Antibodies against Epstein–Barr virus gp78 antigen: a novel marker for serological diagnosis of nasopharyngeal carcinoma detected by xMAP technology

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Immunoglobulin (Ig) A and/or IgG reactivities to several Epstein–Barr virus (EBV) antigens have been used to facilitate diagnosis of nasopharyngeal carcinoma (NPC). However, antibodies against gp78, an EBV membrane glycoprotein, have not been examined to this day. In this study, we utilized Luminex multi-analyte profiling (xMAP) technology to analyse antibody responses to a synthetic peptide of gp78 in sera samples from 95 NPC patients and 91 healthy controls. Our results showed the sensitivity and specificity of IgA-gp78 for NPC diagnosis were 79 and 71 %, respectively, while those of IgG-gp78 were 74 and 73 %, respectively. The IgA and IgG responses to different EBV antigens were not identical within an individual and IgA-gp78 and IgG-gp78 could be complementary to antibodies against viral capsid antigen (VCA), the diffused early antigen (EA-D) and the nuclear antigen EBNA1 for NPC diagnosis. When the six EBV parameters for NPC prediction, i.e. IgA-gp78, IgG-gp78, IgA-VCA, IgA-EBNA1, IgA-EA-D and IgG-EA-D, are combined, the combined predictors were able to reach overall sensitivity and specificity of 91 and 95 %, respectively. Thus, simultaneous detection of these EBV serological markers could improve the predictive values of NPC using xMAP technology.

INTRODUCTION

Epstein–Barr virus (EBV) is a ubiquitous gammaherpes virus composed of a protein core enclosed by DNA, an inner nucleocapsid with 162 capsomeres, a middle protein tegument, and an outer envelope carrying various membrane glycoproteins (Dolyniuk et al., 1976; Epstein et al., 1965; Khanna et al., 1995). EBV infects more than 90 % of the population worldwide and usually establishes a lifelong persistence in the host (Cohen, 2000). Most populations get primary infection in childhood and are asymptomatic, but Westerners whose primary infection is delayed until adolescence may develop infectious mononucleosis (Amon & Farrell, 2005). More strikingly, the virus is associated with a spectrum of cancers presenting endemic features, such as Burkitt’s lymphoma in Africa and nasopharyngeal carcinoma (NPC) in Southern China and South-East Asia (Pattle & Farrell, 2006; Raab-Traub et al., 1987).

EBV is closely correlated with NPC, which could be reflected by consistent expression of EBV gene products in NPC tumour cells and general elevation of serum antibody levels against EBV antigens in NPC patients (Busson et al., 2004; Gastpar et al., 1981; Henle & Henle, 1976; Old et al., 1966; Raab-Traub, 2002). In comparison with healthy EBV carriers, NPC patients typically show strong IgG and especially IgA reactivities to lytic antigens (Fachiroh et al., 2004; Henle & Henle, 1976), so an EBV serological assay could facilitate diagnosis and prognosis of NPC. IgA antibody titres against the EBV viral capsid antigen (VCA) and the diffused early antigens (EA-D) are regularly tested in many clinical centres (Deng et al., 1995; Hadar et al., 1986; Henle & Henle, 1976; Ho et al., 1998). However, the EBV serological spectrum differs among individuals and so other antigens have been further verified as useful indicators of NPC in recent years. These markers include
nuclear antigen EBNA1 (Foong et al., 1990), transactivators Zta and Rta (Dardari et al., 2001; Feng et al., 2001; Yip et al., 1994), replication factors DNA polymerase and alkaline DNase (Littler et al., 1991), nucleotide metabolism factors ribonucleoside reductase and thymidine kinase (Fones-Tan et al., 1994; Littler et al., 1991), and several tegument and capsid antigens (Fachirollo et al., 2004, 2006), which were derived from natural EBV proteins, recombinant products or synthetic peptides.

EBV envelope glycoproteins are of particular interest in studies because they bind to the EBV receptor on the cells and initiate infection. EBV enters B cells by interaction of the major glycoprotein gp350/220 (BLLF1) with CD 21 and then penetrates by the complex of viral glycoproteins gp25 (Pickard et al., 2004), gp42/38 and gp85 (gH) (Tao et al., 2006). Furthermore, EBV glycoproteins play important roles in humoral immune responses and sera against these proteins often neutralize virus. So far, 12 EBV glycoproteins have been characterized (Johannsen et al., 2004). However, few of them have been shown as valuable markers for NPC diagnosis. gp350/220 is the major target of the EBV-neutralizing antibody and several studies detected IgA-gp350/220 in NPC patients with higher titres than healthy controls, but these tests were based upon a relatively small number of samples (Khanna et al., 1999; Littler et al., 1991; Xu et al., 1998).

