFA is increasingly replaced by objective ELISA methods produced with different forms of EBV antigens, either native, recombinant, or synthetic peptide antigens applied as single or multiple antigens. As NPC sera show IgG and IgA antibody reactivity to multiple of EBV antigens, multiple antigen testing in one assay is important for achieving higher sensitivity and specificity. The use of (bio)chemically defined EBV markers adds to improving standardization, handling efficiency and cost aspects. Therefore we developed an EBV IgA ELISA based on combined use of VCA-p18 and EBNA1-derived synthetic peptides, which enables large scale studies. More recent studies confirmed the value of EBNA1 IgA as additional suitable NPC marker besides VCA-p18. The use of this serological tool, possibly combined by simpler finger-prick blood sampling will open the way for further field studies, replacing the IFA-based standard test. Furthermore, the use of EBV IgG immunoblot or recombinant line assay as confirmation assay to the EBV IgA ELISA, may increase the diagnostic value of serological testing. The characteristic aberrant diversity of IgG and IgA anti-EBV responses in NPC patients can be diagnostic in itself.

There is high interest in comparing EBV DNA and EBV-based serology, because the sensitivity of both markers for NPC diagnosis is among the highest reported. The use of EBV DNA quantification in the circulation (whole blood or plasma) for NPC screening has been suggested as a complementary test for a positive serology result but some NPC patients have low to undetectable EBV DNA levels in the circulation at diagnosis. Compared to peripheral blood or plasma, EBV-DNA detection in nasopharyngeal brushings offers a more direct reflection of biological activity at the site of primary tumor development. Since normal nasopharyngeal epithelium does not contain significant amounts of EBV DNA, the CoV between cases and controls can be precisely defined. Sensitivity, specificity, positive and negative predictive values of EBV DNA load tested in nasopharyngeal brushing are reported to be as high as >91%, comparable to EBV IgA serology. Nasopharyngeal brushing has a false negative rate comparable to that of the invasive biopsy but it is more feasible for a screening approach. Importantly, in case of undetermined results during follow-up monitoring, the brushing procedure can be repeated because of its minimal invasive property. However, EBV DNA analysis by PCR is considerably more costly than serological testing. Therefore in our present protocol EBV DNA is used for secondary confirmation in serological
aberrant individuals, in order to minimize the cost burden. Our result (Figure 4) confirmed other reports\textsuperscript{34,36} showing that EBV DNA load and EBV-based serology are independent markers when used in conjunction. The complementary use increases diagnostic sensitivity and minimizes false positivity, which is important in the setting of cancer screening.

Serial follow-up on seropositive individuals in our study showed a variable pattern of EBV IgA ELISA seroreactivity. Almost half of the individuals demonstrated sustained elevated level of EBV IgA, either with or without a high score of EBV IgG in immunoblot or high level of EBV DNA. Antibody levels may change during the progression of NPC as shown by multiple studies from China and Taiwan. Aberrant VCA IgA antibody responses were detected at 16-41 months and even as long as 10-15 years prior to clinical diagnosis of NPC.\textsuperscript{30,40,59} This period is referred to as the serologic window. It implicates that when EBV serology is examined routinely, NPC can be detected in the early stages of evolution. Subjects with increasing EBV-VCA IgA antibody levels have higher risks and shorter time to develop NPC compared to those with a descending pattern giving relative risk of 5.8.\textsuperscript{30,59} The longer the follow-up period, the greater the difference found in the cumulative NPC incidence.\textsuperscript{28} This implies that for patients presenting with a persistent abnormal serologic pattern, closer follow-up and more rigorous clinical examination are recommended. The observation that seronegative subjects may go through EBV IgA seroconversion before developing NPC\textsuperscript{30,42,59} illustrates the relevance of serological screening in non-symptomatic people in high risk regions. However, conducting all EBV-based tests in asymptomatic subjects will raise a high cost burden and may not be feasible in our local region. Therefore in our case finding protocol (Figure 1) a priority for intensive monitoring was given to individuals with symptoms showing seropositive at enrollment.

Among patients presenting with chronic symptoms, there was a weak but significant correlation between age and elevated seropositivity rate (Figure 2). It has previously been shown that anti EBV antibody titers increase with age,\textsuperscript{44,60} presumably because the cumulative exposure to EBV with time. In this study females showed higher seroreactivity compared to males, although significance was not reached. Previous studies support this observation possibly relating to hormonal influences on immune responses.\textsuperscript{44,61,62}

Despite having one advanced case (stage III) in our study population, the goal of early identification for facilitating early treatment and better survival was achieved. Ji et al. also observed that even by routine screening 32% of patients were diagnosed in late stage, decreasing from 79% prior to the program.\textsuperscript{30} Thus close clinical follow-up and EBV-based testing will increase early-stage NPC detection. The prevalence of late stage NPC in our local community may be due to lack of awareness regarding early signs and symptoms of NPC among health workers in primary care centers.\textsuperscript{63} Thus a specific education program is needed and an active screening program with a community-based approach should be designed.
In conclusion, this study verified previous observations that individuals with chronic problems in head and neck area are at risk for NPC. The strategy of combined EBV marker analysis shows promise for identifying early-stage NPC cases in this group of patients. EBV IgA ELISA has proven value for early NPC detection and can be performed in combination with simple finger-prick blood sampling. IgG immunoblot and nasopharyngeal brush EBV DNA load are supportive for confirmation of an aberrant ELISA result, reflecting aberrant EBV behaviour. In a future case finding program, monitoring using the 3-step EBV marker approach is recommended to anticipate early-stage NPC.

Reference

Chapter 3


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