To investigate the values of EBV envelope proteins for NPC diagnosis, we utilized Luminex multi-analyte profiling (xMAP) technology to examine antibodies against several EBV antigens in NPC patients and healthy controls. xMAP technology is based on flow-cytometric analysis of microbeads that act as solid supports for individual assay reactions. Each bead has a unique spectral address and represents a detection reaction. To date, more than 100 distinct reactions could be carried out simultaneously on the various beads in very small sample volumes. The individual beads are identified and their assays are read with a Luminex-100 or -200 instrument (Earley et al., 2002; Elshal & McCoy, 2006; Kettman et al., 1998). After screening antibodies to several glycoproteins, we found antibodies against gp78, a membrane protein with unknown function, were statistically higher in NPC populations than in healthy controls. In this study, we further detected the IgA and/or IgG levels to gp78, VCA, EA-D and EBNA1 in NPC patients and controls and analysed the combination of these EBV serological markers for NPC diagnosis.

**METHODS**

**Patients and controls.** All study participants were Cantonese presenting a high NPC incidence. Ninety-five sera were collected from patients with newly diagnosed and pathologically confirmed NPC. Stage of disease progression was classified according to the 1996 International Union Against Cancer Classification. The serum samples tested included three from patients at cancer stage I, 11 at stage II, 58 at stage III and 23 at stage IV. Normal controls were collected from 91 healthy blood donors (Table 1). Four herpes simplex virus (HSV)-positive sera of children were also detected. Written informed consent was obtained from all study participants.

**Synthetic peptide.** To obtain the potential antigenic epitopes, we examined the protein sequences from the reported EBV proteomes with DNASTAR software (Tarbouriech et al., 2006). Sequences with high possibility of hydrophilicity, surface-orientation and flexibility were chosen. About 20 residues of each peptide were selected and synthesized by adding six carbon and one biotin at the N terminus (Hanyu), and then further purified by HPLC to achieve >90 % purity. The peptide sequences used in this study were gp78 (TSFHRPHRPSKRPHTK, gp350/220 (HHAEMQNPVYLIPE-TVPYIK) and BLRF1 (EADQFYPYTCNADTFS). xMAP analysis.

**Coupling of proteins to beads.** Coupling of EBVVCA-gp125, EA-D and EBNA1 (Biodesign) to the carboxylated beads (Luminex) was performed according to a protocol reported previously (Skogstrand et al., 2005). Briefly, 2.5 × 10⁶ beads were washed with activation buffer (10 mM NaH₂PO₄, pH 6.3), resuspended in 80 μl activation buffer and sonicated for 10 s. N-hydroxysulfosuccinimide (10 μl; Pierce) solution and 10 μl 1-ethyl-3-(3-dimethylaminopropyl) carbodiimide hydrochloride (Pierce) solution, both diluted in activation buffer to 50 mg ml⁻¹, were added to the bead suspension. After mixing, the beads were incubated with rotation for 20 min at room temperature in the dark. The activated beads were subsequently washed twice with coupling buffer (10 mM NaH₂PO₄, 150 mM NaCl, pH 7.4) and then incubated with 500 μl azide-free protein solution (diluted to 250 μg ml⁻¹ in coupling buffer) for 2 h. The beads were washed three times with washing buffer (1 × PBS, 0.05 % Tween-20) and resuspended in 100 μl blocking/storage buffer (1 × PBS, 1 % BSA). Finally, the beads were counted with a haemocytometer, adjusted to a concentration of 1.25 × 10⁶ beads ml⁻¹ with storage buffer and stored protected from light at 4 °C.

**Table 1. Characteristics of study populations**

<table>
<thead>
<tr>
<th>Age</th>
<th>Healthy (n)</th>
<th>NPC (n)</th>
<th>NPC cancer stage</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Male</td>
<td>Female</td>
<td>Male</td>
</tr>
<tr>
<td>&lt;30</td>
<td>4</td>
<td>1</td>
<td>4</td>
</tr>
<tr>
<td>30–39</td>
<td>21</td>
<td>5</td>
<td>17</td>
</tr>
<tr>
<td>40–49</td>
<td>17</td>
<td>3</td>
<td>23</td>
</tr>
<tr>
<td>50–59</td>
<td>19</td>
<td>1</td>
<td>20</td>
</tr>
<tr>
<td>≥60</td>
<td>19</td>
<td>1</td>
<td>9</td>
</tr>
<tr>
<td>Total</td>
<td>80</td>
<td>11</td>
<td>73</td>
</tr>
</tbody>
</table>
Peptide conjugation. Coupling of biotinylated peptides to LumAvidin microspheres (Luminex) was performed according to the manufacturer’s instructions. Briefly, equal volumes of beads and peptide (10 μg ml⁻¹ in storage buffer) were mixed and incubated overnight at 4 °C. After washing twice (with PBS and 0.05% Tween-20) and resuspension in storage buffer (PBS, 1% BSA, 0.05% NaN₃), the beads were counted with a haemocytometer, adjusted to a concentration of 1.25 × 10⁶ beads ml⁻¹ with storage buffer and stored protected from light at 4 °C.

Serum sample test. The conjugated beads were diluted with storage buffer to 1000 beads in 50 μl per reaction well and then added to the 96-well filtration system (Millipore). Sera diluted 1:21 in storage buffer (20 μl per well) were added and incubated with the beads for 30 min and protected from light at room temperature. After washing three times, 150 μl R-phycoerythrin (R-PE)-conjugated goat anti-human IgA or IgG (1:200 in PBS; Jackson ImmunoResearch) was added to each reaction well and incubated for 30 min. The detection analysis was performed with a Luminex multi-analytic system 100 (Bio-Rad). All tests were carried out in duplicate.

Statistical analysis. All statistic analysis was performed with SPSS 13.0 for Windows and the GraphPad Prism 4.0 program. The unpaired t test was used to compare the mean values from NPC and healthy groups, one-way analysis of variance (ANOVA) was used to compare mean fluorescence intensity (FI) of IgA-gp78 or IgG-gp78 among populations with different ages and cancer stages and receiver operating characteristic (ROC) curve analysis was done to determine the cut-off values. Linear correlations between the FI values of antibodies against gp78 and VCA, EA-D or EBNA1 were analysed with Pearson’s method.

RESULTS

High levels of antibodies against EBV gp78 in NPC patients

To evaluate the distribution of antibodies against EBV membrane proteins in NPC and healthy populations, we screened several proteins of this class including gp350, BLRF1 and gp78 in about 50 patients and 50 controls. Previous studies demonstrated that antibodies to three gp350 peptides were able to inhibit EBV binding and invasion (Szakonyi et al., 2006; Urquiza et al., 2005). Our results also revealed that IgA to one of the gp350 peptides was statistically higher in NPC patients than in healthy controls (P=0.005). However, IgG values to gp350 and antibodies to BLRF1 had no significantly distinct distribution in NPC and healthy populations. Interestingly, IgA- and IgG-gp78 reactivities in NPC patients were both higher than controls.

After examining a total of 95 NPC patients and 91 healthy controls, we found the mean xMAP FI values of IgA-gp78 for NPC patients and controls were 1790 and 377, respectively, with high discriminatory value (P<0.0001). Similarly, the difference in IgG FI values was also statistically significant, displaying mean FI values of 12 261 and 4404 for NPC patients and healthy controls, respectively, being statistically significant between NPC and controls (P=0.0001) (Fig. 1a and b).

Sensitivity and specificity of IgA-gp78 and IgG-gp78

Sensitivity and specificity are the most popular parameters for evaluating diagnostic values. Sensitivity is the probability of a positive test among patients with disease, while specificity is probability of a negative test among patients without disease. Here, we appraised the sensitivity and specificity of anti-gp78 antibodies for NPC diagnosis by defining the cut-off values through ROC analysis. The areas under the curve (AUC) of IgA-gp78 and IgG-gp78 were 0.83 [95% confidence interval (CI), 0.77–0.89] and 0.80 (95% CI, 0.73–0.86), respectively (Fig. 1c and d). When

Fig. 1. Distribution frequency and ROC curve analysis of IgA-gp78 and IgG-gp78 in NPC and healthy populations. xMAP technology was used to analyse IgA and IgG reactivity against the synthetic gp78 peptide from Cantonese serum panels consisting of NPC patients (n=95) and healthy blood donors (n=91). (a and b) The percentile distribution of the xMAP FI values in the tested sera, with the mean FI values of both IgA-gp78 (a) and IgG-gp78 (b) being statistically significant between NPC and healthy populations (P<0.0001). The line in the box represents the median xMAP FI value, the box boundaries represent the values for 25% and 75% of the sera, the horizontal lines connected to the boxes with vertical lines represent 5% and 95% of the sera. (c and d) ROC analysis of antibody responses to gp78, showing AUCs of IgA-gp78 (c) and IgG-gp78 (d) were 0.83 (95% CI, 0.77–0.89) and 0.80 (95% CI, 0.73–0.86), respectively.
the FI value 500 was chosen as the cut-off of IgA-gp78, the sensitivity and specificity were 79% (95% CI, 69–87%) and 71% (95% CI, 60–79%), respectively. As for IgG-gp78, if FI values higher than 6000 were defined as positive results, the sensitivity and specificity were 74% (95% CI, 63–82%) and 73% (95% CI, 63–81%), respectively. The positive predictive values (PPV) and negative predictive values (NPV) both exceeded 70% for IgA-gp78 and IgG-gp78 assays (Table 2).

Characteristics of individual IgA- and IgG-gp78 responses

Although both IgA and IgG recognize the synthetic gp78 peptide, individual responses of the two antibodies were not all identical (Supplementary Table S1, available with the online version of this paper). In the NPC population, only 14 persons had lower than cut-off FI values for both IgA-gp78 and IgG-gp78, while six healthy persons had higher values for both. The 14 cases that were false negative for both gp78 antibodies did no have obviously distinct distributions of cancer stages: three cases were stage II, eight cases stage III and three cases stage IV. Furthermore, with respect to all the participants in the study, no statistical difference was observed between IgA-gp78 or IgG-gp78 and age or gender for both NPC and healthy populations.

The distribution of IgA-gp78 and IgG-gp78 levels according to individual cancer stage is shown in Fig. 2, and the relationship between them was assessed by one-way ANOVA. Our results showed no significant relationship for cancer stages with IgA-gp78 ($P=0.587$) or IgG-gp78 ($P=0.440$) levels.

Combination of antibodies responding to gp78, VCA, EA-D and EBNA1 for NPC diagnosis

In this study, IgA antibodies against EBV VCA, EA-D and EBNA1 gp78 were detected in a single xMAP assay, and IgG against EA-D and gp78 were also detected in a single assay. Based on Pearson’s method, it showed positive correlations between the xMAP FI values of the six serological markers, but the $r$ values were not high (Table 3). The data suggested the immunological responses to different EBV antigens varied significantly within an individual, which also resulted in the diversity of EBV antibody profiles among NPC patients. Thus, it is conceivable to improve the diagnostic capacity of NPC by combination of different EBV biomarkers.

The AUCs of IgA-VCA, IgA-EA-D, IgA-EBNA1 and IgG-EA-D were 0.75 (95% CI, 68–82%), 0.88 (95% CI, 83–93%), 0.87(95% CI, 82–93%) and 0.85(95% CI, 79–90%), respectively, comparable to IgA-gp78 and IgG-gp78. According to the optimal sites in their ROC curves, the xMAP FI 350, 300, 6000 and 2000 were selected as cut-offs for IgA-VCA, IgA-EA-D, IgA-EBNA1 and IgG-EA-D, respectively. Hence, 33 NPC patients were positive for all six markers, whereas 25 healthy controls were negative for all markers. In NPC patients, only one had false negative values for all six biomarkers, while no healthy control showed false positive values for any marker, indicating that the six parameters could be complementary for NPC diagnosis. If more than two parameters with higher FI

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**Table 2.** Diagnostic values of IgA and IgG antibodies to EBV gp78 by xMAP analysis

<table>
<thead>
<tr>
<th>Marker tested</th>
<th>Cut-off (FI value)</th>
<th>Sensitivity (%) (95% CI)</th>
<th>Specificity (%) (95% CI)</th>
<th>PPV</th>
<th>NPV</th>
<th>AUC (95% CI)</th>
</tr>
</thead>
<tbody>
<tr>
<td>IgA-gp78</td>
<td>500</td>
<td>79 (69–87)</td>
<td>71 (60–79)</td>
<td>74%</td>
<td>76%</td>
<td>0.83 (0.77–0.89)</td>
</tr>
<tr>
<td>IgG-gp78</td>
<td>6000</td>
<td>74 (63–82)</td>
<td>73 (63–81)</td>
<td>74%</td>
<td>73%</td>
<td>0.80 (0.73–0.86)</td>
</tr>
</tbody>
</table>

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**Fig. 2.** Distribution of antibody values of EBV gp78 according to cancer stage. The xMAP results for pretreatment sera of Cantonese NPC patients having stage I ($n=3$) and II ($n=11$), III ($n=58$), and IV ($n=23$) disease are shown. The mean FI values of IgA- and IgG-gp78 were not significantly different between stages (IgA-gp78, $P=0.587$; IgG-gp78, $P=0.440$).
values than the cut-off were chosen as positive standard for NPC diagnosis, the sensitivity and specificity were 91 % (86 of 95 NPC patients were positive) and 95 % (86 of 91 healthy controls were negative), respectively.

Antibody reactivities against gp78 peptide in HSV-positive sera

Eight members of the family Herpesvirus infect humans. All the herpesviruses have a general similarity in capsid structure and so the genes involved in the lytic cycle are quite similar. However, the proteins involved in cell type-specific infection, such as latent antigens and some surface proteins, are quite unique to each virus (Farrel, 2005). Several envelope proteins of EBV have homologues in HSV, such as pairs of EBV gp110 HSV-1 gB, EBV gp85 and HSV-1 gH (Gong et al., 1987; Heineman et al., 1988).

To investigate whether the antibodies to the gp78 peptide cross-react with HSV, we examined four HSV-positive sera of children. Our results showed the serum of HSV-NO.4 had high IgG-gp78 and IgA-VCA values, with FI reaching 12 106 and 2909, respectively. However, the other three sera had low levels of antibody to the six EBV markers, including IgA- and IgG-gp78 values (Supplementary Table S1). Since we did not find gp78 homologues in the HSV genome, it is possible that individual HSV-NO.4 might be simultaneously infected by HSV and EBV.

DISCUSSION

Elevated serum IgA antibodies against several EBV-encoded proteins, such as VCA, EA-D and EBNA1, is an outstanding feature of NPC patients (Deng et al., 1995; Foong et al., 1990; Xu et al., 1998). However, high titres of EBV IgG are usually not specific for NPC and can be observed in other EBV-infected individuals as well (Gulley, 2001). The major membrane glycoprotein-350/220 in particular could trigger equal antibody responses in both NPC patients and healthy controls, and its IgG antibody was verified to counteract EBV particles (North et al., 1982). In this study, a synthetic peptide of gp78 was used to detect the serum IgA and IgG levels by xMAP analysis. Herein, gp78 is more specific, since both IgA and IgG reactivities to gp78 were higher in the NPC population than in the healthy one.

The sensitivity and specificity of IgA-gp78 and IgG-gp78 for NPC diagnosis both exceeded 70 %, comparable to IgA-VCA and -EA (Henle & Henle, 1976; Leung et al., 2004). Our results suggest that serum IgA and IgG recognize at least one common gp78 epitope, but that the IgA and IgG responses within an individual could differ significantly, as described previously (Fachiroh et al., 2004), implying distinct mechanisms to activate IgA- and IgG-producing B cells. The function of gp78 remains unknown to date. Although the majority of EBV seropositive individuals produce antibodies against it (Mackett et al., 1990), gp78 levels in NPC patients were much higher, suggesting gp78 might be important for EBV replication in epithelial rather than lymphoblastoid cells. Furthermore, EBV gp78 is an envelope protein and the strong immune response induced by gp78 in NPC patients might be involved in counteracting EBV invasion, therefore gp78 might be a vaccine candidate.

To date, an immunoenzymic assay has been employed extensively in South China to detect IgA to EBV VCA and EA-D using EBV-infected cell lines as a target. However, it is only semiquantitative and poorly standardized and a certain amount of expertise is required. In this study, we used xMAP technology to detect EBV serology, which was automatic and the data could be quantified (Chen et al., 1999; Fulton et al., 1997). Simultaneous examination of antibodies to gp78, VCA, EA-D and EBNA1 revealed a positive correlation between them, but with relatively poor r values. It is plausible because these antigens are expressed in EBV lytic replication and their antibody responses have a similar tendency, but distinct individual characteristics. In fact, combination of these six markers could greatly improve the values for NPC diagnosis and mass screening with proper standards. To detect multiple markers simultaneously is the remarkable advantage of xMAP assay compared with ordinary ELISA. Up to now, several diagnostic kits based on xMAP technology have been developed (Biagini et al., 2003; Dunbar et al., 2005; Klutts et al., 2004; Lukacs et al., 2005). Because of the distinct EBV serology spectrum in individual NPC patients, the multiplexed bead assay has powerful potential to allow serological diagnosis of NPC in the future.

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<table>
<thead>
<tr>
<th>Antibody</th>
<th>IgA-gp78</th>
<th>IgG-gp78</th>
<th>IgA-VCA</th>
<th>IgA-EA-D</th>
<th>IgA-EBNA1</th>
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<tbody>
<tr>
<td>IgG-gp78</td>
<td>r=0.394</td>
<td></td>
<td></td>
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<tr>
<td>IgA-VCA</td>
<td>r=0.236</td>
<td>r=0.328</td>
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<td>IgA-EA-D</td>
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<tr>
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<td>r=0.342</td>
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<td>IgG-EA-D</td>
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